

SECTION 3

Aerobic Bacteriology

SECTION EDITOR: *Deirdre L. Church*

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3.1

Introduction to the Section

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The Aerobic Bacteriology section of the handbook has been reorganized to place each part of the procedure together, including collection, specimen processing, supplies, QC, and the actual step-by-step testing. This will allow the user to see an overview of the entire procedure together. When several different methods of testing are acceptable, each option is presented. The users should not reproduce the procedural text of this handbook in its entirety but rather should choose among the various options presented to produce practical procedures applicable to their laboratory.

Procedures are first organized by anatomic site. The user may wish to separate each section of these procedures, as recommended in NCCLS document GP2-A4 (NCCLS is now known as the Clinical and Laboratory Standards Institute) (6). For example, the information in Specimen Collection can be used to provide a separate nursing manual, the information in Quality Control can be used for QC procedure, the information in Materials can be used for an inventory for reagent preparation and procurement of supplies, and the information in the beginning of each Procedure and many of the tables can be used for a specimen inoculation manual and a teaching manual for new employees, etc. Flowcharts and tables within the Procedure can be used to prepare technical bench manuals. References are provided to allow the reader further information for use in decision making when different options are being considered for test methods. Every attempt was made to provide significant original reviewed articles to support the recommended procedures. For procedures 3.3.1 and 3.3.2, general textbooks are listed, which can be purchased for reference material.

Often the laboratory is requested to examine a specimen for only one micro-

organism. To avoid duplication, when a procedure is presented for a specific organism, the procedure is listed following the general procedure for the most common anatomic site of isolation of the organism. For example, the detection of *Neisseria gonorrhoeae* is listed following the genital culture procedure, although *N. gonorrhoeae* may be sought in throat cultures. The procedure for *Brucella* is found following the general blood culture procedure, yet the organism can be found in a variety of other sterile specimens, such as joint and spinal fluid.

Following the procedures by anatomic site are procedures for biochemical testing in alphabetical order. The tests that are listed are the generally accepted tests that laboratories should be able to perform to identify the clinically important microorganisms encountered in the laboratory. Smaller laboratories may choose to perform fewer tests and refer cultures when less common microorganisms are found in culture. Procedures for automated methods and multitest kits are not presented because the list is extensive and manufacturers provide updated package inserts with the details for the performance of their products and preparation of laboratory procedure manuals for their kits. However, tables comparing these kits are presented to allow the user to have information for decision making in the purchase of such kits (also see references 5 and 8). The biochemical tests selected for inclusion in this handbook emphasize those that are rapidly performed. Consequently, the X and V factor procedure is not listed, because laboratories are encouraged to perform the more rapid δ-aminolevulinic acid test in combination with growth on CHOC or the satellite test for *Haemophilus influenzae*. If some me-

dia or biochemical tests are now thought to be less sensitive than other tests, the less sensitive media or tests are *not* listed. For example, V agar is reported to be less sensitive than human blood Tween bilayer media (procedure 3.9.1) for growth of *Gardnerella vaginalis* and *Burkholderia cepacia* selective agar is more sensitive than *Pseudomonas cepacia* agar for *B. cepacia* (procedure 3.11.3). Thus, V agar and *P. cepacia* agar are not listed as choices in the procedures.

Lastly, flowcharts are listed for common and important organisms. These flowcharts are different from any you will encounter, because they emphasize different levels of identification for different anatomic sites and rely on the reported sensitivities and specificities of each test for decision on the need to confirm the results. The flowcharts are designed to rapidly detect clinically important microorganisms with very few tests, but do not yield a species level identification unless it would be clinically relevant. The tables that follow the flowcharts should help further in the identification of both *common organisms and those that are of great clinical importance*. For more extensive identifications in cases of repeated isolation of organisms that do not usually initiate disease and for information on unusual organisms, the reader is referred to other reference material (1, 2, 3, 4, 5, 7, 9).

I would like to thank the original authors of the first edition for their phenomenal work from which the updated handbook was modeled and often duplicated in this edition. Without their procedures and the expert editor of the first edition, Marie Pezzlo, this edition would not have been possible. I am grateful to the many contributors to the first edition and appreciate their thoughtful presentation of the mate-

rial, especially Frank Citron for the many illustrations and Yvonne Ramsay Shea, Paula J. Malloy (deceased), and Joanne J. Bradna for the extensive information on specimen collection and processing, which is used throughout the procedures. The contribution of Patricia Kruezak-Filipov and Roxanne G. Shively to the Gram stain procedure will be a resource to la-

borаторians for many years. Many of the tables and figures that they developed have been retained in this version of the handbook.

Companies that supply media, reagents, and other products tend to change rapidly. Thus, the products listed from these suppliers may not always be available as they are described. Listing is lim-

ited to companies that sell products that are available in the United States. Rather than repeat the details of these companies, a list of common vendors that supply laboratory media and reagents is presented in Appendix 3.1–1. Other vendors' addresses are listed in the specific procedures when they provide a product used for that procedure alone.

REFERENCES

- Forbes, B. A., D. F. Sahm, and A. S. Weissfeld.** 2002. *Bailey and Scott's Diagnostic Microbiology*, 11th ed. Mosby, St. Louis, Mo.
- Holt, J. G., N. R. Krieg, P. H. A. Sneath, J. T. Staley, and S. T. Williams.** 1994. *Bergey's Manual of Determinative Bacteriology*, 9th ed. Williams & Wilkins, Baltimore, Md.
- Koneman, E. W., S. D. Allen, W. M. Janda, P. C. Schreckenberger, and W. C. Winn, Jr. (ed.).** 1997. *Color Atlas and Textbook of Diagnostic Microbiology*, 5th ed. J. B. Lippincott, Philadelphia, Pa.
- Krieg, N. R., and J. G. Holt (ed.).** 1984. *Bergey's Manual of Systematic Bacteriology*, vol. 1. Williams & Wilkins, Baltimore, Md.
- Murray, P. R., E. J. Baron, J. H. Jorgensen, M. A. Pfaller, and R. H. Yolken (ed.).** 2003. *Manual of Clinical Microbiology*, 8th ed. ASM Press, Washington, D.C.
- NCCLS.** 2002. *Clinical Laboratory Technical Procedure Manuals*, 4th ed. Approved guideline GP2-A4. NCCLS, Wayne, Pa.
- Sneath, P. H. A., N. S. Mair, M. E. Sharpe, and J. G. Holt (ed.).** 1986. *Bergey's Manual of Systematic Bacteriology*, vol. 2. Williams & Wilkins, Baltimore, Md.
- Truant, A. L. (ed.).** 2002. *Manual of Commercial Methods in Microbiology*. ASM Press, Washington, D.C.
- Weyant, R. S., C. W. Moss, R. E. Weaver, D. G. Hollis, J. G. Jordan, E. C. Cook, and M. I. Daneshvar.** 1995. *Identification of Unusual Pathogenic Gram-Negative Aerobic and Facultatively Anaerobic Bacteria*, 2nd ed. Williams & Wilkins, Baltimore, Md.

APPENDIX 3.1–1

Vendors for Microbiology Supplies and Equipment

Abbott Diagnostics 100 Abbott Park Rd. Abbott Park, IL 60064 (847) 937-6100 http://www.abbottdiagnostics.com/	Dade Behring, Inc. 1717 Deerfield Rd. Deerfield, IL 60015 (800) 241-0420 http://www.dadebehring.com
BD Diagnostic Systems 7 Loveton Circle Sparks, MD 21152 (800) 638-8663 http://www.bd.com	EY Laboratories 107-127 N. Amphlett Blvd. San Mateo, CA 94401 (800) 821-0044 http://www.eylabs.com
Biolog, Inc. 21124 Cabot Blvd. Hayward, CA 94545 (510) 785-2564 http://www.biolog.com	Focus Diagnostics, Inc. 5785 Corporate Ave. Cypress, CA 90630 (800) 445-0185 http://www.focusdx.com
bioMérieux, Inc. 100 Rodolphe St. Durham, NC 27712 (800) 682-2666 http://www.biomerieux-vitek.com	Gen-Probe Incorporated 10210 Genetic Center Dr. San Diego, CA 92121 (800) 523-5001 http://www.gen-probe.com
Biowhittaker/BMA New name: Cambrex Bio Science Walkersville, Inc. 8830 Biggs Ford Rd. Walkersville, MD 21793 (800) 638-8174 http://www.cambrex.com	Hardy Diagnostics 1430 W McCoy Ln. Santa Maria, CA 93455 (800) 266-2222 http://www.hardydiagnostics.com

APPENDIX 3.1–1 (continued)

Inverness Medical Professional Diagnostics 2 Research Way Princeton, NJ 08540 (800) 257-9525 http://www.invernessmedicalpd.com	Pro-Lab Diagnostics 9701 Dessau Rd., Suite 802 Austin, TX 78754-3941 (800) 522-7740 http://www.pro-lab.com
Invitrogen Corporation 1600 Faraday Ave. Carlsbad, CA 92008 (760) 603-7200 http://www.invitrogen.com	Remel Inc. (Oxoid) 12076 Santa Fe Dr. Lenexa, KS 66215 (800) 255-6730 http://www.remel.com
Key Scientific Products 1402 D Chisholm Trail Round Rock, TX 78681 (800) 843-1539 http://www.keysientific.com	Roche Molecular Systems Inc. 4300 Hacienda Dr. Pleasanton, CA 94588-2722 (925) 730-8000 http://www.roche-diagnostics.com
Meridian Bioscience, Inc. 3471 River Hills Dr. Cincinnati, OH 45244 (513) 271-3700 http://www.mdeur.com	Sigma-Aldrich P.O. Box 14508 St. Louis, MO 63178 (800) 325-3010 http://www.sigmaaldrich.com
MIDI, Inc. 125 Sandy Dr. Newark, DE 19713 (800) 276-8086 http://www.midi-inc.com	TECHLAB, Inc. 2001 Kraft Dr. Blacksburg, VA 24060-6358 (800) TechLab http://www.techlabinc.com
Pharmacia Corporation 1455 F St., NW, Suite 450 Washington, DC (908) 306-8227 http://www.pharmacia.com	Trek Diagnostic Systems, Inc. 982 Keynote Circle, Suite 6 Cleveland, OH 44131 (800) 871-8909 http://www.trekds.com
PML Microbiologicals, Inc. 27120 S.W. 95th Ave. Wilsonville, OR 97071 (800) 547-0659 http://www.pmlmicro.com	

3.2

STAINING PROCEDURES

3.2.1

Gram Stain

[Updated March 2007]

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

The Gram stain is used to classify bacteria on the basis of their forms, sizes, cellular morphologies, and Gram reactions; it is additionally a critical test for the presumptive diagnosis of infectious agents and serves to assess the quality of clinical specimens (12, 16). The test was originally developed by Christian Gram in 1884. The modification currently used for general bacteriology was developed by Hucker in 1921; it provides greater reagent stability and better differentiation of organisms. Other modifications have been specifically developed for staining anaerobes (Kopeloff's modification) and for weakly staining gram-negative organisms (*Legionella* spp., *Campylobacter* spp., *Brucella* spp., etc.) by using a carbol fuchsin or basic

fuchsin counterstain (4, 17). In fact, many laboratories use these counterstains routinely, especially for direct smears of clinical material.

Bacteria stain either gram positive or gram negative on the basis of differences in their cell wall compositions and architectures. Gram-positive species have a thick peptidoglycan layer and large amounts of teichoic acids; they are unaffected by alcohol decolorization and retain the initial stain, appearing deep violet if their cell walls are undamaged by age, antimicrobial agents, or other factors. Gram-negative species have a single peptidoglycan layer attached to an asymmetric lipopolysaccharide-phospholipid bilayer

outer membrane interspersed with protein; the outer membrane is damaged by the alcohol decolorizer, allowing the crystal violet-iodine complex to leak out and be replaced by the counterstain (2, 4).

Interpretation of Gram-stained smears involves consideration of staining characteristics and cell size, shape, and arrangement. These characteristics may be influenced by many factors, including culture age, medium, incubation atmosphere, staining methods (1), and presence of inhibitory substances. Similar considerations apply to the interpretation of smears from clinical specimens, but additional factors include the presence of particular host cell types and phagocytosis.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING

A. Specimens

1. Clinical specimens, generally excluding throat swabs, nasal swabs, sputum from cystic fibrosis patients, fecal material, and prosthetic devices. Direct smears are particularly useful for wounds, eye lesions, sterile fluids, body tissues, and certain discharges.
 2. Broth and blood cultures to determine growth, Gram reaction, or morphology of bacteria
 3. Colonies growing on solid medium
- NOTE:** Young cultures (<24 h old) from noninhibitory media and fresh clinical specimens yield the most favorable results. When morphology is important (e.g., streptococci and gram-positive rods), broth cultures are preferred.

B. Specimen collection

1. Refer to procedures 3.4 to 3.13 for specimen collection by anatomic type.
2. Generally the smear is made in the laboratory; however, when there is a concern that transport will be delayed or that the preservative for culture will alter the specimen, prepare smears and submit slides to the laboratory.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING (continued)

C. Rejection criteria

1. Gram stains are of little value for direct smears of stool, throat samples, and urine and for sputum from cystic fibrosis patients. Gram stains are also not routinely performed on blood cultures due to the low organism density in most samples. However, a direct blood smear may allow rapid detection of the bacteremia type in patients with fulminant sepsis where the organism load is high (e.g., suspected meningococcemia or asplenic patients with suspected pneumococcemia).
2. Stains are not part of standard protocols for evaluation of catheter tip specimens (procedure 3.6).

III. MATERIALS

A. Reagents

1. Methanol, absolute
Store in brown bottles or plastic containers.
2. Crystal violet
 - a. Hucker's modification (1, 4, 10)
 - b. Kopeloff's crystal violet
3. Iodine
 - a. Gram's reagent
 - b. Kopeloff's iodine
4. Decolorizers
 - a. Slowest: ethanol, 95%
 - b. Intermediate: acetone-alcohol
 - (1) Mix 50:50.
 - (2) Combine in brown glass bottle, label with 1-year expiration date, and store at room temperature.
 - c. Fastest: acetone (reagent grade)
Caution: Ethanol and acetone are flammable.
5. Counterstain
 - a. Safranin
 - b. Carbol fuchsin
 - c. Basic fuchsin (0.8, 0.1, or 0.2%)
 - d. Kopeloff's safranin
6. Reagents can be purchased or prepared in-house.
 - a. Refer to Appendix 3.2.1–1 for instructions on preparation of reagents.
 - b. Generally prepare large volumes and prepare working solutions as needed (this is efficient and ensures lot uniformity). Decant reagents into smaller bottles for daily use; however, replace the smaller bottles of the crystal violet and counterstain monthly to avoid the formation of precipitate on the slides.

❑ Indicate the reagent name, preparation date, "in use" date, lot number, expiration date, and storage conditions on the bottles and in the work record. It is not necessary to label the working bottles with this information other than the name of the reagent, if the "in use" date is clearly on the stock bottles and in the control records.

B. Other supplies

1. Wax pencil
2. Precleaned glass slides (25 by 75 mm), frosted ends desirable. Slides with etched rings are an alternative for liquid specimens.
3. Sterile 0.85% NaCl (saline), water, or broth
4. Sterile Pasteur pipettes, wood applicator sticks, inoculating loops, or needles
5. Supplies for disposal of biological waste, including sharps
6. Sterile tubes with caps
7. Sterile scissors, scalpels, and forceps
8. Immersion oil

C. Equipment, depending on specimen source or laboratory protocol

1. Bactericinerator or flame burner with automatic shutoff
2. Electric slide warmer, 45 to 60°C
3. Cytospin centrifuge
4. Vortex mixer
5. Device for collection of toxic stains for chemical hazard disposal

ANALYTICAL CONSIDERATIONS**IV. QUALITY CONTROL**

- A. Check appearance of reagents daily.
 - 1. If crystal violet has precipitate or crystal sediment, refilter before use.
 - 2. Change working solutions regularly if not depleted with normal use. Evaporation may alter effectiveness of reagents.
 - 3. Limit reuse of working stain containers by discarding at least monthly.
- **NOTE:** Stains can become contaminated. When contamination is suspected, use a new lot of stain.
- B. Test laboratory staining procedure prior to use of new lots of each staining and decolorizing reagent and at least weekly thereafter, using a gram-positive and gram-negative microorganism. For laboratory staff that perform Gram stains infrequently, it may be appropriate to have them test a positive and negative control daily or even with each patient specimen tested.
 - 1. Prepare a faintly turbid broth culture of *Escherichia coli* (ATCC 25922) and *Staphylococcus aureus* (ATCC 25923).
 - 2. Make slides using 2 drops per slide spread in the size of a dime.
 - 3. Fix in methanol and store at –20°C.
 - 4. Stain by laboratory Gram stain method.
 - 5. Expected results
 - a. Gram-negative rods, pink
 - b. Gram-positive cocci, deep violet
 - 6. Alternatively, with a broken applicator stick or toothpick, procure material from between teeth and apply to the end of slide used for the specimen, separating this area of the slide with a marker. This method provides a built-in control with gram-positive and gram-negative representatives.
- C. Take corrective action when stained smear preparations show evidence of poor quality, stains are difficult to interpret, or interpretations are inaccurate. Poor staining characteristics (e.g., faintly staining gram-positive organisms, retention of crystal violet by gram-negative organisms, staining only of the edges of a smear, precipitate on slide, etc.) may be due to specimen preparation, reagents, or staining procedure. The following are some common causes of poor Gram stain results.
 - 1. Use of glass slides that have not been precleaned or degreased
 - 2. Smear preparations that are too thick
 - 3. Overheating of smears when heat fixation is used
 - 4. Excessive rinsing during the staining procedure, especially if smear is not properly fixed
 - 5. Precipitate in reagents
- D. Additionally, to ensure accuracy of interpretation, establish a system for reviewing Gram stain reports.
 - 1. Review of selected Gram stains by supervisory personnel to determine training needs and aid in correlating relevant clinical information
 - 2. Compare final culture results with Gram stain reports to check for recovery of morphologies noted in the Gram stain but not recovered in the culture. Similarly, review both the smear and the culture when organisms in 3 to 4+ quantities are recovered in culture but not observed on the Gram stain.
 - **NOTE:** An appreciable number of organisms discerned on a smear can be cultivated. Discrepancies should be investigated for errors in smear evaluation or for indications for further culturing methods (e.g., anaerobic, fungal, or acid-fast bacillus [AFB] culture).
 - 3. Maintain a set of reference slides for competency training.

V. PROCEDURE

The Gram stain is initially used to determine the presence of purulence and bacterial morphotypes in a variety of clinical specimens. Bacterial culture results should also be correlated to the Gram smear results from the original clinical specimen. Organisms that fail to grow in culture but are seen on the Gram stain are typically fastidious or anaerobic bacterial species that require specific media and growth conditions to be viable.

A. Slide preparation

1. General considerations

- a. Place frosted-end glass slides in a container of 95% ethanol (change alcohol solution daily).

- b. Using a forceps, drain excess alcohol and flame slide prior to use.

- c. Label the frosted ends with the information to identify the specimen or culture. Clinical specimens should be processed and handled in a biosafety cabinet, including the preparation of Gram stain slides. When working in a BSL 2 cabinet, personnel protective equipment should be used, including gowns and gloves, and laboratory staff should be trained to follow other BSL 2 recommendations (15).

2. For direct smears, prepare a monolayer of organisms sufficiently dense for easy visualization but sparse enough to reveal characteristic arrangements. As a guideline, newspaper print should be visible through the smear.

NOTE: When working from the same pipette or swab, always prepare the Gram stain and then sequentially inoculate culture plates so that the smear is representative of the original cellular and microbial composition of the clinical specimen.

a. Body fluids, bronchoalveolar lavage fluid (BAL), and CSF

- (1) Place 5 or 6 drops of sample plus 1 drop of 37% formalin into a cytospin specimen chamber. Follow procedure for operation of centrifuge from manufacturer.

NOTE: Use of a cytospin slide centrifuge to concentrate body fluids increases the Gram stain sensitivity and decreases time of standard centrifugation and examination, for more rapid results (13).

- (2) As an alternative when the specimen is viscous or cloudy or the quantity is not sufficient for concentration, use a Pasteur pipette to transfer 1 or 2 drops of the specimen directly to the slide, after marking the location with a wax pencil. Allow the drop(s) to form one large drop. Do not spread the fluid. Optionally, add a second drop of fluid to the same area to increase the concentration of fluid for examination (8).

b. Urine specimens

- (1) Place 10 µl of well-mixed, uncentrifuged urine onto a glass slide marked with a wax pencil to indicate the location of the sample drop. A ring slide may also be used for ease in locating the specimen.

- (2) Air dry without spreading.

c. Specimens received on swabs

- (1) Request a separate swab for adequate smear preparation.

NOTE: Many culturette systems provide two swabs in the container, eliminating problems with sufficient swabs for each test requested.

- (2) Roll the swab gently across the slide to avoid destruction of cellular elements and disruption of bacterial arrangements.



Observe standard precautions.

V. PROCEDURE (continued)

(3) Alternatively, when only one swab is received, place the swab in a small amount of saline or culture broth, cap tube, and vortex. Squeeze the swab against the side of the tube, and use swab to prepare smear. Use the remaining suspension to inoculate culture media.

NOTE: Never mix open tube vigorously. Avoid creation of aerosols.

d. Specimens not received on swabs: aspirates, exudates, sputa, etc.

(1) Transfer specimen to the cleaned slide.

(a) Specimens received by the laboratory in a syringe with the needle still attached should be rejected because of the risk of a needless sharps exposure by laboratory staff. The physician should be immediately phoned to recollect the sample and send it in the proper container.

NOTE: Establish a policy for the proper collection and transport of clinical specimens not collected on swabs. Educate the physicians that needles must be removed from the syringe and the syringe cap secured prior to transport to avoid leakage.

(b) Select purulent or blood-tinged portions of pus or sputum with a sterile applicator stick, pipette, or wire loop.

(c) Spread the sample over a large area of the slide to form a thin film.

(2) For extremely thick or purulent specimens

(a) Dilute in a drop of saline on the slide for easier smear preparation.

(b) Alternately, place the specimen on one slide, cover it with second slide, press the slides together, and pull them apart (Fig. 3.2.1–1). Remove excess material on the side of slides with a disinfectant-soaked paper towel.

e. Dried material or very small amounts of clinical specimen

(1) Emulsify specimen in 0.5 ml of sterile broth. Vortex, if necessary.

(2) Use a sterile Pasteur pipette to transfer 1 drop to a slide.

(3) Use the pipette tip to spread the drop into an even thin film.

f. Biopsy specimens and tissue sections

(1) Touch preparation (Fig. 3.2.1–2)

(a) Place tissue in sterile petri dish, and mince with sterile scissors or surgical scalpel.

(b) With sterile forceps to hold pieces, touch the sides of one or more of the minced fragments to a sterile glass slide, grouping the touches together for easier examination.

(2) Thin-smear preparation (Fig. 3.2.1–1)

(a) When specimen sample is soft tissue or thick exudate, prepare smear by taking a small portion of tissue, and place it on sterile slide with a swab or sterile forceps.

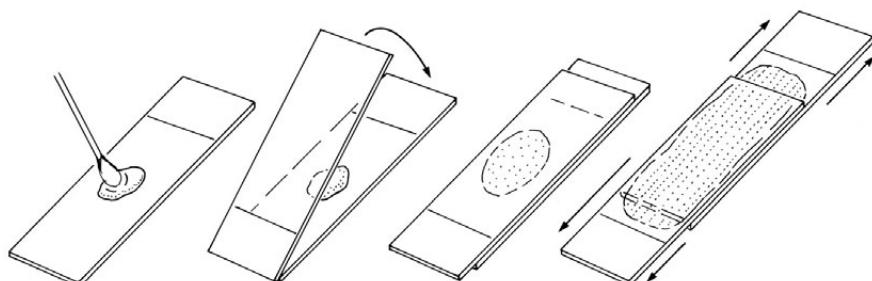


Figure 3.2.1–1 Thin-smear preparation.

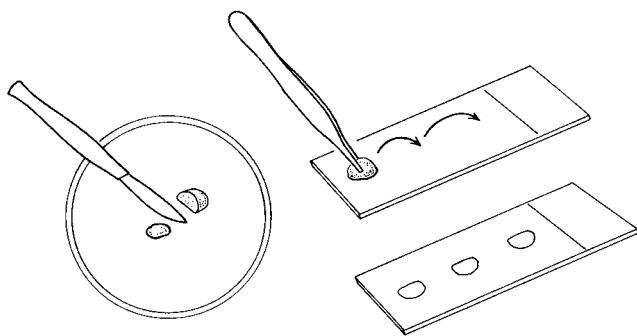


Figure 3.2.1–2 Touch preparation of smear.

V. PROCEDURE (continued)

- (b) Place second sterile slide over sample, and press slides together.
- (c) Separate slide by sliding the two away from each other.
- (3) If there is no other option, use a ground specimen preparation (*see* procedure 3.13.1). Spread 1 drop to the size of a dime.
 - ▣ **NOTE:** Homogenizing or grinding tissue specimens before preparing the smear will often destroy characteristic cellular entities and bacterial arrangements.
3. Broth cultures
 - a. Prepare one smear per slide to avoid washing off of “dried” liquid from one area to another during staining.
 - b. Use a sterile Pasteur pipette (or a venting needle or syringe adapter for containers with septa, such as blood culture bottles, to avoid manipulating a needle and syringe) to transfer 1 or 2 drops to the slide.
 - c. Spread drop into an even thin film.
 - d. For charcoal-containing medium, prepare blood culture smear as for hematology blood count, using a second slide at a 45° angle to spread a thin layer of cells on first slide.
4. Colonies from solid media
 - a. Place a drop of sterile saline or water on slide. Distilled water may distort cellular morphology of fragile organisms.
 - b. Transfer a small portion of colony with a sterile applicator stick, wire needle, or loop.
 - c. Gently mix to emulsify. The resulting smear should be slightly cloudy and homogeneous (adjust saline drop size, area of smear, and/or inoculum to achieve optimum results). If swirl lines are evident as the smear dries, the inoculum was too heavy, the drop of saline was too small, and/or the smear was spread over too small an area.
- B. Smear fixation
 1. Air dry slides in a biosafety cabinet or covered on a slide warmer at 60°C until dry.
 2. Heat fixation
 - a. Pass air-dried slides two or three times through a flame, or hold the slide against the front of a microincinerator for 5 to 10 s. To avoid distortions, do not overheat.
 - b. Allow slide to cool before staining.
 3. Alternatively, fix with methanol to prevent the lysis of RBCs, produce a cleaner background, and prevent washing off of liquid specimens (11).
 - a. Place a few drops of methanol on air-dried slide for 1 min, drain off remaining methanol without rinsing, and allow slide to air dry again.
 - b. Do not use heat before staining.

V. PROCEDURE (continued)

C. Staining procedures (see Table 3.2.1–1 for comparison of methods)

■ NOTE: Do not apply stains, water, or decolorizer directly to specimen area. Apply drops near the frosted end of the slide, allowing reagent to flow over the remaining surface.

1. Hucker's modification

■ NOTE: Hucker's modification is widely used for routine work. The decolorizer chosen will alter decolorization times. Acetone-alcohol gives consistent results, but 95% ethanol is preferred for students and less experienced personnel. Acetone is a more rapid decolorizer with a shorter range of reproducibility and is recommended for experienced personnel only (1). It is most useful for specimens containing an appreciable number of host cells.

- a. Flood the fixed smear with the crystal violet solution. Allow the stain to remain for 30 s.
- b. Decant crystal violet, and rinse slide gently with running tap water.
- NOTE:** Excessive rinsing in this step could cause crystal violet to be washed from gram-positive cells. Apply the flow of water to the underside of the angled slide to ensure a gentle flow across the smeared side.
- c. Rinse off excess water with iodine solution, and then flood the slide with fresh iodine solution. Allow iodine to remain for 30 s.
- d. Rinse off iodine gently with flowing tap water.
- e. Decolorize by letting the reagent flow over the smear while the slide is held at an angle. Stop when the runoff becomes clear. Adjust decolorization time to thickness of smear and type of decolorizer used.
- f. Remove excess decolorizer with gentle flow of tap water.
- g. Flood the slide with counterstain and allow to remain for at least 30 s and for ≥ 1 min with fuchsin stains. Use one of the following counterstains.
 - (1) Safranin
 - (2) 0.1 to 0.2% basic fuchsin (7)
 - (3) Carbol fuchsin or 0.1 to 0.8% basic fuchsin counterstain for detecting faintly staining gram-negative organisms (17). See Table 3.2.1–1 for decolorizer options.

Table 3.2.1–1 Gram stain modifications, recommended reagents, timing, and uses

Stain and use	Hucker's		Carbol fuchsin		Kopeloff's	
	Reagent	Time	Reagent	Time	Reagent	Time
Initial stain	Crystal violet	30 s	Crystal violet	30 s	Alkaline crystal violet: flood with solution A; add 5 drops of solution B	2–3 min
Iodine	Gram's iodine	30 s	Gram's iodine	30 s	Kopeloff's iodine	≥ 2 min
Decolorizer	Acetone-alcohol	$\sim 1\text{--}5$ s	95% ethanol	~ 30 s	3:7 acetone-alcohol: rinse immediately after applying	
Counterstain	Safranin ^a	30 s	Carbol fuchsin or 0.8% basic fuchsin	≥ 1 min	Kopeloff's safranin	10–30 s
Recommended use	General bacteriology		<i>Bacteroides</i> spp. <i>Fusobacterium</i> spp. <i>Legionella</i> spp. <i>Campylobacter</i> spp. <i>Brucella</i> spp. and other faintly staining gram-negative organisms		Anaerobes Diagnosis of bacterial vaginosis (Appendix 3.2.1–3)	

^a Or, preferably, use 0.1 to 0.2% basic fuchsin as a counterstain (7).

V. PROCEDURE (continued)

- h. Remove excess counterstain with a gentle flow of tap water.
 - i. Drain slide, and air dry it in an upright position, or use a commercial slide drier.

2. Kopeloff's modification

■ **NOTE:** Kopeloff's modification is recommended for better visualization and differentiation of anaerobes, which may easily overdecolorize and stain faintly with Hucker's modification (4 to 6). It is recommended for vaginal smears to diagnose bacterial vaginosis (*see Appendix 3.2.1–3*).

- a. Flood fixed smear with solution A (crystal violet). Add approximately 5 drops of solution B (5% sodium bicarbonate). Blow slide to mix. Then allow the stain to remain for 15 to 30 s or up to 2 min, but do not allow it to dry on the slide.
 - b. Rinse slide gently with Kopeloff's iodine.
 - c. Apply fresh Kopeloff's iodine for at least 2 min.
 - d. Hold the slide in a slanted position, and apply decolorizer. Rinse immediately.
 - e. Counterstain with Kopeloff's safranin for at least 30 s.
 - f. Gently rinse excess counterstain with running tap water. Drain slide, and air dry.

D. Examine the direct smear microscopically.

1. Evaluate the general nature of the smear under low power (defined as 10× objective with 10× eyepiece) (9).
 - a. Observe for stain crystals.
 - (1) If an excess of precipitated stain is observed, decolorize and restain slide.
 - (2) Alternatively, prepare another Gram-stained smear.
 - (3) If precipitate continues, use freshly filtered crystal violet or counterstain in a clean container.
 - b. Determine if smear has been properly decolorized.
 - (1) Depending on the source of the specimen, the background should be generally clear or gram negative.
 - (2) If WBCs are present, they should appear completely gram negative.
 - (3) If slide is overdecolorized, completely decolorize and restain slide.
 - c. Determine if thickness of smear is appropriate. For proper interpretation, areas must be no more than one cell thick, with no overlapping of cells. Prepare new slide if unreadable.
 - d. For smears prepared from clinical specimens, examine several fields (10 for urine, 20 to 40 for other specimens) under low power for evidence of inflammation.
 - (1) Observe distribution of organisms and cells.
 - (2) Determine areas representative of inflammation or purulence and areas of apparent contamination with squamous epithelial cells (SECs). If no purulence is seen, choose areas of apparent necrosis, inflammatory cell debris, and mucus.
 2. If cells are present, determine the average count of WBCs and epithelial cells in 20 to 40 representative fields that contain cells.
 3. In a representative area with a preponderance of inflammation or purulence using the oil immersion lens (defined as 100× objective with 10× eyepiece), examine 20 to 40 fields to observe cell morphology and Gram reaction.
 - a. If rare or no organisms are seen from a normally sterile-site specimen, but the specimen appears purulent, or the specimen looks suspicious, perform a more extensive review of the slide.
 - b. Refer to Tables 3.2.1–2, 3.2.1–3, and 3.2.1–4 for characteristic morphologies that may be seen in smears from clinical specimens.

Table 3.2.1–2 Gram-positive organisms found in direct smears from some clinical sources

Organism(s)	Gram stain morphology	Frequent sources	Comments and additional tests or media that may be included
<i>Actinomyces</i> spp.	Thin, beaded, branched gram-positive filaments; may be within sulfur granules with peripheral clubs	Cervicofacial, thoracic, abdominal, and pelvic abscesses and drainages; pleural fluid; bronchial washings	Modified acid-fast stain; if sulfur granules present, wash and crush in THIO
<i>Nocardia</i> spp.	Long, thin, branching, beaded, gram-positive or irregularly staining bacilli; in culture smears, may be pleomorphic with branching and coccoid forms	Sputum, bronchial washings, biopsy material, purulent exudates, CSF, blood	Modified acid-fast stain; if mixed microbiota, mycobacterial decontamination procedures may be used; plate incubated at 45°C may enhance recovery; use CYE ^a or Thayer-Martin agar.
<i>Mycobacterium</i> spp.	Gram-positive beaded or gram-neutral bacilli; often found inside macrophages; bacilli may be short to long, banded or beaded, and/or slightly curved; some species appear pleomorphic and coccoid	Respiratory tract, urine, blood, biopsy material, CSF	Confirm with acid-fast stain. Add AFB culture.
<i>Corynebacterium</i> spp.	Gram-positive pleomorphic, club shaped, irregularly staining bacilli or coccobacilli with palisading and/or angular arrangements	Blood, tissue aspirates, skin lesions, wounds, indwelling catheters, prosthetic heart valves, upper and lower respiratory tracts	Add selective and differential media for <i>Corynebacterium diphtheriae</i> , if suspected.
<i>C. jeikeium</i>	Often small coccobacilli resembling streptococci		
<i>Propionibacterium</i> spp.	Gram-positive, very pleomorphic “diphtheroid” forms that may branch	Blood, CSF, other body fluids, skin lesions	Common skin contaminant during needle aspiration
<i>Listeria monocytogenes</i>	Gram-positive small to medium coccobacilli that may be pleomorphic; occur in short chains or palisades; may be confused with corynebacteria or enterococci	CSF, blood, amniotic fluid, placental or fetal tissue	Direct wet mount for tumbling motility; if mixed microbiota, cold enrichment may be used
<i>Erysipelothrix rhusiopathiae</i>	In tissue, long, slender, gram-positive bacilli; in blood, small “coryneforms”	Skin lesions, biopsy material, tissue aspirates, blood	Associated with occupational or animal contact
<i>Lactobacillus</i> spp.	Medium, straight, uniform gram-positive bacilli with rounded ends; may form chains or spirals; sometimes short and coccobacillary	Usually involved in mixed infections; rarely from blood, CSF	Recovery may be improved by anaerobic incubation; normal vaginal, mouth, and gastrointestinal tract microbiota
<i>Bacillus</i> spp.	Medium to large square-ended gram-positive bacilli with parallel sides with or without spores; some species have spores that swell sides; may stain gram variable or gram negative with age	May be clinically relevant from any source in compromised patient or intravenous-drug abuser; also intraocular	Frequent culture contaminants; may cause ocular infections

Table 3.2.1-2 (continued)

Organism(s)	Gram stain morphology	Frequent sources	Comments and additional tests or media that may be included
<i>Clostridium perfringens</i>	Gram-positive large “boxcars” with no spores; may stain gram negative	Blood, wounds, intra-abdominal	Add egg yolk agar incubated anaerobically; absence of inflammatory cells may indicate clostridial myonecrosis; normal gastrointestinal tract microbiota
<i>Clostridium</i> spp.	Gram-positive, -variable, or -negative bacilli with or without spores; bacilli may be large, slender and short, or long; sometimes form coils; often smaller than <i>Bacillus</i> spp.	Blood, intra-abdominal, wounds, abscesses	Normal gastrointestinal and genital tract microbiota
<i>S. pneumoniae</i>	Gram-positive cocci in pairs, lancet shapes, short chains	Lower respiratory tract, blood, CSF, sterile fluids	Quellung test may be used on selected clinical specimens.
<i>Enterococcus</i> spp.	Gram-positive cocci in pairs, short chains; may resemble pneumococci	Urine, wounds, blood, intra-abdominal abscesses	Normal gastrointestinal tract microbiota; common cause of superinfections in patients treated with expanded-spectrum cephalosporins
<i>Streptococcus</i> spp.	Round to oval gram-positive cocci, occasionally elongated; in pairs and/or short to long chains; nutritionally variant streptococci often seen as highly pleomorphic, gram-variable to gram-negative coccobacilli with pointed ends and spindle shapes	Blood, CSF, respiratory tract, multiple other sources	May be difficult to distinguish from corynebacteria and lactobacilli
<i>Aerococcus viridans</i>	Gram-positive cocci in pairs, tetrads, clusters	Blood, CSF	
<i>Staphylococcus</i> spp.	Gram-positive cocci in pairs, tetrads, clusters	Abscesses, drainages, wounds, respiratory tract, blood, tissue, sterile fluids, indwelling catheters	Normal microbiota, especially skin, nares
<i>S. aureus</i>	May often be characterized by very uniform, geometric clusters of small cocci, whereas coagulase-negative species are often irregular and more pleomorphic, with greater size variation		
<i>Rothia mucilaginosa</i>	Large gram-positive cocci in pairs, tetrads	Blood in compromised patients, peritoneal dialysates	Normal oral microbiota

^a CYE, charcoal-yeast extract agar.

Table 3.2.1–3 Gram-negative organisms seen in direct smears from some clinical sources

Organism(s)	Gram stain morphology	Frequent sources	Comments
<i>Enterobacteriaceae</i>	Straight thick bacilli; short to medium length with rounded ends; antimicrobial agent-affected organisms may be pleomorphic, filamentous, and/or irregularly staining	Urinary tract, multiple other sources	Includes organisms that cause gastroenteritis and bacterial dysentery; also normal gastrointestinal tract microbiota; nosocomial strains may be multiply resistant to antimicrobial agents
<i>Pseudomonas</i> spp.	Thin bacilli; medium length to long with rounded to pointy ends; often in pairs; antimicrobial agent-affected organisms may appear filamentous, coiled, and/or pleomorphic	Lower respiratory tract, wounds, eyes, multiple sites in compromised patients	Nosocomial strains may be multiply resistant to antimicrobial agents
<i>Haemophilus</i> spp., <i>Pasteurella</i> spp., fastidious gram-negative bacilli	Small coccoid to bacillary forms; pleomorphic; often with filamentous forms; may be faintly staining	Blood, sterile fluid (including CSF), lower respiratory tract, abscesses, wounds, eyes, genital tract	Inoculate CHOC.
<i>Legionella</i> spp.	Pleomorphic slender bacilli of variable lengths that may stain pale; may not take stain in clinical specimens	Lower respiratory tract	Add special growth media; direct fluorescent antibody stains and molecular probes available; acid wash method may be used to enhance recovery from specimens with mixed microbiota
<i>Fusobacterium nucleatum</i> , <i>Capnocytophaga</i> spp.	Long slender bacilli with tapered to pointed ends; “needlelike”; may be in pairs, end to end, or filamentous	Respiratory tract, wounds, blood, abscesses	Endogenous microbiota
<i>Fusobacterium necrophorum</i> , <i>Fusobacterium mortiferum</i> , or <i>Fusobacterium varium</i>	Highly pleomorphic bacilli with rounded to tapered ends; pale and irregularly staining, with bizarre forms and round bodies	Wounds, blood, abscesses	Endogenous microbiota
<i>Bacteroides</i> spp.	Pleomorphic straight bacilli with possible irregular to bipolar staining	Wounds, blood, abscesses	Direct fluorescent antibody stain available; endogenous microbiota
<i>Vibrio</i> spp.	Slightly curved to straight bacilli	Stool, wounds	If mixed microbiota, selective medium (TCBS) recommended
<i>Campylobacter</i> spp. (<i>Helicobacter</i> spp.)	Thin, curved bacilli, including S shapes, gull wings, and long spiral forms	Stool, blood, gastric biopsy samples	Microaerophilic or capnophilic atmosphere required; if mixed microbiota, 42°C incubation recommended for recovery of thermophilic species
<i>Acinetobacter</i> spp.	Medium to large cocci in pairs; occasionally coccoid, bacillary, and filamentous forms; often resistant to decolorization	Urine, lower respiratory tract, blood, sterile fluids, wounds, abscesses, tissues, stool	Multiple sites in compromised patients
<i>Neisseria</i> spp., <i>Moraxella catarrhalis</i>	Medium to large cocci in pairs and tetrads, coffee bean shaped; no bacilli seen	Genital tract, urine, lower respiratory tract, blood, sterile fluids, wounds, abscesses	If mixed microbiota, selective medium may be used to enhance recovery of <i>Neisseria gonorrhoeae</i>
<i>Veillonella</i> spp.	Tiny cocci in sheets or clumps	Wounds, blood	Endogenous microbiota

Table 3.2.1-4 (continued)

Organism(s)	Gram stain morphology	Frequent sources	Comments
<i>Pneumocystis carinii</i>	Gram-negative spherical cysts (5–7 µm) often containing rosette of eight gram-negative intracytic bodies, or cluster of gram-negative cysts surrounded by halos in background of amorphous gram-negative material	Open lung and transtracheal biopsy materials, bronchial washings and lavage specimens, sputum	Confirm with Grocott methenamine-silver, toluidine blue O, Gram-Weigert, or direct or indirect fluorescent antibody stain.
<i>Blastomyces dermatitidis</i>	Gram-variable, broad-based, thick-walled yeast cell with figure-eight appearance	Bronchial washings, sputum, purulent exudates, skin lesions	
Microsporidia	Gram-variable spherical cells (1–4 µm), thicker at one end	Stool, respiratory, cornea, urine	Confirm with chromotrope stain
<i>Cryptococcus neoformans</i>	Partially or completely gram-positive round yeast cell with clear or red-orange halo; yeast cells may appear stippled or gram neutral	CSF, blood, biopsy material, sputum, bronchial washings, cutaneous lesions	Confirm capsule with India ink; direct antigen detection procedures may be used on CSF; inoculate urea slant.
<i>Candida</i> spp.	Gram-positive budding yeast cell with or without pseudohyphae; may also appear stippled or gram neutral	Sputum, urine, blood, biopsy material, vaginal discharge, upper respiratory tract	Endogenous microbiota
<i>Malassezia furfur</i>	Bottle-shaped yeast cells in compact clusters, usually with short hyphal elements	Skin scrapings, blood drawn through catheter lines, hyperalimentation fluids	Inoculate lipid-enriched medium.

V. PROCEDURE (continued)

4. Preservation of direct smear slides
 - a. Drain or gently blot excess oil from slide and save slides for further evaluation for a minimum of 1 week to allow a confirmatory review, especially if culture or other laboratory test results are inconsistent.
 - b. Should a slide need to be restained to repeat Gram stain or prepare a special stain to confirm findings suggested by a Gram stain, remove the immersion oil with xylene or a xylene substitute and decolorize smear. Then restain slide.
 - c. For slide libraries and teaching collections that will be stored for longer periods, remove immersion oil with xylene and overlay smear with a sealer to prevent fading.
- E. Examination of broth and plate smears
 1. Specify Gram stain morphology (e.g., gram-positive cocci in clusters).
 2. Specify probable genus or organism group based on Gram stain morphology, colonial morphology, atmospheric requirements (aerobic or anaerobic), and results of any rapid biochemical tests (e.g., probable *Haemophilus* spp. for small pleomorphic aerobic gram-negative bacilli from transparent colonies with a “mousy” odor growing only on CHOC).
 3. Refer to Fig. 3.2.1-3 and 3.2.1-4 for typical morphological descriptions of bacterial genera.
- F. When discarding stained smears, handle as biological waste. Treat slides as sharps, since they may puncture biohazard bags. If cardboard boxes are used, seal with tape prior to discarding.

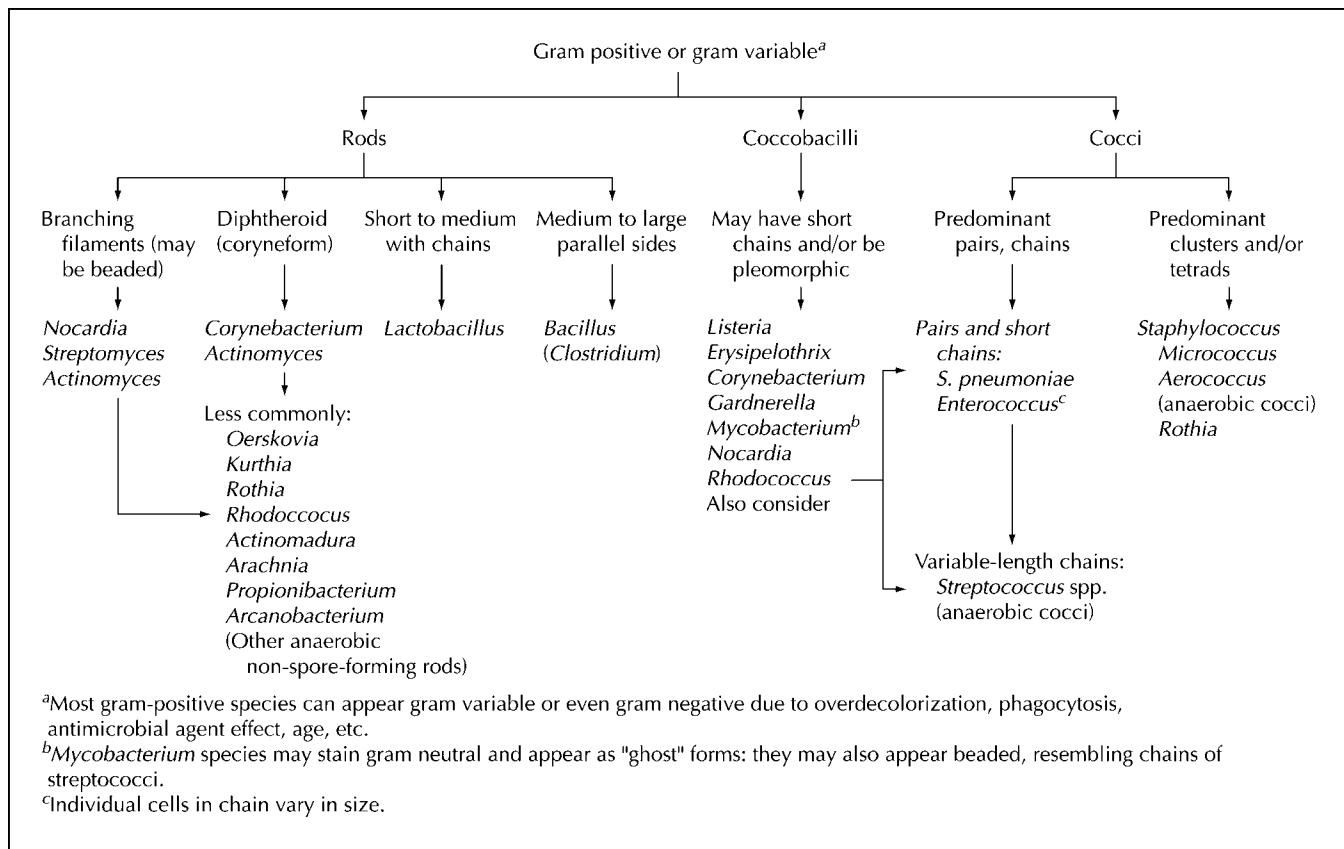


Figure 3.2.1–3 Typical Gram stain morphologies of gram-positive and gram-variable genera.

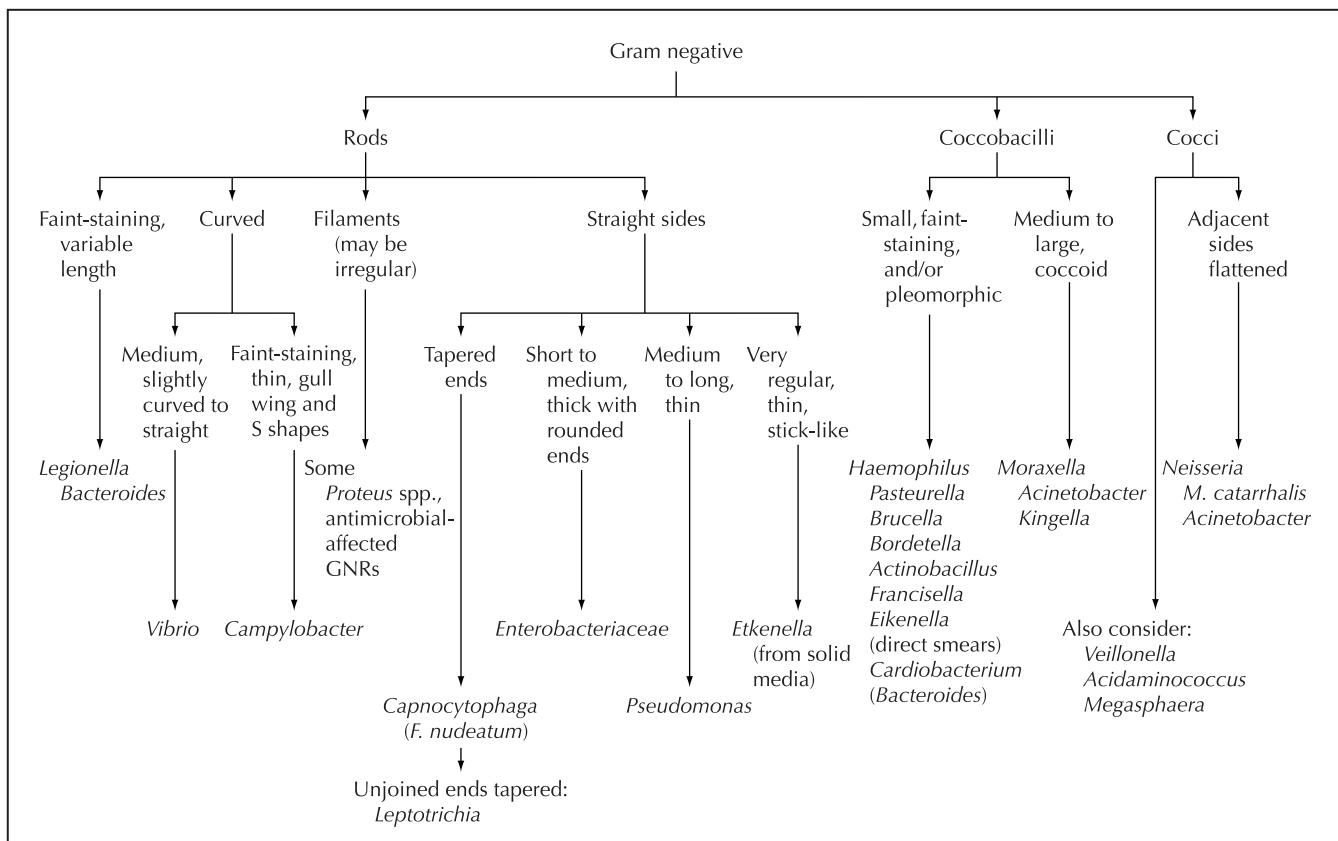


Figure 3.2.1-4 Typical Gram stain morphologies of gram-negative genera.

POSTANALYTICAL CONSIDERATIONS

VI. INTERPRETATION

A. Low-power observations

1. WBCs and RBCs suggest infectious process.
 - **NOTE:** Neutropenic patients may have few WBCs, but they may have necrotic debris and protein in the background.
2. SECs, food debris, etc., suggest an improperly collected specimen (see Appendix 3.2.1-2)
3. Certain microorganisms (parasitic forms, branching hyphae, etc.) indicate infectious process.

B. Oil immersion observations

1. Observe microorganisms for characteristic morphology and presentation. See Fig. 3.2.1-3 and 3.2.1-4 for species consistent with each morphology.
2. Gram reaction
 - a. Gram positive: deep violet
 - b. Gram negative: pink or red (carbol fuchsin counterstains have a more intense color)
 - c. Gram variable: both gram-positive and gram-negative cells with the same morphology
 - **NOTE:** This may result from a smear of uneven thickness, incomplete decolorization, overdecolorization, presence of older cells, damage of cell walls, or gram-variable nature of the particular organism.

VI. INTERPRETATION (continued)

- d. Gram neutral: taking up neither the crystal violet nor the counterstain
 - NOTE:** These cells appear colorless against a generally gram-negative background and may be intracellular. This reaction has been seen in smears of clinical specimens when fungal elements or *Mycobacterium* spp. are present (3).
- 3. Staining characteristics, predominant shapes of microorganisms, relative sizes, and characteristic arrangements: see Table 3.2.1–5
- C. The presence of microorganisms from a normally sterile site is likely to indicate infection with an organism.
- D. Physician offices may perform Gram stains on unspun urine. The presence of microorganisms is likely to indicate a bacterial count of $\geq 10^5$ CFU/ml in unspun urine (procedure 3.12).
- E. The presence of large numbers of a single type of microorganism in a noninvasively collected specimen, especially if associated with WBCs, is likely to indicate infection.
- F. Be wary of interpretations made from observing very few organisms (especially in the absence of inflammation or if the organisms are unevenly distributed), because collection tubes, slides, and media may harbor nonviable bacteria. *For critical specimens, where the results will define an infectious process (e.g., CSF smears), prepare a second smear to confirm rare findings of microorganisms.*

Table 3.2.1–5 Common descriptions of bacterial Gram staining characteristics

Bacterial Gram staining characteristics	Common descriptions
Staining characteristics	Even, bipolar, beaded, stippled, barred, irregular
Predominant shapes	Round, coccoid, coccobacillary, rod, filament, yeastlike
Ends of cells ^a	Rounded, pointed, tapered, flattened, clubbed (swollen), concave
Sides	Parallel, ovoid (bulging), concave, irregular
Axis	Straight, curved, spiral
Pleomorphism ^b	Variation in shape
Relative size ^c	
Overall	Minute or tiny ($<0.3\text{ }\mu\text{m}$), small ($\sim 0.3\text{--}0.5\text{ }\mu\text{m}$), medium, or large
Length	Short ($\sim 0.5\text{--}1\text{ }\mu\text{m}$), medium, long, or filamentous ($10\text{--}30\text{ }\mu\text{m}$)
Width	Thin, medium, or thick
Pleomorphic	Variation in size
Characteristic arrangements	Singles, pairs, chains, tetrads, clusters, palisades, Chinese letters, packets, angular forms (V and L shapes), etc.

^a Swelling of sides can suggest the presence of spores but may also be due to the presence of vacuoles, inclusions, marked pleomorphism, or irregular staining. Phase-contrast microscopy or a spore stain may be helpful in observing bacterial endospores.

^b The descriptive term “diphtheroid” or “coryneform” is used to describe gram-positive bacteria that are pleomorphic, club shaped, or irregularly staining or that have palisading and/or angular arrangements (V and L shapes).

^c Sizes listed are only guidelines. The average size of an RBC is 7 μm , and cytoplasmic granules in neutrophils average 0.2 to 0.3 μm .

VII. REPORTING RESULTS

- A.** If no organisms or cells are detected in a smear of a clinical specimen, report “No organisms seen” or “No cells seen,” respectively.
- ▣ **NOTE:** While there is no scientific basis for a policy on enumeration of cells and bacteria in stained smears, the following standard is the most applicable to all specimens and will provide consistency in the laboratories. The enumeration of cells is based on the publications documenting the relationship between cells and contaminated respiratory specimens (Appendix 3.2.1–2), and the enumeration of bacteria is based on the publications for counts for female genital specimens (Appendix 3.2.1–3). *Counts should be performed only in areas representative of inflammation or necrosis, if present.*
- B.** Determine number of cells and bacteria (Table 3.2.1–6).

Table 3.2.1–6 Reporting Gram stain results^a

Enumeration of cells under low-power objective ^b	Description of the types of cells to report	Enumeration of bacteria under oil immersion objective ^c	Description of the morphology of bacteria ^d
Count each type of cell and report: 1+ (rare or occasional): <1/LPF 2+ (few): 1–9/LPF 3+ (moderate): 10–25/LPF 4+ (heavy): >25/LPF	ECs PMNs RBCs Host cellular material	Count bacteria and yeasts from areas associated with cells and report: 1+ (rare or occasional): <1/OIF 2+ (few): 1–5/OIF 3+ (moderate): 6–30/OIF 4+ (heavy): >30/OIF	Gram positive Cocci in pairs (and chains) Cocci in clusters Large bacilli Small bacilli Branching bacilli Coryneform bacilli Gram negative Diplococci Bacilli Bacilli, filamentous (or pleomorphic) Gram variable: coccobacilli Budding yeast cells Pseudohyphae

^a Determine the number of cells and bacteria in 20 to 40 fields of the smear. Skip fields where there are no cells or bacteria, and do not average these fields in the counts if there are fields where cells and/or bacteria are present.

^b The Gram stain is not a stain that demonstrates host cellular morphology, and the main cells routinely enumerated are WBCs (i.e., polymorphonuclear cells), which determine if the sample is purulent as an indication of infection. For other types of cells, there is no clinically useful reason to report cells present in amounts of <10/40 fields from a Gram stain. LPF, low-power field.

^c Ignore one or two microorganisms on the entire slide, unless the results can be reproduced on a second smear and only then if it is from an invasively collected specimen. OIF, oil immersion field.

^d Microbiologists should be encouraged to specify Gram stain morphology and resemblance to organism group consistent with the source if the presentation is classic; however, use caution when in doubt, as other species may mimic the typical presentations, including (i) gram-positive cocci in pairs, consistent with *Streptococcus pneumoniae*; (ii) small gram-negative coccobacilli, consistent with *Haemophilus*; (iii) tiny gram-positive bacilli, consistent with *Listeria*; and (iv) gram-negative diplococci, consistent with *Neisseria*.

VII. REPORTING RESULTS (continued)

- C. Refer to Appendixes 3.2.1–2 and 3.2.1–3 for special reporting of respiratory specimens and female genital specimens, respectively.
- D. *Notify the caregiver or physician of record, depending on local policy, of any clinically significant findings (any bacteria from a normally sterile site). Document notification, including date and time of notification.*

VIII. LIMITATIONS

- A. The sensitivity of the Gram stain is 10^5 cells/ml or 10^4 /ml if the specimen has been prepared with the cyt centrifuge (13). This is particularly applicable to the smear of a drop of urine, where an average of one bacterial cell per field from an examination of 20 fields corresponds to a count of $\geq 10^5$ CFU/ml.
- B. Gram stain of cyt centrifuged BAL with one or more organisms per OIF correlates with active bacterial pneumonia (14).
- C. Use results of Gram stains in conjunction with other clinical and laboratory findings. Use additional procedures (e.g., special stains, inclusion of selective media, etc.) to confirm findings suggested by Gram-stained smears (3).
- D. Careful adherence to procedure and interpretive criteria is required for accurate results. Accuracy is highly dependent on the training and skill of microscopists (16).
- E. Additional staining procedures, such as acridine orange (procedure 3.2.2), are recommended for purulent clinical specimens in which no organisms are observed by the Gram stain method.
- F. Gram stain-positive, culture-negative specimens may be the result of contamination of reagents and other supplies, presence of antimicrobial agents, or failure of organisms to grow under usual culture conditions (medium, atmosphere, etc.).
- G. False Gram stain results may be related to inadequately collected specimens or delays in transit.

REFERENCES

1. Bartholomew, J. W. 1962. Variables influencing results, and the precise definition of steps in Gram staining as a means of standardizing the results obtained. *Stain Technol.* **37**:139–155.
2. Bottone, E. J. 1988. The Gram stain: the century-old quintessential rapid diagnostic test. *Lab. Med.* **19**:288–291.
3. Brown, M. S., and T. C. Wu. 1986. The Gram stain morphology of fungi, mycobacteria, and *Pneumocystis carinii*. *J. Med. Technol.* **3**:495–499.
4. Clarridge, J. E., and J. M. Mullins. 1987. Microscopy and staining, p. 87–103. In B. J. Howard (ed.), *Clinical and Pathogenic Microbiology*. The C. V. Mosby Co., St. Louis, Mo.
5. Conn, H. J., M. A. Darrow, and V. M. Emmel (ed.). 1960. Stains for microorganisms in smears, p. 226–229. In *Staining Procedures Used by the Biological Stain Commission*, 2nd ed. The Williams & Wilkins Co., Baltimore, Md.
6. Dowell, V. R., and T. M. Hawkins. 1979. *Laboratory Methods in Anaerobic Bacteriology: CDC Laboratory Manual*. Center for Disease Control, Atlanta, Ga.
7. Finegold, S. M., and E. J. Baron. 1990. Appendix B, formulas for commonly used stains, p. A-41. In *Diagnostic Microbiology*, 8th ed. The C. V. Mosby Co., St. Louis, Mo.

REFERENCES (continued)

8. Finegold, S. M., and E. J. Baron. 1990. Microorganisms encountered in cerebrospinal fluid, p. 219. In *Diagnostic Microbiology*, 8th ed. The C. V. Mosby Co., St. Louis, Mo.
9. Finegold, S. M., and E. J. Baron. 1990. Optical methods for laboratory diagnosis of infectious diseases, p. 64–80. In *Diagnostic Microbiology*, 8th ed. The C. V. Mosby Co., St. Louis, Mo.
10. Hendrickson, D. A., and M. M. Krenz. 1991. Reagents and stains, p. 1289–1314. In A. Balows, W. J. Hausler, Jr., K. L. Herrmann, H. D. Isenberg, and H. J. Shadomy (ed.), *Manual of Clinical Microbiology*, 5th ed. American Society for Microbiology, Washington, D.C.
11. Mangels, J. I., M. E. Cox, and L. H. Lindberg. 1984. Methanol fixation: an alternative to heat fixation of smears before staining. *Diagn. Microbiol. Infect. Dis.* **2**:129–137.
12. Murray, P. R., and J. A. Washington. 1975. Microscopic and bacteriologic analysis of sputum. *Mayo Clin. Proc.* **50**:339–344.
13. Shanholzter, C. J., P. Schaper, and L. R. Peterson. 1982. Concentrated Gram stain smears prepared with a cytospin centrifuge. *J. Clin. Microbiol.* **16**:1052–1056.
14. Thorpe, J. E., R. P. Bangham, P. T. Frame, T. A. Wesseler, and J. L. Staneck. 1987. Bronchoalveolar lavage for diagnosing acute bacterial pneumonia. *J. Infect. Dis.* **155**:855–861.
15. U.S. Department of Health and Human Services. 1999. *Biosafety in Microbiology and Biomedical Laboratories*, 4th ed. U.S. Department of Health and Human Services stock no. 017-040-00547-4. U.S. Government Printing Office, Washington, D.C.
16. Washington, J. A. 1986. Rapid diagnosis by microscopy. *Clin. Microbiol. Newslett.* **8**:135–137.
17. Weaver, R. E., and J. C. Feeley. 1979. Cultural and biochemical characterization of the Legionnaires' disease bacterium, p. 20–25. In G. L. Jones and G. A. Hebert (ed.), "Legionnaires": the Disease, the Bacterium and Methodology. Center for Disease Control, Atlanta, Ga.

SUPPLEMENTAL READING

- Chapin-Robertson, K., S. E. Dahlberg, and S. C. Edberg. 1992. Clinical and laboratory analyses of cytospin-prepared Gram stains for recovery and diagnosis of bacteria from sterile body fluids. *J. Clin. Microbiol.* **30**:377–380.
- Church, D., E. Melnyk, and B. Unger. 2000. Quantitative Gram stain interpretation criteria used by microbiology laboratories in Alberta, Canada. *J. Clin. Microbiol.* **38**:4266–4268.

APPENDIX 3.2.1-1



Include QC information on reagent container and in QC records.

Preparation of Gram Stain Reagents

A. Hucker's crystal violet

1. Crystal violet stock solution

crystal violet (90 to 95% dye content) ...	40 g
ethanol, 95%	400 ml

Dissolve (may take overnight) and mix in a glass bottle, label with a 1-year expiration date, and store at room temperature.

Caution: Ethanol is flammable.

2. Ammonium oxalate solution (1%)

ammonium oxalate (reagent grade)	16 g
distilled water	1,600 ml

Dissolve and mix in a brown glass bottle, label with a 1-year expiration date, and store at room temperature.

3. Crystal violet working solution

crystal violet stock solution	40 ml
ammonium oxalate solution (1%)	160 ml

Filter crystal violet stock solution into a glass bottle. Allow to filter completely, and then filter ammonium oxalate solution. Label with earliest expiration date of stock solutions.

B. Gram's iodine

1. Stock Lugol's iodine solution

iodine crystals (reagent grade)	25 g
potassium iodide (reagent grade)	50 g
distilled water	500 ml

Mix or let stand until dissolved in a brown glass bottle, label with a 6-month expiration date, and store at room temperature.

APPENDIX 3.2.1–1 (continued)

2. Sodium bicarbonate, 5% (wt/vol)
- | | |
|-----------------------------------------------------------------|----------|
| sodium bicarbonate (NaHCO_3),
reagent grade | 50 g |
| distilled water | 1,000 ml |

Dissolve in a glass bottle, label with a 1-year expiration date, and store at room temperature.

3. Gram's iodine

stock Lugol's iodine solution	60 ml
distilled water	220 ml
sodium bicarbonate, 5%	60 ml

Mix in a brown glass bottle, label with a 6-month expiration date, and store at room temperature.

Caution: Iodine and potassium iodide are corrosive. Avoid inhalation, ingestion, and skin contact. Do not store near acids.

C. Counterstains

1. Safranin

- a. Safranin stock solution

safranin O (certified)	5.0 g
ethanol, 95%	200 ml

Dissolve in glass bottle, label with a 1-year expiration date, and store at room temperature.

Caution: Ethanol is flammable.

- b. Safranin working solution

safranin stock solution	20 ml
distilled water	180 ml

Combine in a glass bottle, label with a 1-year expiration date, and store at room temperature.

2. Basic fuchsin, 0.1, 0.2, or 0.8% (wt/vol)

basic fuchsin (reagent grade)	1, 2, or 8 g
ethyl alcohol	100 ml
distilled water	900 ml

Add basic fuchsin to a brown glass bottle. Slowly add ethyl alcohol and let sit overnight. Filter through Whatman no. 1 filter paper. Add distilled water. Label with a 1-year expiration date, and store at room temperature.

3. Carbol fuchsin counterstain

- a. Solution A

basic fuchsin (reagent grade)	3 g
ethanol, 95%	100 ml

- b. Solution B

melted phenol crystals	50 ml
distilled water	950 ml

Dissolve basic fuchsin in ethanol in a brown glass bottle (solution A). Add phenol to distilled water in a separate flask (solution B). Add solution B to solution A, label with a 1-year expiration date, and store at room temperature.

Caution: Ethanol is flammable, and phenol is corrosive. Avoid inhalation, ingestion, and skin contact.

D. Kopeloff's modification (for anaerobes)

1. Alkaline crystal violet

- a. Solution A

crystal violet (90 to 95% dye content)	10 g
distilled water	1,000 ml

Dissolve in a glass bottle, label with a 1-year expiration date, and store in glass-stoppered bottle at room temperature.

APPENDIX 3.2.1-1 (continued)

- b. Solution B: sodium bicarbonate, 5% (wt/vol)
- | | |
|-------------------------------------------|----------|
| sodium bicarbonate (NaHCO ₃), | |
| reagent grade | 50 g |
| distilled water | 1,000 ml |

Dissolve in a glass bottle, label with a 1-year expiration date, and store at room temperature.

2. Iodine (Kopeloff's modification)

- | | |
|----------------------------------------|--------|
| sodium hydroxide (NaOH), | |
| reagent grade | 4 g |
| distilled water | .25 ml |
| iodine crystals (reagent grade) | 20 g |
| potassium iodide (reagent grade) | 1 g |
| distilled water | 975 ml |

Dissolve NaOH in 25 ml of distilled water in a brown glass bottle. Add iodine and potassium iodide, and dissolve them well. Gradually add 975 ml of distilled water, mixing well after each addition.

Caution: Iodine and potassium iodide are corrosive. Avoid inhalation, ingestion, and skin contact.

3. Decolorizer: 3:7 acetone-alcohol

- | | |
|-------------------------------|--------|
| ethanol, 95% | 700 ml |
| acetone (reagent grade) | 300 ml |

Combine and mix in a brown glass bottle, label with a 1-year expiration date, and store at room temperature.

Caution: Ethanol and acetone are flammable.

4. Safranin counterstain (Kopeloff's)

- | | |
|-----------------------------|----------|
| safranin O, certified | 20 g |
| ethanol, 95% | 100 ml |
| distilled water | 1,000 ml |

In a 1,000-ml glass bottle, add only enough ethanol to the safranin to dissolve it (approximately 50 ml). Add distilled water to safranin solution, label with a 1-year expiration date, and store at room temperature. (Basic fuchsin, 0.8% [wt/vol], may also be used for counterstain.)

Caution: Ethanol is flammable.

APPENDIX 3.2.1-2**Rejection Criteria for Sputum and Endotracheal Aspirates for Culture****I. RATIONALE**

Despite the frequency of lower respiratory tract infection, diagnostic studies to detect and identify the etiologic agent are insensitive (8). Whether to perform a Gram stain or a culture has been the topic of repeated studies with conflicting conclusions from professional societies, particularly for evaluating cases of community-acquired pneumonia (1, 3, 7). Part of the problem with the Gram stain is the variability of sampling for smears and cultures (2, 6, 9). Everyone does agree that the culture of poorly collected respiratory specimens is a wasteful use of laboratory resources and can lead to erroneous reporting and treatment of patients (1, 5, 10). For laboratories that receive respiratory specimens for smear and culture, the following generally accepted policy should be followed.

II. REJECTION CRITERIA

- A. Do not reject sputum and endotracheal aspirates for culture for *Legionella* or AFB, or specimens from cystic fibrosis patients.
- B. Examine 20 to 40 fields from sputum smears under low power and endotracheal smears under both low power and oil immersion. Average the number of cells in representative fields that contain cells. Reject the following for culture, as poorly collected or not consistent with a bacterial infectious process.

1. Sputum: ≥ 10 SECs/LPF (1, 5, 10)

NOTE: If the number of WBCs is 10 times the number of SECs and there is 3 to 4+ of a single morphotype of bacteria, accept the specimen for culture. Some authors suggest using > 25 SECs/LPF as a criterion, but generally too few sputa are rejected with this policy (10).

APPENDIX 3.2.1–2 (continued)

2. Tracheal aspirates from adults: ≥ 10 SECs/LPF or no organisms seen (4)
3. Tracheal aspirates from pediatric patients: no organisms seen (11)

■ NOTE: if no organisms are seen in a specimen with numerous (4+) WBCs and cellular debris, it might be useful to flood the smear with acridine orange (procedure 3.2.2) and observe using fluorescent microscopy to confirm the absence of organisms in the debris. Both pseudomonads and *Haemophilus* can be missed in such smears, because they cannot be distinguished among the cellular debris (Mary K. York, personal observation). In addition, *Legionella* can be visualized with the acridine orange stain, although WBCs are often lacking in this infectious process.

III. REPORTING

When rejecting the specimen for culture, report one of the following as appropriate.

- A. "Smear contains ≥ 10 squamous epithelial cells per low power field, suggestive of poor quality; culture not performed. Please recollect if clinically indicated."
- B. "Smear is negative for bacteria after examination of 40 fields; culture not performed. Contact laboratory if further studies are clinically indicated."

IV. FOLLOW-UP

- A. Notify the caregiver that the specimen will not be cultured.
- B. Charge for the smear but not for the culture.

■ NOTE: Particularly for outpatient specimens and those that are grossly purulent, the culture should be inoculated immediately upon receipt without waiting for the results of the smear. Plates from rejected specimens can be discarded or separated from the other cultures and examined later to validate the laboratory policy and staff competency.

References

1. Bartlett, J. G., S. F. Dowell, L. A. Mandell, T. M. File, Jr., D. M. Musher, and M. J. Fine. 2000. Guidelines from the Infectious Diseases Society of America—practice guidelines for the management of community-acquired pneumonia in adults. *Clin. Infect. Dis.* **31**:347–382.
2. Cooper, G. M., J. J. Jones, J. C. Arbique, G. J. Flowerdew, and K. R. Forward. 2000. Intra- and inter-technologist variability in the quality assessment of respiratory tract specimens. *Diagn. Microbiol. Infect. Dis.* **37**:231–235.
3. Mandell, L. A., T. J. Marrie, R. F. Grossman, A. W. Chow, R. H. Hyland, and The Canadian Community-Acquired Pneumonia Working Group. 2000. Canadian guidelines for the initial management of community-acquired pneumonia: an evidence-based update by the Canadian Infectious Diseases Society and the Canadian Thoracic Society. *Clin. Infect. Dis.* **31**:383–421.
4. Morris, A. J., D. C. Tanner, and L. B. Reller. 1993. Rejection criteria for endotracheal aspirates from adults. *J. Clin. Microbiol.* **31**:1027–1029.
5. Murray, P. R., and J. A. Washington. 1975. Microscopic and bacteriologic analysis of sputum. *Mayo Clin. Proc.* **50**:339–344.
6. Nagendra, S., P. Bourbeau, S. Brecher, M. Dunne, M. LaRocco, and G. Doern. 2001. Sampling variability in the microbiological evaluation of expectorated sputa and endotracheal aspirates. *J. Clin. Microbiol.* **39**:2344–2347.
7. Niederman, M. S., L. A. Mandell, A. Anzueto, J. B. Bass, W. A. Broughton, G. D. Campbell, N. Dean, T. File, M. J. Fine, P. A. Gross, F. Martinez, T. J. Marrie, J. F. Plouffe, J. Ramirez, G. A. Sarosi, A. Torres, R. Wilson, and V. L. Yu. 2001. Guidelines for the management of adults with community-acquired pneumonia. Diagnosis, assessment of severity, antimicrobial therapy, and prevention. *Am. J. Respir. Crit. Care Med.* **163**:1730–1754.
8. Reimer, L. G., and K. C. Carroll. 1998. Role of the microbiology laboratory in the diagnosis of lower respiratory tract infections. *Clin. Infect. Dis.* **26**:742–748.
9. Roson, B., J. Carratala, R. Verdaguer, J. Dorca, F. Manresa, and F. Gudiol. 2000. Prospective study of the usefulness of sputum Gram stain in the initial approach to community-acquired pneumonia requiring hospitalization. *Clin. Infect. Dis.* **31**:869–874.
10. Wong, L. K., A. L. Barry, and S. M. Morgan. 1982. Comparison of six different criteria for judging the acceptability of sputum specimens. *J. Clin. Microbiol.* **16**:627–631.
11. Zaidi, A. K., and L. B. Reller. 1996. Rejection criteria for endotracheal aspirates from pediatric patients. *J. Clin. Microbiol.* **34**:352–354.

APPENDIX 3.2.1–3

Reporting Gram-Stained Vaginal Smears To Diagnose Bacterial Vaginosis and Vaginitis

I. RATIONALE

Bacterial vaginosis (BV) is a clinical syndrome characterized by an abnormal vaginal discharge in women in childbearing years accompanied by a rise in pH from 4.5 and an amine (fishy) smell, especially after addition of KOH to the discharge (6). The microbiota of the vagina shifts from predominantly lactobacilli to a mixture of *Gardnerella vaginalis*, *Prevotella* spp., *Mobiluncus* spp., and often other anaerobes and *Mycoplasma hominis* (1, 5). Greater than 10^7 CFU of these microorganisms per g of vaginal fluid are present with a decrease in the number of lactobacilli, which are no longer the predominant microorganisms (5). In addition, clue cells, vaginal SECs coated with bacteria such that the cell borders are obliterated, can also be present. BV is a risk factor for obstetric sequelae such as low birth weight and premature delivery. The syndrome has been associated with preterm birth, miscarriage, amniotic infections, and postpartum endometritis (2, 3). Treatment with topical metronidazole or clindamycin is effective, but the syndrome can recur (1). Preliminary diagnosis is usually made in the physician's office by checking the pH of the discharge with an indicator stick and adding KOH to detect the odor, although the usage of these inexpensive tests is reportedly low. The clinic-based microscopic evaluation of the vaginal fluid by wet mount is the most frequently used test, but laboratory-based testing is sometimes desired because no microscope is available or the evaluation of the wet mount is inconclusive.

The Gram stain with scoring of relative amounts of microbial morphotypes is the definitive laboratory method for diagnosis (4). *G. vaginalis* is a small gram-negative to gram-positive pleomorphic bacillus, varying from coccobacillus to longer forms up to 2 to 3 μm ; cells can palisade or appear coryneform and irregularly shaped. *Mobiluncus* organisms are curved gram-negative rods. The relative numbers of lactobacilli (medium to large gram-positive rods) compared to gram-negative curved rods and gram-variable coccobacilli aid in the diagnosis of BV.

The Gram stain can be useful in the diagnosis of candidiasis in women, when the timing does not allow evaluation of the discharge in the physician's office by wet mount, pH, and odor. Vaginal culture for yeasts is more sensitive than Gram stain and may be useful for the detection of yeasts in women with symptoms consistent with yeast vaginitis whose wet mount examinations are negative. Candidiasis is characterized by a white discharge and a normal pH below 4.2. The Gram stain is less helpful in the diagnosis of purulent vaginitis, characterized by a macroscopically yellowish-green, foul-smelling discharge containing ≥ 30 WBCs per high-power field. The most common etiologic agent of this infection is *Trichomonas vaginalis*, which is seen on wet mount but not Gram stain.

II. SPECIMEN

Collect vaginal secretions from posterior fornix using sterile cotton or Dacron swab.

III. METHOD

Perform the Gram stain with Kopeloff's modification and 0.1% basic fuchsin counterstain. After flooding the slide with crystal violet, add 5 drops of sodium bicarbonate and blow slide to mix. Let stand for 15 s. Continue staining as usual for Gram stain. Examine for host cells and bacteria in the same manner as for a routine Gram stain, and, additionally, score as indicated in Table 3.2.1–A1, *only for women in childbearing years and postmenopausal women on estrogen replacement therapy*.

IV. QUALITY CONTROL

To ensure that smears are being read correctly, prepare a collection of smears showing a variety of scores between 0 and 10. Use these smears for competency testing and training. To determine the accuracy of the interpretation in the teaching collection, perform a culture of each specimen that shows either a preponderance of lactobacilli (scores of 0 to 3) or a preponderance of *Gardnerella* (scores of 7 to 10).

- A. Inoculate CHOC and incubate for 48 h.
- B. Determine the relative amount of lactobacilli (catalase negative, greening of the agar) compared to *Gardnerella* (nonhemolytic, catalase-negative, tiny Gram-variable bacilli) in the third and fourth quadrants of the plate.
- C. Do not use selective or differential media to perform the correlation.

APPENDIX 3.2.1–3 (continued)

Table 3.2.1–A1 Standardized scoring method for evaluation of Gram stains for BV

Quantitation of bacterial morphotype ^a	Points scored per morphotype				
	None	1 +	2 +	3 +	4 +
Medium to large gram-positive rods	4	3	2	1	0
Small gram-negative or -variable rods	0	1	2	3	4
Curved gram-negative or -variable rods	0	1	1	2	2

^a Modified from Nugent et al. (4) using quantitation from the Gram stain procedure. Circle in each row the number that corresponds to the quantitation visualized in the smear. Add circled numbers to arrive at total score. Interpret as follows: 0 to 3, normal; 4 to 6, intermediate; and 7 to 10, BV.

D. Cultures with 3 to 4+ lactobacilli should correlate with scores of 0 to 3. Cultures with 3 to 4+ *Gardnerella* should correlate with scores of 7 to 10.

V. REPORTING

Follow routine Gram stain reporting method to enumerate and report the following.

A. WBCs and RBCs

B. Clue cells

C. Yeasts

D. Generally pathogenic morphotypes, such as intracellular gram-negative diplococci consistent with *Neisseria* spp.

Then report one of the following based on the score in Table 3.2.1–A1: 0 to 3, “Morphotypes consistent with normal vaginal microbiota”; 4 to 6, “Mixed morphotypes consistent with transition from normal vaginal microbiota”; or 7 to 10, “Mixed morphotypes consistent with bacterial vaginosis.”

References

1. Hay, P. 2000. Recurrent bacterial vaginosis. *Curr. Infect. Dis. Rep.* **2**:506–512.
2. Holst, E., A. R. Goffeng, and B. Andersch. 1994. Bacterial vaginosis and vaginal microorganisms in idiopathic premature labor and association with pregnancy outcome. *J. Clin. Microbiol.* **32**:176–186.
3. Kimberlin, D. F., and W. W. Andrews. 1998. Bacterial vaginosis: association with adverse pregnancy outcome. *Semin. Perinatol.* **22**:242–250.
4. Nugent, R. P., M. A. Krohn, and S. L. Hillier. 1991. Reliability of diagnosing bacterial vaginosis is improved by a standardized method of Gram stain interpretation. *J. Clin. Microbiol.* **29**:297–301.
5. Roy, S., M. Sharma, A. Ayyagari, and S. A. Malhotra. 1994. A quantitative microbiological study of bacterial vaginosis. *Indian J. Med. Res.* **100**:172–176.
6. Spiegel, C. A. 1999. Bacterial vaginosis: changes in laboratory practice. *Clin. Microbiol. Newslet.* **21**:33–37.

3.2.2

Acridine Orange Stain

[Updated March 2007]

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Acridine orange is a fluorochromatic dye which binds to nucleic acids of bacteria and other cells. Under UV light, acridine orange stains RNA and single-stranded DNA orange; double-stranded DNA appears green, as first described by Strugger

and Hilbrich in 1942 (3). When buffered at pH 3.5 to 4.0, acridine orange differentially stains microorganisms from cellular materials (1). Bacteria and fungi uniformly stain bright orange, whereas human epithelial and inflammatory cells

and background debris stain pale green to yellow. Nuclei of activated leukocytes stain yellow, orange, or red due to increased RNA production resulting from activation. Erythrocytes either do not stain or appear pale green.

II. SPECIMEN HANDLING

NOTE: The acridine orange stain is an optional stain that can be helpful in detecting organisms not visualized by Gram stain, often due to a large amount of host cellular debris.

- A. For apparent plate growth that is not visualized by Gram stain (e.g., *Mycoplasma*), prepare smear as for Gram stain.
- B. Broths
 - 1. Gram stain-negative blood culture bottles that are detected as positive by the instrument
 - 2. Broths that look turbid but are negative by Gram stain
- C. For direct specimens (urine, CSF, body fluids), when WBCs are seen but no organisms are seen, or isolated, and physician requests additional studies for a difficult diagnosis, make a smear from the specimen as for Gram stain.

III. MATERIALS

- A. Acridine orange stain (see Appendix 3.2.2–1; also available from most microbiology stain supply vendors)
 - 1. Use the stain directly from the bottle.
 - 2. Store at 15 to 30°C in the dark.
- B. Methanol, absolute
- C. Glass microscope slides
- D. Heat block at 45 to 60°C (optional)
- E. Immersion oil suitable for fluorescence

F. Fluorescent microscope with the following

- 1. Filter system for fluorescein isothiocyanate, i.e., maximum excitation wavelength of 490 nm and mean emission wavelength of 520 nm
- 2. ×1,000 magnification (×100 oil immersion objective)

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

- A. Examine the acridine orange staining solution for color and clarity. The solution should be clear, orange, and without evidence of precipitate.
- B. Each time of use, stain a prepared slide of known bacteria, such as *Escherichia coli* mixed with staphylococci, and examine for the desired results. Record results and refer out-of-control results to the supervisor.
 1. Gram-negative rods and gram-positive cocci are fluorescent (orange).
 2. Background is nonfluorescent (green-yellow).

V. PROCEDURE



Observe standard precautions.

■ **NOTE:** Acridine orange is a carcinogen when absorbed through the skin. Wear gloves when working with this stain.

- A. Prepare a smear of the specimen on a clean glass slide as for Gram stain (procedure 3.2.1). Spread specimen thinly with a sterile stick.
- B. Allow to air dry.
- C. Fix smear with methanol by flooding the slide, draining the excess; allow to air dry.
- D. Flood slide with acridine orange stain for 2 min.
- E. Drain the excess stain and rinse thoroughly with tap water.
- F. Allow to air dry. The slide may be *gently* blotted on a clean sheet of filter paper or paper towel to decrease drying time.
- G. Read smear on the fluorescent microscope at $\times 400$ to $\times 1,000$ (oil immersion). Look for the distinct morphology of bacteria or fungi. No coverslip is needed.

POSTANALYTICAL CONSIDERATIONS

VI. REPORTING RESULTS

- A. Record results of stain as negative or positive with the morphology of the organisms seen.
- B. Review Gram stain to see if organisms can be recognized.
- C. If organisms are not seen on direct Gram stain, report the following: "Culture (or specimen) positive for bacteria by acridine orange stain; bacteria not seen in Gram-stained preparation."
- D. If the acridine orange stain is positive from a blood culture, subculture for the most likely organism based on the morphology.
- E. If the smear is negative from a direct specimen, report "No bacteria seen by acridine orange-stained smear."

VII. INTERPRETATION

- A. Bacteria and fungi fluoresce bright orange. The background appears black to yellow green. Human epithelial and inflammatory cells and tissue debris fluoresce pale green to yellow. Activated leukocytes will fluoresce yellow, orange, or red, depending on the level of activation and amount of RNA produced, whereas erythrocytes either do not fluoresce or fluoresce pale green.
- B. The presence of one or more organisms per oil immersion field correlates with a colony count of approximately $\geq 10^5$ CFU/ml if unconcentrated specimen has been used to prepare the smear.
- C. *Ureaplasma* and *Mycoplasma* can be visualized by this method.

VIII. LIMITATIONS

- A. Nuclei or granules from disintegrated activated leukocytes, and certain types of debris, may fluoresce in acridine orange-stained smears. These may be differentiated from microorganisms on the basis of morphology.
 - B. Acridine orange staining does not distinguish between gram-negative and gram-positive organisms. The Gram reaction may be determined by Gram staining directly over the acridine orange after removal of the immersion oil. Acridine orange staining may also be done over Gram stain (after removal of oil) if necessary.
 - C. Intracellular organisms may be more difficult to see by the acridine orange stain, due to the staining of cellular nuclei.
 - D. The acridine orange stain will detect some organisms, especially those that are gram negative, when they are poorly visualized by Gram stain.
 - E. The sensitivity of the acridine orange smear is approximately 10^4 bacteria/ml (2).
-

REFERENCES

1. **Kronvall, G., and E. Myhre.** 1977. Differential staining of bacteria in clinical specimens using acridine orange buffered at low pH. *Acta Pathol. Microbiol. Scand. Sect. B* **85**:249–254.
 2. **Lauer, B. A., L. B. Reller, and S. Mirrett.** 1981. Comparison of acridine orange and Gram stains for detection of microorganisms in cerebrospinal fluid and other clinical specimens. *J. Clin. Microbiol.* **14**:201–205.
 3. **Strugger, S., and P. Hilbrich.** 1942. Die fluoreszenzmikroskopische Unterscheidung lebender und toten Bakterienzellen mit Hilfe des Akridinorange-Färbung. *Dtsch. Tierärztl. Wochenschr.* **50**:121–130.
-

SUPPLEMENTAL READING

- De Brauwer, E., J. Jacobs, F. Nieman, C. Bruggeman, and M. Drent.** 1999. Test characteristics of acridine orange, Gram, and May-Grünwald-Giemsa stains for enumeration of intracellular organisms in bronchoalveolar lavage fluid. *J. Clin. Microbiol.* **37**:427–429.
- Hoff, R. G., D. E. Newman, and J. L. Stanek.** 1985. Bacteriuria screening by use of acridine orange-stained smears. *J. Clin. Microbiol.* **21**:513–516.
- Hunter, J. S.** 1993. Acridine orange staining as a replacement for subculturing of false-positive blood cultures with the BACTEC NR 660. *J. Clin. Microbiol.* **31**:465–466.
- Kasten, F. H.** 1967. Cytochemical studies with acridine orange and the influence of dye contaminants in the staining of nucleic acids. *Int. Rev. Cytol.* **21**:141–202.
- Larson, A. M., M. J. Dougherty, D. J. Nowowiejski, D. F. Welch, G. M. Matar, B. Swaminathan, and M. B. Coyle.** 1994. Detection of *Bartonella (Rochalimaea) quintana* by routine acridine orange staining of broth blood cultures. *J. Clin. Microbiol.* **32**:1492–1496.

APPENDIX 3.2.2-1

Include QC information on reagent container and in QC records.

Preparation of Acridine Orange Stain**I. MATERIALS**

- A. Acridine orange powder, 20 mg
- B. Sodium acetate buffer, 190 ml
 1. Solution A: 100 ml of 1 M $\text{CH}_3\text{COONa}\cdot 3\text{H}_2\text{O}$
 2. Solution B: 90 ml of 1 M HCl

Caution: Acridine orange is a carcinogen; avoid all contact with skin. Avoid exposure to powder aerosol by performing reagent preparation in a fume hood and by wearing gloves.

II. PROCEDURE

- A. Acetate buffer: combine solutions A and B.
- B. Add additional solution B as necessary to yield pH between 3.5 and 4.
- C. Dissolve powder in buffer until solution is clear of precipitate.
- D. Store stain away from light (e.g., brown bottle) at 15 to 30°C.

NOTE: The final acridine orange concentration will be about 100 mg/liter.

Supplemental Reading

Lauer, B. A., L. B. Reller, and S. Mirrett. 1981. Comparison of acridine orange and Gram stains for detection of microorganisms in cerebrospinal fluid and other clinical specimens. *J. Clin. Microbiol.* **14**:201–205.

3.2.3

Wet Mount for Detection of Leukocytes and Microorganisms

[Updated March 2007]

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

The presence of WBCs is one factor suggestive of an invasive infectious process. Wet mount examination of noninvasively collected specimens can be useful for rapid, inexpensive evaluation for WBCs and the detection of microorganisms such as yeasts, campylobacters, and *Trichomonas vaginalis*. The method can be done without expensive supplies and equipment, allowing rapid intervention into the disease process in the outpatient setting. The sensitivity of the technique varies with the experience of the microscopist and is generally about 60%.

Fecal leukocytes are produced in response to bacteria that invade the colonic mucosa, e.g., infections caused by *Shigella*, *Campylobacter*, some *Salmonella* organisms, enteroinvasive *Escherichia coli*, and *Yersinia*. Fecal leukocytes are also found in cases of ulcerative colitis, Crohn's disease, amebic dysentery, and antimicrobial agent-associated colitis, associated with *Clostridium difficile* toxin. Leukocytes are not associated with *E. coli* Shiga toxin infections, which is a useful characteristic of the presentation of the disease, since treatment with antimicrobial agents is contraindicated (6). Because of the variability in results for the presence of WBCs in stool and sensitivities of 50 to

60% in gastroenteritis (9) and as low as 14% for *C. difficile* colitis (10), the test cannot be used as a screening test but rather, as one of several tests to evaluate a patient's condition. It is most useful in the outpatient setting for timely evaluation of the patient, since culture confirmation of gastroenteritis is usually delayed several days. In the test, fecal samples from patients are examined microscopically at $\times 400$ magnification for the presence of fecal leukocytes. As an alternative, especially if there is a delay in transport of the specimen, the LEUKO-TEST, a rapid latex agglutination test for the presence of elevated levels of lactoferrin, can be done from stool specimens from adult patients, children, and infants not being breastfed (3, 4, 5). The latex particles are coated with antibody to lactoferrin (a breakdown product of fecal leukocytes).

Urinary leukocytes are present in infectious processes such as cystitis, glomerulonephritis, and catheter-associated urinary tract infection, and the report of their presence (pyuria) is helpful in the determination of disease. When WBCs are counted in a hemacytometer, the test has a high sensitivity for disease in infants (8). Urinary wet mounts can also demonstrate

the presence of motile trichomonads, although the sensitivity is lower than that of vaginal wet prep or culture (7). Additionally, the detection of WBCs in urine can be determined by the dipstick for leukocyte esterase (see Appendix 3.12-1 in procedure 3.12 for detailed method).

The presence of WBCs is one factor suggestive of an invasive reproductive tract infectious process, including pelvic inflammatory disease and cervical infection due to *Chlamydia trachomatis* or *Neisseria gonorrhoeae*. A wet mount of vaginal fluid can demonstrate the presence of leukocytes, specialized epithelial cells coated with bacteria known as "clue cells," *T. vaginalis*, and yeasts. This rapid test is useful in the timely detection of bacterial vaginosis and vaginitis. Large numbers of WBCs are associated with *T. vaginalis* infection. Bacterial vaginosis is a clinical syndrome characterized by a shift in the vaginal microbiota from the dominant microbiota of *Lactobacillus* spp. to a mixed microbiota of *Gardnerella vaginalis*, *Prevotella* spp., *Mobiluncus* spp., and *Mycoplasma hominis*. Budding yeasts and/or pseudohyphae of yeasts can also be identified in the vaginal fluid and are an indicator of yeast vaginitis.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING



Observe standard precautions.

NOTE: Refer to procedure 3.3.1 for additional details. The following can be copied for preparation of a specimen collection instruction manual for caregivers. It is the responsibility of the laboratory director or designee to educate physicians and other caregivers on proper specimen collection.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING (continued)

A. Specimen collection

1. Fresh stool specimen
 - a. Collect stool specimen for wet mount into a sterile, leakproof wide-mouthed container (unpreserved).
 - b. If fresh stool cannot be transported and processed for wet mount within 2 h after collection, place in 10% formalin and/or polyvinyl alcohol (preserved). Preserved stool may be indefinitely transported and stored prior to wet mount exam.
 - c. Store specimen for lactoferrin assay at 4°C for up to 48 h either in no preservative or in Cary-Blair or similar culture transport system. Dilute 1:50 for testing.

NOTE: Stool wet mounts should be discouraged due to their low sensitivity and specificity. A Gram stain or methylene blue stain may be used to detect leukocytes.
2. Urine
 - a. Collect urine specimen into a sterile, leakproof widemouthed container and store at 4°C.
 - b. If fresh urine cannot be transported and processed for wet mount within 2 h after collection, place in a sterile transport tube containing preservative (boric acid). Preserved urine may be indefinitely transported and stored prior to wet mount exam.
3. Vaginal fluid
 - a. Collect vaginal fluid/discharge from the posterior fornix using sterile cotton or Dacron swab.
 - b. Slides for wet mount must be immediately prepared and examined (within 15 to 20 min after collection) for WBC and trichomonad evaluation.
 - c. Vaginal swabs for wet mount that cannot be immediately examined should be placed in Amies' gel transport system (Copan Diagnostics, Inc., Corona, Calif.) for submission to the laboratory (1).
 - d. Vaginal swabs for trichomonad evaluation that cannot be immediately examined by wet mount of smear should be placed in InPouch TV culture medium for submission to the laboratory (1).
4. For information about InPouch TV culture medium, see reference 2.

B. Rejection criteria

1. Reject fresh stool delayed in transport and received greater than 2 h after collection.
2. Reject vaginal swabs that are not received in transport media if they are delayed in transport and received greater than 2 h after collection.
 - a. Vaginal smears prepared at the time of specimen collection are acceptable for WBC evaluation for up to 24 h after collection.
 - b. Vaginal swabs must be submitted in Diamond's culture medium or they should be rejected for trichomonad evaluation (1).

III. MATERIALS

A. Reagents

1. Saline (0.9% NaCl), preferably at 35°C
2. Loeffler's methylene blue (optional)
 - a. Dissolve 0.3 g of methylene blue in 30 ml of ethyl alcohol.
 - b. Add 100 ml of distilled water.
3. Microscope slides and coverslips
4. LEUKO-TEST (TechLab, Blacksburg, Va.)—alternative for fecal specimens

5. Chemstrip LN (Bio-Dynamics, Division of Boehringer Mannheim Diagnostics, Indianapolis, Ind.)—alternative for urine (see Appendix 3.12–1 in procedure 3.12)

B. Equipment

1. Phase-contrast (preferred) or bright-field microscope
2. Hemacytometer for infant urine

ANALYTICAL CONSIDERATIONS**IV. QUALITY CONTROL**

- A. Controls for methylene blue stain and saline
 - 1. Negative control: each day of use, add 1 drop of saline to 1 drop of methylene blue and examine at $\times 400$. No bacteria or cells should be visible.
 - 2. Positive control: each day of use, examine 1 drop of buffy coat from an EDTA-collected blood sample for cellular components.
- B. Positive controls are not needed for wet mounts if no reagents are added, but evaluate competency with an ongoing program using positive and negative smears showing WBCs, clue cells, and trichomonads. Unfortunately, this is generally accomplished by viewing a collection of photographs.
- C. Follow package insert for controls for the *LEUKO-TEST*.

V. PROCEDURE*Observe standard precautions.***A. Specimen preparation***Wear gloves at all times during preparation and reading of smears.*

- 1. Fecal specimens
 - a. Place 2 drops of saline separated by a wax pencil line on a microscope slide.
 - b. Add a small drop of methylene blue to one side and a saline drop to the other.
 - c. Mix each drop with a small portion of stool (preferably liquid stool or sample with blood or mucus).
 - 2. Vaginal and urine specimens
 - a. Place 1 or 2 drops of specimen onto slide.
 - b. If vaginal swab is transported in Amies' gel, suspend into 2 or 3 drops of saline before placing onto the slide.
 - 3. For urine specimens from pediatric patients, place the drop of urine in a hemacytometer (8).
- B.** Apply coverslip and examine by using the high dry objective, preferably under phase-contrast optics with a $10\times$ eyepiece for a magnification of $\times 400$.
- C.** Observe for and count leukocytes, RBCs, monocytes, and epithelial cells (clue cells in vaginal specimens) in 10 to 20 fields to get an accurate determination of the specimen content.
- D.** **NOTE:** Clue cells are squamous epithelial cells with bacteria attached to the top and sides. Clue cells appear as granular cells with an undefined edge.
- E.** Observe for parasites that are motile, such as trichomonads.
- F.** Observe for budding yeast cells and pseudohyphae in vaginal exudate and urine but not in fecal specimens, where they are part of the normal microbiota.
- G.** Dispose of slides in sharps container, wipe microscope with 70% ethanol, and remove gloves.
- H.** Follow the package instructions for the latex agglutination procedure for the *LEUKO-TEST*.

POSTANALYTICAL CONSIDERATIONS**VI. REPORTING RESULTS**

- A. Report wet mount results (Table 3.2.3-1).

VI. REPORTING RESULTS (continued)

Table 3.2.3-1 Outline for reporting wet mount results from different specimens

Specimen	Cells ^a	Microorganisms
Stool	Report WBCs/HPF.	Report the presence of <i>Campylobacter</i> —“Motile organisms suggestive of <i>Campylobacter</i> spp. present.”
Urine	Report WBCs/microliter. Report RBCs/microliter.	Report the presence of bacteria. Report the presence of budding yeasts and pseudohyphae.
Vaginal ^b	Report WBCs/HPF. ■ NOTE: Vaginal infections with <i>T. vaginalis</i> are usually accompanied by >30 WBCs/HPF.	Report the presence of <i>T. vaginalis</i> (see procedures 9.6.6 and 9.9.4 for details). Report the presence of budding yeasts and pseudohyphae. Report the presence of clue cells.

^a Report the number of WBCs and RBCs per high-power field (HPF). Average several fields and report as follows: (i) heavy = $\geq 5/\text{HPF}$, (ii) moderate = 1 to 4/HPF, (iii) few = $< 1/\text{HPF}$, and (iv) none = 0/HPF.

^b If the vaginal specimen is negative for parasites and was not preserved, or was not processed within 15 min, report “Unable to determine the presence of *Trichomonas vaginalis* in unpreserved specimen or because of delay in transport.”

VII. INTERPRETATION

A. Urine

1. Greater than 5/HPF is considered positive for pyuria. Such a result has a specificity of 90% for predicting catheter-associated infection with greater than 10^5 CFU/ml but a sensitivity of only 37% (procedure 3.12).
2. Greater than 10/ μl with the hemacytometer method is reported to have a sensitivity of 84% and a specificity of 90% for predicting pyuria in infants (8).

B. Fecal WBCs

1. The presence of $> 5/\text{HPF}$ had a sensitivity of 63.2% and a specificity of 84.3% in one study (9), but in another study a 52% sensitivity was seen only if $> 1 \text{ WBC}/\text{HPF}$ was used as the cutoff (10).
2. The results of fecal WBCs were not statistically or clinically significantly different from those of lactoferrin testing (9).
3. *If WBCs are not present and RBCs are present in a stool submitted for culture, always perform a culture for E. coli O157 or a Shiga toxin test (6).*
- C. For vaginal specimens, the sensitivity of testing by wet mount for WBCs is not as important as the examination for clue cells, yeasts, and *T. vaginalis*, although *T. vaginalis* is more common in specimens with WBCs.
 1. The presence of budding yeast is associated with candidiasis.
 2. The presence of clue cells is indicative of bacterial vaginosis.

VIII. LIMITATIONS

- A. Activated leukocytes disintegrate easily, decreasing the sensitivity of the assay with delays in transit.
- B. The wet mount is dependent on the expertise of the microscopist, whose work should be reviewed at frequent intervals.
- C. WBCs are often confused with trichomonads that are no longer moving, which can be avoided by careful examination for the characteristics of the parasite.
- D. If there is a question in reading the test, the cells, but not yeasts, will dissolve with the addition of KOH (see procedure 8.3).
- E. Delays in transit decrease the ability to detect trichomonads.

REFERENCES

1. Beverly, A. L., M. Venglarik, B. Cotton, and J. R. Schwebke. 1999. Viability of *Trichomonas vaginalis* in transport medium. *J. Clin. Microbiol.* **37**:3749–3750.
2. Borchardt, K. A., M. Z. Zhang, H. Shing, and K. Flink. 1997. A comparison of the sensitivity of the InPouch™ TV, Diamond's, and TichoSEL media for detection of *Trichomonas vaginalis*. *Genitourin. Med.* **73**:297–298.
3. Fine, K. D., F. Ogunji, J. George, M. D. Niehaus, and R. L. Guerrant. 1998. Utility of a rapid fecal latex agglutination test detecting the neutrophil protein, lactoferrin, for diagnosing inflammatory causes of chronic diarrhea. *Am. J. Gastroenterol.* **93**:1300–1305.
4. Guerrant, R. L., V. Araujo, E. Soares, K. Kotoff, A. A. M. Lima, W. H. Cooper, and A. G. Lee. 1992. Measurement of fecal lactoferrin as a marker for fecal leukocytes. *J. Clin. Microbiol.* **30**:1238–1242.
5. Huicho, L., V. Garaycochea, N. Uchima, R. Zerpa, and R. L. Guerrant. 1997. Fecal lactoferrin, fecal leukocytes and occult blood in the diagnostic approach to childhood invasive diarrhea. *Pediatr. Infect. Dis. J.* **16**:644–647.
6. Iida, T., A. Naka, O. Suthienkul, Y. Sakaue, R. L. Guerrant, and T. Honda. 1997. Measurement of fecal lactoferrin for rapid diagnosis of enterohemorrhagic *Escherichia coli* infection. *Clin. Infect. Dis.* **25**:167.
7. Lawing, L. F., S. R. Hedges, and J. R. Schwebke. 2000. Detection of trichomoniasis in vaginal and urine specimens from women by culture and PCR. *J. Clin. Microbiol.* **38**:3585–3588.
8. Lin, D. S., F. Y. Huang, N. C. Chiu, H. A. Koa, H. Y. Hung, C. H. Hsu, W. S. Hsieh, and D. I. Yang. 2000. Comparison of hemocytometer leukocyte counts and standard urinalyses for predicting urinary tract infections in febrile infants. *Pediatr. Infect. Dis. J.* **19**:223–227.
9. Ruiz-Pelaez, J. G., and S. Mattar. 1999. Accuracy of fecal lactoferrin and other stool tests for diagnosis of invasive diarrhea at a Colombian pediatric hospital. *Pediatr. Infect. Dis. J.* **18**:342–346.
10. Savala, K. L., E. J. Baron, L. S. Tompkins, and D. J. Passaro. 2001. Fecal leukocyte stain has diagnostic value for outpatients but not inpatients. *J. Clin. Microbiol.* **39**:266–269.

APPENDIX 3.2.3-1

Demonstration of *Treponema pallidum* in Specimens Using Dark-Field Microscopy**I. PRINCIPLE**

Dark-field microscopy is used to demonstrate the presence of motile *Treponema pallidum* in lesions or aspirates in early-stage syphilis prior to healing of lesions (1, 2, 3).

II. MATERIALS

- A. Dark-field microscope with parfocal 10×, 40× to 45×, and 100× oil immersion objectives, 10× oculars, dark-field immersion condenser (single or double deflecting), and a 6.0- to 6.5-V high-intensity lamp with variable transformer for regulating light intensity.
- B. Microscope slides, 1 by 3 in.
- C. Coverslip, 22 by 22 mm
- D. Immersion oil, nondrying

III. SPECIMEN

Collect serous fluid from lesion for examination prior to antimicrobial therapy.

- A. Clean surface of lesion with saline, and blot dry.

- B. Gently remove any crusts, and discard.

- C. Abrade superficially until slight bleeding occurs, using a needle, scalpel blade, or broken glass slide. Irrigate with sterile saline and wipe away the first few drops of blood, etc.

- D. Apply gentle pressure at lesion base, touching *clear* exudate in ulcer base with a glass slide.

- E. If no exudate is present, add a drop of saline to the lesion or insert a needle and syringe at lesion base, aspirate, and then draw a drop of saline into the needle. Express the material onto a slide.

- F. Place coverslip immediately, and examine the slide by dark-field microscopy.

IV. MICROSCOPE EXAMINATION PROCEDURE

- A. Search entire specimen with high dry objective for spiral organisms.

- B. Center suspicious objects, and examine them under oil immersion objective.

- C. Upon completion of examination, discard slide into container of appropriate disinfectant.

V. INTERPRETATION

- A. *T. pallidum* organisms appear as delicate, corkscrew-shaped, rigid, uniform, tightly wound, deep spirals; coil appearance is maintained even while organisms are actively motile.



Observe standard precautions.



Observe standard precautions.

APPENDIX 3.2.3–1 (continued)

- B. Observe for rotational motility around longitudinal base; backward and forward movement; flexion, bending, or twisting from side to side; and snapping motion.
- C. Spirochetes are 6 to 14 μm long, which is slightly longer than the diameter of an erythrocyte.

VI. REPORTING RESULTS

- A. When organisms are seen that have characteristic morphology, shape, and motility of *T. pallidum*, report “Treponemas resembling *T. pallidum* observed.”
- B. When no treponemas are observed, report “No treponemas resembling *T. pallidum* observed.”

VII. LIMITATIONS

The specimen must be examined immediately (within 20 min) to observe motile organisms. For optimal sensitivity, up to three separately collected slides should be examined by dark-field microscopy to rule out *T. pallidum*. If immediate dark-field examination is not possible, air dry the slide and submit to a laboratory, such as the CDC Syphilis Diagnostic Immunology Section (<http://www.cdc.gov>; STD hotline, 1-800-232-4636), that has reagents for specific direct fluorescent-antibody examination for treponemes, or purchase commercial reagents (Virostat, Portland, Maine) (3).

References

1. Baron, E. J., G. H. Cassell, D. A. Eschenbach, J. R. Greenwood, S. M. Harvey, N. E. Madinger, E. M. Paterson, and K. B. Waites. 1993. Cumitech 17A, *Laboratory Diagnosis of Female Genital Tract Infections*. Coordinating ed. E. J. Baron. American Society for Microbiology, Washington, D.C.
2. Holmes, K. K., P. A. Mardh, P. F. Sparling, P. J. Wiesner, W. Cates, Jr., S. M. Lemon, and W. E. Stamm. 1990. *Sexually Transmitted Diseases*, 2nd ed. McGraw Hill Book Co., New York, N.Y.
3. Norris, S. J., V. Pope, R. E. Johnson, and S. A. Larsen. 2003. *Treponema* and other human host-associated spirochetes, p. 955–971. In P. R. Murray, E. J. Baron, J. H. Jorgensen, M. A. Pfaffer, and R. H. Yolken (ed.), *Manual of Clinical Microbiology*, 8th ed. ASM Press, Washington, D.C.

3.3

PROCESSING, ISOLATION, DETECTION, AND INTERPRETATION OF AEROBIC BACTERIOLOGY CULTURES

3.3.1

Paratechnical Processing of Specimens for Aerobic Bacteriology

[Updated March 2007]

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

The initial processing of clinical specimens for bacteriology is a multifaceted endeavor involving a number of decision-making steps, including the need for processing the specimen for anaerobic bacteriology, mycology, virology, and parasitology, depending on the nature of the specimen. The need for direct tests, such as Gram stains, must also be consid-

ered. These issues will determine whether the specimen requires any pretreatment before inoculation.

First, one must consider the specimen type and its anatomic origin. The second step is the selection of primary isolation media to be used for each specimen type. The final step is the selection of incubation temperature and atmosphere. A biological

safety cabinet should be used during the processing of all specimens. This procedure covers only general processing instructions for routine cultures. Refer to the Specimen Collection, Transport, and Handling portions of procedures 3.4 through 3.15 for processing by specific anatomic site and the use of specialized media for processing for specific organisms.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING



Observe standard precautions.

- A. Prepare specimen collection instruction manual and make available to physicians and other caregivers for proper collection, timing, and transport of specimens. This can be achieved by copying the Specimen Collection, Transport, and Handling portions from each of the procedures that follow. It is the responsibility of the laboratory director or designee to educate physicians and caregivers on proper specimen collection.
 - NOTE:** Proper specimen collection is critical to isolating the causative agent of infection.
- B. Along with the specimen, design a system to provide the following.
 - 1. Demographics of the patient: name, address, age, sex, location in the hospital or clinic, unique patient identifying number, name of physician of record, name of the physician who is ordering the test, and ICD9 code or diagnosis.
 - 2. Details of the specimen: type of specimen, anatomic site of collection (if variable), whether the specimen was collected from an invasive procedure (e.g., during surgery), and gross description, if variable.
 - 3. Specific culture, stain, and antigen test requests.
- C. Provide the above information on each request form, specimen container, and transport carrier. The patient demographic information, specimen information, and specific test requests provided on the laboratory requisition should match those on the specimen container label and the transport carrier.
- D. Determine acceptability of specimen labeling and collection. See section 2 of this handbook for general rejection criteria and each procedure that follows for specific rejection criteria by specimen type.

III. MATERIALS

- A.** The general media used for routine cultures in bacteriology are listed in Table 3.3.1–1.
- B.** Stain reagents and supplies (see procedures 3.2.1 to 3.2.3.)
- C.** Other supplies
 - 1. Sterile petri dishes
 - 2. Pasteur pipettes
 - 3. Sterile scissors, forceps, and scalpels
- D.** Equipment
 - 1. Biological safety cabinet
 - 2. Incubators (35 to 37°C; both 5% CO₂ and ambient air)
 - 3. Bactericinerator or flame burner with automatic shutoff (optional)

Table 3.3.1–1 Common routine laboratory media^a

Medium	Abbreviation	Type ^b	Atmosphere	Inhibitor(s)	Purpose
Nonselective agars					
Chocolate agar	CHOC	N	5–10% CO ₂	None	To grow most bacteria, including <i>N. gonorrhoeae</i> and <i>Haemophilus</i>
Tryptic soy agar with 5% defibrinated sheep blood or Columbia agar with 5% defibrinated sheep blood	BAP	N	5–10% CO ₂ preferred; O ₂ for special circumstances	None	To grow most bacteria and determine the type of hemolysis: alpha (green), beta (clear), or gamma (none). Will not support <i>N. gonorrhoeae</i> , <i>Haemophilus</i> , <i>Legionella</i> , or <i>Bordetella pertussis</i> .
Gram-negative rod selective agars					
MacConkey agar	MAC	S, D	O ₂	Bile salts, crystal violet, lactose, neutral red	Gram-negative enteric agar that inhibits growth of gram-positive organisms and yeasts and inhibits the spreading of <i>Proteus</i> . Lactose-positive colonies are pink, and lactose-negative colonies are colorless.
Eosin-methylene blue	EMB	S, D	O ₂	Eosin, methylene blue, lactose, sucrose	Gram-negative enteric agar that inhibits (but does not prevent) the growth of gram-positive organisms and some yeasts. Enhances the growth of <i>Candida glabrata</i> and some molds. Enteric rods have various colors. Lactose- and sucrose-fermenting colonies are dark.
Gram-positive selective agars					
Phenylethyl alcohol with 5% defibrinated sheep blood	PEA	S	5–10% CO ₂	Phenylethyl alcohol	Inhibits most gram-negative bacteria. Used for growth of <i>Staphylococcus</i> and <i>Streptococcus</i> in mixed cultures. Has short shelf life.
Colistin-nalidixic acid Columbia agar with 5% defibrinated sheep blood	CNA	S	5–10% CO ₂	Colistin and nalidixic acid	Inhibits most gram-negative bacteria. Used for growth of <i>Staphylococcus</i> and <i>Streptococcus</i> in mixed cultures.
Selective agar for neisseriae	TM	S	5–10% CO ₂	Vancomycin, colistin, nystatin	To select for <i>Neisseria</i> in mixed cultures. For other related agars, see procedure 3.9.3.
Thayer-Martin					

Table 3.3.1-1 (continued)

Medium	Abbreviation	Type ^b	Atmosphere	Inhibitor(s)	Purpose
Broths: support the growth of most aerobic bacteria					
Brain heart infusion	BHI	E	Ambient air		
Tryptic soy broth	TSB				
Brucella broth	BRUB				
Thioglycolate	THIO				
Chopped-meat broth	CMB				
Fastidious anaerobic broth (4)	FAB				
					BHI and TSB can be supplemented with 0.1% agar to support some anaerobic growth and with X and V factor or sheep blood to support growth of <i>Haemophilus</i> and other fastidious facultative anaerobic organisms. THIO does not support the growth of fastidious facultative anaerobic organisms.

^a For enteric selective media for fecal cultures (e.g., Hektoen and XLD), refer to procedure 3.8.1. For specialized media for specific organism requests, refer to the specific procedure for that organism.

^b N, nutrient; D, differential; S, selective; E, enrichment.

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

- A. For agar media, inspect each shipment or batch for cracks in media or plastic petri dishes, thin or unequal fill, hemolysis, evidence of freezing, desiccation, bubbles, and visible contamination and report deficiencies to manufacturer.
- B. Maintain records of date received or prepared, lot number, and expiration date of each medium.
- C. QC media, whether prepared in-house or procured from commercial vendors, with one or more organisms expected to grow and one or more organisms expected to be inhibited, if applicable. See Clinical and Laboratory Standards Institute (CLSI; formerly NCCLS) document M22-A3 (1) and procedure 14.2 for details, including preparation of stock cultures with controlled inocula of microorganisms.
- D. *Exception:* For media listed in Table 2 of CLSI document M22-A3 (1) and procured from commercial sources, omit QC and verify that the vendor has controlled the media according to procedures outlined in document M22-A3.
- E. Test each lot of CHOC and TM for QC, whether purchased commercially or prepared in-house. Refer to procedure 3.9.3 for QC of TM and related selective agars. For QC of CHOC, use the following positive controls; a negative control is not needed. Incubate plates for 24 to 48 h in 5 to 7% CO₂ at 35°C.

Test organism	Result
<i>Neisseria gonorrhoeae</i> ATCC 43069 or ATCC 43070	Growth
<i>Haemophilus influenzae</i> ATCC 10211	Growth

- F. Check and record incubator temperatures daily; include in records readings of humidity and CO₂ concentrations, if applicable.
- G. Set up a QA program to ensure that the specimens received are of the best possible quality (see section 14). Troubleshoot problems and follow with necessary revisions to existing procedures to improve quality.
- H. Prepare an alternative testing policy to be used when the laboratory is unable to perform testing if reagents, personnel, and methods of communication are not available. The following are examples.

IV. QUALITY CONTROL (continued)

1. If media cannot be supplied by vendor, contact alternative vendor. . . .
2. If incubator is inoperable, use alternative incubator located at. . . .
3. If computer system is not operable, send manual reports according to details in the computer crash plan.

V. PROCEDURE



Observe standard precautions.

A. Timing

The most important specimens must be inoculated to media first. Look through the cultures and pull out all those that are invasively collected. The preferred order of setting up cultures is listed in Table 3.3.1–2. The delay of inoculation and processing of certain specimens can affect the quality of the culture and ability to isolate pathogens.

B. Specimen pretreatment

1. Work in a biological safety cabinet. Wear gloves, laboratory coat, and other protective equipment when working with specimens.
 2. Verify that the patient demographic information, specimen information, and specific test requests provided on the laboratory requisition match those on the specimen container label and the transport carrier. Assign a unique identifying number to the specimen at the time of accession.
 3. Generally, look for the most purulent part of the specimen and use that for smears and culture.
 - a. Fluids, except urine
 - (1) For normally sterile body fluids
 - (a) Use the cytocentrifuge to concentrate smears from clear fluids.
 - (b) In addition to plate culture, inoculate broth culture, diluting the specimen 1:10 in broth.
 - (c) For joint and peritoneal fluid specimens, culture at least 10 ml of specimen.
- NOTE:** The culture of large volumes of specimen has been shown to have a higher yield than centrifugation of specimens

Table 3.3.1–2 Order of specimen processing for bacteriology when multiple specimens are received at the same time

Order	Common tests or specimens	Maximum time to processing
1	STATS: specimens from surgery and normally sterile sites are processed before STATs from nonsterile body sites.	20 min after receipt
2	<i>N. gonorrhoeae</i> cultures submitted on plates or unpreserved swabs	20 min after receipt
3	CSF (treat all as STAT)	20 min after receipt
4	BAL ^b	20 min after receipt
5	Tissues	1 h
6	Other body fluids	1 h
7	Abscesses	1 h
8	Unpreserved stools for culture	30 min after collection, or place in transport medium immediately ^a
9	Sputum and other lower respiratory cultures	1 h at room temp, 2 h at 4°C
10	Blood	4 h at room temp after collection
11	Swabs in transport tubes	8 h at 4°C
12	Urine	Up to 24 h at 4°C
13	Group A and B streptococcal cultures	8 h at 4°C

^a *Shigella* sp. viability is compromised unless the specimen is placed in transport medium.

^b BALs, bronchoalveolar lavage samples.

V. PROCEDURE (continued)

for aerobic bacteria. Generally inoculation of up to 10 ml into the aerobic and 10 ml into the anaerobic blood culture bottle will increase the yield of the causative agent from joint and peritoneal body fluids.

- (2) For other liquid specimens, inoculate plates and smears using a swab or pipette dipped into the liquid.

b. Tissues

Refer to procedure 3.13 for details on preparation of tissues for smear and culture.

c. Swabs

- (1) Reject dry swabs not submitted in transport medium.
- (2) Use the swab to streak the first quadrant of the plate. If a second swab is submitted, use for smear preparation.
- (3) If only one swab is received or several plates and smears are to be prepared, place swab into a small amount of broth and vortex. Ream swab to expel all fluid and discard. Then use a pipette to inoculate agar plate media and to prepare smears.

■ NOTE: Use of the CultureSwab EZ II system (BD Diagnostic Systems) omits the need to extract the organisms from the swab.

- d.** For prosthesis specimens and other surgical foreign-object specimens with no visible tissue or purulent fluid that can be cultured, add culture broth to the sterile specimen container and incubate at 35°C for 18 h. Then subculture broth and perform a Gram stain. Hold broth with rest of culture. If the object is from a genital source (e.g., intrauterine device), touch specimen to a CHOC plate and to anaerobic plates prior to adding broth.
- e.** For catheter tips, refer to procedure 3.6 for quantitative and semiquantitative methods of culture.
- f.** Refer to procedure 3.12 for options to inoculate quantitative urine cultures.

4. Saving specimens

- a.** Hold urine and stool not in preservative only long enough to resolve any labeling problems. These specimens should be recollected rather than recultured if there are indications to do so.
- b.** Hold samples from other nonsterile sites for 1 to 2 days, usually at 4°C, to resolve any problems.
- c.** Hold specimens from normally sterile sites for 7 days at 4°C, if possible. Rotate specimens such that older ones are discarded in favor of recently cultured specimens.
- d.** Freeze CSF at -20°C in case PCR testing is requested after evaluation of cultures.

C. Medium choices (see Table 3.3.1-1)

- 1.** Inoculate most specimens onto a BAP.
- 2.** In addition, inoculate specimens from normally sterile sites, genital sites, and respiratory sites to CHOC (or, optionally, horse blood agar for respiratory specimens).
- 3.** Add additional media based on nosocomially significant microorganisms.
- 4.** For specimens from nonsterile sites, inoculate to either MAC or EMB to select for these organisms within normal microbiota.
- 5.** Use Columbia colistin-nalidixic acid agar (CNA) or phenylethyl alcohol agar (PEA) to select for gram-positive organisms in specimens potentially contaminated with gram-negative microbiota.
- 6.** Historically, tissues from surgery and fluids from selected normally sterile sites have been inoculated into broth, but this has been shown to not be

V. PROCEDURE (continued)

- clinically relevant or cost-effective in improving the recovery of pathogenic organisms from these types of specimens (2, 3).
7. Refer to the individual procedures that follow for details of inoculation of stool cultures and cultures for specific organisms to special media.
 - D. Perform Gram stains on most respiratory and wound cultures, all cultures from normally sterile sites, and urine and genital cultures, on request. Gram stains are not performed on throat, nasal, stool, or catheter tip cultures. For smear preparation, see procedure 3.2.1.
 1. If sterile glass slides are not used for the Gram stain, the culture should be inoculated first to avoid contamination. Never touch the nonsterile glass slide with a pipette or swab that will subsequently be used for culture. Alternatively, the Gram stain may be prepared prior to inoculation of the culture if sterile glass slides are used. Preparation of the Gram stain prior to inoculation of the culture may provide a better smear that accurately reflects the clinical cellular and bacterial contents of the original clinical specimen.
 2. If there is insufficient specimen, omit the smear rather than the culture.
 3. *If the smear result indicates a mixed culture and selective medium was not inoculated, add appropriate selective medium to the culture inoculation.*
 4. Use a cytocentrifuge to prepare smears from normally sterile body fluids, especially CSF.
 - E. Inoculation technique
 1. Label plates with at least the identifying number and date of culture. If convenient, label at least one plate with the anatomic site and patient name.
 2. Generally inoculate onto plate by touching specimen to one quadrant with a swab, pipette, or sterile forceps containing the specimen.
 - a. Sterilize the inoculating loop in the microincinerator for 5 to 10 s. Allow to cool. Alternatively, use separate, sterile disposable loops or sticks.
 - b. Streak with gentle pressure onto one-fourth to one-third of the culture plate using the sterile loop, or stick, with a back-and-forth motion several times and without entering the area that was previously streaked. Avoid touching the sides of the petri dish.
 - c. Turn the plate a quarter turn. Pass the loop through the edge of the first quadrant approximately four times, while streaking into the second quadrant. Continue streaking in the second quadrant without going back to the first quadrant.
 - d. Rotate the plate another quarter turn and repeat the above procedure until one or two additional quadrants are streaked, as shown in Fig. 3.3.1–1.

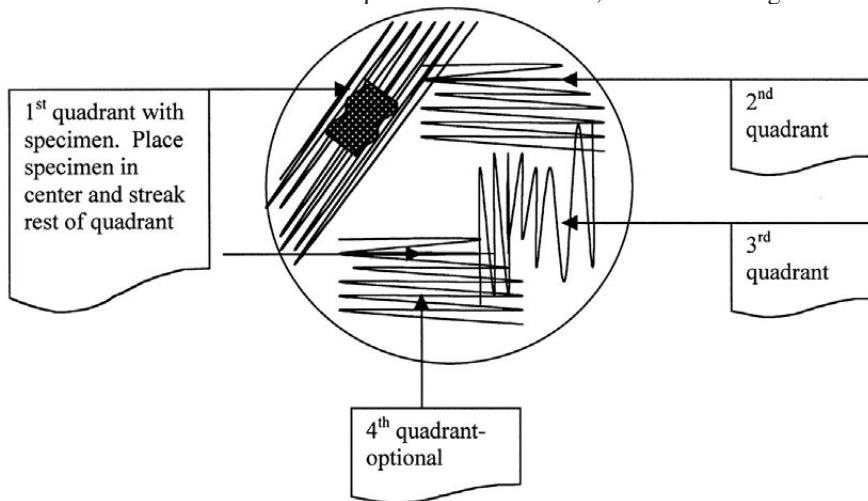


Figure 3.3.1–1 Appropriate method to streak plate for isolation of bacteria. Inoculate first quadrant with a few drops or pieces of specimen or by rolling a swab on a small area.

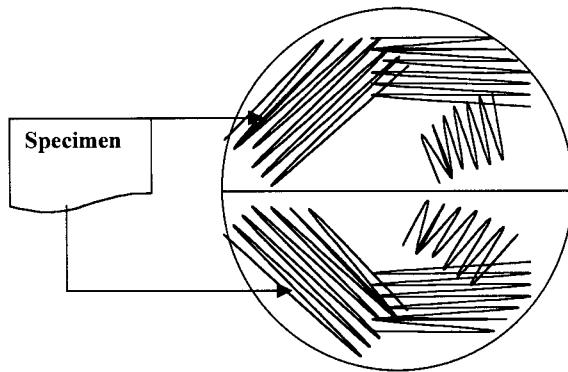


Figure 3.3.1–2 Inoculation of biplate, often used for specimens from sterile sites when blood agar and CHOC are needed. To avoid cross contamination of media with plate contaminants, always use a separate loop or flame loop between inoculation of each side of plate.

V. PROCEDURE (continued)

3. For normally sterile specimens, use separate disposable loops, sticks, or needles for each plate. Alternatively, flame the loop or needle between each plate inoculation. Use biplates to save handling time and space (Fig. 3.3.1–2).
4. For inoculation of plates to detect beta-hemolysis, carefully stab the agar with the same loop both inside and outside the inoculated area to expose the organism to anaerobic conditions (Fig. 3.3.1–3) (usually done for throat cultures).
5. For urine, catheter tips, and quantitative cultures, do not streak in quadrants. Refer to the relevant procedures in this section.
6. Optional: for respiratory specimens or aerobic wound cultures, cross streak or dot the BAP with coagulase-negative staphylococci after the specimen has been inoculated to the plate, to allow for recognition of *Haemophilus* (Fig. 3.3.1–4). This technique may be done instead of or in addition to the CHOC plate inoculation. Refer to procedure 3.17.44 for further details.

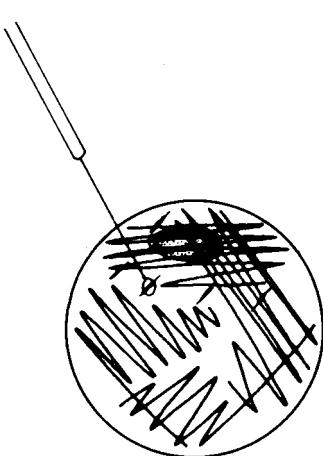


Figure 3.3.1–3 Optional method of streaking plate for throat cultures.

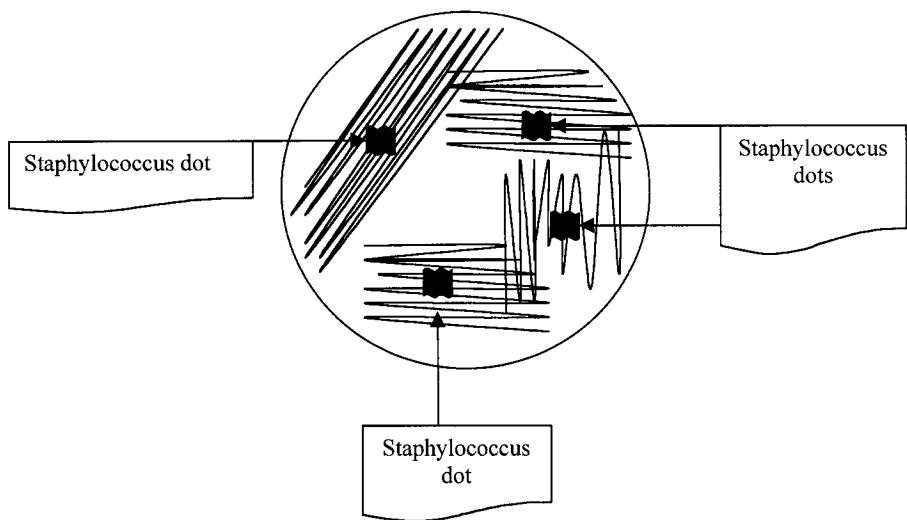


Figure 3.3.1–4 Alternative for detection of *H. influenzae* on BAP (e.g., respiratory specimens).

V. PROCEDURE (continued)

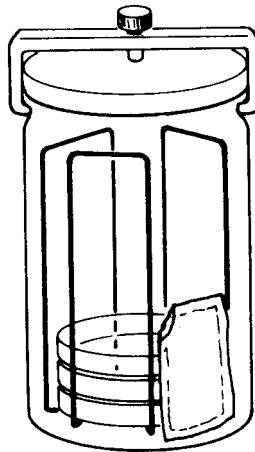


Figure 3.3.1-5 GasPak envelopes in airtight jar, used to produce 5 to 7% CO₂ atmosphere.

F. Incubation

1. Separate all inoculated direct plates and broths according to the sorting system used in the laboratory (e.g., alphabetical by patient name, by specimen type, by ward, etc.).
2. Generally place in order by workstation in canisters and incubate in a humidified incubator at 35°C.
 - a. Supply humidity by an automatic humidifier or by placing a large pan of water at the bottom of the incubator.
 - b. Check humidity control or water level daily to maintain constant humidity.
3. Provide 5 to 7% CO₂ for appropriate cultures, particularly those for possible isolation of *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Neisseria gonorrhoeae*, especially routine respiratory cultures, genital cultures, and normally sterile body fluid cultures. CO₂ can be supplied as follows.
 - a. By incubators with special controls and a CO₂ gas cylinder
 - b. By using a gas-generating envelope in an airtight jar (Fig. 3.3.1-5) or plastic container *or*
 - c. By placing the medium in a bag with a CO₂-generating ampoule or tablet.
4. Do not incubate the following in increased CO₂.
 - a. Stool culture selective agar
 - b. *Legionella* cultures
 - c. *Bordetella pertussis* cultures
 - d. Throat cultures when using BAP without selective media with antimicrobial agents
5. Increased CO₂ is optional for the following.
 - a. MAC or EMB
 - b. Broths and blood cultures
 - c. Group B streptococcal cultures
 - d. Diphtheria cultures
 - e. Urine cultures
6. Cultures for special organisms have special requirements. See the relevant procedures (e.g., for *Campylobacter*, *Bartonella*, and *Haemophilus ducreyi*).
7. Incubate anaerobic plates in an anaerobic environment, as discussed in section 4 of this handbook.

POSTANALYTICAL CONSIDERATIONS

VI. REPORTING SPECIMEN INFORMATION

- A. Document association of specimen identifying number with patient demographics (name, patient identifier, patient location) and information on the specimen (anatomic site, date and time of collection, and name of person ordering the test).
- B. Document type, container, and macroscopic description of specimen (e.g., color, consistency, etc.).
- C. Document all problems with specimens, culturing, and test requests in laboratory report and notify the collecting location of such problems.
- D. If ICD9 code is available, provide access to information to person examining culture.

VII. LIMITATIONS

- A. False-positive cultures result from specimen mix-up and from contamination of media used for culture.
- B. False-negative results are due to improper collection, delays in culture inoculation, inappropriate medium usage, and inappropriate incubation conditions.

REFERENCES

1. Clinical and Laboratory Standards Institute. 2004. *Quality Assurance for Commercially Prepared Microbiological Culture Media*, 3rd ed. Approved standard M22-A3. Clinical and Laboratory Standards Institute, Wayne, Pa.
2. Meredith, F. T., H. K. Phillips, and L. B. Reller. 1997. Clinical utility of broth cultures of cerebrospinal fluid from patients at risk for shunt infections. *J. Clin. Microbiol.* **35**:3109–3111.
3. Morris, A. J., S. J. Wilson, C. E. Marx, M. L. Wilson, S. Mirrett, and L. B. Reller. 1995. Clinical impact of bacteria and fungi recovered only from broth cultures. *J. Clin. Microbiol.* **33**:161–165.
4. Scythes, K. D., M. Louis, and A. E. Simor. 1996. Evaluation of nutritive capacities of 10 broth media. *J. Clin. Microbiol.* **34**:1804–1807.

SUPPLEMENTAL READING

- Bannatyne, R. M., C. Clausen, and L. R. McCarthy. 1979. *Cumitech 10, Laboratory Diagnosis of Upper Respiratory Tract Infections*. Coordinating ed., I. B. R. Duncan. American Society for Microbiology, Washington, D.C.
- Baron, E. J., G. H. Cassell, D. A. Eschenbach, J. R. Greenwood, S. M. Harvey, N. E. Madinger, E. M. Paterson, and K. B. Waites. 1993. *Cumitech 17A, Laboratory Diagnosis of Female Genital Tract Infections*. Coordinating ed., E. J. Baron. American Society for Microbiology, Washington, D.C.
- Difco Laboratories. 1984. *Difco Manual*, 10th ed., p. 546–551, 1025–1026. Difco Laboratories, Detroit, Mich.
- MacFaddin, J. F. 1985. *Media for Isolation-Cultivation-Identification-Maintenance of Medical Bacteria*, vol. 1. The Williams & Wilkins Co., Baltimore, Md.
- Miller, J. M. 1999. *A Guide to Specimen Management in Clinical Microbiology*, 2nd ed. American Society for Microbiology, Washington, D.C.
- Morris, A. J., S. J. Wilson, C. E. Marx, M. L. Wilson, S. Mirrett, and L. B. Reller. 1995. Clinical impact of bacteria and fungi recovered only from broth cultures. *J. Clin. Microbiol.* **33**:161–165.
- Ray, C. G., J. A. Smith, B. L. Wasiluska, and R. J. Zabransky. 1993. *Cumitech 14A, Laboratory Diagnosis of Central Nervous System Infections*. Coordinating ed., A. J. Smith. American Society for Microbiology, Washington, D.C.
- Runyon, B. A., M. R. Antillon, E. A. Akriviadis, and J. G. McHutchison. 1990. Bedside inoculation of blood culture bottles with ascitic fluid is superior to delayed inoculation in the detection of spontaneous bacterial peritonitis. *J. Clin. Microbiol.* **28**:2811–2812.
- Ryan, K. J., T. F. Smith, and W. R. Wilson. 1987. *Cumitech 7A, Laboratory Diagnosis of Lower Respiratory Tract Infections*. Coordinating ed., J. A. Washington II. American Society for Microbiology, Washington, D.C.
- Silletti, R. P., E. Ailey, S. Sun, and D. Tang. 1997. Microbiologic and clinical value of primary broth cultures of wound specimens collected with swabs. *J. Clin. Microbiol.* **35**:2003–2006.
- Simor, A. E., F. J. Roberts, and J. A. Smith. 1988. *Cumitech 23, Infection of the Skin and Subcutaneous Tissues*. Coordinating ed., J. A. Smith. American Society for Microbiology, Washington, D.C.
- Wilhelmus, K. R., T. J. Liesegang, M. S. Osato, and D. B. Jones. 1994. *Cumitech 13A, Laboratory Diagnosis of Ocular Infections*. Coordinating ed., S. C. Specter. American Society for Microbiology, Washington, D.C.

3.3.2

Interpretation and Rapid Identification of Bacterial Growth on Primary Culture Media

[Updated March 2007]

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

The initial interpretation of bacterial growth on primary culture media, which usually follows the first 24 to 48 h of incubation, is an opportunity for the skilled microbiologist to make a preliminary identification and to decide what addi-

tional tests and procedures must be performed to arrive at a definitive identification. Many of the tables in this procedure are useful for a staff training manual, but they need not be a part of the bench manuals for daily use.

II. SPECIMEN

Cultures of specimens incubated for routine bacterial culture at 35°C for 24, 48, or ≥72 h

III. MATERIALS

A. Reagents and biochemical tests

1. Aminolevulinic acid (ALA) test (procedure 3.17.3)
2. Bile solubility (procedure 3.17.6)
3. Bile-esculin (procedure 3.17.5)
4. Butyrate disk test (procedure 3.17.7)
5. Catalase reagent (procedure 3.17.10)
6. Coagulase and agglutination tests for detection of coagulase (procedures 3.17.13 and 3.17.14).
7. Gram stain reagents (procedure 3.2.1)
8. Hippurate test with ninhydrin (procedure 3.17.21)
9. Indole reagent (procedure 3.17.23)
10. Leucine amino peptidase (LAP) (procedure 3.17.26)
11. 4-Methylumbelliferyl-β-D-glucuronide (MUG) (procedure 3.17.34)
12. Mueller-Hinton (MH) agar for growth studies and disks (e.g., novobiocin) (procedure 3.17.4)

13. Optochin (procedure 3.17.38)

14. Ornithine decarboxylase (procedure 3.17.15)
15. Oxidase test reagent (procedure 3.17.39)

16. Pyrrolidonyl-β-naphthylamide (PYR) (procedure 3.17.41)

17. Urea disks (procedure 3.17.48)

18. Other tests for sugar fermentation, pigment enhancement, serology, DNA probes, gelatin, germ tube, H₂S, motility, and various kit identification systems

B. Supplies

1. Microscope
2. Microscope slides
3. UV (Wood's) light
4. Petri dishes and sterile sticks and inoculating loops

ANALYTICAL CONSIDERATIONS**IV. QUALITY CONTROL**

- A. Verify that media meet expiration date and QC parameters per the current Clinical and Laboratory Standards Institute (formerly NCCLS) M22 document. See procedures 14.2 and 3.3.1 for further procedures, especially for in-house-prepared media.
- B. For QC requirements for biochemical tests, refer to the individual tests in procedure 3.17.

V. PROCEDURE

For specimens from lymph nodes and tiny colonies from normally sterile sites, examine cultures in a biological safety cabinet (6).

A. Initial examination of primary plates

- 1. Describe colonial morphology for each agar medium.
 - a. Note the different types of colonies on each agar plate.
 - (1) When making these initial assessments, look at the culture plates from different angles and use direct illumination of the plate.
 - (2) Use a light with a magnifying lens to observe slow-growing or very small colonies, which are often hidden between the much larger rapidly growing colonies.
 - b. Describe the gross colonial morphology of each colony type. Refer to Table 3.3.2–1 for a list of terms.
- 2. Enumerate each colony type per the criteria in Table 3.3.2–2.
 - a. Do not enumerate as CFU in cultures (other than urine, quantitative cultures, and catheter tips) since the specimen has not been quantitatively sampled.
 - b. On the work record, note the number of colonies on each of several media to distinguish suspected plate contamination from true presence of an organism in a culture.
 - c. Be careful when one mold colony is larger than several smaller ones. The smaller ones could have arisen from the larger one; all could be contaminants.
- 3. Interpret colonies on various primary culture media to determine the most likely genus. Refer to Table 3.3.2–3 for a list of common microorganisms and their characteristics on primary media. Refer to procedure 3.8.1 for media specific to the detection of stool pathogens.
 - a. Note the appearance of organisms on general-purpose primary media.
 - b. Be aware of organisms that will grow or are inhibited on various selective primary media. Commonly used primary culture media and the likely pathogens isolated from each medium are described in Table 3.3.2–4.
 - c. Observe medium changes in the vicinity of the bacterial colonies that provide some indication of bacterial activity. Different dyes, sugars, and other ingredients are incorporated into the media to detect end products of bacterial enzymatic activity.
 - d. Note hemolysis, which is helpful in characterizing microorganisms.
 - (1) Alpha-hemolysis is the reduction of hemoglobin to methemoglobin, producing a greenish discoloration; RBC membrane is intact on microscopic inspection.
 - (2) Beta-hemolysis is the lysis of RBCs, resulting in a distinct, clear, colorless zone surrounding and under the colony. The RBC membrane is destroyed.
 - (3) Gamma-hemolysis indicates no hemolysis. No destruction of RBCs occurs, and there is no change in the medium.

Table 3.3.2-1 Terms to describe gross colonial morphology

SIZE (diameter in mm)		Large = greater than 1mm in diameter Medium = 1mm in diameter Small = less than 1mm in diameter					
SHAPE							
	Circular	Filamentous	Irregular	Punctiform	Rhizoid	Spindle	
ELEVATION							
MARGIN (edge of colony)							
COLOR	White, Black, Cream, Orange etc.						
SURFACE APPEARANCE		Glistening	Dull				
		Smooth	Rough				
		Granular	Creamy				
DENSITY (ability to see through the colony)							
	Opaque = can not see through the colony Transparent = can see through the colony Translucent = only with light shining through						
CONSISTENCY (best observed by picking up a colony with a loop or needle)							
	Butyrous (buttery) Brittle Viscid (sticky) Membranous (pliable) Friable (crumbles easily)						

V. PROCEDURE (continued)

- (4) Alpha-prime-hemolysis is a small zone of complete hemolysis that is surrounded by an area of partial lysis with green discoloration. Alpha-prime-hemolysis is best seen when magnification is used to observe the colonies.
- e. Check for pigment production. If needed, take a swab and pick up some colonies to check for pigment against a white background.
 - (1) Water-soluble pigments produce a discoloration of the surrounding medium, best seen on colorless agars.
 - (2) Nondiffusible (carotenoid) pigments are confined to the colonies themselves.

Table 3.3.2–2 Enumeration guidelines

Report	If the observation is growth
Isolated from broth only	In broth only
Scant growth	One to five colonies. For one colony of skin microbiota, may wish to add note regarding possible contamination.
1+ or few	In first quadrant only, ignoring a few colonies in the second quadrant
2+ or moderate	Up to second quadrant, ignoring a few colonies in the next quadrants
3+ or numerous	Up to third quadrant, ignoring a few colonies in the fourth quadrant
4+ or numerous	Up to fourth quadrant
Number + CFU/ml	For urine and quantitative cultures
Number + CFU	For intravascular catheter tips

V. PROCEDURE (continued)

f. Odor may be important in the evaluation of colonies. Many bacteria have distinct odors that are strong clues to their presence and/or identification.

- (1) *Pseudomonas aeruginosa* is described as having a fruity, grape-like odor.
- (2) *Eikenella corrodens* often smells like bleach or crackers.
- (3) *Proteus* species have been described as smelling like devil's food cake or burnt chocolate.

■ NOTE: Because of the danger of laboratory-acquired infections with microorganisms whose aerosols are infectious (6), laboratory personnel should not directly smell colonies. Plates should be opened at arm's length, and odors should be detected by what is obvious during this procedure. In addition, all cultures suspected of containing an organism that requires a higher level of containment should be tape sealed and worked on in a BSL 2 cabinet.

B. Preliminary testing

1. Day 1

- a. Gram stain colonies that do not appear to be normal microbiota.
- b. Perform initial rapid identification procedures (catalase, oxidase, indole, etc.) for clinically relevant colony types as correlated with Gram stain and most likely pathogen. See Table 3.3.2–5 for appropriate testing.
- c. Some specimen types will yield mixed cultures of >3 morphotypes of bacteria. Depending on the source of the specimen and whether these colonies represent potential pathogens, report such cultures with morphologic descriptions and minimal workup.
- d. Set up definitive biochemical tests or other identification protocols on clinically relevant isolates, if sufficient pure culture material is available and identification from rapid tests is not definitive per Table 3.3.2–5.
- NOTE:** The recommendations in Table 3.3.2–5, except as noted in the table, include the minimum required to identify the organisms listed with a greater than 95% accuracy as presented in the guidelines of CLSI (formerly NCCLS) (5). Further testing even from normally sterile sites is not needed if there is a definite identification from performance of the tests listed.
- e. Set up susceptibility tests as appropriate (references 1 and 2 and section 5).

Table 3.3.2–3 Colonial morphology on primary media

Organism(s)	Morphology on:		
	BAP or CNA ^a	CHOC	MAC/EMB ^b
<i>Escherichia coli</i>	Gray, mucoid, flat or convex, not swarming, may be beta-hemolytic ^c	Same as BAP	Pink/dark center and may have green sheen
<i>Proteus</i> spp.	Flat, gray, spreading ^c	Same as BAP	Colorless
<i>Pseudomonas aeruginosa</i>	Flat, gray-green, rough, may have spreading margins, metallic sheen, may be extremely mucoid ^c	Same as BAP	Colorless
<i>Neisseria gonorrhoeae</i>	Inhibited	Small, gray, entire, sticky	Inhibited
<i>Neisseria meningitidis</i>	Medium to large, creamy and gray, alpha-hemolytic ^c	Same as BAP, no hemolysis	Inhibited
<i>Haemophilus</i> spp.	Inhibited	Gray, raised, smooth, may be mucoid	Inhibited
<i>Moraxella catarrhalis</i>	Whitish, medium to large, raised or dome shaped ^c	Same as BAP	Inhibited
<i>Staphylococcus aureus</i>	Large, convex, white-yellow, creamy, opaque, may be beta-hemolytic	Same as BAP, no hemolysis	Inhibited/may be pinpoint
Coagulase-negative staphylococci	White-gray, raised, creamy	Same as BAP	Inhibited
Beta-hemolytic streptococci	Pinpoint to medium, zone of beta-hemolysis (clear zone) translucent, dull, gray	Same as BAP, no hemolysis	Inhibited
Viridans group streptococci	Pinpoint to medium, white-gray, caramel odor, alpha-hemolysis	Same as BAP	Inhibited
<i>Enterococcus</i> spp.	Gray, medium, usually no hemolysis	Same as BAP	Inhibited/may be pinpoint
<i>Streptococcus pneumoniae</i>	Umbilicate, alpha-hemolysis, transparent, may be mucoid, flattened, or teardrop shaped	Same as BAP	Inhibited
<i>Listeria monocytogenes</i>	Whitish gray similar to group B streptococcus, flat, narrow zones of beta-hemolysis	Same as BAP, no hemolysis	Inhibited
<i>Corynebacterium</i> spp.	White, dry, may be sticky	Same as BAP	Inhibited
Yeast cells	White, creamy, bread odor, “feet” extending from colony	Same as BAP	Inhibited/pinpoint

^a CNA, Columbia colistin-nalidixic acid agar.^b EMB reaction is listed after MAC reaction separated by a slash, if they differ.^c Inhibited on CNA.**V. PROCEDURE (continued)**

- f. Make subcultures of nonisolated colonies to appropriate media for later evaluation.
- g. Reincubate all primary and subculture media for an additional 8 to 12 h.
- h. Examine broth cultures by following the procedure below.
- i. Prepare preliminary report.

Table 3.3.2–4 Commonly used primary plating media^a

Medium	Expected isolates	Comments
CHOC	Most microorganisms, including <i>Haemophilus influenzae</i> , <i>Neisseria gonorrhoeae</i> , and <i>N. meningitidis</i>	Low agar content provides increased moisture required by some organisms. Contains heme and often has enrichments, such as IsoVitaleX and cysteine for fastidious organisms. (CHOC without enrichment should not be used for routine cultures.)
BAP with 5% sheep blood	Gram-positive and gram-negative organisms	General-purpose medium. Hemolysis can be observed. Stabbing creates area of reduced O ₂ , which can demonstrate oxygen-labile hemolysin O.
PEA	Gram-positive organisms	Inhibits gram-negative bacilli.
CNA with 5% sheep blood	Gram-positive organisms	Colistin inhibits gram-negative organisms, and nalidixic acid inhibits <i>Proteus</i> spp. Hemolysis may be observed. Columbia agar base is enriched for gram-positive organisms.
EMB	Gram-negative enteric bacilli	Differentiates L+ or Su+ (black, purple, metallic sheen) from L- or Su- (colorless or transparent). <i>Pseudomonas aeruginosa</i> is violet with filamentous margin. L and/or Su fermenters are generally normal enteric bacteria. Gram-positive organisms are inhibited by EMB. The agar concentration can be increased to 5% to inhibit swarming of <i>Proteus</i> spp. Enterococci can appear pinpoint.
MAC	Gram-negative enteric bacilli	Differentiates L+ (pink) from L- (colorless). Gram-positive organisms are inhibited by bile salts. The agar concentration can be increased to 5% to inhibit swarming of <i>Proteus</i> spp.
TM or MTM agar	<i>Neisseria gonorrhoeae</i> , <i>Neisseria meningitidis</i>	Vancomycin inhibits gram-positive organisms, colistin inhibits gram-negative organisms, and nystatin inhibits yeast cells. Trimethoprim lactate is added to inhibit <i>Proteus</i> spp. <i>Neisseria lactamica</i> may grow.

^a Abbreviations: L, lactose; Su, sucrose; TM, Thayer-Martin; MTM, modified Thayer-Martin. For other abbreviations, refer to Table 3.3.1–1.

V. PROCEDURE (continued)

- j. When colonies are too small to determine their characteristics and incubation has been less than 18 h, report as “too young to evaluate.” Reread plates later in the day.
- 2. Day 2
 - a. Read and record reactions and test results from previous day.
 - b. Reexamine plates and broth for changes and for colony morphologies not present the previous day.
 - c. Read anaerobic plates.
 - (1) Distinguish between organisms that are true anaerobes (i.e., those which grow markedly better or only under anaerobic conditions) and those that are facultative anaerobes (i.e., those which grow equally

Table 3.3.2–5 Common pathogens and rapid and conventional methods to identify them when suspected from colony morphology listed in Table 3.3.2–3

Organism	Presumptive identification	Additional tests for definitive identification ^a	Special requirements
Gram-negative rods			
<i>Escherichia coli</i>	1. Oxidase negative 2. Indole positive 3. Hemolytic	If not hemolytic, then either lactose positive and PYR negative or MUG positive	If lactose negative or not known, PYR will not identify. Confirm non-lactose fermenting from sterile sites with kit.
<i>Proteus mirabilis</i>	1. Spreading 2. Indole negative	No other testing if ampicillin susceptible. If ampicillin resistant, could be <i>Proteus penneri</i> .	If ampicillin resistant, confirm with positive ornithine or negative maltose fermentation.
<i>Proteus vulgaris</i>	1. Spreading 2. Indole positive	No other testing for definitive identification	
<i>Pseudomonas aeruginosa</i>	1. Oxidase positive 2. Indole negative 3. Fruity odor of grapes	Odor is definitive. If not fruity odor, blue-green pigment is definitive.	If not fruity or blue-green pigment, has green, fluorescent pigment, and grows at 42°C. ^b If cystic fibrosis patient, check colistin or polymyxin B; must be susceptible. Gelatin or lecithin will separate.
<i>Pseudomonas fluorescens/putida^b</i>	1. Oxidase positive 2. Indole negative	Fluorescent but no odor or growth at 42°C	
<i>Vibrio/Aeromonas^b</i>	1. Oxidase positive 2. Indole positive 3. MAC positive	Do kit and growth on MH agar with and without salt to identify (procedure 3.8.1)	If no growth on MAC, could be <i>Pasteurella</i> (see Table 3.18.2–3)
<i>Eikenella corrodens^b</i>	1. Gram-negative rod 2. Oxidase positive 3. Catalase negative 4. No growth on MAC 5. Nonhemolytic	Odor of bleach; ornithine positive	
<i>Haemophilus influenzae</i>	1. Gram-negative coccobacilli 2. Good growth on CHOC in 24 h and <i>not</i> on BAP, or satellite growth on BAP around staphylococci	Negative ALA test for porphyrin	The nonpathogenic <i>Haemophilus haemolyticus</i> is beta-hemolytic on horse or rabbit blood agar and often on sheep BAP. It is ALA negative but often grows unaided on BAP since it hemolyzes the blood. <i>Francisella tularensis</i> takes 48 h to grow as a smaller colony on CHOC; it does not grow on BAP.
Gram-negative cocci			
<i>Moraxella catarrhalis</i>	1. Gram-negative diplococci 2. Oxidase positive 3. Colonies on BAP move when pushed	Positive butyrate test	Gram stain morphology of “diplococcus” separates from other <i>Moraxella</i> organisms, which are “coccobacilli”
<i>Neisseria gonorrhoeae^b</i>	1. Gram-negative diplococci 2. Oxidase positive 3. No growth on nutrient or MH agar 4. 4+ positive catalase with 30% H ₂ O ₂	Sugar fermentation positive for glucose only, or identification with <i>Neisseria</i> kit, DNA probe, or serology (see procedure 3.9.3)	More than one test needed to confirm if negative result or abuse case
<i>Neisseria meningitidis^b</i>	1. Gram-negative diplococci 2. Oxidase positive 3. Growth on BAP	Sugar fermentation positive for glucose and maltose only, or identification with <i>Neisseria</i> kit	(See Table 3.18.2–1.) Use caution when handling culture.

Table 3.3.2-5 (continued)

Organism	Presumptive identification	Additional tests for definitive identification ^a	Special requirements
Gram-positive cocci			
<i>Staphylococcus aureus</i>	1. Catalase positive 2. Gram-positive cocci in clusters 3. Tube or slide coagulase or latex agglutination test positive	No other testing for definitive identification, except nonhemolytic in urine, which need tube coagulase to confirm agglutination test	For isolates from sterile sites, do tube coagulase test for further accuracy. ^b See procedure 3.18.1.
Coagulase-negative staphylococci	1. Catalase positive 2. Gram-positive cocci in clusters 3. Tube or slide coagulase or latex agglutination test negative	Colony not sticky; check for <i>Staphylococcus saprophyticus</i> in urine (novobiocin resistant) and <i>Staphylococcus lugdunensis</i> in blood (PYR positive; ornithine positive ^b)	For isolates from sterile sites, do tube coagulase test with 24 h of incubation for further accuracy. ^b
<i>Streptococcus pyogenes</i> (group A)	1. Gram-positive spherical cocci in pairs 2. Catalase negative 3. Hemolytic 4. Colony of >0.5 mm in diam with sharp edges	PYR positive	Confirm with serology or negative bile-esculin, if site is not respiratory. ^b
<i>Streptococcus agalactiae</i> (group B)	1. Gram-positive spherical cocci in pairs 2. Catalase negative 3. Small zone of hemolysis around translucent colony	CAMP positive or hippurate positive	If invasively collected specimen or not hemolytic, confirm hippurate with PYR (negative) and CAMP or serology. ^b
Viridans group streptococci ^b	1. Gram-positive cocci in pairs 2. Catalase negative 3. Alpha-hemolytic 4. Bile negative or colony whitish	PYR negative	From sterile sites, do LAP and susceptibility to vancomycin or kit identification. Other genera can mimic streptococci (e.g., <i>Aerococcus</i> and <i>Leuconostoc</i>). ^b
<i>Enterococcus</i> spp.	1. Gram-positive cocci in pairs 2. Catalase negative 3. Nonhemolytic	PYR positive Hemolytic colonies could be group A streptococci. If hemolytic, enterococci are bile-esculin positive. ^b	From sterile sites, do LAP (positive) to verify genus. <i>Lactococcus</i> cannot be ruled out. ^b Do motility to separate species and <i>Vagococcus</i> .
<i>Streptococcus pneumoniae</i>	1. Gram-positive, lancet-shaped cocci in pairs 2. Catalase negative 3. Alpha-hemolytic	Bile soluble or Quellung positive	If bile-resistant but typical colonies, confirm with optochin susceptibility or DNA probe. ^b
Gram-positive rods			
<i>Bacillus</i> spp. (and related spore-forming genera) ^b	1. Large gram-positive rods 2. Catalase positive 3. Spores present	Motile (nonmotile should be checked for <i>Bacillus anthracis</i>)	<i>Bacillus cereus</i> group (not <i>B. anthracis</i>) is beta-hemolytic and penicillin resistant, with cells >1 µm in diam.
Coryneform rods ^b	1. Gram-positive rods, not large 2. Catalase positive 3. Nonhemolytic 4. Not pigmented 5. Nonmotile	Not branching, or partially acid-fast	Kits are useful to identify to the species level. See procedure 3.11.7 for <i>Corynebacterium diphtheriae</i> culture.
<i>Gardnerella vaginalis</i> ^b	1. Tiny gram-variable rod 2. Catalase negative 3. Tiny nonhemolytic colonies grow better on CNA ^c and CHOC than BAP	Beta-hemolytic on human blood or SPS ^c sensitive or hippurate positive	Confirmatory tests not needed for vaginal samples if direct Gram stain is consistent (Appendix 3.2.1–3).
<i>Lactobacillus</i> spp. ^b	1. Gram-positive rods 2. Catalase negative 3. Alpha-hemolytic	H ₂ S negative, vancomycin resistant	<i>Erysipelothrix</i> is H ₂ S positive and vancomycin resistant. Vancomycin susceptible could be <i>Actinomyces</i> .

(continued)

Table 3.3.2–5 Common pathogens and rapid and conventional methods to identify them when suspected from colony morphology listed in Table 3.3.2–3 (*continued*)

Organism	Presumptive identification	Additional tests for definitive identification ^a	Special requirements
<i>Listeria monocytogenes</i> ^b	1. Tiny gram-positive rods without chaining 2. Catalase positive 3. Narrow zone of beta-hemolysis 4. Motile (tumbling) in wet mount	Bile-esculin positive and CAMP positive	If wet mount motility is questionable, check motility at 25°C but not at 35°C in semi-solid agar.
Yeast cells	Budding yeast in smear	1. “Feet” seen on BAP in 48 h or germ tube positive in ≤3h is <i>Candida albicans</i> 2. Tiny yeasts that are rapid trehalose positive or grow better on EMB are <i>Candida glabrata</i> .	Growth at 45°C separates <i>C. albicans</i> from <i>Candida dubliniensis</i> . See section 8 of this handbook for test methods and other identifications.

^a For procedures on biochemical testing, refer to procedure 3.17; for information on multitest biochemical kit tests, refer to procedure 3.18.

^b All testing requirements for identifications are found in NCCLS document M35 (5), *except* those noted by this footnote, which are taken from the references listed in Supplemental Reading and in procedures 3.18.1 and 3.18.2. The identifications from the NCCLS document are greater than 95% accurate without further testing.

^c CNA, Columbia colistin-nalidixic acid agar; SPS, sodium polyanethol sulfonate.

V. PROCEDURE (*continued*)

- well aerobically and anaerobically) by performing Gram stains and comparing colonies on anaerobic plates to those present on aerobic plates.
- (2) Note each colony type of suspected anaerobe, and subculture it to a section of CHOC and a section of an anaerobic medium; incubate the CHOC aerobically and the other anaerobically.
 - (3) Refer to section 4 of this handbook for further identification protocols for anaerobic bacteria.
 - d. Set up additional tests as needed, using references for unusual and difficult identifications (*see* procedures 3.18.1 and 3.18.2 and references listed in Supplemental Reading).
 - e. Prepare updated or final report, notifying appropriate persons of clinically or epidemiologically important results.
 - f. For positive cultures or isolates, whether complete identification is performed or not, hold a representative plate at room temperature for 5 days in case the clinician notifies the laboratory of the need for further studies.
 - g. Get immediate help from a supervisor or laboratory director when the identification or extent of identification is not clear. Delays in identification can affect patient care.
 - h. Perform susceptibility testing (section 5) for those organisms deemed significant, provided that standard methods are available for testing (1, 2).
 - i. Send subculture of significant isolates to reference laboratory when unable to identify or when confirmation is required or desired. See procedure 15.5 for details for packaging and shipping.
 3. For additional days, follow up identification and susceptibility testing procedures until all relevant isolates have been identified; then send a final updated report.
- NOTE:** When a culture of a slow-growing organism, e.g., *Nocardia*, is requested, media should be held and examined for 7 days. (*See* section 6 of this handbook.)

V. PROCEDURE (continued)

4. Isolates from positive cultures of sterile fluids (i.e., cerebrospinal, pleural, synovial, peritoneal, and pericardial), blood cultures, and tissues (i.e., bone, liver, brain, lung, etc.) should be saved for a minimum of 1 year so that further studies can be performed on the organism(s) if required for clinical or epidemiological purposes.
 - a. Save all primary pathogens for each patient.
 - b. Contaminants recovered from sterile-site cultures such as skin microbiota do not need to be saved unless their clinical relevance in a particular infection has not been definitively established.
 - c. Use one of the following methods for temporary isolate storage.
 - (1) Inoculate onto semisolid medium, such as sulfide-indole-motility agar (procedure 3.17.22) or cysteine Trypticase agar.
 - (2) Hold strict anaerobes in chopped meat.
 - (3) Hold fastidious organisms on CHOC slants or deeps overlaid with mineral oil.
 - d. For permanent storage, aerobic bacterial isolates should be frozen in TSB in 15% glycerol or in sterile skim milk at -70°C. Anaerobes should be frozen in rabbit blood or sterile skim milk.
5. Handling of broth cultures

Historically, tissues from surgery and fluids from selected normally sterile sites have been inoculated into broth, but this practice has been shown to be clinically neither relevant nor cost-effective in improving the recovery of pathogenic organisms from these types of specimens (3, 4). Prolonged incubation of broth cultures rarely enhances isolation of a primary pathogen and may result in overgrowth of commensal skin microbiota. The following procedure is included for laboratories continuing to perform broth cultures.

 - a. Incubate at 35°C and examine daily for 4 days.
 - b. For broths with apparent growth indicated by turbidity, fronds of growth, puff balls, pellicles, or sediment in the bottom of an otherwise clear tube, follow procedure below.
 - (1) If the direct plates have growth of fewer than three morphologic types and the broth is turbid, smear the broth for comparison with direct plates. Subculture if there is any suggestion that a different morphology is present. Use appropriate plates to select for aerobes and anaerobes depending on the smear results.
 - (2) If direct plates are negative, smear and subculture turbid broths to BAP and CHOC, using care not to contaminate the broth. If the morphology is suggestive of anaerobes, inoculate anaerobic BAP.
 - (3) If an organism is seen in the broth but not on solid media on subculture, try more nutritive media or special incubation conditions to isolate the organism. Also, evaluate uninoculated broth for evidence of "dead" organisms in medium.
 - (4) If the culture grew coagulase-negative staphylococci and gram-positive cocci are in the broth, subculture the broth to rule out the presence of *Staphylococcus aureus*.
 - (5) When in doubt, resmear a turbid culture, but a subculture need not be repeated. Use of acridine orange stain may be helpful. See procedure 3.2.2.
 - (6) When broth is entered, record on tube the date and indicate that broth was smeared and subcultured. This will allow quick evaluation of broths on subsequent days.

V. PROCEDURE (continued)

(7) Broth cultures should be incubated and examined for growth for a minimum of 4 days at 35°C. Broth cultures that are positive for a primary pathogen within this time may be discarded once the culture report is finalized.

c. Broth cultures that remain negative after 4 days of incubation at 35°C may be held at room temperature for up to 7 days after inoculation and examined for obvious growth only prior to discard.

■ **NOTE:** In the case of a clinically important infectious disease for which a pathogen has not been detected, refer specimen to a reference laboratory for molecular studies.

POSTANALYTICAL CONSIDERATIONS**VI. REPORTING RESULTS**

A. Report results of direct smear within 1 h of receipt for specimens from invasive procedures and 4 h for specimens from other sites.

B. Reporting the presence of pathogens

1. Enumerate each pathogen as indicated in Table 3.3.2–2.

2. Report preliminary results on all cultures, including presumptive identifications.

3. When reporting identifications that are suggestive but for which not all biochemical testing has been completed, preface the organism name with “probable” or “presumptive.”

4. If the identification changes after further testing, make a note of the change in the report.

5. If a species identification is not indicated, inform the physician by adding “NOS” (not otherwise specified) following the genus name and “spp.”

6. Use the following guidelines to report repeated isolation of the same organism.

a. Do not perform full identification and susceptibility testing on microorganisms, if the patient has had a positive culture from the same source within the last (*x*) days with what apparently is the same organism(s) and full identification and susceptibility testing were done on the previous isolate(s).

■ **NOTE:** For determination of (*x*) days, a good general rule is to repeat identifications every 7 days, if the morphology is the same. An exception would be for nonhemolytic staphylococci, all of which should be checked with a coagulase test. Policies on how often to repeat antimicrobial susceptibility testing (AST) vary and should be based on evaluation of local AST results and therapies used to treat disease. General guidelines include 7 days for oxacillin-susceptible staphylococci and most gram-negative rods, 4 days for *P. aeruginosa* and selected other gram-negative rods, and 30 days for vancomycin-resistant enterococci. If extended-spectrum beta-lactamases are present locally, additional susceptibility surveillance may be indicated.

b. Ensure that the current organism is morphologically consistent with the previous isolate(s) prior to reporting them as identical. Perform minimal procedures to confirm the identification (oxidase, indole, catalase, etc.), if possible.

c. Report the genus and species identification.

d. When referring identification to prior identification, indicate in the report that the identification is “presumptive” followed by the following comment after the organism name: “Refer to culture from [date] for complete identification [and susceptibility testing].” Use caution so that referred cultures are not referred to referred cultures.

VI. REPORTING RESULTS (continued)

- e. If susceptibility testing was performed (e.g., not sure it is the same, previous positive overlooked, etc.), record these results but do not report them, unless they differ from the prior result. Such reporting can distort the data in the antibiogram produced by the laboratory for epidemiologic surveys.

7. Possible plate and broth contaminants

- a. Review plates for possible plate contaminants (especially if the broth has no growth) before reporting.

■ NOTE: If a broth turns positive with a gram-positive organism after the broth has been sampled, the organism may have been introduced at the time of sampling.

- b. Do not report clear contaminants (those not on the streak).

- c. For questionable contaminants, add the following notation to the report: “[Organism name] present in culture, cannot distinguish true infection from plate contamination; consider confirmation of isolate with appropriate follow-up culture.”

- d. Investigate repeated isolation of the same mold or skin microbiota, and perform cleaning of laboratory equipment to prevent recurrence. Special attention should be paid to the potential pathogenicity of a “contaminant” isolated repeatedly from the same or different anatomic sites.

C. Reporting mixed cultures

1. When three or more microorganisms of questionable clinical significance are isolated, report as a mixed culture.

Example: “Culture yields abundant growth of >3 colony types of enteric gram-negative bacilli. Please consult microbiology laboratory if more definitive studies are clinically indicated.”

2. If only normal microbiota is observed, report as the following.

- a. Gastrointestinal

- b. Skin

- c. Genital

- d. Oral-nasal

- e. Staphylococcal skin (for wounds with only coagulase-negative staphylococci in adults)

■ NOTE: Reporting coagulase-negative staphylococci may be indicated for cluster epidemics in neonatal units or intensive care units

- f. Mixed anaerobic microbiota

- g. Mixed gram-positive microbiota, NOS (for mixed respiratory microbiota [staphylococci, diphtheroids, and enterococci] without viridans group streptococci)

- h. Alternatively, report as “No significant microorganisms isolated.”

- D. If no growth is observed on all media, send out report as “No growth in x days,” where “x” is the number of days the culture has been incubated.

1. When the culture has been incubated the number of days required based on the source, document that the culture report is final.

2. If incubation is continued beyond the final date but no more reports will be issued unless it turns positive, add the comment “Cultures will be held for _____ days.”

- E. Telephone clinically or epidemiologically critical results to the appropriate persons. Such results include the following.

1. Anything isolated from normally sterile source (blood, CSF, peritoneal fluid, tissue, etc.)

2. Pathogens of serious clinical or epidemiologic concern, for example, *Neisseria gonorrhoeae*, *Streptococcus pyogenes*, *Clostridium difficile* toxin, enteric pathogens, and *Mycobacterium tuberculosis*

VI. REPORTING RESULTS
(continued)

3. Take the initiative to immediately call any unusual, impressive finding that is of critical importance to patient care.
 - F. Document all testing on a hard copy or computerized work record.
 - G. Document reference laboratory identification when performed by reporting "Identification confirmed by" followed by reference laboratory name and address.
 - H. Document all processing and reporting errors in the report, and telephone report of such errors to the person who ordered the test.
-

VII. INTERPRETATION

- A. Report culture results with emphasis on the clinical importance and relevance to the diagnosis and treatment of the patient.
 - B. Reporting normal microbiota, mixed cultures, or questionable contamination with the same level of detail as done for clinically significant pathogens can lead to erroneous diagnoses and treatment of the patient.
 - C. Use the Gram stain of the specimen as a guide to interpretation of results.
 - D. Bacterial cultures from normally sterile sites typically contain a low number of organisms so that recovery of isolates from these types of specimens may be difficult to achieve. The primary specimen Gram stain should be compared to the morphotypes recovered from culture. Anaerobes or fastidious bacteria may be present if an organism is seen in the direct Gram stain but is not recovered on aerobic culture. Other methods may need to be used to isolate the agent, including incubation of cultures under different atmospheric conditions, the use of specialized media and stains, and the use of DNA probes and immunological tests.
 - E. Decisions regarding the performance of susceptibility testing must also consider the significance of the organism and the ability of the susceptibility testing to provide information that is not already available; e.g., *S. pyogenes* organisms in throat cultures are uniformly susceptible to penicillin, and susceptibility testing for this antimicrobial is not indicated.
-

VIII. LIMITATIONS

- A. Accurate reporting of culture results is limited to the expertise of the microbiologist who is able to recognize, evaluate, and pursue significant bacteria in the culture.
- B. False-negative results occur when one of the following occurs.
 1. Cultures are delayed in processing.
 2. Incorrect atmosphere or temperature of incubation is used.
 3. Medium does not support growth of the microorganism.
 4. Organisms are present in low numbers or the volume of specimen cultured is too low to detect them.
 5. WBCs and other body defense factors inhibit growth.
 6. Collection or transport of the specimen was not optimal.
 7. The microorganism cannot be cultured by any method available at the present time.
- C. False-positive results are due to the following.
 1. Mix-up of cultures from different patients
 2. Reporting of contaminants from the laboratory or collection process as pathogens in a culture
- D. The lack of isolation of a pathogen does not necessarily mean that the laboratory was unable to detect the agent, because other diseases can have the same presentations as infectious diseases.

REFERENCES

1. **Clinical and Laboratory Standards Institute.** 2006. *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically*, 7th ed. Approved standard M7-A7. Clinical and Laboratory Standards Institute, Wayne, Pa.
2. **Clinical and Laboratory Standards Institute.** 2006. *Performance Standards for Antimicrobial Disk Susceptibility Tests*, 9th ed. Approved standard M2-A9. Clinical and Laboratory Standards Institute, Wayne, Pa.
3. **Meredith, F. T., H. K. Phillips, and L. B. Reller.** 1997. Clinical utility of broth cultures of cerebrospinal fluid from patients at risk for shunt infections. *J. Clin. Microbiol.* **35**:3109–3111.
4. **Morris, A. J., S. J. Wilson, C. E. Marx, M. L. Wilson, S. Mirrett, and L. B. Reller.** 1995. Clinical impact of bacteria and fungi recovered only from broth cultures. *J. Clin. Microbiol.* **33**:161–165.
5. **NCCLS.** 2002. *Abbreviated Identification of Bacteria and Yeast*. Approved guideline M35-A. NCCLS, Wayne, Pa.
6. **Richmond, J. Y., and R. W. McKinney.** 1999. *Biosafety in Microbiological and Biomedical Laboratories*, 4th ed. Centers for Disease Control and Prevention, National Institutes of Health, Washington, D.C.

SUPPLEMENTAL READING

- Barrow, G. I., and R. K. A. Feltham (ed.).** 1993. *Cowan and Steel's Manual for the Identification of Medical Bacteria*. Cambridge University Press, New York, N.Y.
- Forbes, B. A., D. F. Sahm, and A. S. Weissfeld.** 2002. *Bailey and Scott's Diagnostic Microbiology*, 11th ed. Mosby, St. Louis, Mo.
- Holt, J. G., N. R. Krieg, P. H. A. Sneath, J. T. Staley, and S. T. Williams.** 1994. *Bergey's Manual of Determinative Bacteriology*, 9th ed. Williams & Wilkins, Baltimore, Md.
- Horvath, R. S., and M. E. Ropp.** 1974. Mechanism of action of eosin-methylene blue agar in the differentiation of *Escherichia coli* and *Enterobacter aerogenes*. *Int. J. Syst. Bacteriol.* **24**:221–224.
- Koneman, E. W., S. D. Allen, W. M. Janda, P. C. Schreckenberger, and W. C. Winn, Jr. (ed.).** 1997. *Color Atlas and Textbook of Diagnostic Microbiology*, 5th ed. J. B. Lippincott, Philadelphia, Pa.
- Krieg, N. R., and J. G. Holt (ed.).** 1984. *Bergey's Manual of Systematic Bacteriology*, vol. 1. Williams & Wilkins, Baltimore, Md.
- MacFaddin, J. (ed.).** 1985. *Media for Isolation-Cultivation-Identification-Maintenance of Medical Bacteria*. Williams & Wilkins, Baltimore, Md.
- Murray, P. R., E. J. Baron, J. H. Jorgensen, M. A. Pfaffer, and R. H. Yolken (ed.).** 2003. *Manual of Clinical Microbiology*, 8th ed. ASM Press, Washington, D.C.
- Parks, L. C. (ed.).** 1997. *Handbook of Microbiological Media by Ronald Atlas*, 2nd ed. CRC Press, Boca Raton, Fla.
- Sneath, P. H. A., N. S. Mair, M. E. Sharpe, and J. G. Holt (ed.).** 1986. *Bergey's Manual of Systematic Bacteriology*, vol. 2. Williams & Wilkins, Baltimore, Md.
- Truant, A. L. (ed.).** 2002. *Manual of Commercial Methods in Microbiology*. ASM Press, Washington, D.C.
- Weyant, R. S., C. W. Moss, R. E. Weaver, D. G. Hollis, J. G. Jordan, E. C. Cook, and M. I. Daneshvar.** 1995. *Identification of Unusual Pathogenic Gram-Negative Aerobic and Facultatively Anaerobic Bacteria*, 2nd ed. Williams & Wilkins, Baltimore, Md.

3.4.1

General Detection and Interpretation

[Updated March 2007]

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

When bacteria or fungi overcome the host's normal defense mechanisms and enter the bloodstream through the lymphatics or from extravascular sites, they can quickly disseminate throughout the body, causing severe illness. In addition, the by-products of their metabolism can lead to septic shock, among the most serious complications of infectious diseases. *Rapid recognition and immediate institution of appropriate treatment are essential.* Laboratory diagnosis of bacteremia and fungemia depends on blood cultures, which are probably the most important cultures performed by the microbiology laboratory. Because the culture methods are so sensitive, the procedure must be carefully controlled beginning at the preanalytical stage (collection), to avoid the misinterpretation of a procurement-as-

sociated skin commensal microorganism as an agent of infection.

One blood culture usually consists of blood from a single venipuncture inoculated into two separate bottles to accommodate the volume of blood removed (usually 20 ml for adults), since optimal blood-to-broth ratios are 1:5 to 1:10. The use of more than one formulation of medium (usually one aerobic and one anaerobic bottle) for each blood culture generally maximizes recovery of all possible pathogens. Adequate volume is the single most important factor in the laboratory detection of microorganisms in the bloodstream; the more blood cultured, the more likely a culture will be positive (13, 15, 16, 17, 26). In one study of adults, increasing the total volume cultured from 20 to 40 ml increased the yield by 19%; increas-

ing the volume from 40 to 60 ml increased the yield by an additional 10% (17). The total volume of blood drawn should be divided equally between at least two separate venipunctures, to allow evaluation of the recovery of a skin organism in a single positive set that could have resulted from contamination of the culture during collection (15). Sodium polyanethol sulfonate (SPS) is added as an anticoagulant, an antiphagocytic agent that inactivates complement, and a neutralizing agent to inhibit effects of many antimicrobial agents and antibacterial factors in blood (19). Media should contain 0.025 to 0.05% SPS; however, even at that level it can inhibit the recovery of some bacteria, such as *Neisseria* spp., *Streptobacillus moniliformis*, *Peptostreptococcus*, and *Gardnerella vaginalis* (21).

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING



Observe standard precautions.

■ NOTE: Refer to procedure 3.3.1 for additional details. This portion can be copied for preparation of a specimen collection instruction manual for physicians and caregivers. It is the responsibility of the laboratory director or designee to educate physicians and caregivers on proper specimen collection.

- A. See Appendix 3.4.1–1 for collection and testing for specialized microorganisms.
- B. Collect blood aseptically by venipuncture or from previously placed lines.

■ NOTE: Bone marrow culture should be reserved for culture for specific pathogens such as *Brucella*, *Salmonella*, *Listeria*, and fungi and mycobacteria; it adds little to detection of most other bacteria in the blood. Because of the complex nature of the collection procedure, it is more likely to result in a contaminated bacterial culture than a diagnostic one (M. K. York, unpublished data). Thus, routine cultures of bone marrow without a specific indication should be discouraged.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING (continued)

- C. Recommended total volume and numbers of blood cultures
1. Neonates to 1 year (<4 kg): 0.5 to 1.5 ml/tube, although at least 1.0 ml is preferred (16)
■ NOTE: Two separate venipunctures are generally not possible.
 2. Children 1 to 6 years old: 1 ml per year of age, divided between two blood cultures. For example, for a 3-year-old, draw 1.5 ml from each of two sites, for a total of 3.0 ml of blood. Consult with the physician, who is responsible for ordering the amount to be collected, especially if the child is below normal weight or has had previous venipuncture for other reasons.
 3. Children weighing 30 to 80 lb: 10 to 20 ml, divided between two blood cultures
 4. Adults and children weighing >80 lb: 30 to 40 ml, divided between two blood cultures
■ NOTE: At least 20 to 30 ml of blood in two draws is the minimal requirement (17).
- D. Timing of blood cultures
- NOTE:** Although drawing blood cultures before or during the fever spike is optimal for recovery, volume is more important than timing in the detection of agents of septicemia.
1. When acute sepsis or another condition (osteomyelitis, meningitis, pneumonia, or pyelonephritis) requires immediate institution of antimicrobial agent therapy, draw two blood cultures of maximum volume consecutively from different anatomic sites before starting therapy.
 2. For fever of unknown origin, subacute bacterial endocarditis, or other continuous bacteremia or fungemia, draw a maximum of three blood cultures with maximum volume.
 3. When it is appropriate to draw blood cultures from patients on antimicrobial therapy, they should be drawn when antimicrobial agents are at their lowest concentration.
■ NOTE: Use of a resin- and/or dematiaceous earth-containing medium may enhance recovery of microorganisms, especially staphylococci. However, appropriate dilution and action of some anticoagulants and antifoaming agents may also diminish the effect of antimicrobial agents. *If multiple cultures were drawn prior to the start of therapy, additional blood cultures from patients on antimicrobial therapy during the same febrile episode should be discouraged, since they are rarely positive* (9).
- E. Skin antisepsis and collection of blood from venipuncture
1. Select a different venipuncture site for each blood culture.
 - a. If poor access requires that blood for culture be drawn through a port in an indwelling catheter, the second culture must be from a peripheral site, because cultures drawn through catheters can indicate catheter colonization but may not be indicative of sepsis (1).
 - b. Do not draw blood from a vein into which an intravenous solution is running.
 - c. Except from neonates, draw the two blood cultures in succession. If the phlebotomy must be performed at the same site (usually because of bad veins), perform the second venipuncture at that site.
 2. Prepare the site.
 - a. Vigorously cleanse with 70% isopropyl or ethyl alcohol to remove surface dirt and oils. Allow to dry.
 - b. Swab or wipe concentric circles of tincture of iodine, moving outward from the center of the site.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING (continued)

- c. Allow the iodine to dry (about a minute), and avoid touching the site.
 NOTE: If povidone-iodine is used, it must be allowed to dry completely (about 2 min); 2% chlorhexidine gluconate in isopropyl alcohol may be used in place of tincture of iodine.
 - d. *For pediatric patients, omit the iodine step and clean two additional times with separate preparation pads saturated with 70% isopropyl alcohol or ethyl alcohol (24).*
 3. Prepare the septum of the blood culture bottle and the rubber stoppers on bottles or tubes. Label the bottles with the patient name and the date and time of draw. Site of draw may also be listed.
 4. Vigorously wipe septa with 70% alcohol and allow to dry completely, usually for 30 to 60 s.
 NOTE: Wiping the septum with iodine is usually unnecessary but may be considered if there is a history of problems with *Bacillus* spores or mold contamination.
 5. While wearing gloves, insert the needle into the vein and withdraw the blood. Use a new needle if the first attempt is not successful. *Do not repalpate the skin after it is disinfected.*
 6. *Apply a safety device to protect the phlebotomist from needle exposure.*
 NOTE: Safety devices consist of domes with internal needles that attach either to a syringe or directly to the tubing used in collecting the blood. The external port of these devices will accommodate a syringe or the end of a butterfly needle depending on the product (e.g., blood transfer device [catalog no. 364880; Becton Dickinson and Co., Paramus, N.J.], BacT/Alert blood transfer device [bioMérieux Inc., Hazelwood, Mo.], Angel Wing adapter [Sherwood Davis & Geck, St. Louis, Mo.]).
 7. Inoculate first the aerobic bottle and then the anaerobic bottle with *no more* than the manufacturer's recommended amount of blood.
 - a. For direct inoculation into the bottles from the needle apparatus, mark the side of the bottle with the manufacturer's recommended draw.
 - b. If using a needle and syringe, use the volume markings on the syringe to note the volume. *Hold the syringe plunger during transfer to avoid transfer of excess blood into bottles having a significant vacuum.*
 NOTE: There is no need to change the safety device between bottle inoculations (19).
 8. Thoroughly mix bottles to avoid clotting.
 9. After phlebotomy, dispose of needles in sharps container and remove residual tincture of iodine from the patient's skin by cleansing with alcohol to avoid development of irritation.
- F. Collection of blood from intravascular catheters
- NOTE:** Using either quantitative cultures (Appendix 3.4.1–2) or time to positive signal of cultures processed on an automated instrument, the comparison of cultures that are drawn through an indwelling intravenous catheter and through a peripheral site may be useful for diagnosis of catheter-related sepsis (1).
 1. Label bottles with patient name, site of draw, and date and time of draw.
 2. Disinfect the septum of the blood culture bottle and the rubber stoppers on bottles or tubes with 70% alcohol as for peripheral draw. Allow to dry completely, usually for 30 to 60 s.
 3. Using two separate alcohol preps, scrub catheter hub connection for 15 s with 70% alcohol. Air dry.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING *(continued)*

4. While wearing gloves, disconnect tubing or cap of catheter and attach syringe to collect discard blood (suggested amounts are 3 ml for adults and 0.2 ml for pediatric patients), which is not used for culture.
■ **NOTE:** Avoid drawing from lines within an hour of completion of antimicrobial agent administration.
 5. Using a new syringe, collect blood for culture through the hub. Quickly reconnect tubing.
 6. Connect filled syringe to safety system adapter.
 7. Holding the syringe plunger for control, inoculate the bottles with *no more* than the amount recommended by the manufacturer.
 8. Thoroughly mix bottles to avoid clotting.
- G. In addition to labeling the blood culture bottles or tubes with patient demographic information and collection time, indicate whether the collection was from a peripheral draw or a catheter draw and the initials of the phlebotomist.
- H. Specimen transport
1. Do not refrigerate blood cultures. Generally hold at room temperature until processed, for a maximum of 4 h.
 2. Refer to manufacturer's instructions for the appropriate method to store cultures prior to incubation in automated culture systems.
 3. Provide method of transport that will ensure that bottles are not broken in transit. Ensure that users follow exactly the instructions for sending blood culture bottles through pneumatic tube systems.
- I. Rejection criteria
1. Reject blood cultures that are received unlabeled.
 2. Do not process if the tube or bottle is cracked or broken.
 3. Labeled blood cultures are not rejected even if medium is expired, volume or number of bottles is insufficient, or bottles were received >12 h after collection, but develop an education plan to ensure that cultures are collected appropriately and numbers are not excessive. Document deficiency in report, as well as the effect on the reliability of the culture results.

III. MATERIALS

(See procedure 3.1 for vendor contact information.)

- A. Media (see references 19 and 27 for review of literature on comparison of blood culture media listed below)
1. Usually inoculate one aerobic and one anaerobic medium for each culture. In addition, or instead of the above, inoculate a similar medium containing resin or dematiaceous earth to absorb antimicrobial agents.
■ **NOTE:** For specialized patients (e.g., pediatric patients that do not have an abdominal abnormality), anaerobic cultures may not be indicated (5, 6, 14, 30). However, always inoculate at least two bottles for adults. Always include anaerobic cultures for febrile neutropenic patients and patients with diabetes or wound infections.
 2. Several companies supply media for automated systems, formulated to maximize detection based on the indicator system.
 - a. Bactec (BD Division Instrument Systems)
 - b. ESP (Trek Diagnostics)
 - c. BacT/Alert (bioMérieux Inc.).
■ **NOTE:** Follow manufacturer's instructions for inoculation, special handling, and incubation of blood culture media. Most systems accommodate 10 ml per bottle for adult bottles and less for bottles designed for pediatric use.

III. MATERIALS (continued)

3. Alternatives to automated systems
 - a. Broth media in bottles to be observed manually (TSB, supplemented peptone, THIO, anaerobic BHI)
 - b. Biphasic media (agar and broth in one bottle)
 - (1) BBL Septi-Chek (BD Microbiology Systems)
 - (a) Agar consists of CHOC, MAC, and malt agar.
 - (b) Broth options are BHI, BHI supplemented, TSB, TSB with sucrose, Columbia, THIO, and Schaedler broth.
 - (2) PML biphasic (PML Microbiologicals, Inc.)
 - (a) Agar consists of CHOC and BHI agar.
 - (b) Broth is TSB.
 - c. Lysis-centrifugation system of ISOLATOR (Wampole Laboratories).
 - B. Reagents and media for biochemical tests
 1. Agar plate media listed in Table 3.3.1–1
 2. Gram stain reagents
 3. Antimicrobial susceptibility testing (AST) system and beta-lactamase test (refer to section 5)
 4. δ-Aminolevulinic acid (ALA) reagent (procedure 3.17.3)
 5. Bile-esculin slants (procedure 3.17.5)
 6. 10% Bile (sodium desoxycholate [procedure 3.17.6])
 7. Catalase test reagent (procedure 3.17.10)
 8. Coagulase rabbit plasma and (optionally) staphylococcal agglutination tests (procedures 3.17.13 and 3.17.14)
 9. Disks (procedure 3.17.4)
 - a. 10 U of penicillin
 - b. 30 µg of vancomycin
 - c. 1 µg of oxacillin
 - d. Optochin (procedure 3.17.38)
 - e. 300 U of polymyxin B or 10 µg of colistin
 10. Spot indole reagent (procedure 3.17.23)
 11. Broth for motility (procedure 3.17.31)
 12. Ornithine decarboxylase (procedure 3.17.15)
 13. Oxidase test reagent (procedure 3.17.39).
 14. L-Pyrrolidonyl-β-naphthylamide (PYR) substrate and developer (procedure 3.17.41)
 15. Multitest gram-negative and gram-positive commercial automated, semiautomated, and manual kit identification systems, referred to as “kits.” For detailed information on these products, refer to Evangelista et al. (8).
 16. Media for identification of yeast and mold (see section 8)
 - a. CHROMagar (optional)
 - b. Calf serum for germ tube
 - c. India ink
 - d. Phenol oxidase test (caffein acid disk or birdseed agar)
 - e. Rapid trehalose test (section 8)
 - f. Urea agar (procedure 3.17.48)
 17. Other media as needed for special identifications
 - a. Acridine orange stain (procedure 3.2.2)
 - b. Media for H₂S detection (procedure 3.17.22)
 - c. Quellung test (procedure 3.17.42)
 - d. Leucine aminopeptidase (LAP) (procedure 3.17.26)
 - e. Salt (6.5%) broth (procedure 3.17.43)
 - f. Optional: serologic reagents for grouping *Salmonella* and *Shigella* (Appendix 3.8.1–1) or grouping beta-hemolytic streptococci into the Lancefield groups (procedure 3.11.8)
 - g. Rapid urea disk (procedure 3.17.48)
 - C. Supplies
 1. 3-ml syringes with safety apparatus or venting needle
 2. Alcohol (70 to 95%) and gauze
 3. Microscope slides

ANALYTICAL CONSIDERATIONS**IV. QUALITY CONTROL**

- A. Verify that plate media meet expiration date and QC parameters per current Clinical and Laboratory Standards Institute (CLSI; formerly NCCLS) document M22. See procedures 14.2 and 3.3.1 for further procedures.
- B. See individual tests in procedure 3.17 for biochemical test QC.
- C. Blood culture bottles
 - 1. For in-house-prepared culture media, verify that each lot of media will support the growth of bacteria likely to be present in blood cultures, including, but not limited to, the following microorganisms (as listed in Table 2 of CLSI document M22-A3 [3]).
 - a. Aerobic bottles
 - (1) *Pseudomonas aeruginosa* (ATCC 27853)
 - (2) *Streptococcus pneumoniae* (ATCC 6305)
 - b. Anaerobic bottles
 - (1) *Bacteroides fragilis* (ATCC 25285)
 - (2) *S. pneumoniae* (ATCC 6305)
 - c. Method of testing
 - (1) Prepare a broth culture of each microorganism equivalent to a 0.5 McFarland standard.
 - (2) Inoculate each bottle with 0.01 ml (10 µl) of suspension.
 - (3) Incubate for up to 5 days and observe for visible growth.
 - 2. Maintain records provided by manufacturers of commercial blood culture systems, documenting their QC testing.
 - **NOTE:** Regulatory agencies do not require routine QC checks by the user of commercially purchased blood culture bottles.
- D. Check CHOC on biphasic medium paddles for growth of *Haemophilus influenzae* and *Neisseria gonorrhoeae*.
- E. Prior to institution of a blood culture system, determine that it will support the growth of a variety of microorganisms, including fastidious gram-negative rods and cocci. Use bottles supplemented with human blood from volunteers for testing fastidious microorganisms.
- F. Contamination
 - 1. Have a system in place to determine if a culture has been collected from an intravascular catheter and whether a peripheral collection accompanied the line collection for adults. Educate clinicians on the need to collect both, to properly evaluate the culture results (1, 24).
 - 2. Monitor positive blood culture results regularly for skin contamination.
 - a. For purposes of determination of contamination rate, consider only skin contaminants from venipuncture as significant. These consist of the following.
 - (1) Coagulase-negative staphylococci (excluding pediatric and line collections)
 - (2) *Bacillus* species
 - (3) *Corynebacterium* species
 - (4) *Propionibacterium* species
 - b. Exclude positive cultures as skin contaminants if more than one blood culture from the same patient is positive for any of the microorganisms listed above, provided that for coagulase-negative staphylococci the antibiograms are consistent with the isolates being the same strain.
 - c. Include as skin contaminants all positive cultures, even if both bottles are positive or only one culture was collected (18).
 - d. Calculate the contamination rate by dividing the number of cultures containing skin contaminants by the total number of cultures collected by venipuncture.

IV. QUALITY CONTROL (continued)

3. Initiate additional training of staff involved with blood collection and processing if the contamination rate exceeds the expected rate for the institution. The rate should generally not exceed 3%, although many institutions have a goal to decrease the rate below 1% (24, 25) for cultures collected by venipuncture. When rates are high, separate calculations can be done for each phlebotomist and for each collection location.
- G. Validate true-positive blood culture rates.
 1. Calculate the true-positive culture rate by dividing the number of cultures positive by the total number of blood cultures received.
 2. The rate should be in the 6 to 12% range. If it is too low, too many cultures might have been drawn; if it is too high, not enough cultures may have been collected.
- H. Perform statistical analysis periodically to determine if the length of incubation is sufficient or can be decreased without loss of clinically meaningful information (2, 5, 7, 10, 20, 29).
 1. Tabulate the number of cultures positive on days 4 through 7.
 2. Delete those positives that are considered skin contaminants, as defined above.
 3. Delete those positives that are positive in another culture collected within 24 h before or after the delayed positive culture.
 4. For the remaining positive cultures, determine from chart review if they were considered contributing to true bacteremia.
 5. Review completed results with the infectious disease service to determine what is a reasonable incubation period for cost-effective detection of bacteremia.
- I. Use statistics to determine if bottles in use are appropriate for detection of local pathogens (12).
- J. Monitor compliance with volume and number of bottles submitted and number of cultures collected from catheter lines without an accompanying peripheral culture.

V. PROCEDURE



Observe standard precautions.

A. Laboratory processing of blood cultures

1. Safety
 - a. When working with blood cultures, keep the culture bottles within a biosafety cabinet or behind a shield, or wear a face mask.
 - b. Always wear gloves, because blood cultures contain material from patients that may harbor blood-borne pathogens.
 - c. Use needleless transfer devices or safety needles, and never recap them.
 - d. Dispose of needles and syringes in puncture-proof container.
2. Incubate blood cultures for the predetermined period at 35°C (usually 5 days, unless quality monitors indicate less time). In special circumstances (e.g., *Brucella* cultures), incubate for longer than standard policy. See procedure 3.4.2.
3. Maintain incubation conditions to allow recovery of microorganisms (follow manufacturer's instructions) and maintain rotation or agitation of the media if at all possible.
4. Examine the cultures at least daily, whether detection of positives is by visual inspection or by an automated system. For visual inspection, observe for hemolysis, turbidity, gas production, pellicle formation, "puffballs," and clotting, which are indicative of microbial growth (Table 3.4.1-1).

Table 3.4.1–1 Visible signs of growth caused by organisms commonly encountered in blood cultures

Microscopic observation	Associated microorganisms
Hemolysis	Streptococci, staphylococci, <i>Listeria</i> spp., clostridia, <i>Bacillus</i> spp.
Turbidity	Aerobic gram-negative bacilli, staphylococci, <i>Bac-teroides</i> spp.
Gas formation	Aerobic gram-negative bacilli, anaerobes
Pellicle formation	<i>Pseudomonas</i> spp., <i>Bacillus</i> spp., yeast cells
Clotting	<i>Staphylococcus aureus</i>
Visible colonies (“puffballs”)	Staphylococci, streptococci

V. PROCEDURE (continued)

5. For manual broth systems, perform at least one blind subculture to solid agar from visually negative bottles. Generally subculture after 72 h of incubation to detect most pathogens missed by visual inspection.
■ NOTE: Subculture of automated systems has little clinical utility (11).
6. Biphasic systems
 - a. For systems with agar paddle, remove plastic cap from bottle and securely attach paddle in biological safety cabinet while wearing gloves.
 - b. Invert bottle to inoculate agar, and incubate at 35°C.
 - c. Invert twice daily for the first 2 days and then daily for the remaining days of incubation. Observe broth and paddle for growth prior to each inversion.
7. In special circumstances when cultures appear to be negative, perform a Gram stain (procedure 3.2.1), wet mount (procedure 3.2.3), or acridine orange stain (procedure 3.2.2) from the culture or its sediment to determine the presence of organisms.
8. Discard negative bottles safely in infectious-waste container.

B. Culture methods for positive blood cultures

■ NOTE: Since positive results with biphasic systems and ISOLATOR will be detected by the presence of colonies, subculture protocols for broth systems detailed below are not applicable. However, inoculation of biochemical tests for identification does apply.

1. Gram stain a thin smear from the broth or agar immediately when suggestive of growth 24 h a day for optimal patient care.
2. Following Table 3.4.1–2, subculture to agar media and biochemical tests based on the Gram stain results.
■ NOTE: The extent of identification of isolates considered to be procurement-associated skin microorganisms should be determined in consultation with the institution’s infectious disease specialists.
3. Develop and validate a laboratory protocol to inoculate a kit identification system for gram-negative rods and direct AST. Follow protocol, as indicated in Table 3.4.1–2. Examples of methods are listed below.
■ NOTE: RBCs may interfere with some tests.
 - a. Dilute 2 drops of blood culture in 5 ml of water or saline and inoculate gram-negative rod manual kit test, such as API 20E, that will support a low inoculum (8).
■ NOTE: Citrate result is not reliable.
 - b. Centrifuge in serum separator tube at $1,400 \times g$ for 10 min, and adjust pellet on silicone layer equivalent to a 0.5 McFarland standard. Use inoculum for gram-negative kit identification system and susceptibility test system (23).

Table 3.4.1–2 Initial processing and reporting results from positive blood culture bottles^a

Gram stain result	BAP	BAP with disks ^b	CHOC	Anaerobe plate	Other tests	Report
Positive cocci in clusters	×	Oxacillin Penicillin Vancomycin			1 or 2 drops to coagulase plasma at 35°C ^c ; add AST ^d if coagulase positive or multiple “sets” positive	“Probable <i>Staphylococcus</i> spp.”; update to “Probable <i>Staphylococcus aureus</i> ” and notify physician if coagulase positive!
Positive cocci in chains or pairs	×	Penicillin Vancomycin Optochin CAMP test	×	×	1 drop to bile-esculin slant at 35°C ^c ; AST ^d if bile-esculin positive (Optional: Quellung or slide bile solubility if lancet-shaped pairs)	“Probable <i>Streptococcus</i> spp.”; update to “Probable <i>Enterococcus</i> ” if bile-esculin positive or “Probable <i>Streptococcus pneumoniae</i> ” if Quellung positive or bile soluble, and notify physician!
Small positive rods	×	Penicillin Vancomycin CAMP test	×	×	1 drop to bile-esculin slant at 35°C ^c ; wet mount	“Gram-positive rods”; update to “Probable <i>Listeria</i> ” if tumbling motile or bile-esculin positive
Large positive rods	×	Penicillin Vancomycin	×	Only if anaerobic bottle	For aerobic bottle, wet mount to rule out <i>B. anthracis</i>	“Gram-positive rods”; if diphtheroid-like or large and only in anaerobic bottle, report “Probable anaerobic gram-positive rods”
Negative rods	×	Polymyxin B or colistin disk	×	Only if anaerobic bottle	MAC or EMB; CNA or PEA; kit ^d ; AST ^d ; add BAP microaerobically if morphology is campylobacter-like	“Gram-negative rods” or “Probable <i>Campylobacter</i> ” if smear indicates gull wing shapes, or “Probable <i>Haemophilus</i> ” if morphology is consistent
Negative cocci	×	Penicillin	×	×	Warning: Staphylococci can appear gram negative; <i>Acinetobacter</i> organisms can appear as cocci.	“Gram-negative cocci” or “diplococci”; if tiny in sheets in anaerobic bottle, “anaerobic gram-negative cocci”
Yeast	×		×		CHROMagar; germ tube read at 2 h; India ink if round; 2 drops to urea slant at 35°C ^c	“Yeast”; update to “Probable <i>Cryptococcus</i> ” if urease positive or capsules present, and notify physician! Update to “ <i>Candida albicans</i> ” if germ tube positive.

^a Incubate aerobic plates in 5% CO₂ and anaerobic plates under anaerobic conditions. For any subsequent positive companion bottles or other positive bottles “collected” within the same 48 h as the first positive bottle, subculture only to BAP unless the Gram stain is different or the original culture did not grow on BAP. In addition, for subsequent positive cultures, inoculate MAC or EMB and either CNA (Columbia colistin-nalidixic acid agar) or PEA (phenylethyl alcohol agar) if gram-negative rods are present and a direct coagulase if gram-positive cocci in clusters are present.

^b Disk testing is a screen for identification of *Lactobacillus*, *Erysipelothrix*, *Bacillus*, *Leuconostoc*, *Pediococcus*, and vancomycin-resistant and -dependent *Enterococcus* or *Staphylococcus* organisms that are penicillin susceptible (see procedure 3.17.4 for details on potency and QC). Disks will also detect mixed cultures but are not a substitute for a standard susceptibility test. A streak of *S. aureus* ATCC 25923 perpendicular to but not touching the line of the initial inoculum will aid in identification of group B streptococci and *Listeria*.

^c Read test up to 4 h and update report and notify physician if test result is positive. Remove coagulase from incubator at ≤4 h and incubate at room temperature for remainder of 24 h.

^d For methods for preparation of inoculum for direct testing of automated or manual commercial multitest kit identification systems and for AST, see text.

V. PROCEDURE (continued)

- c. For MIC microtiter tray, place 1 drop of culture in 0.5 ml of BHI and incubate at 35°C for 3 to 4 h, with shaking; assume that it is equivalent to a 0.5 McFarland standard when entire volume is added to 5 ml of inoculation reagent. Check final inoculum with a colony count plate. Repeat AST if count is below $3 \times 10^5/\text{ml}$. Refer to section 5 for details on AST methods.
 - d. For disk method, add 10 drops of the blood culture or the broth of specimen in the case of a sterile fluid to 5 ml of TSB and inoculate Mueller-Hinton agar. If growth is not confluent at 18 h, repeat test or confirm with MIC method.
 - e. Alternatively, subculture to solid medium and incubate for 5 h. Scrape sufficient growth from plate to make inoculum for identification and AST. *This method is preferred for AST for S. pneumoniae but not staphylococci.*
4. Follow-up workup of positive blood culture isolates
 - a. Gram-negative rods growing on MAC or EMB
 - NOTE:** The presence of these isolates in blood cultures is most commonly associated with septic shock. Process and report as quickly as possible.
 - (1) As soon as results are available, read kit identification test and report results if there is a good probability that the result is accurate.
 - (2) Perform spot indole and oxidase to confirm kit results.
 - (3) Immediately report AST when available.
 - (4) Use caution to be sure that the culture is not mixed prior to reporting AST results (e.g., colistin or polymyxin B resistant). If the culture is mixed, contact physician to provide some information.
 - (5) For isolates identified as *Salmonella* or *Shigella*, perform serologic grouping to confirm identification.
 - b. *Haemophilus* or other fastidious gram-negative rods that do not grow on MAC or EMB
 - NOTE:** If colistin or polymyxin B resistant, the organism may be a poorly staining gram-positive rod.
 - (1) Set up ALA test if growth is only on CHOC—*H. influenzae* organisms are negative. See Table 3.18.2–2.
 - (2) Do beta-lactamase test.
 - (3) Do catalase, oxidase, and rapid urea if growing on both BAP and CHOC, and see procedure 3.18.2; for *Brucella* identification, see procedure 3.4.2.
 - c. Staphylococci
 - (1) Do catalase. Should be positive.
 - (2) Confirm that it is not sticky or bright yellow. If clearly yellow, call it a *Micrococcus* sp. See procedure 3.18.1 for other testing. If it is sticky, see Table 3.18.1–4b for possible *Rothia mucilaginosa* (*Stomatococcus mucilaginosus*).
 - (3) If there is a large zone of inhibition (>20 mm) around the *penicillin* disk, perform beta-lactamase test from zone around *oxacillin* disk. Omit testing if the AST is available and shows penicillin resistance.
 - NOTE:** Penicillin-susceptible coagulase-negative strains may be *Micrococcus* (see procedure 3.18.1).
 - (4) Do latex agglutination test for clumping factor and protein A as a confirmation of a *negative* tube coagulase test. See charts for gram-positive cocci in procedure 3.18.1 if results are discrepant or for other confirmatory tests.
 - (5) Do PYR if tube coagulase is negative, regardless of slide coagulase result. If positive, set up ornithine decarboxylase to rule out *Staphylococcus lugdunensis*.



It is imperative that these cultures be handled in a biosafety hood.

V. PROCEDURE (continued)

■ **NOTE:** Isolation of *S. lugdunensis* is more often associated with endocarditis and poor outcome than isolation of other coagulase-negative staphylococci; thus, it should be differentiated from other species (22). It is also problematic for susceptibility testing, since the breakpoints for oxacillin susceptibility of coagulase-negative staphylococci do not correlate well (4). *S. lugdunensis* is rarely, if ever, resistant to oxacillin, although the MIC is generally $\geq 0.5 \mu\text{g/ml}$. See section 5 for options for testing.

- (6) Read and report AST.
- (7) Check vancomycin disk. If resistant, see procedure 3.18.1 for identification. If coagulase positive, see section 5 for further AST on possible vancomycin-intermediate staphylococci.

d. Streptococci and other catalase-negative cocci

- (1) *S. pneumoniae* (should have zones around optochin and vancomycin disks.)
 - (a) Set up AST.
 - (b) If the growth is confluent and the zone size is $> 20 \text{ mm}$ to penicillin, report preliminary result as “Susceptible to penicillin by nonstandard method.”
- (2) If beta-hemolytic
 - (a) Do catalase—should be negative
 - (b) Check CAMP. Report “*Streptococcus agalactiae*” if positive.
 - (c) Do PYR. If PYR positive and bile-esculin negative, report “*Streptococcus pyogenes*.”
 - (d) Otherwise, Lancefield group and report.

- (3) Viridans group streptococci (have no zone around the optochin disk and a zone around the vancomycin disk).

■ **NOTE:** *Leuconostoc* and *Pediococcus* look like streptococci, but they are vancomycin resistant. See Table 3.18.1–4.

- (a) Do catalase—should be negative
- (b) A repeat Gram stain from original broth should show chains and pairs.
- (c) Do PYR (result should be negative). If PYR positive, refer to Table 3.18.1–4.
- (d) Do LAP. If positive, report “Viridans group streptococcus” and identify further according to laboratory policy. If present in only one culture, call physician to see if it is a significant isolate for identification and AST.

■ **NOTE:** For accuracy, strains may require several subcultures anaerobically prior to biochemical testing, especially with kit systems that require preformed enzymes for reactions. They also tend to present with various morphologies on plate culture. If there is any question of identification, set up growth at 42°C and 6.5% salt.

- (e) If there is no zone around the penicillin disk, notify caregiver that strain may be penicillin resistant. Set up MIC method for susceptibility to penicillin and an extended-spectrum cephalosporin (see section 5).
- (f) If there is no growth on BAP but growth on CHOC, set up satellite test (procedure 3.17.44) to determine if it is nutritionally variant streptococcus.

■ **NOTE:** Nutritionally variant streptococci satellite around staphylococci.

V. PROCEDURE (continued)

- (4) Enterococci
 - (a) Do catalase—should be negative or weakly positive
 - (b) Do PYR. If positive and bile-esculin was positive, report “*Enterococcus* species; identification to follow.”
 - (c) Set up species identification and AST per policy.
 - (d) If vancomycin resistant by disk
 - i. Inoculate broth for motility (procedure 3.17.31) and incubate at 30°C for 2 h to rule out motile species. If nonmotile, report “Vancomycin resistant by preliminary test.”
 - ii. Check for yellow pigment by sweeping with a cotton swab from direct BAP; swab will appear bright yellow.
 - iii. Confirm vancomycin result with MIC method.
 - iv. Use kit or sugar fermentation to differentiate *Enterococcus faecium* from *Enterococcus faecalis*.
 - (5) Other cocci: if sticky or vancomycin resistant or PYR positive and catalase negative or weakly positive and do not look like *Enterococcus* on Gram stain, see procedure 3.18.1.
- e. Gram-positive rods
- (1) Do catalase test.
 - (a) Catalase positive
 - i. Small rods with small zone of hemolysis and bile-esculin positive, set up motility tests at 22 and 35°C using semisolid agar. *Listeria* organisms are motile at 22°C. Check CAMP test. *Listeria monocytogenes* is positive.
 - ii. Large rods. Check for spores and motility.
NOTE: *Bacillus cereus* is hemolytic and motile; *Bacillus anthracis* is nonhemolytic and nonmotile.
 - iii. If present in only one culture of several collected, limit workup of diphtheroids (bile-esculin-negative, catalase-positive rods) unless physician indicates that the isolate may be significant. If isolate is significant and is penicillin resistant (no zone around the penicillin disk), notify caregiver, even though the test is not standard. Identify with commercial kit and/or biochemical tests (see procedure 3.18.1).
NOTE: *Corynebacterium jeikeium* is associated with catheter-related sepsis.
 - (b) Catalase-negative and vancomycin resistant: set up H₂S to rule out *Erysipelothrix* organisms, which are H₂S positive.
NOTE: *Erysipelothrix* and often lactobacilli are vancomycin resistant. Notify physician of vancomycin result.
 - (2) Refer to gram-positive rod flowcharts (procedure 3.18.1) for reporting and further testing.
- f. Potential anaerobic microorganisms: refer to section 4.
- g. Yeasts: refer to section 8.
- (1) Do phenol oxidase test if urease positive; omit test if patient has recent positive culture or cryptococcal antigen test.
 - (2) If urea negative
 - (a) Repeat germ tube if it was negative or questionable.
 - i. Call “*Candida albicans*” if germ tube positive.
 - ii. If hyphae are seen, call “*Candida*” species.
 - iii. If hyphae are not seen and isolate grows better on BAP than EMB, call “Yeast, not *Cryptococcus*.”
 - iv. If growth is better on EMB and the yeast is tiny on smear, call “Probable *Candida glabrata*” and set up rapid trehalose test.

V. PROCEDURE (continued)

- (b) Read CHROMagar at 48 h for species identification, or see section 8 for other methods.
- h. If there is no growth on initial subculture
 - (1) Subculture bottle anaerobically if anaerobic plates were not inoculated.
 - (2) Subculture to buffered charcoal-yeast extract agar for growth of *A. ipia* or other fastidious gram-negative rods.
 - (3) Subculture to BAP and incubate microaerobically for campylobacters.
- i. Hold positive cultures for at least a few weeks for further studies. See procedure 3.3.2 for methods to freeze isolates.

POSTANALYTICAL CONSIDERATIONS**VI. REPORTING RESULTS**

- A. For "No growth cultures," indicate the length of incubation: "No growth after x days of incubation" for both preliminary and final reports.
- B. Positive cultures
 1. Immediately report Gram stain results of all positive cultures, or additional organisms found in previously positive cultures, to the physician of record, with as much interpretive information as possible (using Table 3.4.1-2 for guidelines).
 2. Follow immediately with a written or computer-generated report including the following.
 - a. Number of positive cultures compared with total number of specimens collected for specific patient.
 NOTE: There is no justification for telling the physician that one or both bottles in the set are positive, because the number of positive bottles in a set does not reliably differentiate contamination from true infection (18).
 - b. Date and time of collection and receipt
 - c. Date and time positive result is reported and whether it was from a catheter draw or a peripheral draw.
 NOTE: Such information is useful in the diagnosis of catheter-related sepsis (1).
 - d. Name, phone number, and location of person taking report
Example: Positive culture reported to Dr. X on 07/01/04 at 1300 h.
 3. For single positive cultures with microorganisms generally considered skin contaminants (coagulase-negative staphylococci, viridans group streptococci, corynebacteria, *Propionibacterium* [28]), perform only minimal identification and do not perform AST. For single positive cultures with these potential skin contaminants (28), report result with a comment similar to the following: "One set of two positive. Isolation does not necessarily mean infection. No susceptibility tests performed. Contact laboratory for further information."
 4. Provide genus and species identification as soon as possible, using tests in Table 3.3.2-5 and charts in procedures 3.18.1 and 3.18.2.

VI. REPORTING RESULTS (continued)

5. For subsequent positive cultures, it is not necessary to repeat biochemical testing if the microorganism has the same Gram and colony morphology as the first isolate. Perform a few spot tests (catalase, coagulase, indole, PYR, etc.) to verify that it is the same strain. Report as “Probable [genus and species]; refer to prior positive for complete identification and susceptibility testing.”

VII. INTERPRETATION

- A. The report of a positive culture generally means that the patient is bacteremic. However, skin microbiota may contaminate the culture, causing a false-positive result or pseudobacteremia. Pseudobacteremias have many other causes.
 1. If organisms are seen but not cultured, dead organisms can be found in the medium components and produce a positive smear.
 2. *Bacillus* or other bacteria can be present on the nonsterile gloves of the phlebotomist (31).
 3. Laboratory contamination of equipment or supplies used in culture may contaminate the patient specimens.
- B. Mixed cultures can be present and account for a significant number of bacteremias. Be aware of this when examining smears and plates.
- C. Performance and reporting of AST are critical for timely patient care and increase the chance of appropriate therapy and cure.

VIII. LIMITATIONS

- A. Low levels of organisms may not be detected in the incubation interval of the culture.
- B. The media used may not support the growth of some organisms. Use of multiple formulations increases the yield.
- C. SPS may inhibit the growth and viability of the organism.
- D. Other diseases can present similarly to bacteremia, since there are many causes of fever of unknown origin.
- E. Bacterial metabolism may not produce sufficient CO₂ for detection in automated systems.
- F. There are a number of fastidious microorganisms that infect the blood that cannot be grown in routine culture of blood. These are presented further in the appendixes and other procedures that follow.

REFERENCES

1. Blot, F., G. Nitenberg, E. Chachaty, B. Raynard, N. German, S. Antoun, A. Laplanche, C. Brun-Buisson, and C. Tancrede. 1999. Diagnosis of catheter-related bacteremia: a prospective comparison of the time to positivity of hub-blood versus peripheral-blood cultures. *Lancet* **354**:1071–1077.
2. Bourbeau, P. P., and J. K. Pohlman. 2001. Three days of incubation may be sufficient for routine blood cultures with BacT/Alert FAN blood culture bottles. *J. Clin. Microbiol.* **39**:2079–2082.
3. Clinical and Laboratory Standards Institute. 2006. *Quality Assurance for Commercially Prepared Microbiological Culture Media*, 3rd ed. Approved standard M22-A3. Clinical and Laboratory Standards Institute, Wayne, Pa.
4. Clinical and Laboratory Standards Institute. 2006. *Performance Standards for Antimicrobial Susceptibility Testing*. Sixteenth informational supplement. Approved standard M100-S16. Clinical and Laboratory Standards Institute, Wayne, Pa.
5. Cornish, N., B. A. Kirkley, K. A. Easley, and J. A. Washington. 1998. Reassessment of the incubation time in a controlled clinical comparison of the BacT/Alert aerobic FAN bottle and standard anaerobic bottle used aerobically for the detection of bloodstream infections. *Diagn. Microbiol. Infect. Dis.* **32**:1–7.
6. Cornish, N., B. A. Kirkley, K. A. Easley, and J. A. Washington. 1999. Reassessment of the routine anaerobic culture and incubation time in the BacT/Alert FAN blood culture bottles. *Diagn. Microbiol. Infect. Dis.* **35**:93–99.
7. Doern, G. V., A. G. Brueggemann, W. M. Dunne, S. G. Jenkins, D. C. Halstead, and J. McLaughlin. 1997. Four-day incubation period for blood culture bottles processed with the Difco ESP blood culture system. *J. Clin. Microbiol.* **35**:1290–1292.
8. Evangelista, A. T., A. L. Truant, and P. P. Bourbeau. 2002. Rapid systems and instru-

REFERENCES (continued)

- ments for the identification of bacteria, p. 22–49. In A. L. Truant (ed.), *Manual of Commercial Methods in Microbiology*. ASM Press, Washington, D.C.
9. Grace, C. J., J. Lieberman, K. Pierce, and B. Littenberg. 2001. Usefulness of blood culture for hospitalized patients who are receiving antibiotic therapy. *Clin. Infect. Dis.* **32**:1651–1655.
 10. Han, X. Y., and A. L. Truant. 1999. The detection of positive blood cultures by the AccuMed ESP-384 system: the clinical significance of three-day testing. *Diagn. Microbiol. Infect. Dis.* **33**:1–6.
 11. Hardy, D. J., B. B. Hulbert, and P. C. Mignault. 1992. Time to detection of positive BacT/Alert blood cultures and lack of need for routine subculture of 5- to 7-day negative cultures. *J. Clin. Microbiol.* **30**:2743–2745.
 12. Istrup, D. M. 1978. Statistical methods employed in the study of blood culture media, p. 31–39. In J. A. Washington II (ed.), *The Detection of Septicemia*. CRC Press, West Palm Beach, Fla.
 13. Istrup, D. M., and J. A. Washington II. 1983. The importance of volume of blood cultured in the detection of bacteremia and fungemia. *Diagn. Microbiol. Infect. Dis.* **1**:107–110.
 14. James, P. A., and K. M. Al-Shafi. 2000. Clinical value of anaerobic blood culture: a retrospective analysis of positive patient episodes. *J. Clin. Pathol.* **53**:231–233.
 15. Kellogg, J. A., F. L. Ferrentino, J. Liss, S. L. Shapiro, and D. A. Bankert. 1994. Justification and implementation of a policy requiring two blood cultures when one is ordered. *Lab. Med.* **25**:323–330.
 16. Kellogg, J. A., J. P. Manzella, and D. A. Bankert. 2000. Frequency of low-level bacteremia in children from birth to fifteen years of age. *J. Clin. Microbiol.* **38**:2181–2185.
 17. Li, J., J. J. Plorde, and L. G. Carlson. 1994. Effects of volume and periodicity on blood cultures. *J. Clin. Microbiol.* **32**:2829–2831.
 18. Mirrett, S., M. P. Weinstein, L. G. Reimer, M. L. Wilson, and L. B. Reller. 2001. Relevance of the number of positive bottles in determining clinical significance of coagulase-negative staphylococci in blood cultures. *J. Clin. Microbiol.* **39**:3279–3281.
 19. Reimer, L. G., M. L. Wilson, and M. P. Weinstein. 1997. Update on detection of bacteremia and fungemia. *Clin. Microbiol. Rev.* **10**:444–465.
 20. Reisner, B. S., and G. L. Wood. 1999. Times to detection of bacteria and yeasts in BACTEC 9240 blood culture bottles. *J. Clin. Microbiol.* **37**:2024–2026.
 21. Reller, L. B., P. R. Murray, and J. D. MacLowry. 1982. *Cumitech 1A, Blood Cultures II*. Coordinating ed., J. A. Washington II. American Society for Microbiology, Washington, D.C.
 22. Teong, H. H., Y. S. Leo, S. Y. Wong, L. H. Sng, and Z. P. Ding. 2000. Case report of *Staphylococcus lugdunensis* native valve endocarditis and review of the literature. *Ann. Acad. Med. Singapore* **29**:673–677.
 23. Waites, K. B., E. S. Brookings, S. A. Moser, and B. L. Zimmer. 1998. Direct bacterial identification from positive BacT/Alert blood cultures using MicroScan overnight and rapid panels. *Diagn. Microbiol. Infect. Dis.* **32**:21–26.
 24. Waltzman, M. L., and M. Harper. 2001. Financial and clinical impact of false-positive blood culture results. *Clin. Infect. Dis.* **33**:296–299.
 25. Weinbaum, F. I., S. Lavie, M. Danek, D. Sissmith, G. F. Heinrich, and S. S. Mills. 1997. Doing it right the first time: quality improvement and the contaminant blood culture. *J. Clin. Microbiol.* **35**:563–565.
 26. Weinstein, M. P., S. Mirrett, M. L. Wilson, L. G. Reimer, and L. B. Reller. 1994. Controlled evaluation of 5 versus 10 milliliters of blood cultured in aerobic BacT/Alert blood culture bottles. *J. Clin. Microbiol.* **32**:2103–2106.
 27. Weinstein, M. P., and B. Reller. 2002. Commercial blood culture systems and methods, p. 12–21. In A. L. Truant (ed.), *Manual of Commercial Methods in Microbiology*. ASM Press, Washington, D.C.
 28. Weinstein, M. P., M. L. Towns, S. M. Quarey, S. Mirrett, L. G. Reimer, G. Parmigiani, and L. B. Reller. 1997. The clinical significance of positive blood cultures in the 1990s: a prospective comprehensive evaluation of the microbiology, epidemiology, and outcome of bacteremia and fungemia in adults. *Clin. Infect. Dis.* **24**:584–602.
 29. Wilson, M. L., S. Mirrett, L. B. Reller, M. P. Weinstein, and L. G. Reimer. 1993. Recovery of clinically important microorganisms from the BacT/Alert blood culture system does not require testing for seven days. *Diagn. Microbiol. Infect. Dis.* **16**:31–33.
 30. Wilson, M. L., M. P. Weinstein, S. Mirrett, L. G. Reimer, R. J. Feldman, C. R. Chuard, and L. B. Reller. 1995. Controlled evaluation of BacT/Alert standard anaerobic and FAN anaerobic blood culture bottles for detection of bacteremia and fungemia. *J. Clin. Microbiol.* **33**:2265–2270.
 31. York, M. K. 1990. *Bacillus* species pseudobacteremia in AIDS patients traced to contaminated gloves in blood collection. *J. Clin. Microbiol.* **28**:2114–2116.

SUPPLEMENTAL READING

- Barenfanger, J., C. Drake, and G. Kacich.** 1999. Clinical and financial benefits of rapid bacterial identification and antimicrobial susceptibility testing. *J. Clin. Microbiol.* **37**:1415–1418.
- Barenfanger, J., M. Short, and A. Groesch.** 2001. Improved antimicrobial interventions have benefits. *J. Clin. Microbiol.* **39**:2823–2828.
- Doern, G., R. Vautour, M. Gaudet, and B. Levy.** 1994. Clinical impact of rapid in vitro susceptibility testing and bacterial identification. *J. Clin. Microbiol.* **32**:1757–1762.
- Dunne, W. M., F. S. Nolte, and M. L. Wilson.** 1997. *Cumitech 1B, Blood Cultures III*. Coordinating ed., J. A. Hindler. American Society for Microbiology, Washington, D.C.
- O'Grady, N. P., P. S. Barie, J. G. Bartlett, T. Bleck, G. Garvey, J. Jacobi, P. Linden, D. G. Maki, M. Nam, W. Pasculle, M. Pasquale, D. L. Tribbett, and H. Masur.** 1998. Practice guidelines for evaluating new fever in critically ill patients. *Clin. Infect. Dis.* **26**:1042–1059.

APPENDIX 3.4.1-1**Specialized Processing of Blood for Detection of Unusual Microorganisms or Conditions****A. Transfusion reactions**

Collect blood cultures both from the patient and from the transfused product.

1. See procedure 13.13 for processing transfused blood.
2. Never discard the transfused product; return it to the transfusion service.
3. Always keep good records of what was cultured.

B. If the laboratory is notified that the isolation of unusual pathogens in blood is sought, perform an acridine orange stain (procedure 3.2.2) from the blood cultures prior to discarding bottles that do not indicate growth.

C. Inoculate special media or perform special testing listed below for the following organisms (listed alphabetically).

1. *Bartonella* spp. (formerly *Rochalimaea*)

Collect ISOLATOR tube and see procedure 3.4.3.

2. *Borrelia* spp. (relapsing fever)

- a. Visualize in a Wright or Giemsa smear of citrated blood. Prepare smears as for malaria (see procedure 9.8). *Borrelia* organisms are 10 to 20 µm long and 0.2 to 0.5 µm wide, with uneven coils. Scan at $\times 400$ and verify identification at $\times 1,000$ magnification. Read at least 60 fields on a thin smear and 20 fields on a thick smear.

- b. Look for twisting motion in a dark-field exam.

- c. Concentrate by centrifugation at $200 \times g$, remove plasma, and examine buffy coat as described above.

- d. Culture and mouse inoculation are done in research laboratories. Blood is generally shipped cold.

NOTE: *Borrelia burgdorferi* is best detected by PCR analysis of urine, blood, CSF, or lymph node aspirate.

3. *Brucella* spp.

Most blood culture systems will grow *Brucella* if incubation is extended with blind subculture; see procedure 3.4.2 for optional techniques and details.

4. *Ehrlichia*

- a. This parasite can occasionally be detected as an intracellular morula or intracytoplasmic inclusion in the WBCs visualized in a Giemsa or Wright stain of blood or buffy coat smear.

- b. PCR and serologic methods are better tests.

5. *Histoplasma* (see section 8)

NOTE: Although yeast cells are usually detected in aerobic broth systems, culture using biphasic media, an ISOLATOR tube concentrate, or the buffy coat from an SPS or heparin tube to specialized fungal media may allow faster and more extensive recovery of *Histoplasma* (2). For *Cryptococcus*, the cryptococcal antigen test is recommended for rapid diagnosis. The Myco/F lytic bottle (BD Diagnostic Systems) is designed for enhanced recovery of yeasts and mycobacteria. Bone marrow specimens are excellent for recovery of *Histoplasma*.

6. *Legionella* spp.

Refer to procedure 3.11.4.

7. *Leptospira* spp.

Refer to procedure 3.14 prior to collection of specimens.

APPENDIX 3.4.1-1 (continued)

8. *Malassezia furfur* (usually seen in catheter-related infections in infants receiving lipid-rich total parenteral nutrition) (1)
 - a. Automated systems may not give a positive signal for *M. furfur* (2).
 - b. Preferred method: collect a few milliliters of blood from the potentially infected catheter port.
 - (1) Prepare slide by spreading blood in a thin film and perform Gram stain.
 - (2) Streak BAP or noninhibitory fungal medium for isolated colonies.
 - (3) Add a drop of pure virgin olive oil (sterilization not necessary) the size of a dime onto the agar surface to provide essential nutrients.
9. Mycobacteria (*see section 7*)

■ NOTE: Collect specimen in special culture media supplied by the manufacturer of automated instruments or culture buffy coat from either SPS- or heparin-collected blood. ISOLATOR can also be used to concentrate the organisms. Testing should generally be limited to those patients with CD4 counts of less than 200 cells/ μ l, since the yield of mycobacteria is low from immunocompetent individuals. However, bone marrow is an appropriate specimen for culture from all patients.
10. Mycoplasmas (*see procedure 3.15*)
11. *Streptococcus moniliformis* (cause of “rat bite fever” and “Haverhill fever”)
 - a. The specimens of choice are blood and joint fluid. Because this organism is inhibited by SPS and requires more blood to grow, inoculate *twice* the amount of blood or joint fluid recommended by the manufacturer into the blood culture bottle. Pediatric bottles are good for this. Growth appears as crumbs in the liquid. Alternatively, collect citrated blood and inoculate it into several THIO tubes (2 ml/10 ml of THIO).

■ NOTE: Lymph nodes and abscesses are also good specimens for isolation of *S. moniliformis*. Inoculate as indicated below for subcultures.
 - b. Culture specimen or subculture positive blood culture broths as follows.
 - (1) Prepare agar with 20% rabbit, calf, or horse serum.
 - (a) Melt 20 ml of BHI, TSA, or heart infusion agar.
 - (b) Cool to 45 to 50°C.
 - (c) Add 4 to 5 ml of rabbit, calf, or horse serum.
 - (d) Mix, pour into petri dish, and allow to solidify.
 - (e) Inoculate with a few drops from the specimen or the positive culture.
 - (2) To THIO with 20% rabbit, calf, or horse serum added (2 ml/10 ml of broth), inoculate with a few drops from the positive culture or up to 2 ml of the specimen.
 - c. Incubate plates in high humidity with 5 to 10% CO₂.
 - d. *S. moniliformis* is gram negative and catalase, indole, nitrate, and oxidase negative and does not grow on MAC (3). It may be pleomorphic and show long filaments, chains, and swollen cells in broth culture. It is arginine positive. Confirmatory identification is usually done in a reference laboratory.

References

1. Marcon, M. J., and D. A. Powell. 1992. Human infections due to *Malassezia* spp. *Clin. Microbiol. Rev.* **5**:101–119.
2. Reimer, L. G., M. L. Wilson, and M. P. Weinstein. 1997. Update on detection of bacteremia and fungemia. *Clin. Microbiol. Rev.* **10**:444–465.
3. Weyant, R. S., C. W. Moss, R. E. Weaver, D. G. Hollis, J. G. Jordan, E. C. Cook, and M. I. Daneshvar. 1995. *Identification of Unusual Pathogenic Gram-Negative Aerobic and Facultatively Anaerobic Bacteria*, 2nd ed. Williams & Wilkins, Baltimore, Md.

Supplemental Reading

- Beebe, J. L., and E. W. Koneman. 1995. Recovery of uncommon bacteria from blood: association with neoplastic disease. *Clin. Microbiol. Rev.* **8**:336–356.
- Brouqui, P., and D. Raoult. 2001. Endocarditis due to rare and fastidious bacteria. *Clin. Microbiol. Rev.* **14**:177–207.

APPENDIX 3.4.1–2

Processing of the Lysis-Centrifugation System of ISOLATOR

I. PRINCIPLE

The ISOLATOR system (Wampole Laboratories, Cranbury, N.J.) consists of Vacutainer tubes for blood collection that contain lysing, antifoaming, and anticoagulating agents. The lysis of cells followed by centrifugation results in concentration of the microorganisms from the blood specimen into liquid Fluorinert, a water-immiscible substance. The Fluorinert mixture is cultured on plate media, resulting in simultaneous detection, enumeration, and isolation of organisms (2, 3). Because the system utilizes plates, it is not a sealed system; airborne contamination can be a problem in the inoculation and evaluation of the cultures (1, 4).

II. SPECIMEN

Collect specimen as for other blood cultures, and fill tube after careful disinfection of the stopper. Process within 8 h of collection.

III. MATERIALS

- A. Collection tube contains a lysis fluid of saponin, polypropylene, SPS, Fluorinert, and EDTA. Draw is either 1.5 ml for pediatric and bone marrow patients or 10 ml for adults.
- B. ISOSTAT press and rack
- C. ISOSTAT supernatant and concentrate pipettes and white cap
- D. Fixed-angle centrifuge
- E. Plate media for desired culture

IV. PROCEDURE

- A. Invert the tube several times to ensure adequate mixing at the time of collection.
- B. Centrifuge at $3,000 \times g$ for 30 min in a fixed-angle rotor.
- C. Place tube in ISOSTAT press.
- D. Wipe spun ISOLATOR tube, especially the top, with alcohol. Be generous with the alcohol; leave a drop on the top of the cap. Allow to dry. Do not disturb the microbial concentrate during the transfer. If it is disturbed, respin the tubes for another 30 min and allow the centrifuge to stop manually.
- E. Disinfect ISOSTAT press hammer with alcohol and allow to dry.
- F. Aseptically position cap over tube, and press down with ISOSTAT press. Do not touch top of tube. Gently pull the handle down as far as possible. The spike will penetrate the stopper and firmly seat the cap.
- G. Don gloves and place tube and plates for inoculation in biologic safety cabinet.
- H. Aseptically remove a supernatant pipette from the pouch. Squeeze bulb of pipette, collapsing it completely, and insert pipette into tube through the cap membrane. Do not touch pipette stem. Release bulb slowly, allowing the supernatant blood to be drawn into the pipette. When bubbles appear, remove the pipette *without* squeezing the bulb, and discard in a biohazardous-waste container. If bubbles were not present in the pipette, repeat the procedure with another pipette.
- I. Vortex the contents for at least 20 s.
- J. Aseptically remove a concentrate pipette from the package. Squeeze the pipette bulb, collapsing it completely, and insert into the tube through the cap membrane so that the stem reaches the bottom of the tube. Release bulb slowly, allowing the pellet to be drawn into the pipette.
- K. Remove pipette from tube and distribute the entire concentrate equally on media that will allow recovery of the organism sought. Spread evenly over surface of each plate, using separate loops or spreaders. Avoid the edges of the plates.
- L. If the lysis-centrifugation system is used alone, inoculate a portion of the sediment onto anaerobic plates or into anaerobic broth for recovery of anaerobes.
- M. Incubate the media under the correct conditions and for sufficient time to recover all pathogens sought.
- N. Incubate agar side down for the first 24 h of incubation to maintain contact of the inoculum with agar and to prevent concentrate from dripping onto the lid. After the initial incubation, incubate plates agar side up. Generally plates are incubated for 4 days unless unusual fastidious organisms are suspected.



It is imperative that these cultures be handled in a biosafety hood.

APPENDIX 3.4.1–2 (continued)**V. INTERPRETATION**

- A. The ISOLATOR system can be used for quantitative counting of microorganisms in blood. Use the formula below.

CFU/ml = total number of CFU on all plates/total number of plates on which organism is expected to grow \times (times) number of plates inoculated per blood volume

Example: Number of colonies: BAP—2; CHOC—3; CHOC—2; CHOC—1; CHOC—2. Therefore: $10 \text{ CFU}/5 \text{ plates} \times 5 \text{ plates}/1.5 \text{ ml} = 7 \text{ CFU/ml}$ (pediatric blood) or BAP—5; CHOC—7; CHOC—7; fungal selective agar—0. Therefore: $19/3 \times 4/10 = 2.5 \text{ CFU/ml}$ adult (10-ml tube).

- B. The number of plate contaminants can be problematic in the interpretation of the results with this system. Generally one colony of coagulase-negative staphylococci is ignored, but the laboratory should work to achieve no contamination and monitor the results to reduce false-positive reporting of larger numbers of skin microbiota.

References

1. Arpi, M., B. Gahrn-Hansen, and V. T. Rosdahl. 1988. Contaminating coagulase-negative staphylococci isolated in a lysis-centrifugation (Isolator) blood culture system. Application of different epidemiological markers for deduction of mode of contamination. *APMIS* **96**:611–617.
2. Henry, N. K., C. A. McLimans, A. J. Wright, R. L. Thompson, W. R. Wilson, and J. A. Washington II. 1983. Microbiological and clinical evaluation of the Isolator lysis-centrifugation blood culture tube. *J. Clin. Microbiol.* **17**:864–869.
3. Isenberg, H. D. 1983. Clinical laboratory comparison of the lysis-centrifugation blood culture technique with radiometric and broth approaches, p. 38–54. In A. Balows and A. Sonnenwirth (ed.), *Bacteremia—Laboratory and Clinical Aspects*. Charles C Thomas, Springfield, Ill.
4. Thomson, R. B., Jr., S. J. Vanzo, N. K. Henry, K. L. Guenther, and J. A. Washington II. 1984. Contamination of cultures processed with the Isolator lysis-centrifugation blood culture tube. *J. Clin. Microbiol.* **19**:97–99.

3.4.2

Brucella Cultures

[Updated March 2007]

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Brucella is a fastidious, aerobic, small, gram-negative coccobacillus that is slow growing and difficult to isolate. It is zoonotic, with four species being recognized as causing infection in humans: *Brucella abortus* (cattle), *Brucella melitensis* (goats, sheep, and camels), *Brucella suis* (pigs), and *Brucella canis* (dogs). Although species identification is of interest, it is *not* clinically important and is difficult to perform. Key characteristics of the species can be found in Shapiro and Wong (8).

Infections are seen in essentially two patient populations. The first group is individuals who work with animals and who have not been vaccinated against brucellosis. This patient population includes farmers, veterinarians, and slaughterhouse workers. *B. abortus* and *B. suis* are the agents most likely to cause infections in this group of individuals. They become infected either by direct contact or by aerosol from infected animal tissues.

Brucellosis is also seen in individuals who ingest unpasteurized dairy products

contaminated with brucellae. This is most likely to occur in individuals who travel to or migrate from rural areas of Latin American and the Middle East where disease is endemic in dairy animals, particularly goats and camels. *B. melitensis* is the most common agent seen in this patient population. Brucellae are included in the microorganisms at risk for being used in a bioterrorist event; refer to procedure 16.6 for further information on this agent's role in bioterrorism.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING



It is imperative that these cultures be handled in a biosafety hood.

III. MATERIALS

A. Media

1. Primary media
 - a. Biphasic blood culture
 - (1) Septi-Chek (CHOC, MAC, and malt agar with BHI or Columbia broth) (BD Diagnostic Systems)
 - (2) PML biphasic media (CHOC and BHI agar with TSB) (PML Microbiologica, Inc.)
 - (3) Castaneda bottles (TSB or brucella broth and agar)
(2)
2. Media for blind subculture or for positive cultures
 - a. BAP preferably with BHI base
 - b. CHOC
 - c. Brucella agar

b. Automated blood culture system

NOTE: Automated systems may require subculture to detect growth. The pediatric lysis-centrifugation method using a volume of 1.5 ml was not as sensitive as automated methods in one study (13).

2. Media for blind subculture or for positive cultures
 - a. BAP preferably with BHI base
 - b. CHOC
 - c. Brucella agar

III. MATERIALS (continued)

B. Reagents

1. Gram stain reagents
2. Catalase test (procedure 3.17.10)
3. Oxidase test (procedure 3.17.39)
4. Rapid urea-phenylalanine deaminase (PDA) disks (4) (Remel Inc.; Hardy Diagnostics) (procedures 3.17.40 and 3.17.48)

5. 10% Ferric chloride

6. Antiserum to *Brucella* from febrile agglutination test

C. Supplies

1. Incubator with 5 to 10% CO₂
2. Microscope slides

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

- A. Verify that plate media meet expiration date and QC parameters per current Clinical and Laboratory Standards Institute (formerly NCCLS) document M22. See procedure 14.2 and procedure 3.3.1 for further procedures.
 - B. For QC of blood culture broths, refer to procedure 3.4.1.
 - C. See individual tests in procedure 3.17 for biochemical test QC.
- NOTE:** Specific QC using *Brucella* organisms is not recommended because of the danger to laboratory workers handling such cultures. The confirmation of the identification of suspicious isolates generally should be done in a reference laboratory to avoid exposure of laboratory personnel to the organism.

V. PROCEDURE



Observe standard precautions.



It is imperative that these cultures be handled in a biosafety hood.

A. Laboratory processing of blood cultures

When working with suspected *Brucella* isolates, all manipulations of the culture, including oxidase and catalase tests and Gram staining, should be performed in a biological safety cabinet to minimize the possibility of exposure to aerosols of this organism. Gloves should be worn to protect from skin exposure to the culture. Laboratory-acquired cases of *Brucella* are not uncommon and include infections resulting by absorption of culture droplets through intact skin (5, 8, 9).

1. For biphasic systems, invert twice daily for the first 2 days and then daily for the remaining days of incubation. Incubate for 21 days.
2. Automated broth systems
 - a. Incubate broths for a minimum of 7 days and a maximum of 10 days.
 - b. Subculture an aliquot at day 10 to BAP, CHOC, or brucella agar and incubate plates in 5 to 10% CO₂ for at least 72 h.

NOTE: When using the BACTEC 9240 system in one study, no positives were detected by the system after an incubation time of 10 days (1). Subcultures were not done in this study. In a study using Peds Plus medium, all but 1 of 42 positive cultures were detected by the BACTEC instrument in 6 days; blind subculture yielded only the 1 additional positive (11). The success of this medium may be due to the decreased amount of sodium polyanethol sulfonate in the pediatric bottle. In another study with the BacT/Alert system, a terminal subculture at 10 days detected all but one of the 7 positive cultures that were not detected by the instrument prior to that time (11).

B. Laboratory processing of positive cultures from any site

1. *Brucella* is most often isolated from blood cultures and sterile fluids (e.g., synovial, cerebrospinal, and pleural fluids) and tissues (e.g., bone marrow, lymph nodes, and lung). Rarely is *Brucella* isolated from semen and urine cultures.
2. *Brucella* has been listed as a potential agent of bioterrorism by the CDC (see section 16). Sentinel laboratories (level A laboratories) in the CDC Laboratory Referral Network should follow the identification guidelines outlined below for the presumptive identification of this organism (3). *Brucella* iso-

V. PROCEDURE (continued)

lates from any clinical site or source should be immediately referred to a reference laboratory for further analysis.

3. Brucellae are class 3 organisms, and laboratory staff may acquire infections when working on positive cultures if the proper containment procedures are not followed. All cultures suspected or confirmed to contain *Brucella* spp. should be opened and handled in a BSL2 cabinet. Culture plates should never be smelled. Staff should wear personal protective equipment, including gloves, gowns, and goggles.
4. Look for colonies typically showing “dust-like” growth after overnight incubation on BAP and CHOC. Colonies are tiny but smooth, glistening, raised with an entire edge, and white to cream color at 48 to 72 h.
 - a. Gram stain suspicious colonies or broth; brucellae are tiny, gram-negative coccobacilli.
 - b. Perform catalase, oxidase, and rapid urea tests. Brucellae are *positive* by all three tests.
 - c. Optional: if the urea test is positive and the disk or tablet contains phenylalanine, do the following.
 - (1) Add 2 drops of 1 N HCl to acidify the alkaline urea reaction (optional).
 - (2) Perform PDA test by adding ferric chloride to tube. Brucellae are negative for PDA.
- **NOTE:** If a microorganism having the above reactions is isolated from a normally sterile site and is growing on BAP throughout the inocula, rather than just satelliting around a colony of staphylococci, it is presumptively identified as *Brucella* at this point and the physician should be informed.
5. If the colony is growing only on CHOC, subculture to CHOC, MAC, and BAP with a spot of *Staphylococcus aureus* ATCC 25923 added after inoculation of BAP with the suspected colony. If equivalent growth is seen on CHOC and BAP, but *not* on MAC or EMB, and the organism does not satellite around the staphylococcus, it is likely to be *Brucella* (procedure 3.17.44).
■ **NOTE:** The most common organisms misidentified as *Brucella* spp. are *Haemophilus* spp., which will satellite around the staphylococcus on the BAP. Brucellae grow on the entire BAP without inhibition.
6. Confirm identification with serologic test or send to a reference laboratory for serologic confirmation.
 - a. Prepare a suspension of the isolate in 1.0% formalinized saline to match a no. 2.0 McFarland standard.
 - b. Let sit overnight to kill the organisms.
 - c. Set up tube agglutination test using the positive control antiserum from the febrile agglutinin test and normal rabbit serum as a negative control.
 - d. As a positive control, test the positive control antiserum with the antigen in the febrile agglutinin test.

POSTANALYTICAL CONSIDERATIONS**VI. INTERPRETATION**

- A. If a gram-negative coccobacillus is isolated from a blood, lymph node, tissue, or joint culture and meets the following criteria, it is preliminarily identified as *Brucella*.
 1. Catalase positive
 2. Oxidase positive
 3. Urea positive (may be delayed), PDA negative
 4. Grows on both BAP and CHOC without satelliting around staphylococcus

Table 3.4.2-1 Urea-positive fastidious gram-negative coccobacilli similar to *Brucella*^a

Characteristic or test	<i>Brucella</i>	EO-2, EO-4 <i>Psychrobacter</i> <i>immobilis</i>	<i>Psychrobacter</i> <i>phenylpyruvicus</i>	<i>Oligella</i> <i>ureolytica</i> ^b	<i>Actinobacillus</i> spp. ^c	<i>Bordetella bronchiseptica</i> , <i>Ralstonia paucula</i> (IV c2)	<i>Bordetella</i> <i>hinzii</i>	<i>Haemophilus</i> spp. ^d
Gram stain morphology	Tiny CCB, stains faintly	Small CCB, rods, EO-2 in packets	CCB	Tiny CCB	CCB, rods	CCB, rods	CCB, rods	CCB
Catalase	+	+	+	+	V	+	+	+
Oxidase	+	+	+	+	+	+	+	V
Urea ^e	+	V	+	+	+	+	14% Positive	V
Motility	-	-	-	+ delayed	-	+	+	-
PDA	-	-	+	+	-	V	-	-
Nitrate	+	V	68%	+	+	V	-	NA
Nitrite	-	V	-	+	-	-	-	NA
TSP	Alkaline	Alkaline	Alkaline	Acid/acid	Alkaline	Alkaline	Alkaline	No growth
MAC, 48 h	-, poor	-, poor	-, poor	-, poor	-	+	+	-

^a Reactions extracted from references 8 and 10. NA, not applicable; V, variable; CCB, coccobacilli.^b *O. ureolytica* is primarily a uropathogen.^c *A. actinomycetemcomitans* is urea negative and rarely oxidase positive. Urea-positive *Actinobacillus* organisms are from animal sources.^d Grows only on CHOC, or on blood agar associated with staphylococcus colony.^e Use rapid urea test to increase sensitivity.^f TSI, triple sugar iron agar.

VI. INTERPRETATION (continued)

5. Does not grow on MAC
 - **NOTE:** If the microorganism is not identified as *Brucella* by these tests, consider other fastidious gram-negative rods, such as *Haemophilus aphrophilus*, *Eikenella*, *Actinobacillus actinomycetemcomitans*, *Capnocytophaga*, and *Kingella kingae* (HACEK group), which are urea negative (Tables 3.18.2–4 and 3.18.2–5). However, some brucellae are urea negative.
- B. The identification is confirmed if the isolate agglutinates with specific antiserum.
- C. Organisms confused with *Brucella* (see Table 3.4.2–1)
 1. If the isolate is from a urine culture, it could be *Oligella ureolytica*, a uropathogen with the same phenotypic characteristics as *Brucella*.
 2. *Psychrobacter phenylpyruvicus* has the same phenotypic characteristics but is rare and is PDA positive.
 3. *Haemophilus* does not grow on BAP without association with a staphylococcus colony.
 4. Some *Bordetella* spp. are urea positive but grow on MAC and are motile.

VII. REPORTING RESULTS

- A. For negative cultures, indicate the length of incubation: e.g., “No growth of *Brucella* after x days of incubation” for both preliminary and final reports, where “ x ” indicates the number of days of incubation.
- B. Positive cultures
 1. Report suspected cases to the physician and to infection control.
 2. Report preliminary and final identification to the genus level as soon as available following the testing listed in this procedure.
 3. Since brucellosis is a reportable disease in most states, notify the local or state health department of a positive culture (also see procedure 16.6).

VIII. LIMITATIONS

- A. When evaluating patients, leukocyte counts are often within normal limits and liver function tests are not abnormal. Such a nonspecific presentation makes it difficult to consider this pathogen.
- B. *Brucella* isolation is often delayed compared to that of other bloodstream pathogens, with peak isolation occurring at 3 to 4 days, compared to 6 to 36 h for most other pathogens.
- C. The laboratory diagnosis of brucellosis is made primarily by recovery of organisms from blood cultures. Overall blood cultures are positive in 50 to 90% of cases, but the chances of recovery decrease over time. Joint and bone marrow specimens may also be a good source of *Brucella*.
- D. In suspected cases, serologic testing of serum for *Brucella* agglutinins is a rapid method to diagnose brucellosis. Options include agglutination tests (Rose Bengal, Wright’s tube, Wright’s card, and Wright-Coombs), indirect immunofluorescence, complement fixation, and ELISAs. The standard with which all other methods are compared is Wright’s tube agglutination test. A titer equal to or greater than 1/160 is considered significant (7). These tests can have variable sensitivity since there is no standard for antigen preparations and methodology, even for the “standard” Wright’s tube agglutination test.
- E. Antimicrobial susceptibility tests are not necessary and should not be reported, since they erroneously show susceptibility to some agents. The correct identification is sufficient information for appropriate therapy. Tetracyclines (generally doxycycline) are the most active drugs and should be used in combination with streptomycin (or gentamicin or rifampin, if streptomycin is unavailable) to prevent relapse.

REFERENCES

1. **Bannatyne, R. M., M. C. Jackson, and Z. Memish.** 1997. Rapid detection of *Brucella* bacteremia by using the BACTEC 9240 system. *J. Clin. Microbiol.* **35**:2673–2674.
2. **Castaneda, M. R.** 1947. A practical method for routine blood culture in brucellosis. *Proc. Soc. Biol. Med.* **64**:114.
3. **Centers for Disease Control and Prevention.** 2000. Biological and chemical terrorism: strategic plan for preparedness and response (recommendations of the CDC Strategic Planning Workgroup). *Morb. Mortal. Wkly. Rep.* **49**(RR-4):1–14.
4. **Ederer, G. M., J. H. Chu, and D. J. Blazevic.** 1971. Rapid test for urease and phenylalanine deaminase production. *Appl. Microbiol.* **21**:545.
5. **Fiori, P. L., S. Mastrandrea, P. Rappelli, and P. Cappuccenelli.** 2000. *Brucella abortus* infection acquired in microbiology laboratories. *J. Clin. Microbiol.* **38**:2005–2006.
6. **Gotuzzo, E., C. Carrillo, J. Guerra, and L. Llosa.** 1986. An evaluation of diagnostic methods for brucellosis—the value of bone marrow culture. *J. Infect. Dis.* **153**:122–125.
7. **Moyer, N. P., G. M. Evans, N. E. Pigott, J. D. Hudson, C. E. Farshy, J. C. Feeley, and W. J. Hausler, Jr.** 1987. Comparison of serologic screening tests for brucellosis. *J. Clin. Microbiol.* **25**:1969–1972.
8. **Shapiro, D. S., and J. D. Wong.** 1999. *Brucella*, p. 625–631. In P. R. Murray, E. J. Baron, M. A. Pfaller, F. C. Tenover, and R. H. Yolken (ed.), *Manual of Clinical Microbiology*, 7th ed. ASM Press, Washington, D.C.
9. **Staszkiewicz, J., C. M. Lewis, J. Colvile, M. Zervos, and J. Band.** 1991. Outbreak of *Brucella melitensis* among microbiology laboratory workers in a community hospital. *J. Clin. Microbiol.* **29**:287–290.
10. **Weyant, R. S., C. W. Moss, R. E. Weaver, D. G. Hollis, J. G. Jordan, E. C. Cook, and M. I. Daneshvar.** 1995. *Identification of Unusual Pathogenic Gram-Negative Aerobic and Facultatively Anaerobic Bacteria*, 2nd ed. Williams & Wilkins, Baltimore, Md.
11. **Yagupsky, P.** 1999. Detection of brucellae in blood cultures. *J. Clin. Microbiol.* **37**:3437–3442.
12. **Yagupsky, P., and E. J. Baron.** 2005. Laboratory exposures to brucellae and implications for bioterrorism. *Emerg. Infect. Dis.* **11**:1180–1185.
13. **Yagupsky, P., N. Peled, J. Press, O. Abramson, and M. Abu-Rashid.** 1997. Comparison of BACTEC 9240 Peds Plus medium and Iso-lator 1.5 microbial tube for detection of *Brucella melitensis* from blood cultures. *J. Clin. Microbiol.* **35**:1382–1384.

3.4.3

Bartonella Cultures

[Updated March 2007]

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

The genus *Bartonella* comprises at least 19 species of gram-negative short (0.25 to 0.75 by 1 to 3 μm) rod-shaped bacteria, 7 of which have been implicated in human diseases (17). Arthropod vectors transmit the organism, and cats constitute the major reservoir of the species most commonly recognized (*Bartonella henselae*). *B. henselae* is the classic agent of cat scratch disease (CSD) and is globally endemic. It is also the agent of bacillary angiomatosis (BA) and peliosis (BP) in immunocompromised patients as well as in the immunocompetent (1, 6, 13). *Bartonella quintana* was historically responsible for outbreaks of trench fever in World War I, but it has more recently been found to cause BA and BP (2, 6). Both species have been associated with bacteremia and, not uncommonly, endocarditis. *Bartonella elizabethae*, *Bartonella clarridgeiae*, *Bartonella grahamii*, *Bartonella vinsonii* subsp. *arupensis*, and *Bartonella vinsonii* subsp. *berkhoffii* have been rarely associated with bacteremic or CSD-like illnesses (17).

Bartonellosis (or Carrión's disease), caused by *Bartonella bacilliformis*, can be

acquired by persons traveling to the Andes mountains of Peru, Colombia, and Ecuador. It has been found only in humans and is associated with exposure to sand fly vectors found on the western slopes of the Andes at elevations of approximately 2,500 to 9,000 ft (3). South American bartonellosis occurs in two forms, i.e., Oroya fever and verruga peruana, or Peruvian wart. Oroya fever is characterized by a severe acute-onset anemia, at the peak of which a majority of RBCs are infected with one to six organisms each, and massed intracellular bartonellae in vascular endothelial cells produce a diagnostic lesion that bulges into the lumen of the vessel. Verruga peruana is characterized by cutaneous eruptions ranging from small disseminated elevations to distinctive red-purple "cranberries." Verruga peruana is visually alarming but is benign and self-limiting. Conversely, Oroya fever is often life-threatening, and if not treated (with oral chloramphenicol), all of the erythrocytes become infected by the bacteria (15). In survivors of Oroya fever, verruga per-

uana may occur after the anemia and bartonellemia have subsided. In areas where the organism is endemic, verruga peruana is common but Oroya fever is rarely seen. The converse is true in areas of nonendemicity (3). Asymptomatic human infection may account for the continued presence of the disease. This organism remains the biggest threat to human health of all the *Bartonella* species (3). The causative agent, *B. bacilliformis*, is found in or on RBCs and vascular endothelial cells of infected patients. Diagnosis is generally made by serology, Wright stain of the peripheral blood, or culture.

Isolation of all *Bartonella* species is usually from blood and requires special procedures and long incubation (6, 9, 13). Contamination of cultures is common, and efforts must be taken to avoid loss of cultures from overgrowth with molds. Diagnosis is more often made serologically (4) or by histologic examination of biopsy samples of nodes, skin, or liver. PCR is also helpful for diagnosis (10).

II. SPECIMENS

NOTE: Cultures should only be performed by or submitted to a laboratory that is familiar with the culture and identification of the organism. Freshly prepared culture media optimize the chance for isolation of *Bartonella* spp.

- A. Process aseptically collected tissues, such as lymph nodes or liver, by direct smear, by inoculation into endothelial tissue culture cells, or by plating on blood agar or CHOC. Direct detection of *Bartonella* using molecular methods is usually performed in a reference laboratory (10). Smears may also be stained with Wright or Giemsa stain. Excised verrugae can also be stained or examined with specific antiserum.

II. SPECIMENS (continued)

- B. In cases of Oroya fever, collect thick and thin blood smears (preferably before antimicrobial therapy) from fresh drops of peripheral blood for staining with Wright or Giemsa stain (procedure 9.8.6 or 9.8.5, respectively).
 - C. Collect 10 ml of blood for culture into an ISOLATOR tube using sterile technique (see Appendix 3.4.1–2). Prior antimicrobial treatment negates the ability to culture the organism. Collection of more than one specimen may increase the yield of a culture.
- NOTE:** Automated blood culture systems have yielded *Bartonella*, but the organism typically does not produce sufficient CO₂ to be detected by the instrument. On day 8, remove a small aliquot of blood culture broth and stain with acridine orange (procedure 3.2.2) or observe motile organisms by wet mount using phase-contrast. If growth is observed, subculture to CHOC (13). La Scola and Raoult successfully cultured 1 ml of blood collected into a Vacutainer tube containing lithium heparin (Becton Dickinson Systems, Rutherford, N.J.) and plated it onto Columbia sheep blood agar (9). The ISOLATOR method in combination with rabbit blood agar has the best yield for most species (7, 16).

III. MATERIALS

- A. ISOLATOR system (Appendix 3.4.1–2)
 - B. Anaerobic jar or bag and CO₂-generating system
 - C. Sterile M199 tissue culture medium (Gibco, Invitrogen, Carlsbad, Calif.) for dilutions
 - D. Media
 - 1. Fresh CHOC (double poured [40 ml per plate] and less than 3 weeks old has higher yield)
 - 2. Heart infusion agar with 5% rabbit blood
 - 3. In addition to the media listed above, the following media have been used successfully for culture of *B. bacilliformis*.
 - a. Columbia agar with 10% whole horse, rabbit, or sheep blood (2, 3)
 - b. Biphasic media consisting of the following
 - (1) A solid medium of 10% defibrinated sheep blood, glucose, tryptose, NaCl, and agar
 - (2) A liquid RPMI 1640 medium (Mediatech, Fisher Scientific) enriched with HEPES buffer, sodium bicarbonate, and 10% fetal bovine serum (8)
- NOTE:** In some cases successful culture of *Bartonella* can only be achieved by initial cultivation in an endothelial tissue culture cell line or in liquid RPMI 1640 medium (2).

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

- A. Test each medium with a known *Bartonella* species (e.g., *B. henselae* ATCC 49793, *B. quintana* ATCC 51694, *B. bacilliformis* ATCC 35685 or ATCC 35686) to verify the ability to grow the organism.
- B. Check each medium for sterility by incubation of sample plates and animal blood products prior to culture.
- C. Refer to procedure 3.3.1 for other QC requirements.

V. PROCEDURE



It is imperative that these cultures be handled in a biosafety hood.

- A. Process ISOLATOR as in Appendix 3.4.1–2, with the following exceptions.
 1. Use gloves, and wash gloves with alcohol during each step to avoid contamination of the culture.
 2. Centrifuge ISOLATOR tube for 45 min at 3,000 × g in a fixed-angle rotor.
 3. Pop the top of the ISOLATOR tube using alcohol-soaked wipes with plastic backs for protection.
 4. Using the ISOLATOR supernatant pipette, remove all the supernatant blood without disturbing the Fluorinert. Discard blood in infectious-waste container.
 5. Add 0.2 ml (4 drops) of M199 to the pellet with a sterile pipette. Mix well with the pipette.
 6. Prior to inoculation, expose the plates and lids to UV light in a biological safety cabinet while drying completely.
 7. Inoculate the plates with the entire volume of Fluorinert. Use 0.3 ml/plate of chocolate and blood media. Streak for isolation.
 8. Place the plates in the anaerobe jar or bag, moisten with sterile water and sterile gauze, and activate a CO₂-generating system.
 9. Incubate bag or jar at 37°C for most species; however, incubate at 25 to 30°C without increased CO₂ for *B. bacilliformis* and *B. claridgeiae*.
- B. Examine plates for growth weekly for up to 5 weeks.
 1. Wear sterile gloves and swab often with alcohol. Work quickly and subculture if growth is observed or if contaminants are growing on the plate.
 2. Tiny colonies may be visible as early as day 4 under ×60 to ×100 magnification.
 3. *B. bacilliformis* colonies may be friable, small, dark, and round with an entire edge and may appear to have a halo under certain lighting conditions. A second colony type which may also be present is lighter, larger with an irregular edge, and not friable. Both cause an indentation of the agar under the center of the colonies (14).
- C. If tiny colonies are observed, perform a Gram stain with extended exposure to safranin or using carbol fuchsin as a counterstain. Examine for the presence of small, gram-negative, curved bacilli. These will be suggestive of *Campylobacter* or *Helicobacter* in morphology. Colonies visualized with a stereoscope take two forms: irregular, dry, and cauliflower-like or small, circular, tan, moist colonies that pit the agar.
- D. Perform wet mount motility and observe for twitching motility.
- E. Perform catalase and oxidase tests, which are either negative or weakly positive. Perform urea test, which should be negative (13).

■ NOTE: *Bartonella* organisms do not ferment or oxidize common carbohydrates or grow anaerobically. Other organisms, including *Mycobacterium tuberculosis*, could grow on the media used for culture. They are likely to be detected by Gram stain or wet mount and should be identified using charts and tables in other sections of this handbook.
- F. Confirm identification, using specific genetic or immunofluorescence tests and cellular fatty acid analysis.

■ NOTE: There are no Food and Drug Administration-cleared assays to identify this organism.

POSTANALYTICAL CONSIDERATIONS**VI. REPORTING AND INTERPRETATION OF RESULTS**

- A. Very slowly growing, small, gram-negative, curved bacilli with twitching motion, that are catalase and oxidase negative or weakly reacting but are urea negative, isolated from a blood culture are likely to be one of the *Bartonella* spp.
- B. Confirm identification usually by submitting the isolate to a reference laboratory. Serologic testing of the patient may be useful pending confirmation of the isolate's identity.
- C. For blood smears stained with Wright or Giemsa stain for *B. bacilliformis*, a presumptive diagnosis of bartonellosis can be made if all of the following three conditions are met.
 - 1. The patient has lived in or visited an area in which the disease is endemic.
 - 2. The patient's clinical signs are consistent with bartonellosis.
 - 3. Large numbers of RBCs are infected with multiple organisms. The organisms stain reddish violet and are pleomorphic and may be seen as slender rods (straight or bent) or as oval to round forms; they may be found singly or in segmenting chains. Forms range from tiny dots to large irregular shapes and spindles. Vascular endothelial cells swollen with rounded masses of organisms are diagnostic of Oroya fever.
- NOTE:** Excellent color plates are available in reference 15. The severe anemia is macrocytic and often hypochromic, with various aberrations; Howell-Jolly bodies and basophilic granules may be seen in addition to the bartonellae. In verruga peruana, the bartonellae are scattered among young cells and are definitive in the presence of the distinctive histology of the verrugae.

VII. LIMITATIONS

- A. Culture is generally unsuccessful if the patient has been treated with macrolide or aminoglycoside antimicrobial agents.
- B. Contamination of cultures is common, and every precaution should be taken to guard against mold growth. Exposure of plates and equipment to UV light and wearing gloves during all steps in the culture inoculation and observation of the cultures are important.
- C. Caution must be used in the interpretation of growth on the media, since mycobacteria, which are gram positive, and the yeast *Histoplasma* may be isolated from patients with similar symptoms.
- D. Many species of *Bartonella* are difficult to grow and identify. See reference 16.
- E. *Bartonella*-like structures due to other pathological conditions and other agents (e.g., *Haemobartonella* and *Eperythrozoon* spp.) may cause confusion in the diagnosis of Oroya fever. These other causes of RBC inclusions should be suspected in patients who have had no contact with an area of endemicity.
- F. Smears and cultures of specimens taken very early and late in the disease may be negative.
- G. Asymptomatic individuals may yield positive cultures or test positive for *Bartonella* antibodies.
- H. Reference laboratories offer serologic testing by either immunofluorescence assay (IFA) or EIA methods. Immunoglobulin G and M antibodies to crude and partially purified *Bartonella* antigens have been detected by EIAs. Antigens in both the IFA and EIA cross-react with *Chlamydia* and *Coxiella* antibodies, as well as with species within the *Bartonella* genus (5).
- I. Reference laboratories offer molecular testing because *Bartonella* culture is very slow and has a low sensitivity. The most common types of clinical specimens referred for *Bartonella* detection include lymph node and skin tissue and pus or

VII. LIMITATIONS (continued)

fluid from abscesses. The reference nucleic acid amplification method targets the 16S rRNA gene using a universal primer set that is neither species nor genus specific. Amplified products are characterized either by nucleic acid sequencing or hybridization probes (11). Amplification of the *rpoB* gene followed by restriction analysis of the products with three enzymes has also been used to identify *Bartonella* species in clinical specimens with the same sensitivity as the reference method (12).

REFERENCES

- Adal, K. A., C. J. Cockerell, and W. A. Petri. 1994. Cat scratch disease, bacillary angiomatosis, and other infections due to *Rochalimaea*. *N. Engl. J. Med.* **330**:1509–1515.
- Anderson, B. E., and M. A. Neuman. 1997. *Bartonella* spp. as emerging human pathogens. *Clin. Microbiol. Rev.* **10**:203–219.
- Birtles, R. J., N. K. Fry, P. Ventosilla, A. G. Cáceres, E. Sánchez, H. Vizcarra, and D. Raoult. 2002. Identification of *Bartonella bacilliformis* genotypes and their relevance to epidemiological investigations of human bartonellosis. *J. Clin. Microbiol.* **40**:3606–3612.
- Chamberlin, J., L. Laughlin, S. Gordon, S. Romero, N. Solorzano, and R. L. Regnery. 2000. Serodiagnosis of *Bartonella bacilliformis* infection by indirect fluorescence antibody assay: test development and application to a population in an area of bartonellosis endemicity. *J. Clin. Microbiol.* **38**:4269–4271.
- Knobloch, J., L. Solano, O. Alvarez, and E. Delgado. 1985. Antibodies to *Bartonella bacilliformis* as determined by fluorescence antibody test, indirect hemagglutination and ELISA. *Trop. Med. Parasitol.* **36**:183–185.
- Koehler, J. E. 1996. *Bartonella* infections. *Adv. Pediatr. Infect. Dis.* **11**:1–27.
- Koehler, J. E., F. D. Quinn, T. G. Berger, P. E. LeBoit, and J. W. Tappero. 1992. Isolation of *Rochalimaea* species from cutaneous and osseous lesions of bacillary angiomatosis. *N. Engl. J. Med.* **327**:1625–1631.
- Kosek, M., R. Lavarello, R. H. Gilman, J. Delgado, C. Magaña, M. Verastegui, A. G. Lescano, V. Mallqui, J. C. Kosek, S. Recavarren, and L. Cabrera. 2000. Natural history of infection with *Bartonella bacilliformis* in a non-endemic population. *J. Infect. Dis.* **182**:865–872.
- La Scola, B., and D. Raoult. 1999. Culture of *Bartonella quintana* and *Bartonella henselae* from human samples: a 5-year experience (1993 to 1998). *J. Clin. Microbiol.* **37**:1899–1905.
- Matar, G. M., J. E. Koehler, G. Malcolm, M. A. Lambert-Fair, J. Tappero, S. B. Hunter, and B. Swaminathan. 1999. Identification of *Bartonella* species directly in clinical specimens by PCR-restriction fragment length polymorphism analysis of a 16S rRNA gene fragment. *J. Clin. Microbiol.* **37**:4045–4047.
- Relman, D. A., J. S. Loutit, T. M. Schmidt, S. Falkow, and L. S. Tompkins. 1990. The agent of bacillary angiomatosis. An approach to the identification of uncultured pathogens. *N. Engl. J. Med.* **323**:1573–1580.
- Renesto, P., J. Gouvernet, M. Drancourt, V. Roux, and D. Raoult. 2001. Use of *rpoB* gene analysis for detection and identification of *Bartonella* species. *J. Clin. Microbiol.* **39**:430–437.
- Spach, D. H., A. S. Kanter, M. J. Dougherty, A. M. Larson, M. B. Coyle, D. J. Brenner, B. Swaminathan, G. M. Matar, D. F. Welch, R. K. Root, and W. E. Stamm. 1995. *Bartonella (Rochalimaea) quintana* bacteremia in inner-city patients with chronic alcoholism. *N. Engl. J. Med.* **332**:424–428.
- Walker, T. S., and H. H. Winkler. 1981. *Bartonella bacilliformis*: colonial types and erythrocyte adherence. *Infect. Immun.* **31**:480–486.
- Weinman, D. 1981. Bartonellosis and anemias associated with bartonella-like structures, p. 235–248. In A. Balows and W. J. Hausler, Jr. (ed.), *Diagnostic Procedures for Bacterial, Mycotic and Parasitic Infections*, 6th ed. American Public Health Association, Washington, D.C.
- Welch, D. F., and L. N. Slater. 2003. *Bartonella* and *Afipia*, p. 824–834. In P. R. Murray, E. J. Baron, J. H. Jorgensen, M. A. Pfaffer, and R. H. Yolken (ed.), *Manual of Clinical Microbiology*, 8th ed. ASM Press, Washington, D.C.
- Zeaiter, Z., Z. Liang, and D. Raoult. 2002. Genetic classification and differentiation of *Bartonella* species based on comparison of partial *ftsZ* gene sequences. *J. Clin. Microbiol.* **40**:3641–3647.

SUPPLEMENTAL READING

- Brouqui, P., and D. Raoult. 2001. Endocarditis due to rare and fastidious bacteria. *Clin. Microbiol. Rev.* **14**:177–207.
- Daly, J. S., M. G. Worthington, D. J. Brenner, C. W. Moss, D. G. Hollis, R. S. Weyant, A. G. Steigerwalt, R. E. Weaver, M. I. Daneshvar, and S. P. O'Connor. 1993. *Rochalimaea elizabethae* sp. nov. isolated from a patient with endocarditis. *J. Clin. Microbiol.* **31**:872–881.
- Ellis, B. A., L. D. Rotz, J. A. D. Leake, F. Samalvides, J. Bernable, G. Ventura, C. Padilla, P. Villaseca, L. Beati, R. Regnery, J. E. Childs, J. G. Olsen, and C. P. Carrillo. 1999. An outbreak of acute bartonellosis (Oroya fever) in the Urubamba region of Peru, 1998. *Am. J. Trop. Med. Hyg.* **61**:344–349.

3.5

Body Fluid Cultures (Excluding Blood, Cerebrospinal Fluid, and Urine)

[Updated March 2007]

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Infection of normally sterile body fluids often results in severe morbidity and mortality; therefore, rapid and accurate microbiological assessment of these samples is important to successful patient management. Most organisms infecting these sites are not difficult to culture, but determining the significance of low numbers of commensal cutaneous microorganisms does present a challenge (11). With the increased use of prostheses, immunosuppressive therapeutic regimens, and long-term care of individuals with chronic debilitating disease, the likelihood of true infection with commensal organisms has increased, making accurate diagnoses difficult. Care must be taken during specimen collection and transport to ensure that the specimen is not contaminated. Any microorganism found in a normally sterile site must be considered significant, and all isolates must be reported.

Culture of the specimen should include the most likely organisms to cause infec-

tion. Joint infections are commonly caused by *Staphylococcus aureus*, but *Streptococcus pyogenes*, *Neisseria gonorrhoeae*, anaerobes, *Kingella kingae*, and *Brucella* spp. may be likely causes of infectious arthritis (2, 6, 10, 11, 12, 20, 21). The lysis-centrifugation method (Appendix 3.4.1–2) is more sensitive than plate culture without lysis, but automated blood culture systems have been shown to be faster than lysis-centrifugation and have a higher yield (10, 18, 20, 21, 22). Pediatric bottles have the advantage of containing less sodium polyanethol sulfonate (SPS), which inhibits some organisms (20, 21). For prosthetic-joint infections, the importance of preoperative joint fluid cultures cannot be overemphasized. The diagnosis is increased by culture of large volumes, by culturing anaerobically, and by culturing more than one specimen (2, 5, 6, 9, 12, 18). Typically the Gram stain shows inflammation, without the presence of bacteria. In fact, a British collaborative study group has recom-

mended that Gram stains be abandoned for evaluation of elective revision arthroplasty, since they are not helpful to diagnosis (2).

Peritoneal fluid can be contaminated with numerous mixed gastrointestinal microbiota in cases of ruptured intestine, but in patients with chronic ambulatory peritoneal dialysis (CAPD) or spontaneous bacterial peritonitis (SBP), the likely pathogen is usually present in very low numbers. In CAPD the pathogens are staphylococci; viridans group streptococci; non-glucose-fermenting, gram-negative rods; *Candida albicans* and other *Candida* spp.; and fungi (19). Numerous studies have shown that culture of large volumes of fluid in blood culture bottles, rather than concentration by centrifugation, will result in a higher yield (1, 4, 5, 9, 14, 17, 19). Blood culture bottles are superior to the lysis-centrifugation system for diagnosis of SBP (17).

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING

A. Refer to Table 3.5-1 for commonly submitted body fluids and synonyms.

- NOTE:** Because the body may respond to infections with infiltration of fluid, some of these sites may have fluid accumulation only during infection.
- B. There are many other drainage devices that are named for the person or company inventing or popularizing them, e.g., Axiom sump drain, Shirley sump drain, Tenchoff catheter, Abramson drain, Chaffin drain, Lakey tube biliary drain, Wheaton T-tube biliary drain, etc. Since improved versions are introduced continually, each laboratory should familiarize itself with the systems used locally. Calling the departments of surgery and invasive radiology and communicating with the hospital's distribution department would provide the names and descriptions of drainage devices used to collect specimens.

Table 3.5-1 Types of body fluids submitted for culture

Fluid	Location	Synonym	Definition of synonym
Joint	At the union of two bones	Synovial	Viscid fluid of the joint cavity
Pleural	Within the membrane surrounding the lungs	Empyema Thoracentesis	Fluid with purulent exudate Fluid collected by aspiration following puncture of chest wall
Peritoneal	Within the membrane lining the abdominal cavity	Abdominal Ascites Paracentesis CAPD PV fluid	Same as peritoneal Abnormal accumulation of fluid in the cavity Fluid collected by aspiration following puncture of cavity Fluid from peritoneum of patient on CAPD Fluid from peritoneum of patient with a shunt tube inserted from the ventricles of the brain, under the skin into the peritoneal cavity
Pericardial	Within the membrane lining the cavity of the heart		
Cul-de-sac	A blind pouch between the anterior wall of the rectum and the posterior wall of the uterus	Culdocentesis	Fluid obtained by transvaginal puncture and aspiration of the cul-de-sac
Amniotic	Within the membrane of the fetus	Amniocentesis	Fluid obtained by puncture and aspiration of amniotic fluid

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING (continued)



Observe standard precautions.

- C.** Specimen collection: body fluid specimens collected by percutaneous aspiration for pleural, pericardial, peritoneal, amniotic, and synovial fluids

▣ **NOTE:** Refer to procedure 3.3.1 for additional details. The following can be copied for preparation of a specimen collection instruction manual for physicians and other caregivers. It is the responsibility of the laboratory director or designee to educate physicians and caregivers on proper specimen collection. Use care to avoid contamination with commensal microbiota.

1. Clean the needle puncture site with alcohol, and disinfect it with an iodine solution (1 to 2% tincture of iodine or a 10% solution of povidone-iodine [$\% \text{ free iodine}$]) to prevent introduction of specimen contamination or infection of patient. (If tincture of iodine is used, remove with 70% ethanol after the procedure to avoid burn.)
2. Aseptically perform percutaneous aspiration with syringe and needle to obtain pleural, pericardial, peritoneal, or synovial fluid. Use safety devices to protect from needle exposure.
3. Immediately place a portion of the joint fluid or peritoneal fluid collected from patients with CAPD or SBP into aerobic and anaerobic blood culture bottles, retaining some (0.5 ml) in syringe for Gram stain and direct plating.
 - a. Use the minimum and maximum volumes recommended by the bottle manufacturer (generally up to 10 ml is the maximum for each bottle).
 - b. Alternatively, inoculate the blood culture bottles after receipt in the laboratory.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING (continued)

4. Submit other fluids and the remainder of specimens placed in blood culture bottles in one of the following
 - a. A sterile, gassed-out tube or a sterile blood collection tube without preservative (e.g., sterile Vacutainer brand red-top tube); however, fluids in such tubes may clot during transport. Alternatively, fluids may be placed in a purple-top tube containing an anticoagulant such as heparin or SPS, although some fastidious organisms may be inhibited if the specimen is delayed in transport. EDTA and citrate anticoagulants are the most likely to inhibit microorganisms in fluid specimens and should not be used.
 - b. Anaerobic transport vial (for small-volume specimens)
 - c. Specimens received by the laboratory in a syringe with the needle still attached should be rejected because of the risk of a needless sharps exposure by laboratory staff. The physician should be immediately phoned to recollect the sample and send it in the proper container.
■ NOTE: Establish a policy for the proper collection and transport of clinical specimens not collected on swabs. Educate the physicians that needles must be removed from the syringe and the syringe cap secured prior to transport to avoid leakage.
 - d. Syringes that have been capped with a Luer-Lok (with needle removed) prior to transport may be accepted for culture provided the specimen has not clotted inside the syringe and there is no leakage during transport which could result in contamination of the culture. The laboratory may reject specimens that have clotted in a capped syringe because they cannot be processed for culture without inadvertently contaminating the specimen.
- D. Specimen transport
 1. Submit to laboratory as soon as possible and, if from a normally sterile site, alert laboratory that specimen has been submitted.
 2. Do not refrigerate.
 3. Label specimens with patient demographics and date, time, and site of collection: e.g., left knee joint fluid.
 4. Record the patient diagnosis for improved processing of specimen.
- E. Rejection criteria
 1. If only blood culture bottles are received, a Gram stain cannot be performed.
 2. Collect prior to antimicrobial therapy for greatest diagnostic sensitivity.
 3. Do not submit specimens from drains after they have been infused with antimicrobial agents.
 4. Call physician when fluid specimens are received on a swab.
■ NOTE: Swabs afford the least desirable sample for culture of body fluids and should be discouraged as devices for transport, since the quantity of sample may not be sufficient to ensure recovery of a small number of organisms.
 5. Contact physician if specimen is insufficient for the number of tests requested.
■ NOTE: Routine bacterial culture is sufficient for culture for *Candida* species, if blood culture bottles are used or specimen is centrifuged. Fungal cultures of joint and abdominal specimens are occasionally indicated (especially for *Blastomyces dermatitidis* and *Histoplasma capsulatum*) but should be discouraged routinely. AFB cultures should not be routine but should be limited to those with a clinical indication.
 6. Invasively collected specimens in leaky containers must be processed, but alert the physician of the possibility of contamination.

III. MATERIALS

- A. Media**
- 1. BAP
 - 2. CHOC
 - 3. MAC
 - 4. Nonselective anaerobic medium, e.g., CDC anaerobic sheep blood agar. See section 4 for media and methods.
 - 5. Special selective media for recovery of fastidious or unusual organisms (e.g., charcoal-yeast extract agar for *Legionella*). Refer to the table of contents for procedures for specific microorganisms.
 - 6. Add media selective for gram-negative or gram-positive organisms (see Table 3.3.1–1) based on Gram-stained smear of the specimen, which shows multiple morphologies of microorganisms.
 - 7. Broth known to support the growth of both fastidious aerobic organisms and anaerobes, to detect small numbers of organisms (6, 11, 12, 13, 15). Common broth culture media are as follows.
 - a. Blood culture bottles (added supplements have not been shown to be important [5, 9])
- b. Anaerobic BHI or TSB with 0.1% agar with or without yeast extract**
- c. Fastidious anaerobe broth or fastidious broth (Quelab Laboratories, Inc., Montreal, Quebec, Canada; Remel, Inc., Lenexa, Kans.) (15)**
- d. Brucella broth**
- e. THIO is least desirable as a broth to grow low numbers of aerobic organisms and yeasts. It is excellent for anaerobic organisms (13, 15).**
- 8. See procedure 3.17 for biochemical test reagents and procedure 3.3.1 for medium descriptions.**
- B. Gram stain reagents**
- C. Other supplies**
- 1. Cytocentrifuge and holders
 - 2. Cleaned glass slides
 - 3. Sterile Pasteur pipettes
 - 4. CO₂-generating system and 35°C incubator

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

- A. Verify that media meet expiration date and QC parameters per current Clinical and Laboratory Standards Institute (CLSI; formerly NCCLS) M22 document. See procedures 14.2 and 3.3.1 for further procedures, especially for in-house-prepared media.**
- B. For QC of blood culture broths, refer to procedure 3.4.1.**
- C. See individual tests in procedure 3.17 for biochemical test QC.**
- D. Periodically check laboratory processing to ensure there is no break in sterile technique and that media, stain reagents, collection tubes, slides, and other supplies are not contaminated with dead organisms.**
- 1. Filter sterilize (through a 0.22-μm-pore-size membrane filter) fluid from saved specimens, and periodically process along with patient samples.**
 - 2. No organisms should be detected on Gram stain, and these cultures should yield no growth.**

V. PROCEDURE



Observe standard precautions.

- A. Inoculation**
- 1. Process specimen as soon as it is received.**
 NOTE: Use of biosafety cabinet will avoid contamination of the culture or specimen as well as protect laboratory processing personnel.
 - 2. Record volume of specimen.**
 - 3. Record gross appearance, i.e., color, viscosity, light-transmitting properties, and presence of a clot.**

V. PROCEDURE (continued)

4. Inoculate BAP and CHOC with 2 or 3 drops of specimen.
 - a. If only blood culture bottles were received, subculture immediately to CHOC to isolate microorganisms that prefer agar plates for growth (e.g., *N. gonorrhoeae*).
 - b. If little specimen is received (1 or 2 drops), inoculate only CHOC and rinse tube with broth culture medium. Omit Gram stain. Note on report the volume received.
 5. If greater than 0.5 ml of specimen is received, do the following.
 - a. Inoculate up to 1 ml into 10 ml of broth culture media.
 - b. For greater than 2 ml of pericardial, peritoneal, amniotic, or synovial fluid specimens not received in blood culture bottles, inoculate aerobic and, if there is sufficient volume of specimen, anaerobic blood culture bottles. Do not add less than the amount recommended by the manufacturer, as the excess SPS may be inhibitory to growth of the infecting organism.
 6. Inoculate anaerobic medium with 2 or 3 drops of specimen, if an anaerobic broth or blood culture bottle was not inoculated. For pleural fluid or drainage fluid, perform only on request and if specimen was transported anaerobically,
 7. For peritoneal fluid contaminated with bowel contents, add MAC or EMB, Columbia colistin-nalidixic acid (CNA) or phenylethyl alcohol agar, and selective media for anaerobic isolation of mixed anaerobic microbiota, and omit broth cultures.
 8. For requests to culture for microorganisms that do not grow on routine laboratory media (e.g., *Legionella*), inoculate special media as appropriate (refer to Appendix 3.4.1–1 for details on isolation and detection of these microorganisms).
 9. Dispersion of clots
 - a. Pour the sediment containing clotted material into a sterile tissue grinder.
 - b. Add a small volume (<0.5 ml) of sterile broth to the grinder, and gently homogenize this mixture to disperse the clots and release any trapped bacteria.
- NOTE:** Do not grind the clots if a fungal culture is also requested for the sample. Tease the clots apart. Vigorous grinding can kill hyphal filaments.
- B. Prepare Gram stain by placing 1 or 2 drops of fluid specimen on an alcohol-rinsed slide.
 1. Allow the drop(s) to form one large drop. Do not spread the fluid.
 2. Air dry the slide in a biosafety cabinet or covered on a slide warmer.
 3. Fix smear with methanol and stain (see procedure 3.2.1 for staining details).
- NOTE:** The use of a cytocentrifuge for preparation of the Gram stain from clear aspirates or nonviscous body fluids is highly recommended (16).
- C. Incubation
 1. Incubate plates at 35 to 37°C in 5% CO₂. Alternatively, use a CO₂-generating system to provide the proper atmosphere if a CO₂ incubator is unavailable.
 2. Incubate broths at 35 to 37°C in ambient air.
 - D. Save some specimen at 4°C for 1 week for further testing.
 - E. Gram stain
 1. Interpret Gram stains immediately.
 2. If positive
 - a. Notify the physician immediately.
 - b. For invasively collected specimens, refer to positive blood culture workup (Table 3.4.1–2) for additional tests that can be performed on the direct specimen.
 - c. If mixed morphologies are seen in Gram stain, retrieve the specimen and inoculate selective aerobic (e.g., MAC and CNA) and anaerobic plates to select for mixed pathogens.

V. PROCEDURE (continued)**F. Culture examination**

1. Examine all plated and broth media for macroscopic evidence of growth at 24 h.
2. If no visible growth is observed on the culture media, reincubate.
 - a. Read aerobic plates daily for 4 days for invasively collected specimens and 2 days for drainage cultures.
■ NOTE: For specific requests or where patient history or clinical state suggests a slow-growing pathogen (e.g., *Brucella*), additional incubation time is appropriate.
 - b. For broth cultures, refer to procedure 3.3.2 for general guidelines and incubation.
 - c. Incubate blood culture bottles for 5 to 7 days. Extend incubation if *Brucella* culture is requested.
3. Cultures with growth
 - a. Notify physician of positive culture findings.
 - b. Correlate culture results with those of the direct Gram stain.
 - c. Identify all organisms, using the rapid tests listed in Table 3.3.2–5. For less common pathogens refer to procedures 3.18.1 and 3.18.2.
 - d. Do not perform complete identification if the physician indicates that the organism is a probable contaminant or that the isolate is one or two colonies of a coagulase-negative staphylococcus on one plate medium with no growth in the broth.
 - e. For peritoneal specimens that contain mixed gastrointestinal microbiota and no predominant organism, generally group organisms into “enteric,” “anaerobic,” and “skin” microbiota and do not identify further. However, screen the culture for the usual fecal pathogens (see procedures 3.8.1 and 3.8.2). Detection of yeasts, *S. aureus*, *Pseudomonas aeruginosa*, or, possibly, vancomycin-resistant enterococci may represent etiologies not covered by empiric regimens and should be listed individually.
■ NOTE: Empiric antimicrobial therapies are selected for the treatment of gastrointestinal tract microbiota, including anaerobes, enteric gram-negative bacilli, and enterococci. To attempt to isolate and report each of these agents is labor-intensive and does not add to the requirement to treat the patient with agents that are effective against all the usual microbiota.
 - f. Perform antimicrobial susceptibility testing as appropriate per CLSI standards (7).
 - g. Hold positive culture plates or tubes for at least 7 days or, preferably, freeze isolates for long-term retrieval.
 - h. Isolates from broth cultures may be contaminants. Analysis should be directed by the clinical picture.

POSTANALYTICAL CONSIDERATIONS**VI. REPORTING RESULTS**

- A. Refer to procedure 3.3.2 for general reporting procedure.
- B. When reporting negative results, indicate the incubation time in the report.
- C. If a plate contaminant is suspected, add a notation: “Unable to differentiate contamination of plate media from true infection; suggest repeat culture of appropriately collected specimen.”
- D. Report the probable genus and species as soon as preliminary tests are completed. For mixed abdominal microbiota, a general statement listing the groups of organisms may be sufficient, e.g., “Numerous enteric rods, numerous mixed anaerobic microbiota, including *Clostridium perfringens* and *Staphylococcus aureus*, present.”
- E. Document notification of positive findings.

VII. INTERPRETATION

- A. Generally, a positive culture indicates infection with the organism.
- B. WBCs are usually present with infections of body fluids.

VIII. LIMITATIONS

- A. False-positive cultures can result from contamination of the specimen with skin microbiota.
- B. False-negative results can be caused by low numbers of organisms, prior antimicrobial treatment, or the fastidious nature of the infective organism.
- C. Many organisms that cannot be easily cultured cause arthritis. These include *Borrelia burgdorferi*, the agent of Lyme disease.

REFERENCES

1. Alfa, M. J., P. Degagne, N. Olson, and G. K. M. Harding. 1997. Improved detection of bacterial growth in continuous ambulatory peritoneal dialysis effluent by the use of BacT/Alert FAN bottles. *J. Clin. Microbiol.* **35**:862–866.
2. Atkins, B. L., N. Athanasou, J. J. Deeks, D. W. M. Crook, H. Simpson, T. E. A. Peto, P. McLardy-Smith, A. R. Berendt, and the Osiris Collaborative Study Group. 1998. Prospective evaluation of criteria for microbiological diagnosis of prosthetic-joint infection at revision arthroplasty. *J. Clin. Microbiol.* **36**:2932–2939.
3. Bernard, L., B. Pron, A. Vuagnat, V. Gleizes, F. Signoret, P. Denormandie, A. Si-Ali, C. Perrone, J. M. Feron, J. L. Gaillard, and the Groupe d'Etude sur l'Ostéite. 2002. The value of suction drainage fluid culture during aseptic and septic orthopedic surgery: a prospective study of 901 patients. *Clin. Infect. Dis.* **34**:46–49.
4. Blondeau, J. M., G. B. Pylypchuk, J. E. Kappel, R. B. Baltzan, Y. Yaschuk, and A. J. Adolph. 1995. Evaluation of aerobic Bactec 6A non-resin- and 16A resin-containing media for the recovery of microorganisms causing peritonitis. *Diagn. Microbiol. Infect. Dis.* **22**:361–368.
5. Bourbeau, P., J. Riley, B. J. Heiter, R. Master, C. Young, and C. Pierson. 1998. Use of the BacT/Alert blood culture system for culture of sterile body fluids other than blood. *J. Clin. Microbiol.* **36**:3273–3277.
6. Brook, I., and E. H. Frazier. 1993. Anaerobic osteomyelitis and arthritis in a military hospital: a 10-year experience. *Am. J. Med.* **94**:21–28.
7. Clinical and Laboratory Standards Institute. 2006. *Performance Standards for Antimicrobial Susceptibility Testing*. Sixteenth informational supplement. Approved standard M100-S16. Clinical and Laboratory Standards Institute, Wayne, Pa.
8. Everts, R. J., J. P. Heneghan, P. O. Adholla, and L. B. Reller. 2001. Validity of cultures of fluid collected through drainage catheters versus those obtained by direct aspiration. *J. Clin. Microbiol.* **39**:66–68.
9. Fuller, D. D., T. E. Davis, P. C. Kibsey, L. Rosmus, L. W. Ayers, M. Ott, M. A. Saubolle, and D. L. Sewell. 1994. Comparison of BACTEC Plus 26 and 27 media with and without fastidious organism supplement with conventional methods for culture of sterile body fluids. *J. Clin. Microbiol.* **32**:1488–1491.
10. Lejbkowicz, F., L. Cohn, N. Hashman, and I. Kassis. 1999. Recovery of *Kingella kingae* from blood and synovial fluid of two pediatric patients by using the BacT/Alert system. *J. Clin. Microbiol.* **37**:878. (Letter.)
11. Morris, A. J., S. J. Wilson, C. E. Marx, M. L. Wilson, S. Mirrett, and L. B. Reller. 1995. Clinical impact of bacteria and fungi recovered only from broth cultures. *J. Clin. Microbiol.* **33**:161–165.
12. Nakata, M. M., and R. P. Lewis. 1984. Anaerobic bacteria in bone and joint infections. *Rev. Infect. Dis.* **6**(Suppl. 1):S165–S170.
13. Rinehold, C. E., D. J. Nickolai, T. E. Piccinni, B. A. Byford, M. K. York, and G. F. Brooks. 1988. Evaluation of broth media for routine culture of cerebrospinal and joint fluid specimens. *Am. J. Clin. Pathol.* **89**:671–674.
14. Runyon, B. A., M. R. Antillon, E. A. Akriavidis, and J. G. McHutchison. 1990. Bedside inoculation of blood culture bottles with ascitic fluid is superior to delayed inoculation in the detection of spontaneous bacterial peritonitis. *J. Clin. Microbiol.* **28**:2811–2812.
15. Seythes, K. D., M. Louis, and A. E. Simor. 1996. Evaluation of nutritive capacities of 10 broth media. *J. Clin. Microbiol.* **34**:1804–1807.
16. Shanholtzer, C. J., P. J. Schaper, and L. R. Peterson. 1982. Concentrated Gram stain smears prepared with a cytopsin centrifuge. *J. Clin. Microbiol.* **16**:1052–1056.
17. Siersema, P. D., S. de Marie, J. H. van Zeijl, D. J. Bac, and J. H. Wilson. 1992. Blood culture bottles are superior to lysis-centrifugation tubes for bacteriological diagnosis of spontaneous bacterial peritonitis. *J. Clin. Microbiol.* **30**:667–669.
18. von Essen, R., and A. Holtta. 1986. Improved method of isolating bacteria from joint fluids by the use of blood culture bottles. *Ann. Rheum. Dis.* **45**:454–457.

REFERENCES (continued)

19. von Graevenitz, A., and D. Amsterdam. 1992. Microbiological aspects of peritonitis associated with continuous ambulatory peritoneal dialysis. *Clin. Microbiol. Rev.* **5**:36–48.
20. Yagupsky, P. 1999. Use of blood culture systems for isolation of *Kingella kingae* from synovial fluid. *J. Clin. Microbiol.* **37**:3785. (Letter.)
21. Yagupsky, P., N. Peled, and J. Press. 2001. Use of BACTEC 9240 blood culture system for detection of *Brucella melitensis* in synovial fluid. *J. Clin. Microbiol.* **39**:738–739.
22. Yagupsky, P., and J. Press. 1997. Use of the Isolator 1.5 microbial tube for culture of synovial fluid from patients with septic arthritis. *J. Clin. Microbiol.* **35**:2410–2412.

SUPPLEMENTAL READING

- Smith, J. W., and E. A. Piercy. 1995. Infectious arthritis. *Clin. Infect. Dis.* **20**:225–231.

3.6

Catheter Tip Cultures

[Updated March 2007]

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Intravascular (intra-arterial or intravenous) catheter insertions cause a break in the skin barrier amenable to infection. The continued presence of this foreign body predisposes further to infection, which can result from either colonization of the catheter by the cutaneous microbiota or, less commonly, hematogenous seeding due to hub contamination. Since infected catheters are usually exposed directly to sterile spaces, there is a risk that the infection will result in bacteremia. Intravascular catheter-related infections are a major cause of morbidity and mortality in the United States. The Infectious Disease Society of America practice guidelines for management of these infections include culture of both catheters and blood (8). Central catheter infection may manifest as infection at the skin insertion site, as cellulitis along the soft tissues overlying the tunneled portion, or as bacteremia without evidence of external infection at either of these superficial sites. Bacteremia occurs secondary to infection of the central catheter or as a manifestation of more serious complications, including septic thrombophlebitis or endocarditis. Laboratory confirmation of central catheter infection requires recovery of the same organism from a patient's blood and from cultures of the catheter tip and/or intracutaneous catheter segment. Clinical policy should instruct physicians to submit both catheter segments and blood cultures to the laboratory. The most common infecting organisms are *Staphylococcus aureus*, enterococci, *Candida*

spp., *Pseudomonas aeruginosa*, *Enterobacteriaceae*, and resident skin organisms, such as coagulase-negative staphylococci and *Corynebacterium* spp. The significance of this last group of organisms is not always clear, since the catheter is removed through the skin.

Several methods have been used to diagnose catheter-related sepsis. Semiquantitative (roll plate) cultures, catheter flush cultures, and quantitative (sonication) cultures are more reliable diagnostic methods than qualitative cultures, where the tip is cultured in broth and a single contaminating microbe can give a positive result (8, 13). The semiquantitative technique is reported to distinguish infection from contamination, with counts of ≥ 15 CFU considered significant (6). The quantitative method and flush culture method use $\geq 10^2$ CFU as the significant count for most organisms. However, lower counts of particular organisms, including *S. aureus* and *Candida albicans*, may be clinically significant if there is associated bacteremia (12). If such counts are accompanied by signs of local or systemic infection, they are indicative of catheter-related infection (8). Quantitative and semiquantitative methods have high specificity in the diagnosis of catheter-related septicemia, but the quantitative sonication method is reported to be 20% more sensitive than the semiquantitative culture method in the diagnosis of catheter-related bloodstream infection (11). Quantitation with sonication

(see Appendix 3.6-1) is thought to be more sensitive because it is able to detect lumen colonization. At this time it is unclear whether the greater sensitivity of the quantitative method is clinically significant (8). Most laboratories perform the semiquantitative method, because of its ease of use and because there is a lack of scientific studies showing that the quantitative method offers added benefit.

The submission of two blood cultures (one peripheral and one through the catheter) is an alternate method to diagnose catheter-related bacteremia without removal of the catheter. A blood culture from a peripheral site and a blood culture through the catheter are submitted simultaneously, for comparison of time to positivity. If the culture through the catheter is positive >2 h before the peripheral culture is positive with the same microorganism, it is likely that the infection is catheter related (1, 7). Quantitative blood cultures can also be performed using the ISOLATOR system (Appendix 3.4.1-2). If the blood collected through the catheter has a count that is 5- to 10-fold greater than the count of the same microorganism from the peripheral vein, there is evidence of catheter-related sepsis (8). Alternatively, for staphylococci, comparison of the clone from the catheter and the peripheral site can also predict catheter-related sepsis. (See procedure 13.12 for further information on catheter cultures from an infection control perspective.)

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING



Observe standard precautions.

NOTE: Refer to procedure 3.3.1 for additional details. This portion can be copied for preparation of a specimen collection instruction manual for caregivers. It is the responsibility of the laboratory director or designee to educate physicians and caregivers on proper specimen collection.

- A. Collect two blood cultures, one through the catheter and one from a peripheral site at the time the catheter is submitted for culture.
- B. Clean the skin with 70% alcohol prior to catheter removal.
- C. Observing aseptic technique, hold the exposed end of the catheter and carefully remove the catheter from the patient with a sterile instrument, taking care to avoid contact with exposed skin. Holding the distal end over a sterile container, cut the tip with a sterile scissors, dropping the last 2 to 3 in. into the container.
- D. Avoid drying by sealing the tube and submit to the laboratory as soon as possible.
- E. Rejection criteria
 1. Do not accept urinary Foley catheter tips, chest tube tips, or abdominal drainage tips.
 2. Catheter tips should not be placed in saline or transport medium.
 3. Smears of catheter tips add little to the diagnosis of catheter-related sepsis.
 4. Submit catheter tips for culture only if there are signs of infection, i.e., inflammation at the insertion site, fever, signs of sepsis, or documented bacteremia in which the source is not apparent. Patients suspected of having a central line infection should also have blood cultures done (3).
 5. For ventricular-peritoneal shunts, peritoneal or spinal fluid is preferred to the catheter tip. If tip is submitted, culture it, but request submission of fluid. Refer to procedure 3.7 for workup of such cultures.
 6. If there is evidence of pus and local tissue infection at the central catheter insertion site, the skin surface should be cleansed of debris and pus collected from the deep wound using a sterile swab. There is rarely enough pus at the catheter exit site to collect an adequate sample by aspiration. Aspirates of pus or fluid expressed from an infected subcutaneous catheter tunnel track may be sent.

III. MATERIALS

- A. BAP
- B. MAC or EMB (optional)
- C. Forceps

D. Olive oil

Decant olive oil into a sterile container for use, but there is no need to sterilize the oil.

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

Verify that media meet expiration date and QC parameters per current Clinical and Laboratory Standards Institute (formerly NCCLS) M22 document. See procedures 14.2 and 3.3.1 for further procedures, especially for in-house-prepared media.

V. PROCEDURE



Observe standard precautions.

Semiquantitative method

- A. Using a sterile forceps, remove catheter tip from transport tube.
- B. Lay catheter tip on BAP.
- C. Roll the tip back and forth across the entire surface of a BAP (and, optionally, either MAC or EMB, in addition to the BAP) using sterile forceps and exerting slight downward pressure.
- D. If the tip is too long, using sterile scissors, cut the end closest to the top of the tube (proximal end) prior to rolling on the plates (Fig. 3.6–1). The proximal end may be rolled on a second plate, if desired.

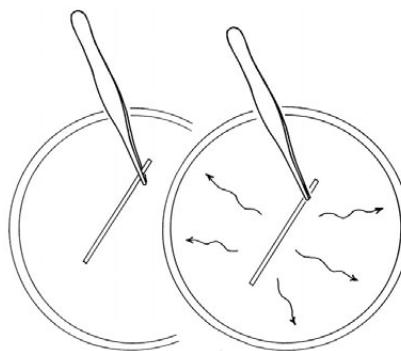


Figure 3.6–1 Inoculation of catheter tip to agar plate.

V. PROCEDURE (continued)

- E. If the specimen is from the total parenteral nutrition or is the catheter tip from a hyperalimentation line, culture also for *Malassezia furfur*.
 - 1. Using a sterile pipette, add a small drop of olive oil to the initial area of roll after inoculation of the blood plate.
 - 2. Do not allow the oil to spread beyond a small area of the plate.
- **NOTE:** The diagnosis of catheter infection with *M. furfur* is difficult with most blood culture systems. However, the organism load in the catheter is high and a Gram stain of a drop of blood from the catheter hub will generally demonstrate the infecting organisms, without removal of the catheter.
- F. Incubate plates at 35°C in CO₂.
- G. Certain types of catheter tips require prolonged incubation. Cultures from central line and vascular catheter tips should be held for up to 4 days, mainly to look for yeasts, including *M. furfur*, which grow as pinpoint colonies in 3 days. Cultures from central nervous system shunt tips (e.g., ventriculoperitoneal and ventriculoatrial shunts) should be incubated for up to 14 days in order to recover important slow-growing pathogens such as *Propionibacterium acnes*.
- H. Read the semiquantitative plates at 24, 48, 72, and 96 h.
 - 1. Count each type of colony isolated, comparing growth on each medium. Only enumerate the growth on the BAP; MAC or EMB is used only to provide separation of colony types.
 - 2. Identify to at least the genus level any of the following.
 - a. Each organism present from vascular catheter tips with colony counts of >15 CFU, including gram-positive rods.
 - b. For counts of <15 CFU, identify only significant pathogens (e.g., *Candida albicans*, group A streptococci, and gram-negative rods).
 - 3. Save plates with growth for 1 week for comparison in case blood cultures become positive.
 - 4. Antimicrobial susceptibility testing should be performed on all significant isolates from vascular catheters or grafts and central nervous system shunts even when the same organism is identified from the patient's blood. Since catheter tip infections are due to the formation of a biofilm on the device, different morphotypes of the same organism (e.g., two or more species of coagulase-negative *Staphylococcus*) may be recovered from the blood and the device. These morphotypes may have different antibiotic susceptibility profiles, and antibiotic therapy should be directed against the most resistant isolate.

POSTANALYTICAL CONSIDERATIONS**VI. REPORTING RESULTS**

- A. For any morphotype with a count of >15 CFU
 - 1. Enter the number of CFU isolated for each organism; i.e., 18 CFU followed by the organism name, at least to the genus level.
 - **NOTE:** Organisms are *not* enumerated in catheter tip cultures as few, moderate, and numerous.
 - 2. For gram-positive rods, report as “Coryneform rods; not otherwise specified” if reactions are appropriate per Table 3.3.2–5.
 - 3. If different morphologies of coagulase-negative staphylococci are present, report “[number] CFU of coagulase-negative staphylococci (mixed morphologies).”
- B. If any morphotype has <15 CFU
 - 1. Report significant pathogens by name.
 - 2. Group together mixed skin microbiota organisms (i.e., coagulase-negative *Staphylococcus*, diphtheroids, non-*Candida albicans* yeasts, *Acinetobacter*, or viridans group streptococci) as, e.g., “25 CFU of mixed skin microbiota.”
 - 3. Report minimal identification of pure cultures of skin microbiota, e.g., staphylococci or gram-positive rods.
- C. If organisms are too numerous to count, report as “>100 CFU.”
- D. Report preliminary negative cultures as “No growth at *x* days,” where “*x*” is the number of days of incubation. Report final negative cultures as, e.g., “No growth at 4 days.”
- E. If gram-negative rods or *S. aureus* is isolated and no blood culture was submitted, add the following note to the report: “Submit blood cultures to diagnose catheter-related sepsis.”

VII. INTERPRETATION

- A. Antimicrobial susceptibility testing should be performed on isolates both from blood and from the catheter tip and other segments submitted to the laboratory for culture. Different bacterial morphotypes isolated from the blood and the catheter may only be recognized by variations in their antibiogram profiles. If a central catheter cannot be immediately removed for clinical reasons (e.g., risk of bleeding), then antimicrobial therapy is directed against the antibiotic susceptibility profile of the blood isolate. In cases where the antibiotic profiles of the blood and catheter isolate(s) are different, then antimicrobial therapy is directed against the most resistant morphotype isolated from either site.
- B. The presence of >15 CFU suggests the catheter as a potential source of bacteremia, which occurs with about 10% of colonized catheters.

VIII. LIMITATIONS

- A. Semiquantitative catheter tip cultures are estimated to have a sensitivity of 85% in diagnosis of catheter-related bacteremia, but the specificity to diagnose catheter-related sepsis is low (13). Blood cultures collected from peripheral sites are helpful in confirmation of the diagnosis of catheter-related sepsis (1, 4, 6).
- B. Infections of the catheter hub lumen may be missed by culture of only the tip.
- C. Efforts to diagnose catheter-related sepsis using unpaired quantitative blood cultures drawn from the catheter are less sensitive than tip cultures (13).
- D. Catheter cultures can be helpful in determination of the cause of fever.
- E. Some authors have shown that quantitative catheter tip cultures have a greater sensitivity and specificity than the semiquantitative method of Maki et al. (6) in the diagnosis of catheter-related sepsis; however, these methods are more labor-intensive (2, 5, 9, 11, 12, 13). See Appendix 3.6–1 for method.

VIII. LIMITATIONS (continued)

- F. Siegman-Igra et al. (13) suggest that antimicrobial treatment of patients with positive catheter tip cultures but negative blood cultures increases medical costs unnecessarily, because of the low specificity of catheter tip cultures. For this reason, susceptibility testing of catheter tip isolates is not indicated. If concomitant blood cultures are positive, antimicrobial testing of the blood isolate is appropriate. Exceptions include performing antimicrobial testing when specifically requested by a physician and when testing the catheter tip isolate would provide more rapid results than waiting for the companion blood culture isolate to be tested (8).
- G. In simulated studies, rolling catheters, impregnated with antiseptics, on a blood plate inhibited the ability of the organisms to grow on the agar (10). This suggests that alternative methods must be found to diagnose infections in antiseptic-impregnated catheters.

REFERENCES

1. Blot, F., G. Nitenberg, E. Chachaty, B. Raynard, N. Germann, S. Antoun, A. Laplanche, C. Brun-Buisson, and C. Tancrede. 1999. Diagnosis of catheter-related bacteremia: a prospective comparison of the time to positivity of hub-blood versus peripheral-blood cultures. *Lancet* **354**:1071–1077.
2. Brun-Buisson, C., F. Abrouk, P. Legrand, Y. Huet, S. Larabi, and M. Rapin. 1987. Diagnosis of central venous catheter-related sepsis. Critical level of quantitative tip cultures. *Arch. Intern. Med.* **147**:873–877.
3. Centers for Disease Control and Prevention. 2002. Guidelines for the prevention of intravascular catheter-related infections. *Morb. Mortal. Wkly. Rep.* **51**(RR-10):1–26.
4. Dooley, D. P., A. Garcia, J. W. Kelly, R. N. Longfield, and L. Harrison. 1996. Validation of catheter semiquantitative culture technique for nonstaphylococcal organisms. *J. Clin. Microbiol.* **34**:409–412.
5. Gutierrez, J., C. Leon, R. Matamoros, C. Nogales, and E. Martin. 1992. Catheter-related bacteremia and fungemia. Reliability of two methods for catheter culture. *Diagn. Microbiol. Infect. Dis.* **15**:575–578.
6. Maki, D. G., C. E. Weise, and H. W. Sarafin. 1977. A semiquantitative culture method for identifying intravenous catheter-related infection. *N. Engl. J. Med.* **296**:1305–1309.
7. Malgrange, V. B., M. C. Escande, and S. Theobald. 2001. Validity of earlier positivity of central venous blood cultures in comparison with peripheral blood cultures for diagnosing catheter-related bacteremia in cancer patients. *J. Clin. Microbiol.* **39**:274–278.
8. Mermel, L. A., B. M. Farr, R. J. Sherertz, I. I. Raad, N. O'Grady, J. S. Harris, and D. E. Craven. 2001. Guidelines for the management of intravascular catheter related infections. *Clin. Infect. Dis.* **32**:1249–1272.
9. Raad, I. I., M. F. Sabbagh, K. H. Rand, and R. J. Sherertz. 1992. Quantitative tip culture methods and the diagnosis of central venous catheter-related infections. *Diagn. Microbiol. Infect. Dis.* **15**:13–20.
10. Schmitt, S. K., C. Knapp, G. S. Hall, D. L. Longworth, J. T. McMahon, and J. A. Washington. 1996. Impact of chlorhexidine-silver sulfadiazine-impregnated central venous catheters on in vitro quantitation of catheter-associated bacteria. *J. Clin. Microbiol.* **34**:508–511.
11. Sherertz, R. J., S. O. Heard, and I. I. Raad. 1997. Diagnosis of triple-lumen catheter infection: comparison of roll plate, sonication, and flushing methodologies. *J. Clin. Microbiol.* **35**:641–646.
12. Sherertz, R. J., I. I. Raad, A. Belani, L. C. Koo, K. H. Rand, D. L. Pickett, S. A. Straub, and L. L. Fauerbach. 1990. Three-year experience with sonicated vascular catheter cultures in a clinical microbiology laboratory. *J. Clin. Microbiol.* **28**:76–82.
13. Siegman-Igra, Y., A. M. Anglim, D. E. Shapiro, K. A. Adal, B. A. Strain, and B. M. Farr. 1997. Diagnosis of vascular catheter-related bloodstream infection: a meta-analysis. *J. Clin. Microbiol.* **35**:928–936.

APPENDIX 3.6-1

Sonication Method for Culture of Catheter Tips (1)

I. PROCEDURE

- A. Place catheter tip in 10 ml of TSB.
- B. Sonicate for 1 min at 55,000 Hz and 125 W.
- C. Vortex for 15 s.
- D. Add 0.1 ml of broth to 9.9 ml of saline. Vortex.
- E. Drop 0.1 ml of broth and 0.1 ml of saline suspension onto separate BAP and MAC (or EMB). Spread with spreader (Excel Scientific, Rightwood, Calif.).
- F. Incubate for 48 h in 5% CO₂ and count colonies.

II. INTERPRETATION

- A. Multiply the number of colonies on the broth culture plate by 10² CFU.
- B. If too numerous to count, multiply the number of colonies on the saline culture plate by 10⁴ CFU.
- C. A count of greater than 10² CFU is considered significant for catheter-related infection.

III. REPORTING RESULTS

- A. Report the genus and species of organisms present preceded by their count in CFU. If organisms are too numerous to count on the higher dilution plate, report as "Greater than 10⁶ CFU." See item VI in procedure 3.6 for further details.
- B. If no organisms are present, report "No growth at 1:100 dilution."

IV. LIMITATION

Although this method is more reliable than the roll plate method (2), it is not clear whether the difference is clinically significant. Blood cultures remain a very important part of detection of catheter-related sepsis.

References

1. Sherertz, R. J., I. I. Raad, A. Belani, L. C. Koo, K. H. Rand, D. L. Pickett, S. A. Straub, and L. L. Fauerbach. 1990. Three-year experience with sonicated vascular catheter cultures in a clinical microbiology laboratory. *J. Clin. Microbiol.* **28**:76–82.
2. Siegman-Igra, Y., A. M. Anglim, D. E. Shapiro, K. A. Adal, B. A. Strain, and B. M. Farr. 1997. Diagnosis of vascular catheter-related bloodstream infection: a meta-analysis. *J. Clin. Microbiol.* **35**:928–936.

3.7

Cerebrospinal Fluid Cultures

[Updated March 2007]

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Bacterial meningitis is the result of infection of the meninges. Identification of the infecting agents is one of the most important functions of the diagnostic microbiology laboratory because acute meningitis is life-threatening. *CSF from a patient suspected of meningitis is an emergency specimen that requires immediate processing to determine the etiologic agent.*

CSF is obtained by transcutaneous aspiration, and therefore, all organisms recovered from the culture are potential pathogens and must be reported to the physician immediately. Because the number of organisms in the CSF can be as low as 10^3 CFU/ml (7), concentration of the Gram stain by cytocentrifugation is important for rapid diagnosis. Cytospin concentration can increase the sensitivity up to 100-fold compared with both uncentrifuged and conventional centrifuged fluid (13). Concentration for culture is unnecessary, since the plate inoculum is suffi-

cient to detect the usual low numbers of organisms (4, 7, 10).

Aerobic bacteria commonly cause bacterial meningitis (Table 3.7-1), but anaerobes may be present in CSF when a meningeal abscess or a similar infectious process is adjacent to the meninges. These include traumatic head injury or prostheses, such as metal cranial plates and shunt drains. Inoculation of anaerobic media is not recommended for diagnosis of community-acquired meningitis. For shunt infections a backup broth that will grow anaerobes and aerobes in low numbers is recommended (4, 9).

Direct antigen testing is rarely performed because the cost-benefit is low. The sensitivity of testing for certain serogroups of *Neisseria meningitidis* is poor. While the sensitivity of testing for *Haemophilus influenzae* serogroup B is high, the disease has virtually disappeared in

countries with active neonatal vaccination programs. For streptococcal and *Escherichia coli* K-12 infections, the Gram stain is usually positive, except possibly in cases of partially treated meningitis. To maintain reagents for the latter indication is of low benefit. In a study of 103 episodes of meningitis, antigen tests had a sensitivity of 9% for Gram stain-negative specimens, with a 33% sensitivity overall (6).

PCR is becoming the method of choice for rapid, sensitive diagnosis of meningitis, especially for organisms that are present in low numbers or for organisms that are difficult to grow, particularly when the patient is partially treated with antimicrobial agents, e.g., *Borrelia*, *Ehrlichia*, *Mycobacterium tuberculosis*, *Mycoplasma*, and *Streptococcus pneumoniae* (3, 5, 8). Unfortunately, many of these tests are not available commercially.

Table 3.7-1 Common bacterial organisms causing acute meningitis by age or condition

Age or condition	Organism(s)
Neonate	<i>E. coli</i> , <i>S. agalactiae</i> (group B streptococci), <i>Listeria monocytogenes</i>
<2 mo	<i>S. agalactiae</i> , <i>L. monocytogenes</i> , <i>E. coli</i>
<10 yr	<i>H. influenzae</i> , <i>S. pneumoniae</i> , <i>N. meningitidis</i>
Young adult	<i>N. meningitidis</i>
Adult	<i>S. pneumoniae</i> , <i>N. meningitidis</i>
Elderly	<i>S. pneumoniae</i> , gram-negative bacilli, <i>L. monocytogenes</i>
Central nervous system shunt infection/ventriculitis	CoNS, <i>S. aureus</i> , <i>Corynebacterium</i> spp., <i>Propionibacterium acnes</i> , aerobic gram-negative bacilli, other anaerobes

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING



Observe standard precautions.

NOTE: Refer to procedure 3.3.1 for additional details. The following can be copied for preparation of a specimen collection instruction manual for physicians and other caregivers. It is the responsibility of the laboratory director or designee to educate physicians and caregivers on proper specimen collection.

A. Specimen collection

NOTE: This is a medical procedure that is performed by a physician guided by appropriate precautions.

1. Lumbar puncture

- a. Disinfect the puncture site with antiseptic solution and alcohol in a manner identical to phlebotomy skin preparation for blood culture to prevent specimen contamination and introduction of infection.
- b. Insert a needle with stylet at the L3-L4, L4-L5, or L5-S1 interspace. When the subarachnoid space is reached, remove the stylet; spinal fluid will appear in the needle hub.
- c. Measure the hydrostatic pressure with a manometer.
- d. Sequentially collect the CSF into five calibrated sterile tubes labeled no. 1 to no. 5.
- e. Physicians should be instructed to sequentially collect 2.0 ml of CSF into three sterile calibrated tubes if only routine chemistry (total protein and glucose), bacteriology (C&S), and hematology (cell count) are required.
- f. Table 3.7-2 outlines the collection and test ordering guidelines that should be used by physicians who collect CSF samples from adults, children, and neonates. The laboratory should include specific collection guidelines for CSF samples in its guide to services, including the minimal volume of samples required to perform specific tests, including those that will be referred out. Up to five sequentially collected sterile calibrated tubes should be collected depending on the extent of CSF testing required. Physicians should be instructed to collect CSF into a fifth tube if a portion of the sample needs to be sent to a reference laboratory for specialized microbiology tests as outlined in Table 3.7-2.

2. Ommaya reservoir fluid or ventricular shunt fluid

- a. Clean the reservoir site with antiseptic solution and alcohol prior to removal of fluid to prevent introduction of infection.
- b. Remove fluid by aspiration of CSF from the Ommaya reservoir or by collection from the ventricular drain or shunt. Sequentially collect CSF into a minimum of three sterile calibrated tubes if only routine chemistry (total protein and glucose) (tube no. 1), bacteriology (C&S) (tube no. 2), and hematology (cell count) (tube no. 3) are required.
3. An initial CSF sample should be collected prior to antimicrobial therapy for highest diagnostic sensitivity. Subsequent CSF samples are then collected every 2 to 3 days once antimicrobial therapy is started to monitor for resolution of the infection.

**II. SPECIMEN COLLECTION,
TRANSPORT, AND HANDLING**
*(continued)***B. Specimen transport**

1. Submit to laboratory as soon as possible and alert laboratory that specimen is in transit.
2. Do not refrigerate.
3. Each sterile calibrated tube containing CSF must be properly labeled with the patient's name, birthdate, and/or unique identification number, and the date and time of collection. The laboratory requisition or electronic order entry system should also request physicians to indicate the site of collections including lumbar puncture, Ommaya reservoir, ventricular shunt, ventricular drain, or ventricular puncture.
4. Complete requisition with demographic and specimen collection information. Record the patient diagnosis for improved processing of specimen.
5. Limit requests to those reflecting patient's condition.

C. Rejection criteria

1. Call physician to prioritize requests if there is insufficient volume.
 NOTE: Fungal and acid-fast bacillus (AFB) cultures of the CSF are infrequently indicated in acute community-acquired meningitis. Refer to Appendix 3.7–1 for a sample laboratory policy to communicate to caregivers. Since the fungal pathogens in CSF (*Cryptococcus neoformans*, *Coccidioides immitis*, and *Histoplasma capsulatum*) are best and most rapidly diagnosed by serologic methods or cultures of other sites, fungal CSF culture should be discouraged. However, the fungi that cause CSF disease grow well on the media inoculated for routine culture. For fungal requests, incubate the routine culture plates for a longer period and inoculate a fungal broth (Sabouraud) with a large volume of specimen to increase the yield of *Cryptococcus* and *Coccidioides*. *M. tuberculosis* is best diagnosed by PCR. Refer to sections 7 and 8 of this handbook.
2. Specimens in leaky containers must be processed, but alert the physician of the possibility of contamination.
3. Direct antigen testing is not recommended.

Table 3.7–2 CSF collection and test ordering guidelines for CSF specimens

Collection tube no.	Laboratory tests performed	Minimum CSF vol (ml)/test for adults and children	Minimum CSF vol (ml)/test for neonates	Comments
1 (chemistry)	Total protein	0.2	0.1	Chemistry immediately performs total protein and glucose tests and splits the remaining CSF sample in tube no. 1 as required for the other tests.
	Glucose	0.2	0.1	
	Immunoglobulin index	0.2	NA ^a	
	Oligoclonal banding (electrophoresis)	3.0	NA	
2 (microbiology)	Bacterial culture and sensitivity (includes Gram stain)	1.0	0.5	Tube no. 2 should be transported STAT to the clinical microbiology laboratory. A minimum of 3.0 ml of CSF should be cultured for optimal recovery of fungi from this site. Up to three separate CSF samples may need to be submitted for fungal culture from patients suspected of chronic meningitis to definitively rule out a fungal etiology.
	Viral culture	0.5	0.2	
	Cryptococcal antigen	1.0	NA	
	Fungal culture (includes calcofluor white stain)	3.0	NA	
3 (microbiology)	AFB culture (includes special stains)	3.0	NA	AFB culture is usually done by a reference laboratory. A minimum of 3.0 ml of CSF should be cultured for optimal recovery of AFB from this site. Up to three separate CSF samples may need to be submitted for AFB culture from patients suspected of chronic meningitis to definitively rule out an AFB infection.
4 (hematology)	Cell count	1.0	0.5	Hematology immediately performs the cell count and splits the remaining CSF for distribution to the flow cytometry and cytology laboratories. CSF samples for flow cytometry and/or cytology testing must be transported STAT and be received by the laboratory within 1 h after collection. The CSF cytology sample is immediately placed in a sterile container with fixative (e.g., Cytolyt) if transport has been delayed. A separate portion of the CSF sample is placed into RPMI/TTM solution for flow cytometry.
	Flow cytometry	2.0–10.0	NA	
	Cytology	2.0		
5 (special pathogens)	Creutzfeldt-Jakob disease (14-3-3 protein)	3.0	NA	These tests are usually performed by a reference laboratory. CSF samples for <i>Acanthamoeba</i> testing should be placed into a separate sterile calibrated tube containing Pages Amoeba saline and transported to the laboratory within 1 h of collection. CSF for anaerobic culture should be placed into a tube containing anaerobic transport medium (e.g., prereduced anaerobically sterilized media). Specialized viral tests should not be done unless the patient CSF total protein and the CSF WBC count are increased. CSF samples for Creutzfeldt-Jakob disease, West Nile virus, or viral PCR testing should each be collected into separate tubes. Common viral pathogens detected by PCR include herpesviruses (primarily herpes simplex virus) and enteroviruses.
	Viral PCR	1.0		
	West Nile virus	1.0		
	<i>Acanthamoeba</i>	10.0		
	Anaerobic culture (includes Gram stain)	1.0		

^a NA, not applicable.

III. MATERIALS

A. Media

1. BAP
2. CHOC
3. MAC
4. Broth known to support the growth of both fastidious aerobic organisms and anaerobes, such as anaerobic BHI or TSB with 0.1% agar and 5% sheep blood or fastidious anaerobe broth or fastidious broth (Quelab Laboratories, Inc., Montreal, Quebec, Canada; Remel, Inc., Lenexa, Kans.) (1, 11, 12).

5. See procedure 3.3.2 for common biochemical tests and media and procedure 3.17 for detailed procedures for biochemical tests.

B. Gram stain reagents

C. Other supplies

1. Cytocentrifuge
2. Sterile Pasteur pipettes

■ NOTE: Since THIO is mainly a broth for anaerobes and does not support the growth of the most common pathogens in CSF, it cannot be recommended for CSF culture (11, 12).

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

- A. Verify that plate media meet expiration date and QC parameters per current Clinical and Laboratory Standards Institute (formerly NCCLS) document M22. See procedures 14.2 and 3.3.1 for further procedures, especially for in-house-prepared media.
- B. Periodically check laboratory processing to ensure that there is no break in sterile technique and that media, stain reagents, collection tubes, slides, and other supplies are not contaminated with dead organisms.

V. PROCEDURE



Observe standard precautions.

A. Inoculation

1. Process specimen as soon as received.
■ NOTE: Use of biosafety cabinet will avoid contamination of the culture or specimen as well as protect laboratory processing personnel.
2. Verify patient name on both label and requisition.
3. Record the following.
 - a. Volume of CSF
 - b. Gross appearance of CSF, i.e., clear, bloody, cloudy, xanthochromic
4. Inoculate media.
 - a. Using a sterile pipette, aspirate fluid from the bottom of the collection tube.
 - b. Place 2 or 3 drops each onto BAP, CHOC, and MAC. Streak in quadrants using a separate loop or flamed loop for streaking each plate.
 - c. If greater than 1 ml is available for routine culture from a ventricular source or shunt, inoculate 1 ml to broth. If the site of the collection is unknown, use the patient location as an aid to determination of the need for anaerobic culture or use of a backup broth.
 - (1) Specimens from the emergency department usually are from community-acquired meningitis.
 - (2) Specimens from the neurology service likely represent those requiring broth culture.
 - (3) If large volumes of specimen are received, they usually are from shunts or reservoirs.

V. PROCEDURE (continued)

5. If a CSF specimen tube appears to be empty or contains only 1 to 5 drops of fluid and if more specimen cannot be obtained steriley from other laboratory departments, proceed as follows.
 - a. If 1 drop is available, use a sterile Pasteur pipette and prepare a smear for Gram stain from a portion of the specimen.
 - b. Using a sterile Pasteur pipette, place about 0.5 ml of broth medium into the specimen tube. Recap the tube, and invert it to mix the contents.
 - c. Use all the broth to inoculate media and do smear.
 - d. Note on report the volume received.
- B. Gram stain
 1. Place 5 or 6 drops of sample plus 1 drop of 37% formalin into a cytocentrifuge specimen chamber. Follow procedure for operation of centrifuge from manufacturer.
 2. As an alternative, when the specimen is cloudy or the quantity is not sufficient for concentration, prepare the smear by placing 1 or 2 drops of CSF on an alcohol-rinsed slide.
 - a. Allow the drop(s) to form one large drop. Do not spread the fluid.
 - b. Air dry the slide in a biosafety cabinet or covered on a slide warmer.
 3. Fix smear with methanol. (See procedure 3.2.1 for staining and reading smear.)
 4. Interpret CSF Gram stains immediately.
 5. Any bacteria seen are considered significant. However, confirm low numbers only seen in one or two fields with a second smear. If positive, do the following.
 - a. Notify the physician immediately.
 - b. If sufficient specimen is left, refer to blood culture workup of positive broths (Table 3.4.1–2) for additional tests that can be performed on the direct specimen.
- C. Save some CSF at 4°C and/or at –20°C for 1 week for PCR or other subsequent requests.
- D. Incubation
 1. Incubate plates at 35 to 37°C in 5% CO₂. Alternatively, use a CO₂-generating system to provide the proper atmosphere if a CO₂ incubator is unavailable.
 2. Incubate broths at 35 to 37°C in ambient air.
- E. Culture examination
 1. Examine all plate and broth media for macroscopic evidence of growth at 24 h.
 2. If no visible growth is observed on the culture media, reincubate.
 - a. Read aerobic plates daily for 4 days.
 - b. If the Gram stain is positive and there is no growth on the plates or a fungal culture was ordered, hold all plates for at least 1 week.
 - c. Examine broth media daily for 4 days and hold for 7 days before discarding.
 - d. Refer to procedure 3.3.2 for general guidelines.
 3. Cultures with growth
 - a. Notify physician of positive culture findings.
 - b. Identify all organisms, using the rapid tests listed in Table 3.3.2–5.
 - (1) Perform rapid bile solubility spot test (procedure 3.17.6) on all alpha-hemolytic streptococci to identify *S. pneumoniae*. If positive, report *S. pneumoniae*.
 - (2) Perform catalase and Gram stain of organisms growing on BAP and/or CHOC. Identify further according to Gram stain and rapid tests in Table 3.3.2–5. *Listeria* and group B streptococci are significant CSF pathogens.

V. PROCEDURE (continued)



It is imperative that these cultures be handled in a biosafety hood.

- (3) Perform oxidase test on gram-negative diplococci. If positive and colony is grayish to white, perform commercial kit containing δ -glutamyl-aminopeptidase (see Table 3.18.2-1). If positive, report *N. meningitidis*.
- (4) For all staphylococci, perform the tube coagulase for confirmation of slide or latex coagulase results.
- (5) For less common pathogens, refer to procedures 3.18.1 and 3.18.2.
- (6) *Generally determine the probable genus and usually the species identification of most CSF pathogens within 2 h of visible growth on the plates.*
- c. Do not perform complete identification or antimicrobial susceptibility testing (AST) if the isolate is clearly a plate contaminant or the isolate is a coagulase-negative staphylococcus (CoNS) isolated from broth only.
 - NOTE:** Isolates of CoNS and *Corynebacterium* are probably contaminants in community-acquired infection but may or may not be a cause of infection in shunt infections and those with head injuries (4, 9, 10). A few colonies of catalase-positive, gram-positive rods growing only on CHOC should be subcultured to BAP to check hemolysis and rule out *Listeria* before being reported as corynebacteria.
- d. Perform AST on enteric and nonfermenting gram-negative rods, enterococci, *S. pneumoniae*, *Staphylococcus aureus*, and other significant staphylococci.
 - (1) For *H. influenzae*, perform beta-lactamase test. Perform AST to penicillin or ampicillin if beta-lactamase test is negative and either agent will be used for therapy.
 - (2) For *Listeria*, *Streptococcus agalactiae*, and *N. meningitidis*, do not perform AST or beta-lactamase testing, which can lead to erroneous results. Resistance to penicillin in these isolates is rare; confirm any non-penicillin-susceptible isolates in a reference laboratory (2). For the penicillin-allergic patient, consult with the physician to guide AST.
- e. Hold positive culture plates for at least 7 days or, preferably, freeze isolates for long-term retrieval.

POSTANALYTICAL CONSIDERATIONS

VI. REPORTING RESULTS

- A. Report the Gram stain results as soon as possible, usually within 1 h of receipt.
- B. Report probable genus and species as soon as preliminary tests are completed.
- C. Refer to procedure 3.3.2 for general reporting.
- D. Document notification of physician of positive findings.

VII. INTERPRETATION

- A. Generally a positive culture indicates infection with the organism.
- B. Lack of WBCs in CSF does not rule out infection, especially in listeriosis (6).
- C. The most common cause of community-acquired bacterial meningitis is *S. pneumoniae*. Performance of the rapid bile solubility spot test (procedure 3.17.6) or the Quellung test (procedure 3.17.42) on all alpha-hemolytic streptococci seen in Gram stain is key to rapid diagnosis.
- D. Isolation of enterococci from CSF is always a cause for concern. The presence of the organism may be an indication of strongyloidiasis.

VIII. LIMITATIONS

- A. False-positive results can result from contamination of the specimen or the culture with skin microbiota.
- B. False-negative results can be caused by low numbers of organisms, prior antimicrobial treatment, or the fastidious nature of the infective organism.

REFERENCES

1. Cartwright, C. P., F. Stock, and V. J. Gill. 1994. Improved enrichment broth for cultivation of fastidious organisms. *J. Clin. Microbiol.* **32**:1825–1826.
2. Clinical and Laboratory Standards Institute. 2006. *Performance Standards for Antimicrobial Susceptibility Testing*. Sixteenth informational supplement. Approved standard M100-16. Clinical and Laboratory Standards Institute, Wayne, Pa.
3. Dumler, J. S. 2003. Algorithms for identification of *Mycoplasma*, *Ureaplasma*, and obligate intracellular bacteria, p. 349–353. In P. R. Murray, E. J. Baron, J. H. Jorgensen, M. A. Pfaller, and R. H. Yolken (ed.), *Manual of Clinical Microbiology*, 8th ed. ASM Press, Washington, D.C.
4. Dunbar, S. A., R. A. Eason, D. M. Musher, and J. E. Clarridge. 1998. Microscopic examination and broth culture of cerebrospinal fluid in diagnosis of meningitis. *J. Clin. Microbiol.* **36**:1617–1620.
5. Gillespie, S. H. 1999. The role of the molecular laboratory in the investigation of *Streptococcus pneumoniae* infections. *Semin. Respir. Infect.* **14**:269–275.
6. Hussein, A. S., and S. D. Shafran. 2000. Acute bacterial meningitis in adults. A 12-year review. *Medicine (Baltimore)* **79**:360–368.
7. LaScolea, L. J., Jr., and D. Dryja. 1984. Quantitation of bacteria in cerebrospinal fluid and blood of children with meningitis and its diagnostic significance. *J. Clin. Microbiol.* **19**:187–190.
8. Luft, B. J., C. R. Steinman, H. C. Neimark, B. Muralidhar, T. Rush, M. F. Finkel, M. Kunkel, and R. J. Dattwyler. 1992. Invasion of the central nervous system by *Borrelia burgdorferi* in acute disseminated infection. *JAMA* **267**:1364–1367.
9. Meredith, F. T., H. K. Phillips, and L. B. Reller. 1997. Clinical utility of broth cultures of cerebrospinal fluid from patients at risk for shunt infections. *J. Clin. Microbiol.* **35**:3109–3111.
10. Morris, A. J., S. J. Wilson, C. E. Marx, M. L. Wilson, S. Mirrett, and L. B. Reller. 1995. Clinical impact of bacteria and fungi recovered only from broth cultures. *J. Clin. Microbiol.* **33**:161–165.
11. Rinehold, C. E., D. J. Nickolai, T. E. Piccinni, B. A. Byford, M. K. York, and G. F. Brooks. 1988. Evaluation of broth media for routine culture of cerebrospinal and joint fluid specimens. *Am. J. Clin. Pathol.* **89**:671–674.
12. Scythes, K. D., M. Louis, and A. E. Simor. 1996. Evaluation of nutritive capacities of 10 broth media. *J. Clin. Microbiol.* **34**:1804–1807.
13. Shanholzter, C. J., P. J. Schaper, and L. R. Peterson. 1982. Concentrated gram stain smears prepared with a cytopsin centrifuge. *J. Clin. Microbiol.* **16**:1052–1056.

SUPPLEMENTAL READING

- Gray, L. D., and D. P. Fedorko. 1992. Laboratory diagnosis of bacterial meningitis. *Clin. Microbiol. Rev.* **5**:130–145.
- Mandell, G. 1995. *Principles and Practice of Infectious Diseases*, 4th ed., p. 950–1136. Churchill Livingstone Inc., New York, N.Y.
- Ray, C. G., J. A. Smith, B. L. Wasilauskas, and R. J. Zabransky. 1993. *Cumitech 14A, Laboratory Diagnosis of Central Nervous System Infections*. Coordinating ed., A. J. Smith. American Society for Microbiology, Washington, D.C.

APPENDIX 3.7-1

Suggested Policy for Caregivers for Microbiological Examination of Cerebrospinal Fluid

- I. ROUTINE MICROBIOLOGY CSF TESTS
The following tests should be routinely ordered to detect bacterial meningitis.
 - A. Total protein and glucose (chemistry)—tube no. 1
 - B. Bacterial C&S—the laboratory routinely performs a Gram stain using cytopsin concentration when a bacterial culture is ordered—tube no. 2
 - C. All CSF samples will be held at 4°C for 7 days after receipt by the laboratory for further testing as clinically required—tube no. 3.
- II. RATIONALE FOR SPECIALIZED MICROBIOLOGY CSF TESTS
A. Acute bacterial meningitis is a medically urgent condition that requires prompt institution of appropriate antimicrobial therapy. CSF abnormalities associated with this condition typically include an elevated total protein, decreased glucose compared to the level in serum, an elevated count of WBCs that are predominantly polymorpho-

APPENDIX 3.7-1 (continued)

- nuclear cells, and a positive Gram stain and bacterial culture of the CSF. Since this is the most common type of infection, routine microbiology CSF tests should always include a bacterial culture. Although anaerobes rarely cause acute bacterial meningitis, anaerobic culture of the CSF should be done for patients with a parameningeal infection, particularly in cases where there has been leak or rupture into the cerebrospinal space (e.g., brain abscess, suppurative thrombophlebitis, or mycotic aneurysm). CSF should also be cultured for anaerobes, particularly *P. acnes*, for patients with central nervous system shunt infections.
- B. Patients with acute aseptic meningitis due to a viral infection may present with a picture similar to that of patients with a bacterial infection. Viral tests should be done on the CSF if the total protein and WBC count are elevated and the CSF Gram stain and bacterial culture are negative. Enterovirus infection occurs during the late summer and fall months in most temperate climates. CSF viral culture or PCR for enteroviruses should be done during this period. West Nile virus is a flavivirus that is seasonally transmitted by infected mosquitoes that has recently been introduced to North America and causes acute meningitis or encephalitis in susceptible patients during the summer and early fall months. West Nile virus may be detected in the CSF using a combination of ELISA and PCR methods. Herpes simplex virus (HSV) encephalitis may produce hemorrhagic necrosis of the temporal lobe resulting in an increased CSF RBC count. PCR for HSV should be done for patients suspected of having meningitis or encephalitis.
- C. Chronic meningitis due to yeast or fungal infection of the central nervous system is uncommon, and requests for specialized tests should be restricted to patients with exposure to systemic fungal pathogens (*C. immitis*, *H. capsulatum*, or *Blastomyces dermatitidis*) and those who are immunocompromised. Cryptococcal meningitis is an opportunistic infection that indicates that a human immunodeficiency virus-infected patient has AIDS. Microbiology laboratories should be able to detect the presence of *Cryptococcus* on a CSF Gram stain, and a cryptococcal antigen (CRAG) test should routinely be done in this case and on CSF samples collected from immunocompromised patients. CRAG testing should also be routinely done whenever a yeast or fungal culture is ordered. The sensitivity of CRAG testing approaches 100% from serum and 98% from CSF in diagnosing disseminated cryptococcal infection. The sensitivity of the CSF India ink wet mount stain is only 50%, so laboratories should be performing CRAG tests. Although CRAG testing may not be performed routinely as a STAT procedure, it should be urgently done when *Cryptococcus* meningitis is clinically suspected in an immunocompromised patient and when *Cryptococcus* infection is suspected on the basis of CSF Gram stain or India ink wet mount stain. The sensitivity of CSF fungal culture for systemic mycosis agents is only ~30% even when large volumes of specimen (e.g., 10 ml) are cultured. Serology should be performed on the CSF for *C. immitis* since it has a sensitivity of 100%.
- D. Chronic meningitis due to *Mycobacterium tuberculosis* infection of the central nervous system is also uncommon, and requests for AFB testing should be restricted to patients with a history of exposure or risk of *M. tuberculosis* infection and those with a positive purified protein derivative skin test, those with miliary tuberculosis (TB), and immunocompromised patients. The clinical symptoms that suggest TB meningitis include mental status changes over a period of time, chronic headache and neck stiffness, and cranial nerve palsy since the infection is mainly located along the base of the brain. Characteristic CSF abnormalities include an elevated total protein to the extent that CSF will form a protein precipitate of pellet in a tube, a decreased glucose compared to levels in serum, and an elevated WBC count that is predominantly lymphocytic. The sensitivity of AFB culture is only ~30% even if 10 ml of CSF is submitted for culture, and a result is not available for 4 to 8 weeks. The current recommendation is to perform a PCR assay if TB meningitis is suspected. The PCR TB assay has a sensitivity that approaches 100%, it requires a small sample volume (0.5 ml), and it can be performed within 2 to 4 days of receipt of the sample.
- E. Acanthamoebic meningitis is a life-threatening condition with a high mortality rate. CSF tests for detection of *Acanthamoeba* should be done for patients who develop acute meningitis and who have a recent history of swimming in freshwater. Differentiation between *Naegleria fowleri* infection and *Acanthamoeba* infection requires wet mount examination and culture of a large volume of CSF.

3.8.1

Fecal Culture for Aerobic Pathogens of Gastroenteritis

[Updated March 2007]

■ NOTE: For isolation of *Campylobacter jejuni/coli*, see procedure 3.8.2.

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Gastroenteritis can be caused by bacteria, parasites, or viruses. With such a wide array of pathogens and the need for cost containment, physician input and practice guidelines (12) can help the laboratory determine which tests are appropriate for detecting the etiological agent of diarrhea. Microbiology laboratories should review the local epidemiology of bacterial enterocolitis and implement routine stool culture methods that will allow recovery and detection of all of the major pathogens causing most of the cases in their geographic area. All microbiology laboratories should routinely test for the presence of *Salmonella* spp., *Shigella* spp., and *Campylobacter* spp. on all stool cultures. Other major pathogens, such as Shiga-toxin-producing *Escherichia coli*, particularly *E. coli* O157 or enterohemorrhagic *E. coli* (EHEC), should also be routinely tested for on bloody stool samples during the spring, summer, and early fall months in geographic areas where the prevalence of these strains has been shown to be increased. Microbiology laboratories situated in or near coastal communities may also test for *Aeromonas* and *Vibrio* spp. since the prevalence of these types of infections is increased with exposure to water or contaminated food such as shellfish.

Salmonella and *Shigella* are screened by using differential and selective direct plating media (Table 3.8.1–1), which are based on lactose fermentation and H₂S production (29). Since some nonpathogenic, gram-negative rods found in normal feces may give the same reactions as en-

teric pathogens, biochemical screening and agglutination are necessary for identification. Routinely, culture for *Campylobacter jejuni* is performed, but because the method for isolation is different from the method to detect other pathogens, details are presented in a separate procedure (see procedure 3.8.2).

Other pathogens, such as *Yersinia enterocolitica*, *Aeromonas* spp., *Plesiomonas shigelloides*, *Vibrio cholerae*, and other *Vibrio* spp., including *Vibrio parahaemolyticus*, may be detected in routine culture, but there is a better chance of detection if the physician specifies the suspect organism. For many of these cultures, selective media for these agents are inoculated. If requests for culture for these organisms are infrequent, a reference or public health laboratory may be used for testing. In addition, several organisms produce toxin-mediated disease. While the organism may be detected in culture, detection of the toxin that is responsible requires other laboratory tests. These organisms include verocytotoxin-producing *E. coli* (VTEC), enterotoxigenic *E. coli*, *Clostridium botulinum*, *Clostridium difficile*, *Bacillus cereus*, and *Staphylococcus aureus*. Commercial tests are available for VTEC and for *C. difficile*. For other toxins, testing is available only from public health or reference laboratories. In addition, other genera of gram-negative rods (*Hafnia*, *Edwardsiella*, DF-3, and even *Klebsiella*) have been implicated in gastroenteritis and may be isolated in culture.

VTEC, also known as Shiga toxin-producing or EHEC, produces a toxin mediated by a bacteriophage, which has been implicated in hemorrhagic colitis (HC) and has been associated with hemolytic-uremic syndrome (HUS) (17). VTEC is the fourth leading cause of diarrhea in the United States, with severe complications from HUS and HC, especially in children. Over 60 *E. coli* serotypes produce the toxin, but *E. coli* O157:H7 is responsible for at least half of the cases (11, 18, 22). Since it has a unique biotype, *E. coli* O157 can easily be detected on selective medium (13). A direct assay for the verocytotoxin can detect the presence of toxin in stools infected with other strains or confirm that an *E. coli* O157 strain is a toxin producer (see procedure 11.8). At a minimum, testing for *E. coli* O157 or, preferably, verocytotoxin should be performed routinely on bloody stools from all patients, especially children. Testing on all stools sent for diagnosis of diarrhea is preferred from a public health standpoint but may not be cost-effective for hospital laboratories if the prevalence of disease is low (28).

Fecal cultures should not be performed for patients being treated with broad-spectrum antimicrobial agents, because it is likely that the antimicrobial therapy is responsible for the diarrhea (12). They may have overgrowth with other bacteria, including *Pseudomonas aeruginosa*, and *Candida* spp., the role of which in disease production is not clear. Their presence

Table 3.8.1-1 Commonly used primary plating and broth media for isolation of *Salmonella* and *Shigella*^a

Medium (abbreviation)	Type	Expected isolates	Inhibitors or indicators	Reactions of lactose fermenters	Reactions of pathogens	Comments
Hektoen enteric agar (HEK) (20)	D, S plate	<i>Salmonella</i> and <i>Shigella</i> spp. (especially for <i>Shigella</i> spp.)	Bile salts, ferric ammonium citrate, sodium thiosulfate, lactose, sucrose, salicin, bromthymol blue, fuchsin	Yellow-orange or salmon pink; pink precipitate around colonies, may have black centers.	<i>Shigella</i> is green. <i>Salmonella</i> is blue or green; may have black centers.	Inhibits <i>Citrobacter</i> but is small and blue-green, if present. <i>Proteus</i> and <i>Providencia</i> are yellow or green; may have black centers. Detects H ₂ S.
MacConkey agar (MAC)	D, S plate	Gram-negative enteric bacilli	Bile salts, crystal violet, lactose, neutral red	Pink	Colorless or transparent	5% Agar will inhibit swarming of <i>Proteus</i> spp.
Salmonella-shigella agar (SS)	D, highly S plate	<i>Salmonella</i> and <i>Shigella</i> spp. (<i>S. sonnei</i> inhibited)	Bile salts, lactose, citrate, thiosulfate, ferric citrate, brilliant green, neutral red	Pink, red; may have black centers.	Colorless or transparent; may have black centers.	Detects H ₂ S.
Xylose, lysine, deoxycholate agar (XLD) (29)	D, S plate	<i>Salmonella</i> and <i>Shigella</i> spp. (especially for <i>Shigella</i> spp.)	Deoxycholate, thiosulfate, ferric ammonium citrate, xylose, lactose, sucrose, lysine, phenol red	Yellow; may have black centers.	<i>Salmonella</i> and <i>Shigella</i> are red. <i>Edwardsiella</i> and <i>Salmonella</i> may be red with black centers.	<i>Providencia rettgeri</i> , <i>Morganella morganii</i> , and <i>Proteus</i> spp. are yellow even though they are lactose negative. Detects H ₂ S.
Gram-negative (GN) broth	E broth	<i>Shigella</i> and possibly <i>Salmonella</i> spp.	Deoxycholate, citrate, dextrose, mannitol	Initially enhances growth of mannitol-fermenting rods		Subculture at 6–8 h.
Selenite-F	E broth	<i>Salmonella</i> and <i>Shigella</i> spp. (some shigellae may be inhibited)	Selenite, lactose	Selenite is toxic to <i>Escherichia coli</i> and some other enteric bacteria.		Subculture at 18–24 h. Selenite broth with cysteine may inhibit some salmonellae.

^a Either bile salts, deoxycholate, or Selenite is present in each medium to inhibit gram-positive microbiota. Abbreviations: D, differential; E, enriched; S, selective. Ferric ammonium citrate reacts with hydrogen sulfide (H₂S) from organism to produce black color of colony.

may be reported, along with a statement indicating that the organism was the predominant organism recovered and that expected enteric organisms were not present,

suggesting antimicrobial inhibition. In such cases, the *C. difficile* toxin assay (procedure 3.8.3) is more meaningful. Generally these patients have been hospi-

talized for more than 3 days; after that time requests for routine stool cultures should be rejected.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING

A. Specimen collection

1. Have patient obtain stool specimen.

- a. Patient should pass the stool into a clean, dry pan or special container mounted on the toilet for this purpose.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING

(continued)

- b. Transfer at least 5 ml of diarrheal stool, 1 g of material or a walnut-sized portion of stool, or the amount of fecal specimen that displaces to the line of commercial transport vials to one of the following:
 - (1) Clean, leakproof container with a tight-fitting lid *or*
 - (2) Buffered glycerol saline (preferred for *Shigella* spp. but inhibits *Campylobacter*)
 - (3) Modified Cary-Blair medium (modified to contain 1.6 g of agar per liter rather than 5 g/liter)
 - (a) Purchase from most commercial medium sources *or*
 - (b) Prepare as follows.

Dispense the following ingredients into 991 ml of H₂O.

sodium thioglycolate	1.5 g
disodium phosphate	1.1 g
sodium chloride	5.0 g
phenol red	0.003 g
agar	1.6 g

Heat to dissolve and cool to 50°C. Then add 9 ml of 1% CaCl₂. Adjust pH to 8.4; dispense in vials and steam for 15 min. Cool and tighten caps.

- (c) Whether purchased or prepared in-house, QC transport medium to determine if it will support the viability of *Shigella* and *Campylobacter*. See section 14, Table 14.2–2, for details; medium is listed under “Transport.”

■ NOTE: Do not fill commercial transport vials above indicator line. Overfilling of transport vial results in improper specimen preservation.

- (4) Stool enrichment broths (*see* Table 3.8.1–1).

■ NOTE: Generally, fecal specimens are not placed directly into these broths at collection, but it can be done.

- (5) Anaerobic transport tube for *C. difficile* culture, not toxin assay (culture is only for epidemiologic or nosocomial studies).

■ NOTE: Do not use toilet paper to collect stool, because it may be impregnated with barium salts, which are inhibitory to some fecal pathogens. The specimen should not be mixed with urine, but semisolid to solid feces can be scooped out of urine, if necessary.

2. Rectal swabs

- a. Pass the tip of a sterile swab approximately 1 in. beyond the anal sphincter.
- b. Carefully rotate the swab to sample the anal crypts, and withdraw the swab.
- c. Send the swab in Cary-Blair medium or buffered glycerol saline.

- 3. Submit duodenal, colostomy, or ileostomy contents in leakproof cup or transport vial.

B. Timing and transport

- 1. Submit specimen during the acute stage of infection (usually 5 to 7 days), because pathogens decrease in number with time.
- 2. If fresh stool is submitted for culture that is not in transport medium, the specimen should be transported to laboratory and processed within 2 h after collection.
- 3. If fresh stool is submitted for culture in transport medium, the specimen may be refrigerated at 4°C and transported to the laboratory within 24 h for the best recovery of pathogens.
- 4. Studies with adult and pediatric patients have shown that culture of a single stool specimen has a sensitivity of >95% for detection of the enteric bacterial

**II. SPECIMEN COLLECTION,
TRANSPORT, AND HANDLING**
(continued)

pathogen causing enterocolitis provided the laboratory culture method detects all of the major pathogens prevalent in a given geographic area. If the initial stool culture is negative, then additional fecal samples may be submitted for testing provided the patient collects them from different defecations on successive days.

C. Labeling

1. Provide a place on laboratory order form or computer entry screen to indicate if the stool is bloody.
2. Information on the laboratory requisition and specimen label must match. In both places provide the patient's full name, birthdate, a unique patient identifier number (i.e., hospital number), physician's name, type of sample, and the date and time of collection.
3. Document when specimen is received; if it is liquid, formed, or solid; or if it contains mucus.

D. Rejection criteria

1. Reject stools not in transport medium received >2 h after collection, as changes occur that are detrimental to most *Shigella* spp. If recollection is not possible and the doctor requests that the stool be processed, set up the culture and enter a note: "Delay in specimen receipt by laboratory may compromise recovery of pathogens."
2. If specimen in transport medium is delayed for more than 48 h at 4°C or is delayed more than 24 h at 25°C , request immediate recollection since the specimen may be compromised. If another sample cannot be submitted, then proceed to culture the sample but place a comment on the report stating, "Culture may be falsely negative because of a delay in transport."
3. Multiple stool samples should not be cultured from the same patient that were submitted on the same day.
4. Reject fecal cultures received from adults and pediatric patients hospitalized for >3 to 4 days, unless the patient is known to be human immunodeficiency virus positive or in cases of a cluster epidemic within the institution. Do not reject stool samples from infants and toddlers until after the fourth day of hospitalization since studies have shown that it may take longer to collect a stool sample from pediatric patients admitted with gastroenteritis that are placed on bowel rest and are not eating a normal diet (5).
5. Notify caregiver if transport tube is filled above line, indicating that too much specimen was submitted in vial.
6. If transport vial indicator has turned yellow, refrigerate it at 4°C and notify the physician that a fresh stool sample should be recollected. If another sample cannot be submitted, then proceed to culture the sample but place a comment on the report stating, "Culture may be falsely negative because of a delay in transport."
7. Do not process hard, solid stools that cannot be sampled for inoculation.
8. Do not process dry swabs.
9. Do not process stools with barium.
10. Reject more than three stools from the same patient in a 3-week period or multiple specimens received on the same day.
11. Do not use specimens submitted in bacteriology transport tubes for parasitology examination.

III. MATERIALS

- A. Media for routine stool culture** (see Table 3.8.1-1 for descriptions and abbreviations)
1. BAP
 2. MAC
 3. Choose one or more (to increase yield) of the following to select for *Salmonella* and *Shigella*.
 - a. HEK
 - b. XLD
 - c. SS (do not use as only selective medium)
 4. Enrichment broth (choose one)
 - a. Selenite-F (generally for *Salmonella* enrichment)
 - b. GN broth (for both *Salmonella* and *Shigella* enrichment, but requires subculture at 6 to 8 h)

■ NOTE: The use of enrichment broths for detecting small numbers of *Salmonella* or *Shigella* spp. can be justified for culturing stools of workers in sensitive occupations, such as day care workers and food service employees. Laboratories that have historical data showing very poor recovery of additional pathogens not seen on initial plates can make a case for abandoning routine use of enrichment broths. Generally enrichment broth increases the yield of *Salmonella* and *Shigella* by 10%.
 5. If selected other pathogens are requested or indicated, choose additional selective media from Table 3.8.1-2 to enhance recovery of these stool pathogens. Also use these media based on prevalence of disease and public health requirements in specific geographic areas (e.g., use bismuth sulfite agar for cluster epidemics of *Salmonella*).
- B. Biochemical tests**
1. For screening and detection of *Salmonella*, *Shigella*, and *Yersinia*
 - a. Spot indole reagent (procedure 3.17.23)
 - b. Oxidase reagent (procedure 3.17.39)

■ NOTE: False-negative results are common if indole or oxidase test is done from selective medium. Test only from BAP.

 - c. Urea agar slants or disks (procedure 3.17.48)
 - d. Kligler's iron agar (KIA) or triple sugar iron agar (TSI) slants (procedures 3.17.22 and 3.17.25)
 - e. Andrade's glucose with a Durham tube for gas production (optional) (procedure 3.17.9)
- NOTE:** This medium allows easy reading of gas production and can be used for motility and Voges-Proskauer (VP) reaction.
- f. Pyrrolidonyl-β-naphthylamide (PYR) (procedure 3.17.41)
 - g. Semisolid motility medium (procedure 3.17.31)
 - h. Kit identification system (API 20E, IDS panel, Vitek GNI Plus card [bioMérieux, Inc.]; MicroScan gram-negative identification panels [Dade Behring MicroScan Microbiology Systems]; BD CRYSTAL [BD Diagnostic Systems]; etc.)
 - i. Acetate for problematic *Shigella* identifications (procedure 3.17.2)
 - j. Antisera for grouping (see Appendix 3.8.1-1)
 - (1) *Salmonella* polyvalent and Vi (optional, A, B, C1, C2, and D)
 - (2) *Shigella* groups A, B, C, and D or
 - (3) *Salmonella* and *Shigella* colored latex agglutination reagents (Wellcolex; Remel Inc., Lenexa, Kans.)
- 2. For *Vibrio* and *Aeromonas***
- a. O/129 disks—150 µg (procedure 3.17.36)
 - b. Mueller-Hinton agar (MH) with and without 4% salt
 - c. Esculin slants or broth (optional for identification of *Aeromonas* to species level) (procedure 3.17.5)
 - d. O1 antiserum and toxigenicity kit (optional)
- NOTE:** *Vibrio* toxin testing is performed by latex test, DNA probe, EIA, or tissue culture. One source is Unipath Co., Oxoid, Ogdensburg, N.Y. (*V. cholerae* toxigenicity kit, VET-RPLA).
- 3. For *E. coli* O157**
- a. 4-Methylumbelliferyl-β-D-glucuronide test (MUG) (procedure 3.17.34)
 - b. *E. coli* O157 (and H7) antiserum or latex antibody test (see Appendix 3.8.1-2)
- 4. For *E. coli* O157 and non-O157 VTEC**
- a. MAC broth
 - b. EHEC EIA (see procedure 11.8)
- C. Supplies**
1. Incubator at 35°C
 2. Petri dishes, slides, or disposable black cards for agglutination tests
 3. Sticks and loops

Table 3.8.1–2 Special highly selective media for specific pathogen requests

Medium	Abbreviation	Inhibitors or indicators	Pathogen selected and colony description	Notes ^a
BAP with ampicillin (19)	BAP-A	20 µg of ampicillin/ml	<i>Aeromonas</i> is oxidase positive and may be hemolytic	Increases the yield of isolation of pathogen.
Bismuth sulfite		Brilliant green, bismuth sulfite, ferrous sulfate, dextrose	<i>Salmonella</i> (especially <i>S. typhi</i>) is black, with or without brownish-black zones, or green with no zones.	Especially good for <i>Salmonella</i> serovar Typhi outbreaks; <i>Morganella</i> is not inhibited but appears green.
Brilliant green agar		Brilliant green, lactose, sucrose, phenol red	<i>Salmonella</i> is red, pink, or white surrounded by red zone.	Fermenters are yellow to yellow-green; agar is inhibitory to <i>Salmonella</i> serovars Typhi and Paratyphi.
Inositol-brilliant green-bile salt agar	IBB	Brilliant green, bile salts, inositol	<i>P. shigelloides</i> forms white to pink colonies (6).	Coliforms are either green or pink.
Cefsulodin-Irgasan-novobiocin (19, 26)	CIN	Deoxycholate, crystal violet, cefsulodin, Irgasan, novobiocin, mannitol, neutral red	<i>Y. enterocolitica</i> , <i>Aeromonas</i> spp., and other yersiniae have deep red center with a transparent margin, or “bull’s-eye” appearance.	Incubate at 25°C for 48 h. <i>Citrobacter</i> , <i>Pantoea agglomerans</i> , and <i>Serratia liquefaciens</i> are red. <i>Enterobacter cloacae</i> and <i>S. marcescens</i> colonies are raised and mucoid, with diffuse, pink coloration.
Cycloserine-cefoxitin-egg yolk agar ^b	CCEY ^b	Cycloserine, cefoxitin, cholic acid, egg yolk, p-hydroxyphenylacetic acid, lysed horse or sheep blood	<i>Clostridium difficile</i> colonies are large (4 mm) and gray with whitish centers; they fluoresce chartreuse under long-wave UV light on anaerobic BAP or CCEY; typical barnyard odor.	Incubate under anaerobic conditions for 48–72 h; gram-positive rod with rare spores.
MAC-sorbitol with cefixime and tellurite	CT-SMAC	MAC with sorbitol (rather than lactose), cefixime, and tellurite	<i>E. coli</i> O157:H7 is colorless; other coliforms are pink or inhibited.	Better selection than SMAC, which can also be used (11).
MAC broth	MAC broth	MAC without agar	VTEC, especially non-O157 strains	Use 50 µl of overnight culture to perform EHEC EIA. May incubate at 25°C to enrich for <i>Yersinia</i> (27).
Thiosulfate citrate bile salts ^c	TCBS ^c	Bile salts, citrate, thiosulfate, 1% NaCl, sucrose, bromthymol blue	<i>V. cholerae</i> is yellow; <i>V. parahaemolyticus</i> is green or blue. Some <i>Vibrio</i> spp. are inhibited.	<i>Proteus</i> is yellow; other enteric bacteria are inhibited but blue to translucent if they grow. Enterococci may also grow.
Alkaline peptone water	APW	1% NaCl, pH 8.5	Vibrios are selected.	Subculture to TCBS at 24 h (21).

^a Incubate at 37°C in O₂ unless otherwise stated.^b Since as many as 20% of asymptomatic hospitalized patients may be colonized with *C. difficile*, tests for presence of toxin in stool are more specific for diagnosis of *C. difficile*-associated diarrhea. Isolation of the organism should only be done for epidemiologic studies, with confirmation that the isolated strain is a toxin producer. CCFA (containing cycloserine, cefoxitin, fructose, egg yolk, and neutral red) can also be used for isolation. Do not use a medium with neutral red to demonstrate colonial fluorescence (3).^c Prepare TCBS fresh for use from powder or by melting previously prepared or purchased “deeps.” If made from powder, boil but do not autoclave prior to use.

ANALYTICAL CONSIDERATIONS**IV. QUALITY CONTROL**

- A. Verify that media meet expiration date and QC parameters per current Clinical and Laboratory Standards Institute (CLSI; formerly NCCLS) M22 document. See procedures 14.2 and 3.3.1 for further procedures, especially for in-house-prepared media.
1. Visual inspection
 - a. Inspect for cracks in media or plastic petri dishes, unequal fill, hemolysis, evidence of freezing, bubbles, and contamination.
 - b. Check XLD; it should be reddish and relatively clear prior to inoculation. Excessive heating causes precipitation.
 - c. Record and report deficiencies to manufacturer.
 2. Most agar media that are purchased from commercial sources are not required to have additional user control with microorganisms expected to give appropriate reactions.
 - a. These media currently include BAP, CIN, MAC, HEK, XLD, SS, Selenite-F, and GN broth
 - b. For QC of these media, if prepared in-house, refer to section 14 of this handbook, except for CIN.
 - c. For QC of CIN, see Table 3.8.1–3.
 - d. Refer to CLSI document M22-A3 (7) or section 14 for instructions on preparation of QC microorganisms for testing.
 3. Each lot of media for specialized organisms must have QC testing with appropriate microorganisms, regardless of whether it is prepared in-house or purchased from a commercial source.
 - a. These media include BAP-A, brilliant green agar, bismuth sulfite, IBB, campylobacter agars, CT-SMAC, CCFA, and TCBS (see Table 3.8.1–2).
 - b. Refer to Table 3.8.1–3 for list of QC microorganisms and their reactions.
 4. QC each lot of transport media with *Campylobacter jejuni* ATCC 33291, *Shigella flexneri* ATCC 12022, and *Y. enterocolitica* ATCC 9610 by following the method outlined in Table 14.2–2 for QC listed under “Transport.”
- B. Test antisera with known positively and negatively reacting organisms or commercial somatic O or H antigens prior to use of each new lot and every 6 months thereafter. See Appendix 3.8.1–1.
- C. For QC of specific reagents or biochemical media, refer to individual procedures.



Include QC information on reagent container and in QC records.

Table 3.8.1–3 QC of specialized media for detection of fecal pathogens

Test medium	Test organism	Inoculation method ^a	Incubation			Result
			Time (h)	Temp (°C)	Atmosphere	
BAP-A <i>Note:</i> Proposed in 2003 for exemption from user QC for commercially prepared media (8)	<i>Aeromonas hydrophila</i> ATCC 7965	A	18–24	35	Aerobic	Growth
	<i>Escherichia coli</i> ATCC 25922	B	18–24	35	Aerobic	Partial to complete inhibition
Bismuth sulfite	<i>Salmonella enteritidis</i> ATCC 13076	A	18–24	35	Aerobic	Growth; black with metallic sheen
	<i>Escherichia coli</i> ATCC 25922	B	18–24	35	Aerobic	Partial inhibition; small brown to yellow-green colonies
Brilliant green	<i>Salmonella enteritidis</i> ATCC 13076	A	18–24	35	Aerobic	Growth; colorless to light pink
	<i>Escherichia coli</i> ATCC 25922	B	18–24	35	Aerobic	Partial inhibition; yellow-green colonies
	<i>Staphylococcus aureus</i> ATCC 25923	B	18–24	35	Aerobic	Inhibition
Campylobacter agars	<i>Campylobacter jejuni</i> ATCC 33291	A	24–48	42	Microaerobic	Growth
	<i>Escherichia coli</i> ATCC 25922	B	24–48	42	Microaerobic	Partial to complete inhibition
CCEY or CCFA	<i>Clostridium difficile</i> ATCC 9689	A	24–48	35	Anaerobic	Growth; large, gray (CCEY) or yellow (CCFA) colonies that fluoresce chartreuse under UV light (CCEY only)
	<i>Clostridium perfringens</i> ATCC 13124	B	24–48	35	Anaerobic	Partial to complete inhibition
	<i>Bacteroides fragilis</i> ATCC 25285	B	24–48	35	Anaerobic	Partial to complete inhibition
	<i>Escherichia coli</i> ATCC 25922	B	24–48	35	Aerobic	Partial to complete inhibition
	<i>Staphylococcus aureus</i> ATCC 25923	B	24–48	35	Aerobic	Partial to complete inhibition
	<i>Yersinia enterocolitica</i> ATCC 9610	A	24–48	25	Aerobic	Growth; red center, transparent border
CIN <i>Note:</i> Exempt from user QC for commercially prepared media (7)	<i>Aeromonas hydrophila</i> ATCC 7965	A	24–48	35	Aerobic	Growth; red center, transparent border
	<i>Enterococcus faecalis</i> ATCC 29212	B	24–48	35	Aerobic	Partial to complete inhibition
	<i>Pseudomonas aeruginosa</i> ATCC 27853	B	24–48	35	Aerobic	Partial to complete inhibition
	<i>Escherichia coli</i> ATCC 25922	B	24–48	35	Aerobic	Partial to complete inhibition
	<i>Escherichia coli</i> O157:H7 ATCC 35150	A	24	35	Aerobic	Clear colonies seen; no fermentation of sorbitol
	<i>Escherichia coli</i> ATCC 25922	B	24	35	Aerobic	Partial to complete inhibition. Pink colonies seen; fermentation of sorbitol
CT-SMAC	<i>Proteus mirabilis</i> ATCC 12453	B	24	35	Aerobic	Partial to complete inhibition
	<i>Vibrio parahaemolyticus</i> ATCC 17802	A	18–24	35	Aerobic	Growth; blue-green-centered colonies
	<i>Vibrio alginolyticus</i> ATCC 17749	A	18–24	35	Aerobic	Growth, yellow colonies
	<i>Escherichia coli</i> ATCC 25922	B	18–24	35	Aerobic	Partial inhibition; small, clear colonies

^aAbbreviations are as follows. A, for testing nutritive properties. Inoculate each medium with 10 µl of a 1:100 dilution of standardized cell suspension (0.5 McFarland). If isolated colonies are not obtained, use a 10-fold-lighter inoculum. B, for testing selective properties. Inoculate each medium with 10 µl of a 1:10 dilution of standardized cell suspension (0.5 McFarland). Although ATCC strains are listed, any organism that will yield the identical result is acceptable. For medium abbreviations, refer to Table 3.8.1–2.

V. PROCEDURE



Observe standard precautions.

- A. Perform wet prep for fecal leukocytes from fresh stools on request from outpatient specimens (*see* procedure 3.2.3).
 - ▣ **NOTE:** The presence of WBCs is one factor suggestive of invasive infection in cases of community-acquired gastroenteritis (*see* Table 3.8.1–4 for expected results). They are not helpful for diagnosis from hospitalized inpatients (25). Placement into transport medium destroys the laboratory's ability to determine relative numbers of WBCs or the presence of blood in the stool.
 - B. Culture methods
 1. Inoculation of media
 - a. Inoculate plates using a swab dipped into the transport tube.
 - b. Roll swab over one small area of the BAP and MAC media and streak in quadrants for isolated colonies.
 - c. Use larger amounts of the specimen for HEK, XLD, and SS agars and streak with a heavier-handed method to account for the inhibitory activity of the media.
 - d. Inoculate Selenite-F or GN broth with the swab that has been heavily saturated with stool from the transport vial.
 - e. For bloody stools and stools from pediatric patients, inoculate CT-SMAC plate to detect *E. coli* O157 and/or MAC broth to detect non-O157 VTEC.
 - f. Refer to Table 3.8.1–2 for selective media and enrichment broths for specific organism requests.
 2. Incubate all agars, except CIN, in ambient air at 35 to 37°C for 24 h.
 - a. Incubate CIN and MAC broth for *Yersinia* requests in ambient air at 25°C for 48 h.
 - b. Incubate Selenite-F for 18 to 24 h in ambient air at 35 to 37°C. Then subculture to selective media, e.g., MAC and HEK or XLD.
 - c. Incubate GN broth for 6 to 8 h. Then subculture broth to MAC and HEK or XLD.
- ▣ **NOTE:** If enrichment broths are incubated for excessive periods, the nonpathogenic enteric bacteria can overgrow the pathogens and negate the value of the enrichment procedure. If it is not possible to subculture broths at the appropriate time interval, subculture as soon as feasible, and closely monitor recovery rates. Alternatively, test enrichment broth directly for the presence of *Salmonella* and *Shigella* using commercial colored latex agglutination reagents.

Table 3.8.1–4 Microscopic and gross observations of fecal specimens associated with various infections^a

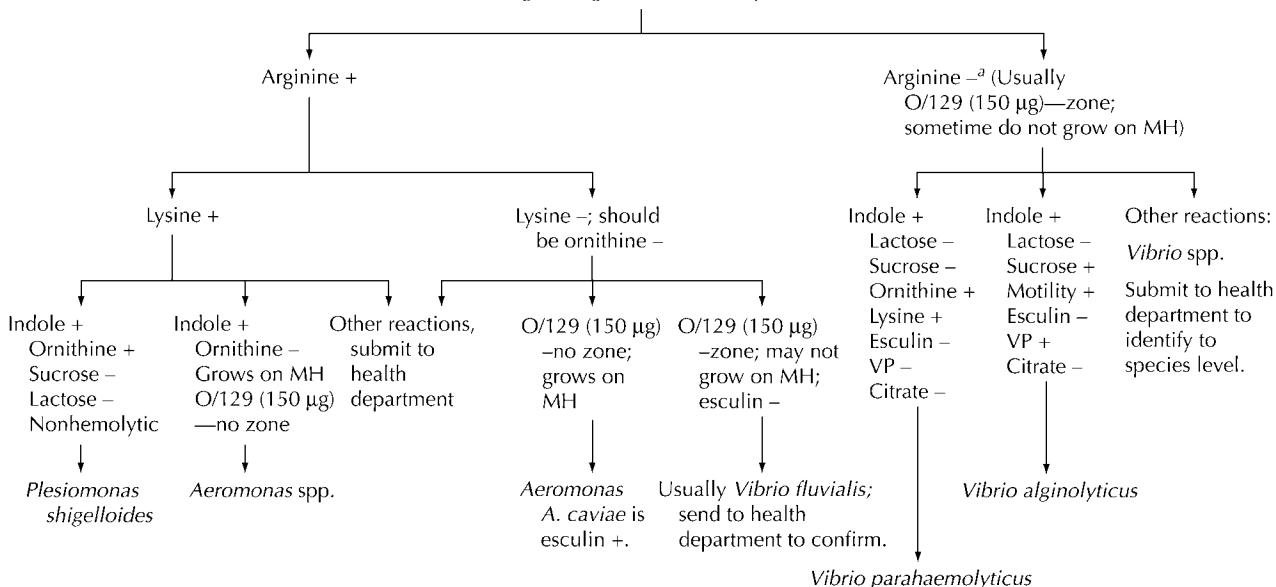
Organism or toxin	Other observations	Cells seen in smear	
		PMNs	RBCs
<i>Campylobacter</i>	Darting motile rods	Yes	Yes
<i>Clostridium difficile</i> toxin		Yes	Yes
<i>Escherichia coli</i> O157:H7, enterohemorrhagic	Watery	No	Yes
<i>Escherichia coli</i> , enteroinvasive	Mucous	Yes	Yes
<i>Escherichia coli</i> , enterotoxigenic	Watery	No	No
<i>Salmonella</i> spp.	Motile rods	Few	Yes
<i>Shigella</i> spp.	Lack of motile rods	Yes	No
<i>Vibrio cholerae</i>	Rice water	No	No
<i>Staphylococcus</i> toxin		No	No
Viruses		No	No

^a Data are only a guideline, and in any infection, observations are variable. For example, only 50% of *C. difficile*-associated cases of diarrhea demonstrate the presence of PMNs.

V. PROCEDURE (continued)

- d. Incubate MAC broth for 24 h at 35 to 37°C for VTEC assay (procedure 11.8).
- 3. Save all stools in transport vial for 24 to 48 h at 4°C in case a toxin assay is later requested.
- C. Examination of culture media
 - 1. BAP and BAP-A
 - a. Sweep through colonies to test for oxidase production. Pursue all oxidase-positive colonies, unless colony smells and looks like *P. aeruginosa*.
 - NOTE:** Most *Vibrio*, *Plesiomonas*, and *Aeromonas* organisms, but not all, are indole positive and beta-hemolytic (14, 15, 16). Despite their salt requirement, most vibrios grow on BAP and MAC.
 - (1) Subculture any oxidase-positive colonies to BAP and either TSI or KIA.
 - (2) Identify further all those with TSI or KIA reactions of acid/acid or alkaline/acid (see Fig. 3.8.1-1).
 - (3) Set up kit identification (use saline as diluent, if needed, for strains that require salt for growth).

Set up multitest kit with decarboxylase and fermentation tests; submit questionable identifications to local health department. Set up MH with 150-µg O/129 disk and ciprofloxacin, sulfamethoxazole-trimethoprim, ampicillin, colistin (or polymyxin B), and cefotaxime for nonstandard disk testing. If no growth on MH, repeat on MH with 4% NaCl.



^a*Aeromonas veronii* bv. Veronii is arginine - and esculin + and will be O/129 resistant; *Vibrio cholerae* may be O/129 resistant.

Figure 3.8.1-1 Flowchart for the identification of oxidase-positive stool pathogens from BAP or from either TCBS or CIN. Most are also indole positive. Biochemical reactions for species identification are available on many commercial kits. Note: Growth on TCBS implies that the organism is a *Vibrio* sp., but not all *Vibrio* spp. grow on TCBS. Abbreviations: MH, Mueller-Hinton agar; ID, identification; K, alkaline; A, acid; r/o, rule out.

V. PROCEDURE (continued)

- (4) Inoculate O/129 disks to both MH with and without 4% salt, as needed to verify identification.
▣ **NOTE:** Kits can misidentify *Vibrio vulnificus* as *V. parahaemolyticus*, *Aeromonas* as *Vibrio fluvialis*, and *Vibrio damselae* as *V. cholerae* (1). Use O/129 disk tests and salt tolerance to prevent initial misidentifications and potential public health consequences (see Fig. 3.8.1–1 and Table 3.18.2–8). *Aeromonas* organisms need not be identified to the species level, since it is difficult and may not be clinically important. For complete identifications, see references 14, 15, and 16.
 - (5) Unless reagents are available, submit *V. cholerae* to the health department for O1 serogrouping and toxin testing.
 - (6) Submit other *Vibrio* spp. if identification is questionable. Not all *Vibrio* spp. grow on TCBS or are indole positive.
 - (7) Perform antimicrobial susceptibility testing (AST) on isolates, since results are variable. Use disk method with MH with salt if isolate will not grow in routine method.
- b. Identify numerous *P. aeruginosa* and *S. aureus* organisms; do not perform AST.
 - c. Identify and report any amount of *B. cereus* organisms, if present.
 - (1) Screen beta-hemolytic colonies for large spore-forming, gram-positive rods.
 - (2) *B. cereus* is also catalase positive, motile, lecithinase positive, and penicillin resistant.
 - d. Look for overgrowth of numerous or pure yeast, or other unusual findings (e.g., DF-3), and report without AST.
▣ **NOTE:** DF-3 has been associated with diarrheal disease (2). It is a nonhemolytic, catalase-negative, oxidase-negative, indole-negative, nonmotile, gram-negative coccobacillus that does not grow on MAC. It ferments glucose without gas and is nitrate reductase negative.
 - e. Note the absence of enteric gram-negative rods.
 - f. Do not report enterococci in stool.
2. HEK, XLD, SS, and MAC
 - a. Work up potential pathogens directly from HEK, XLD, SS, and MAC plates at 24 h and from broth subculture plates at 36 to 48 h (18 to 24 h of incubation). *Screen plates for lactose-negative and/or H₂S-positive colonies.* Refer to Table 3.8.1–1 for colony morphologies of stool pathogens.
▣ **NOTE:** On days when stool cultures are not examined, place plates in refrigerator until they can be examined. After 24 h, alkaline reversion of colonies may make them appear lactose negative. Do not pick colonies after 24 h.
 - b. MAC or SS: pick one representative colony of each morphologic type of colorless colonies.
 - c. XLD: pick one representative colony of each morphologic type of red to red-orange colony and any colony with a black center.
▣ **NOTE:** Species of *Edwardsiella* and *Salmonella* decarboxylate lysine and ferment xylose but not sucrose or lactose. The xylose/lysine ratio in XLD permits these organisms to exhaust the xylose and then attack the lysine, causing a reversion to an alkaline pH, and thus produce red colonies (29).

V. PROCEDURE (continued)

- d. HEK: pick one representative colony of each morphologic type of green and blue colonies that are not pinpoint and any colony with a black center.
- NOTE: *Salmonella* and *Shigella* spp., when surrounded by many bright fermenting *Enterobacteriaceae*, may appear to be faint pink with a green tinge, but there is usually a clear halo around the colonies in the area of precipitated bile. This halo is most apparent when the plates are held up to a light. The colonies are more translucent than those of other *Enterobacteriaceae* (20).
- e. Subculture each colony picked to BAP, KIA or TSI, urea agar (optional; may use rapid urea disks instead), and Andrade's glucose (optional).
- NOTE: For laboratories with few stool culture requests or when the colony morphology indicates typical *Salmonella* or *Shigella*, use of kit identifications initially, rather than screening, may be more economical.
- f. Next day, read biochemical tests. Follow the flowchart in Fig. 3.8.1-2.

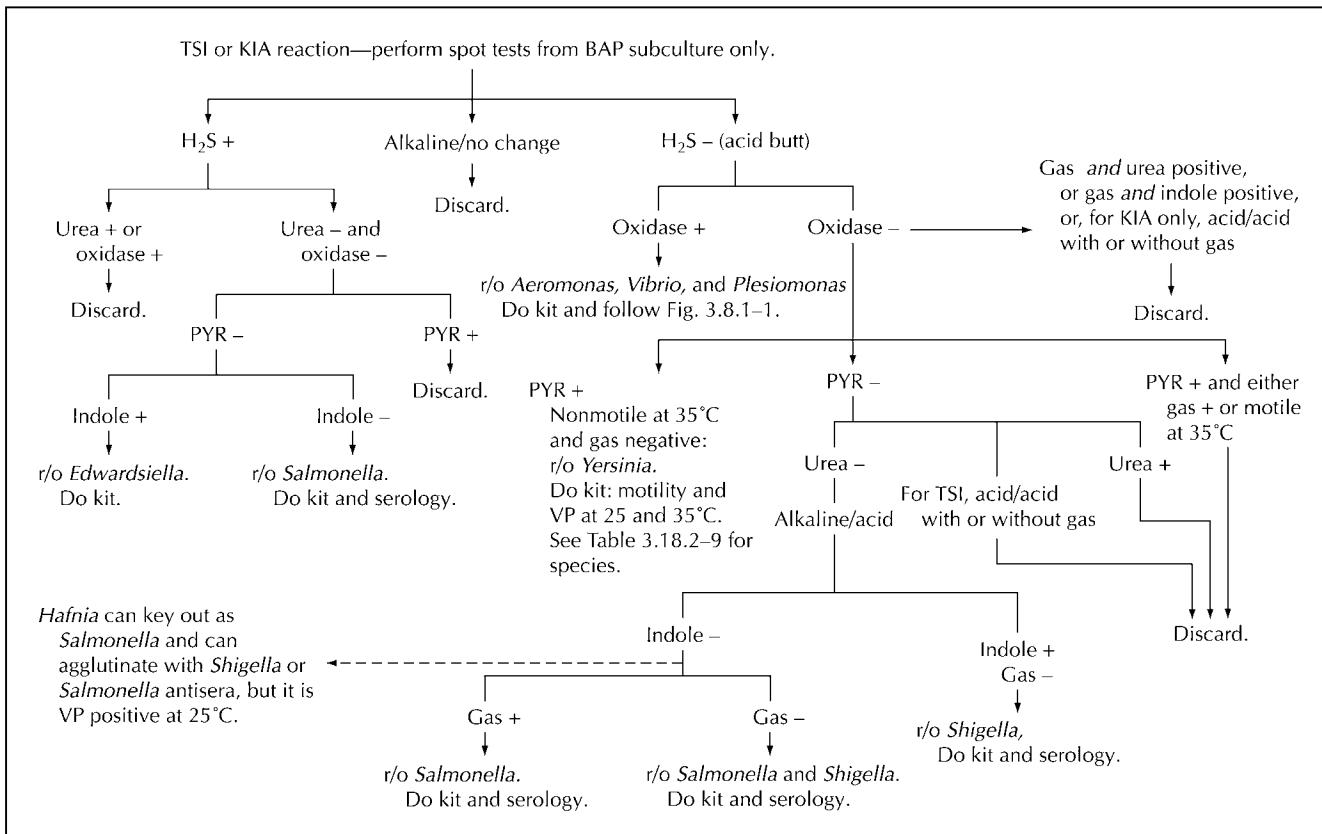


Figure 3.8.1-2 Flowchart for identification of stool pathogens from routine stool cultures. Set up either TSI or KIA, BAP, and urea agar (or rapid urea tube) from all lactose-negative or H₂S-positive colonies on enteric selective agars. Reactions of the slant are listed with a slash before the butt reaction. Optionally for H₂S-negative colonies, Andrade's glucose tube with Durham tube for gas will eliminate most questionable production of gas and provide a broth for VP testing. Perform spot tests (indole, oxidase, PYR) only from BAP. r/o, rule out.

V. PROCEDURE (continued)

- g.** Test for agglutination for *Salmonella* and *Shigella* somatic (O) antigens when the screening biochemical tests fit. See Appendix 3.8.1–1 for procedure. Alternatively, use Wellcolex latex kit or send isolate to reference laboratory for typing.
- NOTE:** *Shigella* spp. are genetically *E. coli*. If there is a question between *E. coli* and *Shigella*, 80% of *E. coli* organisms are acetate positive; shigellae are 100% negative (10). *Shigella* spp. are also negative for motility, citrate, and lysine.
- h.** If the kit gives a genus and species name, be sure that it corresponds to the serologic grouping.
- i.** For *Shigella* spp., identification to the species level is performed using the grouping sera, in combination with the kit biochemical tests.
- (1) Group A—*Shigella dysenteriae*
 - (2) Group B—*S. flexneri* (often indole positive)
 - (3) Group C—*Shigella boydii*
 - (4) Group D—*Shigella sonnei* (indole negative and *o*-nitrophenyl-β-D-galactopyranoside positive)
- j.** If the kit identifies the isolate as *Salmonella* or *Shigella*, but it fails to serogroup, repeat typing, using a boiled suspension of the organism in saline.
- k.** Check the following.
- (1) Mannitol-negative *S. flexneri* may be identified as *S. dysenteriae* but type as group B.
 - (2) If kit codes as *Salmonella* serovar Pullorum or *Salmonella* serovar Gallinarium, perform additional biochemical tests, including VP at 25°C, as these are often *Hafnia* spp. Serovar Pullorum agglutinates with *Salmonella* group D antiserum.
- l.** Check biochemical reactions and serogroup to determine species identification of *Salmonella* serovar Typhi, *Salmonella* serovar Choleraesuis, or *Salmonella* serovar Paratyphi A. Confirm with appropriate tests as indicated in Table 3.8.1–5.
- (1) Only serovar Typhi will type in both group D and in Vi antisera, is ornithine decarboxylase negative, and produces no gas and little H₂S from carbohydrates in KIA or TSI.
- NOTE:** Serovar Paratyphi C will also agglutinate in Vi antiserum.
- (2) Serovar Paratyphi A will type in group A antiserum and is citrate and lysine decarboxylase negative.
 - (3) Serovar Choleraesuis will type in group C antiserum and is arabinose and trehalose negative.

Table 3.8.1–5 Biochemical differentiation of selected members of the *Salmonella* group^a

Test	Serogroup Choleraesuis	Serogroup Paratyphi A	Serogroup Typhi	Other
<i>Salmonella</i> group	C	A	D	A–E
Arabinose fermentation	–	+	–	+
Citrate utilization	V	–	–	+
Glucose gas production	+	+	–	+
Lysine decarboxylase	+	–	+	+
Ornithine decarboxylase	+	+	–	+
Rhamnose fermentation	+	+	–	+
Trehalose fermentation	–	+	+	+

^a Symbols: –, ≤9% of strains positive; V, 10 to 89% of strains positive; +, ≥90% of strains positive.

V. PROCEDURE (continued)

- m. Submit all *Salmonella* (and *Shigella*, if typing is not available or if submission is required by local policy) isolates to the health department for confirmatory identification and for typing.
 - n. Perform AST on all enteric pathogens except *E. coli* O157, because treatment has been shown to predispose children to HUS (23, 24). Since enteric bacterial gastroenteritis is a self-limited illness in otherwise healthy children and adults, AST results should not be routinely reported except for in the following clinical situations:
 - (1) At the request of the physician
 - (2) Patient has disseminated infection with positive urine, blood, or other sterile fluid cultures.
 - (3) *Salmonella* serovar Typhi infection regardless of patient's age
 - (4) *Shigella* infection
 - (5) *Salmonella* infection in a child <1 year of age
 - (6) *Salmonella* infection in an adult >65 years of age
 - (7) Immunocompromised patients, including human immunodeficiency virus/AIDS
 - 3. Screen CIN at 24 and 48 h.
 - a. Look for any colonies with a deep red center with a sharp border surrounded by a translucent zone.
 - b. Inoculate BAP and identification kit.
 - c. Check oxidase reaction. If positive, follow Fig. 3.8.1–1 to identify *Aeromonas*.
 - d. If oxidase negative, inoculate Andrade's glucose (or methyl red-VP [MR-VP] broth) and motility semisolid medium at both 25 and 35°C.
 - e. Read VP and motility at 24 h to confirm kit identification.
 - (1) *Y. enterocolitica* and *Yersinia pseudotuberculosis* are nonmotile and VP negative at 35°C and motile at 25°C (9).
 - (2) *Y. enterocolitica* is VP positive at 25°C, but *Y. pseudotuberculosis* is VP negative.
 - (3) Both are usually urease positive.
 - (4) See Table 3.18.2–9 for biochemical reactions of significant members of this genus.
 - (5) Confirm atypical reactions by submitting species to reference laboratory or health department.
 - f. Perform AST on *Aeromonas* and *Yersinia* spp.
 - 4. Screen TCBS at 24 and 48 h.
 - a. Look for yellow colonies (potential *V. cholerae*, *V. fluvialis*, and *Vibrio furnissii*) and blue colonies (potential *V. parahaemolyticus*, *Vibrio mimicus*, and *Vibrio hollisae*). Pinpoint colonies should also be examined.
 - b. Subculture one representative colony of each morphologic type of any colony to BAP and KIA or TSI.
 - c. Follow Fig. 3.8.1–1 for identification of oxidase-positive colonies that ferment glucose.
 - d. Expediently send cultures with suspected *V. cholerae* to the local public health laboratory for typing and toxin testing, unless reagents are available.
 - e. Refer to item V.C.1 for other details of testing.
- NOTE:** For outbreaks, TCBS, in conjunction with enrichment in alkaline peptone water (APW), incubated for 24 h, followed by subculture onto TCBS significantly increased the yield of *V. cholerae*. Subculture of APW at 5 h is not necessary or practical (21).

V. PROCEDURE (continued)

5. Screen CT-SMAC plate for *E. coli* O157 at 24 h of incubation.
 - a. Pick at least four sorbitol-negative (transparent or colorless) colonies.
 - b. Subculture to BAP.
 - c. If isolate is oxidase negative and indole positive, perform MUG test.
 - d. If isolate is MUG negative, screen using the *E. coli* O157 latex agglutination kit (see Appendix 3.8.1–2).
 - e. If agglutination positive, confirm as *E. coli* by kit identification.
6. Test 50 µl of MAC broth or plate sweep of culture with one of the EHEC serologic assays 24 h after inoculation with stool. See procedure 11.8.
7. Depending on state requirements, submit *V. cholerae*, *Salmonella* spp., *Shigella* spp., *E. coli* O157, or other stool pathogens to the health department. Send repeat isolates of *Salmonella* spp. per local health department policy.

POSTANALYTICAL CONSIDERATIONS**VI. REPORTING RESULTS**

- A. Record biochemical results on colonies being screened on appropriate worksheets, noting media sampled and enumeration.
- B. Negative cultures
 1. For all stool cultures, report a pathogen-negative comment that specifies all of the enteric bacterial pathogens the laboratory has tested for. For example, report “No *Salmonella*, *Shigella*, or *Campylobacter* sp. isolate” if these are the only pathogens routinely tested.
 2. If additional pathogens have been ruled out, the comment should be expanded to also list other culture-negative results. Include a comment such as “No *E. coli* O157, *Yersinia*, *Aeromonas*, or *Vibrio* detected.”
 3. Add “to date” for preliminary reports and delete for final reports.
- C. Additional comments
 1. If gram-negative enteric microbiota are not present in the culture, add a comment: “No normal enteric gram-negative rods isolated.”
 2. Report overgrowth of predominant *S. aureus* and *P. aeruginosa* as “Predominating or pure culture of [organism name].”
 NOTE: The presence of *S. aureus* may or may not indicate gastrointestinal disease or food poisoning. Toxin must be detected in stool or food for disease, which is rarely done.
 3. Report yeast, if found in pure or predominating culture, without genus or species identification.
 4. In the setting of diarrhea and no other likely cause, report a pure culture of unusual microbiota, such as DF-3 or *Klebsiella* spp.
- D. Positive cultures
 1. Report presumptive presence of any enteric pathogens listed in Table 3.8.1–6 with or without quantitation, depending on laboratory’s policy to track positive cultures from enrichment broth only.
 2. Report the pathogen with the preliminary designation as “probable” until the identification is confirmed by both biochemical testing and serology.

Table 3.8.1–6 Summary of detection media and identification methods for fecal pathogens^a

Pathogen(s)	Media	Initial observation	Initial testing	Confirmatory	Comments
<i>Salmonella</i> and <i>Shigella</i>	MAC, HEK, XLD	See Table 3.8.1–1	Follow Fig. 3.8.1–2	Serology	
<i>Yersinia enterocolitica</i>	MAC, CIN	Red on CIN, colorless on MAC	Kit	Motility and VP at 25 and 35°C	
<i>Aeromonas</i> spp.	BAP, BAP-A, MAC, CIN	Oxidase positive, usually indole positive, red on CIN	Kit; follow Fig. 3.8.1–1	O/129 disk Esculin (optional)	Lactose positive or negative
<i>Plesiomonas shigelloides</i>	BAP, MAC	Oxidase positive, indole positive	Kit; follow Fig. 3.8.1–1		Lactose negative
<i>Vibrio</i>	BAP, MAC, TCBS	Oxidase positive, may be colorless on MAC, yellow or blue on TCBS	Follow Fig. 3.8.1–1; kit; may need to add salt to inoculum.	O/129 disk, salt tolerance	<i>V. mimicus</i> is VP negative, colistin susceptible; <i>V. cholerae</i> is VP positive, colistin resistant.
<i>Edwardsiella tarda</i>	MAC, HEK, XLD	Colorless on MAC, black center on HEK or XLD	Kit; indole positive	None	
<i>Bacillus cereus</i>	BAP	Hemolytic	Large gram-positive rods	Catalase positive, motile, lecithinase positive, and penicillin resistant	Toxin testing may be done by health department.
EHEC	CT-SMAC	Colorless	Kit; MUG negative	Serotyping	Toxin assay more sensitive to detect other strains.
Other <i>E. coli</i> strains Enteropathogenic Enteroinvasive Adherent Enterotoxigenic	NA	NA	NA	NA	Send stool to reference laboratory or health department for toxin, invasion, or adherence testing.
<i>Clostridium difficile</i>	CCEY or CCFA	Large, gray (CCEY) or opalescent yellow (CCFA) colonies that fluoresce chartreuse on anaerobic BAP or CCEY	Gram stain showing large gram-positive rods, “horse barn” odor	None	Perform toxin testing. (See procedure 3.8.3.)
<i>Clostridium botulinum</i>	NA	NA	NA	NA	Send stool and serum to public health department.

^a For *Campylobacter* species, see procedure 3.8.2. NA, not applicable.

VI. REPORTING RESULTS (continued)

3. Enter preliminary note indicating that “Isolate has been sent for confirmatory identification to [name and location]” when isolate has been sent for serologic testing.
4. *Salmonella* reporting
 - **NOTE:** *Salmonella* nomenclature is a topic of worldwide debate at the present time (4). There is only one species of *Salmonella* that infects humans; some call this species *Salmonella enterica* and others refer to it as *Salmonella choleraesuis*. In addition, the species is divided into six subspecies and over 2,000 serotypes. Serotypes are grouped into several serogroups noted alphabetically as A, B, C₁, C₂, etc. Serotype names are called either serovars or serotypes and are not in italics. Some texts capitalize the serotype name and others do not. Because the long names are cumbersome and laboratory computer systems do not italicize, *Salmonella enterica* subsp. *enterica* serotype Typhimurium is usually called *Salmonella typhimurium* or *Salmonella* serovar Typhimurium on laboratory reports.
 - a. Report the following to the serotype level: *Salmonella* serovar Paratyphi A, *Salmonella* serovar Choleraesuis, or *Salmonella* serovar Typhi, using Table 3.8.1–5.
 - b. Report isolates that are serologically A, C, or D, but are *not* biochemically serovar Paratyphi A, serovar Choleraesuis, or serovar Typhi, as follows.
 - (1) *Salmonella* serogroup A—not Paratyphi A
 - (2) *Salmonella* serogroup C—not Choleraesuis
 - (3) *Salmonella* serogroup D—not Typhi
 - c. Report other *Salmonella* isolates by group, if tested, or by genus only until the species name is available from the reference laboratory.
 - d. By accepted convention, report final identifications of *Salmonella*, followed by the serovar name only (4).
 - 5. When confirmation is obtained, report *V. cholerae*, including the serogroup (O1 or non-O1), serotype of O1 strains (Inaba, Ogawa, and Hikojima), biotype (e.g., classical or El Tor), and presence of toxin.
 - 6. Report sorbitol-negative colonies which show positive agglutination with O157 latex test reagent and a positive identification of *E. coli* as “*E. coli* serotype O157.” If tested for toxin, report as, e.g., “*E. coli* O157, toxin producer.”
 - 7. Other pathogens listed in Table 3.8.1–6 are generally reported without confirmation by a reference laboratory, unless there is a question.

E. Notification

1. For hospitalized patients, notify infection control and the nursing care unit immediately by telephone with presumptive identification (agglutination positive without biochemical confirmation) of suspicious enteric pathogens so that proper patient isolation protocols can be instituted.
 - **NOTE:** Patients shedding pathogens in their stool are placed on both standard precautions and contact isolation.
2. For patients who have been discharged from the hospital or whose specimens were received as outpatient specimens, communicate the final result of the presence of any of the enteric pathogens to the person responsible for the patient’s care.
3. Because of its life-threatening consequences, contact the physician expeditiously if *V. cholerae*, *E. coli* O157, *Salmonella* serovar Typhi, or *Salmonella* serovar Paratyphi C is isolated.
4. For hospitalized patients, notify the physician if there will be a delay in reporting a highly suspicious culture (e.g., problems with agglutination, etc.).
5. Report all reportable enteric pathogens to the appropriate public health agency as required by the local regulatory and government jurisdictions.

VI. REPORTING RESULTS (continued)

Usually these include *Salmonella*, *Shigella*, *Yersinia*, *Vibrio*, *E. coli* O157, and *Campylobacter*.

- a. Check with local regulations to determine which agents are reportable in your state.
- b. Because of their epidemiologic consequences, *immediately* notify the local health department of suspected *V. cholerae*, *Salmonella* serovar Typhi, *Salmonella* serovar Paratyphi A, or *Salmonella* serovar Choleraesuis.

F. AST

1. Since enterocolitis is a self-limited infection in otherwise healthy children and adults, AST should be performed on enteric pathogens but only selectively reported. Patients should not be routinely treated with antimicrobial agents unless they are <1 year of age, elderly, or immunocompromised.
2. For *Shigella*, *Salmonella*, *Aeromonas*, *Plesiomonas*, *Edwardsiella*, *Vibrio*, and *Yersinia*, report only ampicillin, sulfamethoxazole-trimethoprim, and a quinolone. For patients with invasive infection (i.e., positive blood cultures) and those with multiply resistant strains, a third-generation cephalosporin should also be reported. A comment should be placed on reports of *Salmonella* and *Shigella* that first- and second-generation cephalosporins and aminoglycosides are not appropriate treatment of these pathogens.
3. Suppress quinolone results for children under the age of 14 years, since these drugs inhibit bone growth.
4. For *V. cholerae*, also report doxycycline or tetracycline.
5. Susceptibility testing is contraindicated for *E. coli* O157 and other EHEC organisms, since treatment of enterohemorrhagic colitis with antimicrobial agents can induce bacterial cell lysis with the release of toxin, which may increase the risk of developing HUS. Add a comment to the report such as, “Antimicrobial therapy is not indicated for this pathogen.”

VII. INTERPRETATION

- A. The isolation of a stool pathogen may not identify the cause of the disease. For example, *Salmonella* is present in the carrier state, without disease, and *Plesiomonas* is a questionable pathogen.
- B. Isolation of an organism whose toxin is responsible for disease may not indicate that the organism possesses the genetic ability to produce the toxin or that the toxin was produced in the patient.

VIII. LIMITATIONS

- A. Because the media used for culture are highly selective, some pathogens that should grow may be inhibited. The more media inoculated, the more likely a pathogen will be isolated.
- B. Use of enrichment broth or the use of both XLD and HEK will detect more pathogens, but the cost-benefit may not make the expense and time practical.
- C. Without a specific request for *Vibrio* or *Yersinia*, selective medium will not be inoculated and these pathogens may or may not be detected in culture.
- D. Pathogens present in small numbers may not be detected. Testing of two separate specimens increases the yield.
- E. Many pathogenic *E. coli* organisms are difficult to detect, since there is no medium for their selection.
- F. CT-SMAC will not detect VTEC strains other than *E. coli* O157.
- G. The agent that is causing the patient's diarrheal disease may not have yet been recognized as a stool pathogen and will thus go undetected.
- H. The physician must consider other agents, such as viruses and parasites, that can be responsible for disease.

REFERENCES

1. Abbott, S. L., L. S. Seli, M. Catino, Jr., M. A. Hartley, and J. M. Janda. 1998. Misidentification of unusual *Aeromonas* species as members of the genus *Vibrio*: a continuing problem. *J. Clin. Microbiol.* **36**:1103–1104.
2. Blum, R. N., C. D. Berry, M. G. Phillips, D. L. Hamilos, and E. W. Koneman. 1992. Clinical illnesses associated with isolation of dysgonic fermenter 3 from stool samples. *J. Clin. Microbiol.* **30**:396–400.
3. Brazier, J. S. 1993. Role of the laboratory in investigations of *Clostridium difficile* diarrhea. *Clin. Infect. Dis.* **16**(S4):228–233.
4. Brenner, F. W., R. G. Villar, F. J. Angulo, R. Tauxe, and B. Swaminathan. 2000. *Salmonella* nomenclature. *J. Clin. Microbiol.* **38**:2465–2467.
5. Church, D. L., G. Cadrain, A. Kabani, T. Javaji, and C. Trevenen. 1995. Practice guideline for ordering stool cultures in a pediatric population. Alberta Children's Hospital, Calgary, Alberta, Canada. *Am. J. Clin. Pathol.* **103**:149–153.
6. Clark, R. B., and J. M. Janda. 1991. *Plesiomonas* and human disease. *Clin. Microbiol. Newslett.* **13**:49–52.
7. Clinical and Laboratory Standards Institute. 2004. *Quality Assurance for Commercially Prepared Microbiological Culture Media*, 3rd ed. Approved standard M22-A3. Clinical and Laboratory Standards Institute, Wayne, Pa.
8. Clinical and Laboratory Standards Institute. 2006. *Performance Standards for Antimicrobial Susceptibility Testing*. Sixteenth informational supplement. Approved standard M100-S16. Clinical and Laboratory Standards Institute, Wayne, Pa.
9. Darland, G., W. H. Ewing, and B. R. Davis. 1974. *The Biochemical Characteristics of Yersinia enterocolitica and Yersinia pseudotuberculosis*. Center for Disease Control, Atlanta, Ga.
10. Farmer, J. J., III, B. R. Davis, F. W. Hickman-Brenner, A. McWhorter, G. P. Huntley-Carter, M. A. Asbury, C. Riddle, H. G. Wathen-Grady, C. Elias, G. R. Fanning, A. G. Steigerwalt, C. M. O'Hara, G. K. Morris, P. B. Smith, and D. J. Brenner. 1985. Biochemical identification of new species and biogroups of *Enterobacteriaceae* isolated from clinical specimens. *J. Clin. Microbiol.* **21**:46–76.
11. Fey, P. D., R. S. Wickert, M. E. Rupp, T. J. Safranek, and S. H. Hinrichs. 2000. Prevalence of non-O157:H7 Shiga toxin-producing *Escherichia coli* in diarrheal stool samples from Nebraska. *Emerg. Infect. Dis.* **6**:530–533.
12. Guerrant, R. L., T. Van Gilder, T. S. Steiner, N. M. Thielman, L. Slutsker, R. V. Tauxe, T. Hennessy, P. M. Griffin, H. DuPont, R. B. Sack, P. Tarr, M. Neill, I. Nachamkin, L. B. Reller, M. T. Osterholm, M. L. Bennish, and L. K. Pickering. 2001. Practice guidelines for the management of infectious diarrhea. *Clin. Infect. Dis.* **32**:331–351.
13. Hayes, P. S., K. Blom, P. Feng, J. Lewis, N. A. Strockbine, and B. Swaminathan. 1995. Isolation and characterization of a β-D-glucuronidase-producing strain of *Escherichia coli* serotype O157:H7 in the United States. *J. Clin. Microbiol.* **33**:3347–3348.
14. Huys, G., P. Kampfer, M. Altwegg, I. Kersters, A. Lamb, R. Coopman, J. Luthy-Hottenstein, M. Vancanneyt, P. Janssen, and K. Kersters. 1997. *Aeromonas popoffii* sp. nov., a mesophilic bacterium isolated from drinking water production plants and reservoirs. *Int. J. Syst. Bacteriol.* **47**:1165–1171.
15. Janda, J. M., C. Powers, R. G. Bryant, and S. L. Abbott. 1988. Current perspectives on the epidemiology and pathogenesis of clinically significant *Vibrio* spp. *Clin. Microbiol. Rev.* **1**:245–267.
16. Janda, J. M. 1991. Recent advances in the study of the taxonomy, pathogenicity, and infectious syndromes associated with the genus *Aeromonas*. *Clin. Microbiol. Rev.* **4**:397–410.
17. Karmali, M. 1989. Infection by verocytotoxin-producing *Escherichia coli*. *Clin. Microbiol. Rev.* **2**:15–38.
18. Kehl, K. S., P. Havens, C. E. Behnke, and D. W. Acheson. 1997. Evaluation of the Premier EHEC assay for detection of Shiga toxin-producing *Escherichia coli*. *J. Clin. Microbiol.* **35**:2051–2054.
19. Kelly, M. T., E. M. Stroh, and J. Jessop. 1988. Comparison of blood agar, ampicillin blood agar, MacConkey-ampicillin-Tween agar, and modified cefsulodin-Irgasan-novobiocin agar for isolation of *Aeromonas* spp. from stool specimens. *J. Clin. Microbiol.* **26**:1738–1740.
20. King, S., and W. I. Metzger. 1968. A new plating medium for the isolation of enteric pathogens. I. Hektoen enteric agar. *Appl. Microbiol.* **16**:577–578.
21. Lesmana, M., E. Richie, D. Subekti, C. Simanjuntak, and S. E. Walz. 1997. Comparison of direct-plating and enrichment methods for isolation of *Vibrio cholerae* from diarrhea patients. *J. Clin. Microbiol.* **35**:1856–1858.
22. Mackenzie, A. M. R., P. Lebel, E. Orrbine, P. C. Rowe, L. Hyde, F. Chan, W. Johnson, P. N. McLaine, and The Synsorb Pk Study Investigators. 1998. Sensitivities and specificities of Premier *E. coli* O157 and Premier EHEC enzyme immunoassays for diagnosis of infection with verotoxin (Shiga-like toxin)-producing *Escherichia coli*. *J. Clin. Microbiol.* **36**:1608–1611.
23. Molbak, K., P. S. Mead, and P. M. Griffin. 2002. Antimicrobial therapy in patients with *Escherichia coli* O157:H7 infection. *JAMA* **288**:1014–1016.
24. Safdar, N., A. Said, R. E. Gangnon, and D. G. Maki. 2002. Risk of hemolytic uremic syndrome after antibiotic treatment of *Escherichia coli* O157:H7. A meta-analysis. *JAMA* **288**:996–1001.
25. Savola, K. L., E. J. Baron, L. S. Tompkins, and D. J. Passaro. 2001. Fecal leukocyte stain has diagnostic value for outpatients but not inpatients. *J. Clin. Microbiol.* **39**:266–269.

REFERENCES (continued)

26. Schiemann, D. A. 1979. Synthesis of a selective agar medium for *Yersinia enterocolitica*. *Can. J. Microbiol.* **25**:1298.
27. Schiemann, D. A. 1982. Development of a two-step enrichment procedure for recovery of *Yersinia enterocolitica* from food. *Appl. Environ. Microbiol.* **43**:14.
28. Slutsker, L., A. A. Ries, K. D. Greene, J. G. Wells, L. Hutwagner, and P. M. Griffin. 1997. *Escherichia coli* O157:H7 diarrhea in the United States: clinical and epidemiologic features. *Ann. Intern. Med.* **126**:505–513.
29. Taylor, W. I. 1965. Isolation of shigellae. I. Xylose lysine agars: new media for isolation of enteric pathogens. *Am. J. Clin. Pathol.* **44**:471–475.

SUPPLEMENTAL READING

- Ewing, W. H. (ed.). 1986. *Edwards and Ewing's Identification of Enterobacteriaceae*, 4th ed. Elsevier Science Publishing Co., New York, N.Y.
- Gilligan, P. H., J. M. Janda, M. A. Karmali, and J. M. Miller. 1992. Cumitech 12A, *Laboratory Diagnosis of Bacterial Diarrhea*. Coordinating ed., F. S. Nolte. American Society for Microbiology, Washington, D.C.
- Jeppesen, C. 1995. Media for *Aeromonas* spp., *Plesiomonas shigelloides* and *Pseudomonas* spp. from food and environment. *Int. J. Food Microbiol.* **26**:25–41.
- Karch, H., and M. Bielaszewska. 2001. Sorbitol-fermenting Shiga toxin-producing *Escherichia coli* O157:H(−) strains: epidemiology, phenotypic and molecular characteristics, and microbiological diagnosis. *J. Clin. Microbiol.* **39**:2043–2049.
- McFaddin, J. F. 1985. *Media for Isolation, Cultivation, Identification, and Maintenance of Bacteria*, vol. I. Williams & Wilkins, Baltimore, Md.
- Mead, P. S., L. Slutsker, V. Dietz, L. F. McCaig, J. S. Bresce, C. Shapiro, P. M. Griffin, and R. V. Tauxe. 1999. Food-related illness and death in the United States. *Emerg. Infect. Dis.* **5**:607–625.

APPENDIX 3.8.1-1



Observe standard precautions.

Detection of Somatic O Antigen Serogroups of Bacteria

- I. PRINCIPLE

Gram-negative rods possess somatic antigens in their cell walls, called O antigens, that are useful in the identification of specific serovars or serotypes. These antigens are polysaccharide side chains of endotoxin in the outer membrane of the cell wall. Antibody to these antigens will agglutinate a suspension of the organism.
- II. SPECIMEN

Prepare milky suspension of pure culture from fresh plate in normal sterile saline. If culture is not pure, erroneous results can occur.
- III. MATERIALS
 - A. Antisera
 1. *Salmonella* polyvalent, A, B, C1, C2, D, E, etc., and Vi; *Shigella* groups A, B, C, and D; *E. coli* O157; *V. cholerae* O1, or any other serotype
 2. Store antisera at 4°C.
 - B. Petri dishes and either glass slides or disposable cards with black background (preferred)
- IV. QUALITY CONTROL

Test each lot of reagent with a known positively and negatively reacting organism prior to putting into use it and every 6 months thereafter. Organisms can be stored in formalin at 4°C for QC use.
- V. PROCEDURE

Mix drop of antiserum with drop of suspension of organism. Rotate slide for 1 min and observe for agglutination.
- VI. INTERPRETATION

Positive agglutination is an indication that the strain contains O antigen.
- VII. REPORTING RESULTS

Report presumptive identification as “[organism name]” followed by serotype alpha and numeric designation.

APPENDIX 3.8.1-1 (continued)**VIII. LIMITATIONS**

- A. Some organisms with either capsules or flagella can give a false-negative result. Boil the suspension for 5 min or test *Salmonella* using Vi antigen for the capsule of this genus.
- B. Serological identification should be confirmed with biochemical testing, since serotypes can be shared or cross-react with other species.

Supplemental Reading

Ewing, W. H. 1986. *Edwards and Ewing's Identification of Enterobacteriaceae*, 4th ed. Elsevier Science Publishing Co., New York, N.Y.

APPENDIX 3.8.1-2

Detection of *Escherichia coli* O157 by Latex Agglutination**I. PRINCIPLE**

The *Escherichia coli* serotype most frequently isolated in cases of HC and HUS is O157:H7. Detection of this serotype from a strain of *E. coli* is presumptive for the agent of HUS and HC. Cytotoxin testing confirms the identification as a strain that produces verocytotoxin. Colonies are mixed with latex particles coated with antibody against the cell wall O antigen of *E. coli* O157. *E. coli* O157 will bind with the antibody, causing the latex particles to visibly agglutinate. Bacteria which are not O157 will not bind to the antiserum and will not result in agglutination (1, 2, 3).

II. SPECIMEN

Indole-positive, oxidase-negative, MUG-negative, non-sorbitol-fermenting colonies from CT-SMAC medium. Test multiple colonies if a negative result is obtained.

III. MATERIALS

A. *E. coli* O157 kit stored at 2 to 8°C. Some suppliers are listed below.

1. Oxoid Incorporated, Ogdensburg, N.Y.
2. Pro-Lab Diagnostics, Austin, Tex.
3. Remel Microbiology Products, Lenexa, Kans.
4. Meridian Diagnostics, Cincinnati, Ohio

B. Kits usually contain the following.

1. One dropper vial of *E. coli* O157 latex reagent containing latex particles coated with purified rabbit immunoglobulin G (IgG) which reacts with *E. coli* somatic antigen O157
2. One dropper vial of negative control latex containing latex particles coated with purified rabbit IgG which does not react with *E. coli* serotype O157
3. One dropper vial of positive control suspension containing *E. coli* serotype O157:H7 antigen
4. A negative control *E. coli* suspension (a culture of *E. coli* ATCC 25922 can be used in place of the negative control)
5. Test cards
6. Mixing sticks

IV. QUALITY CONTROL

Check each lot of reagents prior to use and every 6 months thereafter with the suspension of *E. coli* O157:H7 (positive control) and a suspension of *E. coli* (ATCC 25922) (negative control).

APPENDIX 3.8.1–2 (continued)**V. PROCEDURE**

Follow manufacturer's instructions and observe for agglutination. An example is below.

- A. Bring reagents to room temperature before testing.
- B. Resuspend colony in 0.5 ml of normal sterile saline in a culture tube to McFarland 3 to 5.
- C. Place 1 drop of *E. coli* O157 latex reagent onto a test circle.
- D. Using a sterile Pasteur pipette, add 1 drop of the colony suspension to the test circle. Alternatively, add a drop of saline to the card and emulsify the colony directly in the saline until a smooth suspension is obtained.
- E. Mix reagent with the bacterial suspension in the test circle.
- F. Rock card gently and examine for agglutination over a 1- to 2-min period.
- G. Specimens showing positive agglutination within 2 min must be examined further.
- H. Test positive specimens again by repeating the procedure using negative control latex reagent.

VI. INTERPRETATION

- A. Positive agglutination indicates the presence of *E. coli* serogroup O157 in the recommended time. Grade the agglutination as follows.
 1. 4+: large clumps against a very clear background
 2. 3+: large and small clumps against a clear background
 3. 2+: small but definite clumps against a slightly clouded background
 4. 1+: fine granulation against a milky background
 5. Negative: a homogeneous suspension of particles with no visible clumping.
- B. A lack of agglutination indicates the absence of *E. coli* serogroup O157.
- C. Results cannot be interpreted if there is agglutination of both the test and control latex.

VII. REPORTING RESULTS

- A. Report positive agglutination with O157 latex test reagent, no agglutination with negative control reagent, and identification as *E. coli* as "*E. coli* serotype O157."
- B. Testing for the H antigen or VTEC may be needed. See procedure 3.8.1 for further testing.

VIII. LIMITATIONS

- A. Some strains of *E. coli* are difficult to emulsify in saline and may give a stringy-type reaction with both the test and the control reagents. If this stringiness is found, suspend the colony in 0.5 ml of saline. Allow the lumps to settle, and retest the smooth supernatant.
- B. Neither growth of colorless colonies on CT-SMAC nor a positive reaction in the *E. coli* O157 latex test will directly confirm the isolate as a toxin-producing strain.
- C. The latex test does not confirm the isolate as having the H7 flagellar type or that the strain is a toxin producer. Further studies are needed to determine these characteristics.
- D. Other serotypes which produce verocytotoxin are not detected in this assay.
- E. Some strains of *Escherichia hermannii* may cross-react with *E. coli* O157 antiserum and the latex test due to a shared antigen. *E. hermannii* may be differentiated from *E. coli* by the former's ability to ferment cellobiose and yellow pigmentation, which may be delayed.

APPENDIX 3.8.1-2 (continued)**References**

1. Mackenzie, A. M. R., P. Lebel, E. Orrbine, P. C. Rowe, L. Hyde, F. Chan, W. Johnson, P. N. McLaine, and The Synsorb Pk Study Investigators. 1998. Sensitivities and specificities of Premier *E. coli* O157 and Premier EHEC enzyme immunoassays for diagnosis of infection with verotoxin (Shiga-like toxin)-producing *Escherichia coli*. *J. Clin. Microbiol.* **36**:1608–1611.
2. Sowers, E. G., J. G. Wells, and N. A. Strockbine. 1996. Evaluation of commercial latex reagents for identification of O157 and H7 antigens of *Escherichia coli*. *J. Clin. Microbiol.* **34**:1286–1289.
3. Thompson, J. S., D. S. Hodge, and A. A. Borczyk. 1990. Rapid biochemical test to identify verocytotoxin-positive strains of *Escherichia coli* serotype O157. *J. Clin. Microbiol.* **28**:2165–2168.

3.8.2

Fecal Culture for *Campylobacter* and Related Organisms

[Updated March 2007]

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Using recently developed genetic techniques, the genus *Campylobacter* was divided into three genera based on 16S rRNA homology tests (22). The groups *Campylobacter*, *Helicobacter*, and *Arcobacter* are microaerobic to anaerobic organisms. These curved, helical, gram-negative rods are among the most challenging bacteria for the clinical microbiologist to cultivate and identify. In addition, the disease spectrum with these genera ranges from "well established" (as in the association of *Campylobacter jejuni* and diarrhea) to "speculative" (associated with clinical strains such as *Arcobacter* species that have unproven etiology) to "emerging" (such as *C. upsaliensis* and *Helicobacter rappini* ["*Flexispira rappini*"]).

Table 3.8.2-1 lists the taxonomic positions, known sources, and common disease associations for campylobacters, helicobacters, arcobacters, and other, unrelated genera that are sometimes confused with campylobacters (16). In Table 3.8.2-2, clinical diseases and their possi-

ble causative agents are listed. Because of diverse growth requirements, such as temperature, atmosphere of incubation, etc., most clinical laboratory methods are designed to recover only the most common pathogenic strains of these genera. While this procedure focuses on fecal cultures, these basic methods can be used to identify campylobacters from most cultures. For *Helicobacter pylori*, refer to procedure 3.8.4.

It is estimated that *Campylobacter* causes approximately 2.4 million cases of disease each year in the United States. *C. jejuni* is the most commonly identified bacterial pathogen isolated from fecal cultures, with greater than 90% of campylobacter infections caused by *C. jejuni* (7). *C. jejuni* gastroenteritis is a self-limiting disease, but severe infections occur in the young and immunocompromised. The procedure that follows is designed to detect and identify *C. jejuni*. Although other species have been associated with gastroenteritis, they are more difficult to isolate

and identify to the species level. Consequently, culture of these other species cannot be justified in most clinical laboratories.

Because *Campylobacter* spp. and *Escherichia coli* O157:H7 are the major contributors to blood in the stool, it is important to detect the presence of the infecting organism, since the use of antimicrobial treatment for *E. coli* O157 infection is thought to increase the risk of hemolytic-uremic syndrome. The drug of choice for treatment of campylobacters was ciprofloxacin, because it is effective for both *Campylobacter* and other enteric pathogens. The use of quinolones in the poultry industry has increased resistance of campylobacters to quinolones, with rates of 10% (1) to as high as 40% (15). Erythromycin is returning as the drug of choice for treatment, although occasional resistance is also seen with this agent. Newer macrolides are also effective but are more expensive.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING

A. Specimen collection

1. See procedure 3.8.1 for fecal and rectal swab collection.
2. *Campylobacter* can be isolated from routine anaerobic cultures of blood or wound specimens. If isolate does not grow on subculture, incubate in a microaerobic atmosphere at 35°C for possible campylobacter or helicobacter isolation.

Table 3.8.2-1 Taxonomic position, known sources, and common disease associations of *Campylobacter*, *Arcobacter*, *Helicobacter*, and related species^a

Taxon ^b	Known source(s)	Disease association(s)	
		Human	Veterinary
rRNA homology group I			
<i>Campylobacter fetus</i> subsp. <i>fetus</i> ('Campylobacter fetus' subsp. <i>intestinalis</i>)	Cattle, sheep	Septicemia, gastroenteritis, abortion, meningitis	Bovine and ovine spontaneous abortion
<i>Campylobacter fetus</i> subsp. <i>veneralis</i> ('Campylobacter fetus' subsp. <i>fetus</i>)	Cattle	Septicemia	Bovine infectious infertility
<i>Campylobacter hyoilei</i> subsp. <i>hyoilei</i> ^c	Pigs, cattle, hamsters, deer	Gastroenteritis	Porcine and bovine enteritis
<i>Campylobacter hyoilei</i> subsp. <i>lawsonii</i> ^b	Pigs	None at present	Unknown
<i>Campylobacter concisus</i>	Humans	Periodontal disease, gastroenteritis	None at present
<i>Campylobacter mucosalis</i> ('Campylobacter sputorum' subsp. <i>mucosalis</i>)	Pigs	None at present	Porcine necrotic enteritis and ileitis
<i>Campylobacter sputorum</i> bv. <i>Sputorum</i> (incorporating bv. <i>Bubulus</i> ^d)	Humans, cattle, pigs	Abscesses, gastroenteritis	None at present
<i>Campylobacter sputorum</i> bv. <i>Faecalis</i> ('Campylobacter <i>fecalis</i> ')	Sheep, bulls	None at present	None at present
<i>Campylobacter curvus</i> (' <i>Wolinella curva</i> ')	Humans	Periodontal disease, gastroenteritis	None at present
<i>Campylobacter rectus</i> (' <i>Wolinella recta</i> ')	Humans	Periodontal disease	None at present
<i>Campylobacter showae</i> ^b (' <i>Wolinella curva</i> subsp. <i>intermedius</i>)	Humans	Periodontal disease	None at present
<i>Campylobacter gracilis</i> ^d (' <i>Bacteroides gracilis</i> ')	Humans	Periodontal disease, empyema, abscesses	None at present
<i>Campylobacter upsaliensis</i> ('CNW <i>Campylobacter</i>)	Dogs, cats	Gastroenteritis, septicemia, abscesses	Canine and feline gastroenteritis
<i>Campylobacter helveticus</i>	Cats, dogs	None at present	Feline and canine gastroenteritis
<i>Campylobacter hyoilei</i> ^{c,h}	Pigs	None at present	Porcine proliferative enteritis
<i>Campylobacter coli</i>	Pigs, poultry, bulls, sheep, birds	Gastroenteritis, septicemia	Gastroenteritis
<i>Campylobacter jejuni</i> subsp. <i>jejuni</i>	Poultry, pigs, bulls, dogs, cats, water, birds, mink, rabbits, insects	Gastroenteritis, septicemia, meningitis, abortion, proctitis, Guillain-Barré syndrome	Gastroenteritis, avian hepatitis
<i>Campylobacter jejuni</i> subsp. <i>doylei</i> ('NNC group')	Humans	Gastroenteritis, gastritis, septicemia	None at present
<i>Campylobacter lari</i> (' <i>Campylobacter laridis</i> .' 'NARTC')	Birds (including poultry), river water and seawater, dogs, cats, monkeys, horses, fur seals	Gastroenteritis, septicemia	Avian gastroenteritis
<i>Bacteroides</i> [<i>Campylobacter</i>] <i>ureolyticus</i> ^e	Humans	Nongonococcal urethritis, necrotic tissue lesions, infected amniotic fluid, wound infections	None at present
"rRNA homology group Ia"			
<i>Campylobacter</i> -like, unnamed free-living species	Anaerobic sludge	None at present	None at present

(continued)

Table 3.8.2-1 Taxonomic position, known sources, and common disease associations of *Campylobacter*, *Arcobacter*, *Helicobacter*, and related species^a (*continued*)

Taxon ^b	Known source(s)	Disease association(s)	
		Human	Veterinary
rRNA homology group II			
<i>Arcobacter nitrofigilis</i> (" <i>Campylobacter nitrofigilis</i> ")	Plant roots and associated sediment	None at present	None at present
<i>Arcobacter butzleri</i> (" <i>Campylobacter butzleri</i> ")	Pigs, bulls, monkeys, humans, poultry, drinking water, river water, sewage, horses	Gastroenteritis, septicemia	Porcine, bovine, and primate gastroenteritis; porcine abortion
<i>Arcobacter skirrowii</i>	Sheep, bulls, pigs	None at present	Ovine and bovine gastroenteritis, porcine and bovine abortion
<i>Arcobacter cryaerophilus</i> (" <i>Campylobacter cryaerophila</i> ")	Pigs, bulls, sheep, horses, sewage	Gastroenteritis, septicemia	Porcine, bovine, ovine, and equine abortion
rRNA homology group III			
<i>Wolinella succinogenes</i> (" <i>Vibrio succinogenes</i> ")	Bulls	None at present	None at present
<i>Helicobacter pylori</i> (" <i>Campylobacter pylori</i> ," " <i>Campylobacter pyloridis</i> ")	Humans, nonhuman primates	Gastritis, peptic ulcer disease, gastric lymphoma, gastric adenocarcinoma	Gastritis in rhesus monkeys
<i>Helicobacter acinonychis</i>	Cheetahs	None at present	Gastritis in cheetahs
<i>Helicobacter nemestrinae</i> ^c	Nonhuman primates	None at present	Gastritis in macaque monkeys
<i>Gastospirillum lemur</i> ^c	Lemurs (presumed)	Unknown	Unknown
<i>Helicobacter heilmannii</i> (" <i>Gastospirillum hominis</i> 1") ^y	Humans	Gastritis	None at present
<i>Helicobacter heilmannii</i> (" <i>Gastospirillum hominis</i> 2") ^y	Humans	Gastritis	None at present
<i>Gastospirillum suis</i> ^c	Pigs	None at present	Porcine gastritis
<i>Helicobacter bizzozeronii</i> ^s (" <i>Helicobacter bizazzoro</i> ")	Dogs	None at present	Canine gastritis
<i>Helicobacter felis</i> ^c	Cats, dogs	None at present	Feline and canine gastritis
<i>Helicobacter pullorum</i> ⁱ	Poultry	Gastroenteritis	Avian hepatitis
<i>Helicobacter CLO-3</i> ("CLO-3")	Humans	Proctitis	None at present
<i>Helicobacter fennelliae</i> (" <i>Campylobacter fennelliae</i> ")	Humans	Gastroenteritis, septicemia, proctocolitis	None at present
<i>Helicobacter</i> sp. strain Mainz ^c	Humans	Septic arthritis	None at present
<i>Helicobacter cinaedi</i> (" <i>Campylobacter cinaedi</i> ")	Humans, hamsters	Gastroenteritis, septicemia, proctocolitis	Hamster enteritis
' <i>Helicobacter westmeadii</i> '	Unknown	Bacteremia	Unknown
<i>Helicobacter bilis</i> ^c	Mice	None at present	Murine chronic hepatitis and hepatocellular tumors
" <i>Flexispira rappini</i> " ^c (" <i>Helicobacter rappini</i> ")	Humans, sheep, mice	Gastroenteritis, bacteremia	Ovine abortion
<i>Helicobacter canis</i>	Dogs	Gastroenteritis	Canine gastroenteritis
<i>Helicobacter muridarum</i> ^c (" <i>Helicobacter muridae</i> ")	Rodents	None at present	Murine gastritis
<i>Helicobacter hepaticus</i> ^c	Mice	None at present	Murine necrotizing hepatitis
<i>Helicobacter pametensis</i> ^c	Birds, pigs	None at present	None at present
<i>Helicobacter</i> sp. strain Bird-B ^c	Birds	None at present	None at present
<i>Helicobacter</i> sp. strain Bird-C ^c	Birds	None at present	None at present
<i>Helicobacter mustelae</i> ^e (" <i>Campylobacter pylori</i> subsp. <i>mustelae</i>)	Ferrets, mink	None at present	Gastritis in ferrets

Table 3.8.2-1 (continued)

Taxon ^b	Known source(s)	Disease association(s)	
		Human	Veterinary
Other campylobacter-like organisms phylogenetically distinct from rRNA superfamily VI			
<i>Anaerobiospirillum succiniciproducens</i>	Dogs	Gastroenteritis, septicemia	None at present
<i>Anaerobiospirillum</i> sp. strain Malnick et al. 1983	Dogs, cats	Gastroenteritis	None at present
<i>Lawsonia intracellularis</i> ("ileal symbiont intracellularis," " <i>Campylobacter intracellularare</i> ")	Pigs, hamsters, ferrets	None at present	Proliferative enteritis in pigs and hamsters

^a Table adapted from reference 16.^b All organisms listed belong to rRNA superfamily VI. Organisms in boldface type are associated with human sources. Validly published or most commonly used nomenclature of the taxa described are given priority, with superseded (in quotation marks) or less common nomenclature given in parentheses. Names that have not been validly published are given in single quotation marks. Abbreviations: CNW, catalase negative—weak; NNC, nitrate-negative *Campylobacter*; NARTC, NA-resistant thermophilic *Campylobacter*; CLO, *Campylobacter*-like organism; IDO, intracellular *Desulfovibrio* organism.^c Likely phylogenetic position of taxon in rRNA superfamily VI based on 16S rRNA sequence comparisons or DNA-DNA hybridization data.^d Biovar descriptions conform with recent suggestions by On (16).^e Original phylogenetic position in rRNA superfamily VI emended with reference to available 16S rRNA sequence comparisons. Brackets indicate that the taxon is generically misnamed.^f The proposed name of '*H. heilmannii*' did not distinguish between the two phylogenetically distinct taxa referred to as '*G. hominis* 1' and '*G. hominis* 2.'^g Taxonomic position based upon marked morphological similarity to '*Gastrospirillum*' spp.^h Most current taxonomy indicates *C. hyoilei* to be indistinguishable from *C. coli* (23).ⁱ *Helicobacter canadensis* is a newly described agent of human gastroenteritis and is closely related to *H. pullorum* (6).

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING (continued)

B. Specimen transport

1. Process within 1 to 2 h or place in transport medium.

NOTE: *C. jejuni* is stable in feces for 3 days at 4°C and 2 days at 25°C and is unstable at freezing temperatures. It has a similar stability at the respective temperatures in modified Cary-Blair medium, which will inhibit the growth of normal fecal microbiota (11).

2. Transport in modified Cary-Blair medium (formulation modified with reduced agar content of 1.6 g/liter rather than 5 g/liter).

- a. Purchase from commercial source *or*
- b. Prepare as follows.

- (1) Dispense the following ingredients into 991 ml of H₂O.

sodium thioglycolate	1.5 g
disodium phosphate	1.1 g
sodium chloride	5.0 g
phenol red	0.003 g
agar	1.6 g

- (2) Heat to dissolve and cool to 50°C. Then add 9 ml of 1% CaCl₂.

- (3) Adjust pH to 8.4; dispense in vials and steam for 15 min.

- (4) Cool and tighten caps.

3. Avoid phosphate-buffered glycerol transport media.

4. If there is a delay in transport, refrigerate transport vial at 4°C.

- C. Refer to procedure 3.8.1 for other collection, labeling, transport, QC, and rejection criteria.

Table 3.8.2–2 Human disease associations of *Campylobacter* species by clinical syndrome

Organism(s)	Human disease association						
	GI ^a	Bacteremia	Meningitis	Abortion	Periodontitis	Wounds/abscesses	Other
<i>C. jejuni</i>	×	×	×	×		×	Guillain-Barré syndrome
<i>C. jejuni</i> subsp. <i>doylei</i>	×						
<i>C. coli</i>	×						
<i>C. fetus</i> (subsp. <i>fetus</i> and <i>venerealis</i>)	×	×	×	×		×	
<i>C. upsaliensis</i>	×	×				×	
<i>C. hyoilealis</i>	×						
<i>C. showae</i>				×			
<i>C. concisus</i>	×				×		
<i>C. curvus</i>					×		
<i>C. mucosalis</i>	×						
<i>C. rectus</i>				×		×	
<i>C. sputorum</i>						×	
<i>C. gracilis</i> (<i>Sutterella wadsworthensis</i>)				×		×	
<i>C. lari</i>		×					Urinary tract infections
<i>C. sputorum</i>						×	
<i>C. ureolyticum</i>				×			
<i>Arcobacter butzleri</i>	×	×					
<i>A. cryaerophilus</i>	×	×					
<i>H. fennelliae</i>	×	×					Proctocolitis
<i>H. cinaedi</i>	×	×				×	Cellulitis, proctocolitis
<i>H. pullorum</i>	×						
<i>H. canadensis</i>	×						
<i>H. westmeadii</i>		×					
<i>H. pylori</i>							Duodenal and gastric ulcer, mucosa-associated lymphoid tissue, gastric carcinoma
“ <i>Flexispira rappini</i> ”	×	×					

^a GI, gastrointestinal.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING (continued)

D. Other rejection criteria

1. Reject fecal specimen if it has been frozen.
2. Reject fecal specimen in transport media other than *modified Cary-Blair* medium.

III. MATERIALS

A. Media

■ **NOTE:** The use of more than one type of selective medium increases the yield from stools by as much as 15% (4). Cephalothin, colistin, and polymyxin B in some selective media are inhibitory to *C. fetus*, *C. jejuni* subsp. *doylei*, and *C. upsaliensis* and may inhibit some strains of *C. jejuni* and *C. coli*. Cefoperazone is now considered

to be the preferred antimicrobial agent, as it inhibits *Pseudomonas* species and other gram-negative normal microbiota but does not inhibit growth of campylobacters

1. Blood-free charcoal contains Columbia agar, charcoal, pyruvate, and hemin and cefoperazone, vancomycin, and amphotericin B (or cycloheximide) (4).

III. MATERIALS (continued)

2. Blood-free charcoal-cefoperazone-desoxycholate agar (CCDA) contains desoxycholate, charcoal base (CM 739; Oxoid), and cefoperazone (9).
 3. Campy-CVA contains brucella agar with 5% sheep blood, cefoperazone, vancomycin, and amphotericin B.
- B. Stain reagents**
1. Gram stain (procedure 3.2.1) with carbol fuchsin or 0.1% basic fuchsin for colony smears
 2. Acridine orange (procedure 3.2.2) for problem cultures
 3. Wet mount (procedure 3.2.3) for direct specimen
- C. Other supplies**
1. A microaerobic environment

■ **NOTE:** Most campylobacters require a microaerobic environment of approximately 5% O₂ and 85% N₂ for optimal growth. Increased H₂ appears to be a growth requirement for the more fastidious campylobacters (*C. sputorum*, *C. concisus*, *C. curvus*, *C. mucosalis*, and some strains of *C. hyoilectinialis*). These strains are not of significance in fecal cultures, but they may be isolated from blood and may require anaerobic incubation for growth.

 - a. Use an anaerobic jar, without a catalyst, and a gas mixture containing 10% CO₂–5% H₂–balance N₂. Evacuate and fill jar two times to achieve a final atmosphere of 5% O₂, 7% CO₂, and 4% H₂.
 - b. Alternatively, use a leakproof Saranex zipper bag (Associated Bag Company, [800] 926-6100) and fill once with 5% O₂, 10% CO₂, balance N₂ (Campy Gas Cylinder, catalog no. S0730; PML Microbiologicals, Inc., Wilsonville, Oreg., [800] 547-0659, <http://www.pmlmicro.com>)

c. See Table 3.8.2–3 for commercial gas systems.
 2. Incubator at 42 and 35°C
 3. Identification—media and biochemical tests
 - a. Heart infusion agar with 5% rabbit blood (BBL catalog no. 297472; BD Diagnostic Systems), BAP, or brucella agar with 5% sheep blood for disk tests and temperature growth tests
 - b. Heart infusion broth (HIB) or BHI for inoculum preparation (BD Diagnostic Systems)
 - c. Oxidase (procedure 3.17.39)
 - d. Catalase (3% H₂O₂) (procedure 3.17.10)
 - e. Hippurate (procedure 3.17.21 and reference 13)
 - f. Indoxyl acetate disks (procedure 3.17.24 and reference 19)
 - g. Rapid urea hydrolysis (procedure 3.17.48 and references 12 and 17)
 - h. Disks—store at –20°C (BD Diagnostic Systems; Remel, Inc.).
 - (1) Nalidixic acid (NA) disks, 30 µg
 - (2) Cephalothin or cefazolin (CF [abbreviation stands for either drug]) disks, 30 µg
 - (3) Ciprofloxacin, 5 µg, and erythromycin, 15 µg, disks (optional)
 - i. Nitrate test (procedure 3.17.35)
 - j. Triple sugar iron (TSI) (procedure 3.17.22)

■ **NOTE:** For use in the identification of campylobacters, TSI must be freshly prepared and used within 1 week (*CDC Campylobacter Reference Lab Procedure Manual*). Incubate test at 35°C for 72 h before calling result negative.

Table 3.8.2–3 Commercial systems for generating microaerobic environments and the approximate atmospheric content

System	Final atmosphere
BBL Campy Pak	6.9% O ₂ , 5.5–7.0% CO ₂ , 0.2% H ₂
BBL Campy Pouch	7.5–9.0% O ₂ , 11.5–14% CO ₂ , 0.5–1.5% H ₂
BBL Bio Bag Cfj	10.5–15% O ₂ , 7–15% CO ₂ , 6.5–12.5% H ₂
Oxoid CampyGen ^a	6% O ₂ , 15% CO ₂
Mitsubishi Pack-Campylo ^a	6% O ₂ , 14% CO ₂

^a This system produces negligible H₂ and may not grow H₂-requiring species.

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

- A. Verify that media meet expiration date and QC parameters per current Clinical and Laboratory Standards Institute (CLSI; formerly NCCLS) M22 document. See procedures 14.2 and 3.3.1 for further procedures, especially for in-house-prepared media.
- B. QC test *each lot* of campylobacter medium for growth requirements and inhibition of fecal microbiota, per CLSI document M22-A3 (3). See Table 3.8.1-3 for QC organisms and testing details.
- C. QC each lot of transport media with *C. jejuni* ATCC 33291 by following the method outlined in section 14, Table 14.2-2, for QC listed under "Transport."
- D. Test each new lot of CF and NA disks before use in the manner described in item V.D.6 below.
 - 1. *E. coli* ATCC 25922; zone size range for NA = 22 to 28 mm; that for CF = 15 to 21 mm
 - 2. *C. jejuni* ATCC 33291; >20-mm zone for NA; no zone for CF
 - NOTE: QC erythromycin and ciprofloxacin disks as part of the QC for susceptibility testing, using the usual QC organisms listed in section 5.
- E. Test each lot of gas system to ensure that it supports the growth of *C. jejuni*.
- F. For testing the ability of an organism to grow in a microaerobic atmosphere at various temperatures, perform QC on each incubator or temperature condition, when put into use for this test, with the following to determine if incubator temperatures and atmospheric conditions are in control.
 - 1. *Arcobacter cryaerophilus* ATCC 43158 (aerobic growth; no microaerobic growth at 42°C)
 - 2. *C. jejuni* ATCC 33291 (no microaerobic growth at 25°C)
 - 3. *C. fetus* subsp. *fetus* ATCC 27374 (microaerobic growth at 25, 35 to 37, and 42°C)

V. PROCEDURE



Observe standard precautions.

A. Microscopic (rarely performed but can be helpful in isolated cases)

- 1. Perform Gram stain in selected cases during the acute phase of diarrhea for visualization of campylobacters (sensitivity, 66 to 94%) (18, 20).
- 2. Visualize motile campylobacters in wet mounts of very fresh stools (<30 min old) as darting across the field in a zigzag fashion (procedure 3.2.3).
- 3. Use acridine orange stain for problem cultures (procedure 3.2.2).

B. Direct antigen detection

ProSpecT *Campylobacter* microplate assay for stool specimens (Remel, Inc.) is an EIA for detection of *Campylobacter* specific antigen found in *C. jejuni* and *C. coli*. There is no differentiation between the two species. The test cross-reacts with *C. upsaliensis* but not other species of *Campylobacter* (8). Performance analysis demonstrated the assay to be 96 and 89% sensitive and 99% specific in two independent studies (8, 21), with a cost in excess of that of culture. See Appendix 3.8.2-1 for details.

C. Culture

NOTE: Use of enrichment broth cultures (Preston enrichment, Campy-THIO, Campylobacter enrichment) has been reported to increase isolation of campylobacters when specimens are obtained after the acute stage of diarrhea and the broth is incubated overnight at 42°C and subcultured to selective media. However, the value of these enrichments has not been studied adequately, although the use of two primary media or the addition of the filtration technique also increases recovery.

1. Feces—routine plating

- a. Place 1 to 3 drops of stool or broth from transport vial on one or two of the following selective agars: blood-free charcoal, CCDA, or Campy-

V. PROCEDURE (continued)

CVA. Alternatively inoculate plates using a swab dipped into the transport vial, and completely roll the swab on the medium plate in an area the size of a nickel.

- b. Streak in four quadrants.
- c. Place the plate *immediately* in a microaerobic environment.
- d. Incubate at 42°C for 72 h to screen for thermotolerant *Campylobacter* and *Helicobacter* species.

NOTE: If detection of species other than *C. jejuni* and *C. coli* is desired, incubate a second plate at 35 to 37°C. Since most species that do not grow at 42°C will grow at 40°C, use of a 40°C incubator will allow one temperature to be used for all species.

2. Feces—filtration technique

NOTE: This technique has been successful for isolation of campylobacters that are susceptible to antimicrobial agents in media. Passage of motile campylobacters through the pores of the filter allows for selective separation of campylobacters from the nonmotile stool constituents. This method will yield a large variety of *Campylobacter* species, not all of which have been clearly linked with disease.

- a. Place a sterile cellulose acetate membrane filter, with a 47-mm diameter and a 0.65-µm pore size (Sartorius Corp, Edgewood, N.Y., [800] 635-2906, <http://www.sartorius.com>), directly on the surface of a BAP.
- b. Add 6 to 8 drops of the stool directly on the filter.
- c. Incubate for 45 min to 1 h in ambient air at 35 to 37°C.
- d. Remove filter with sterile forceps, taking care not to allow any residual stool on the filter to drip on the plate. *Do not streak for isolation.*
- e. Immediately place the plate in a microaerobic environment at 35 to 37°C for a *minimum of 72 h or up to 6 days* for recovery of fastidious and slow-growing organisms (5).

D. Identification methods

1. Examine direct plates for growth of campylobacters.
 - a. On freshly prepared media, colonies are gray, flat, irregular, and spreading; sometimes they may appear mucoid.
 - b. Some strains will appear as a thin film on the medium and require careful observation to detect growth (best done by scraping a loop over the surface of the medium). They can also appear as colonies that tail along the line of streaking.
 - c. On less fresh medium, colonies, 1 to 2 mm in diameter, appear as gray, round, convex, and glistening, with little or no spreading.
 - d. Colonies can be yellowish to gray or pinkish and are nonhemolytic.
 - e. *Helicobacter cinaedi* and *Helicobacter fennelliae* growth on fresh media in a moist environment may spread as a uniform film over the entire surface of the plate. *H. fennelliae* has the distinct odor of hypochlorite. They require 5 to 10% hydrogen for growth and generally do not grow at 42°C.
 - f. Check aerobic BAP (procedure 3.8.1) for oxidase-positive colonies at 24 h, which are likely *Pseudomonas* or *Aeromonas*. Then reincubate if negative and recheck at 96 h. If positive at 4 to 5 days and not at 48 h, *Arcobacter* could be present.
2. Perform Gram stain on suspicious colonies using basic fuchsin or carbol fuchsin counterstain promptly after removal from the microaerobic environment.
 - a. Look for small gram-negative rods shaped like spirals, gull wings, archery bows, or commas, consistent with *Campylobacter* species. Coccoidal forms may be seen in the Gram stain, especially in older cultures or cultures exposed to ambient air for prolonged periods.

V. PROCEDURE (continued)

- b. *H. cinaedi* and *H. fennelliae* Gram stain as long, wavy, gram-negative rods.
 - c. “*Flexispira rappini*,” also called *Helicobacter* sp. strain *flexispira*, closely associated with helicobacters, is a long, fusiform, gram-negative rod, slightly curved.
 - (1) Perform urea test if Gram stain suggests fusiform rods.
 - (2) *Helicobacter* sp. strain *flexispira* as a cause of gastroenteritis is generally catalase negative.
 - (3) *Helicobacter* sp. strain *flexispira* grows at 42°C and is urea positive.
3. Perform direct wet mount examination for motility (see procedure 3.17.31, with the following caveats).
 - a. Emulsify a loopful of 24- to 48-h bacterial growth in broth (not saline or distilled water).
 - b. Observe microscopically for darting motility typical of spiral rods or vibrio-like organisms.
4. Perform the following rapid assays from colonies isolated at 42°C and use Fig. 3.8.2-1 to identify to the species level.
 - a. Oxidase (procedure 3.17.39)
 - b. Catalase (procedure 3.17.10)
 - c. Hippurate hydrolysis (13); use a 48-h culture if growth is poor at 24 h (procedure 3.17.21)
5. Incubate a plate aerobically at 35°C; *C. jejuni* should *not* grow under these conditions.
6. Perform disk susceptibility tests (14).
 - a. Prepare a bacterial suspension of a 24- to 48-h culture in HIB or BHI to a turbidity equal to a McFarland no. 1 standard.
 - b. Dip cotton- or polyester-tipped swab in the suspension, drain against side of tube, and inoculate entire surface of BAP.
 - c. Place NA and CF disks in separate quadrants of the plate surface.
 - d. It is helpful for both identification and treatment to add a 5-µg ciprofloxacin and 15-µg erythromycin disk to the plate.
 - e. Incubate the plate overnight at 40 to 42°C (use 35 to 37°C if the isolate does not grow at 40 to 42°C) in a microaerobic atmosphere.
 - f. Read and record presence (susceptible) or absence (resistant) of zone of inhibition. If background growth is poorly defined, reincubate the plate and read in 48 h.
 - g. If species can be determined from Fig. 3.8.2-1, omit further testing.
7. If isolate is hippurate negative, perform rapid indoxylo acetate hydrolysis test (procedure 3.17.24 and reference 19), and identify using Fig. 3.8.2-1.
8. For all other identifications, inoculate the following biochemical tests and use Table 3.8.2-4 to identify species.
 - a. Nitrate (procedure 3.17.35)
 - b. Urease (procedure 3.17.48)
 - c. TSI (procedure 3.17.22)
 - d. Prepare inoculum from a 24- to 48-h culture on heart infusion agar with 5% rabbit blood or BAP at 35 to 37°C in a microaerobic atmosphere. Alternatively, use growth from a 72-h culture, if the organism grows slowly. If primary isolation was at 42°C, the biochemical tests may be performed at 42°C.
 - e. Test for the ability of an organism to grow in a microaerobic atmosphere at various temperatures (2).
 - (1) Prepare a bacterial suspension of a 24- to 48-h culture in HIB or BHI to a turbidity equal to a McFarland no. 1 standard.
 - (2) Label four BAPs: microaerobic, 25°C; microaerobic, 35°C; microaerobic, 42°C; and aerobic, 35°C.

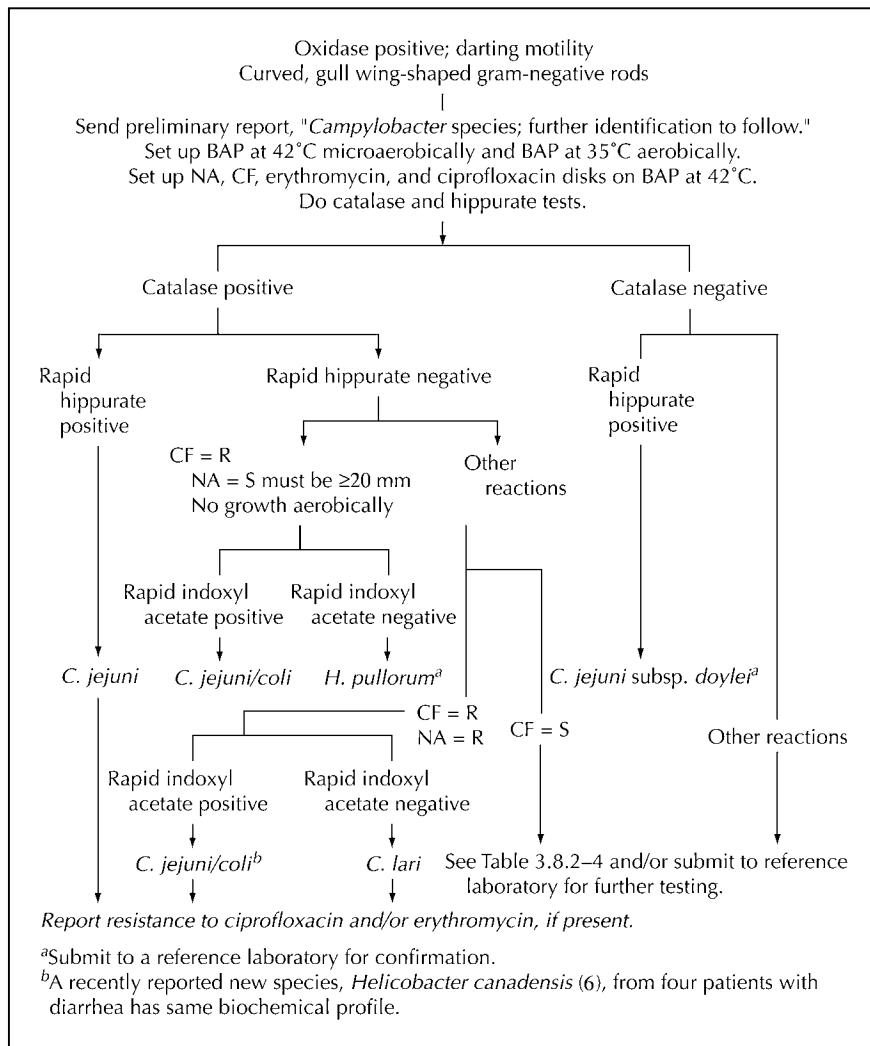


Figure 3.8.2-1 *Campylobacter* identification flowchart for minimum identification of *C. jejuni* from stool specimens. Abbreviations: R, no zone; S, zone.

V. PROCEDURE (continued)

- (3) Mark lines across the bottom of each plate approximately 3/4 in. apart to indicate where inoculum will be placed. Three cultures can be streaked on each agar plate. Label lines with culture numbers.
- (4) Moisten a cotton swab in the bacterial suspension and make a single streak across each plate, rotating the swab one-third of a turn before inoculating the next plate.
- (5) Incubate each plate under its stated temperature and atmospheric conditions for 3 days.
 - (a) Aerobic at 35°C (to screen for *Arcobacter* species)
 - (b) Microaerobic at 35°C (to screen for nonthermotolerant *Campylobacter* and *Helicobacter* species)
 - (c) Microaerobic at 40 to 42°C (to screen for thermotolerant *Campylobacter* and *Helicobacter* species)
 - (d) Microaerobic at 25°C (to screen for *C. fetus*).

Table 3.8.2-4 Phenotypic reactions of clinically important *Campylobacter* and *Helicobacter* species^e

Identification test	<i>C. jejuni</i> subsp. <i>doylei</i>	<i>C. coli</i>	<i>C. jejuni</i> subsp. <i>jejuni</i>	<i>C. lari</i> ^b	<i>C. fetus</i> subsp. <i>fetus</i>	<i>C. upsaliensis</i>	<i>A. cyaerophilus</i> ^c	<i>A. butzleri</i> ^f	<i>C. hyoilealis</i>	<i>C. cinaedi</i> ^d	<i>C. CLO1B</i> ^d	<i>H. fennelliae</i> ^d	<i>H. pullorum</i>
Oxidase	+	+	+	+	+	+	+	+	+	+	+	+	+
Catalase	+	V	+	+	+	+	W	V	+	+	+	+	+
Aerobic growth, 35–37°C	0	0	0	0	V ^e	0	+	0	0	0	0	0	0
Microaerobic growth, 25°C	0	0	0	0	V	0	+	V	0	0	0	0	0
Microaerobic growth, 35–37°C	+	+	+	+	+	+	+	+	+	+	+	+	+
Microaerobic growth, 40–42°C	+	V	+	+	V	+	V	V	V	V	V	V	+
Hippurate hydrolysis	+	+	0	0	0	0	0	0	0	0	0	0	0
Indoxyl acetate	+	+	+	0	0	0	+	+	0	0	0	0	0
NA resistant	0 ^f	0	0 ^f	+	+	0 ^f	V	V	+	0	0	0	0
CF resistant	+	V	+	+	V	0	V	V	0	0	0	V	+
Nitrate reduction	+	0	+	+	+	+	V	V	+	+	0	0	+
H ₂ S in TSI agar	0	0	V ^g	V	0	0	0	0	+	0	0	0	0
Urea hydrolysis ^h	0	0	0	0	0	0	0	0	0	0	0	0	0

^a +, positive reaction; 0, negative reaction; w, weakly positive; V, variable reaction, NA, not available. See procedure 3.8.4 for *H. pylori* identification.^b Urease-positive thermophilic campylobacters or *C. lari*-like strains may be found (12).^c Growth at 42°C; catalase negativity suggests *A. butzleri*.^d *H. cinaedi/CLO1B*, *H. fennelliae*, and *H. pylori* can be definitively identified by cellular fatty acid analysis (10).^e Rare *C. fetus* subsp.*fetus* strains are aerobic.^f These species are historically sensitive to NA; however, resistant strains are seen in as high as 35% of isolates due to acquired fluorquinolone resistance, which may make this assay less useful in identification.^g H₂S in TSI suggests *C. coli*.^h There are isolated reports of *Helicobacter* species that are urease producing other than *H. pylori* (24).

V. PROCEDURE (continued)

(6) Interpretation: record the amount of 72-h growth as follows.

- (a) No growth
- (b) Trace
- (c) 1+ (light)
- (d) 2+ (moderate)
- (e) 3+ (heavy)

9. Other tests

a. Cellular fatty acid analysis (MIDI Laboratories, Newark, Del., [302] 737-4297, <http://www.midi-inc.com/>) (10)

■ NOTE: This method cannot differentiate *C. jejuni* and *C. coli*, the two most common species.

b. Species-specific PCR or 16S rDNA sequencing (CDC Campylobacter Laboratory, Atlanta, Ga.; MIDI Laboratories)

c. *C. jejuni*-specific DNA probe (16) (BioProbe; Enzo Diagnostics, Farmingdale, N.Y., [631] 694-7070, <http://www.enzobio.com>).

d. AccuProbe *Campylobacter* culture identification test (Gen-Probe, Inc., <http://www.gen-probe.com/>) for DNA identification of *C. jejuni/coli/lari* colonies does not differentiate among these three species.

■ NOTE: The AccuProbe system uses a single-stranded DNA probe with a chemiluminescent label that recognizes RNA sequences that are unique to *C. jejuni*, *C. coli*, and *C. lari*. Following the release of the target RNA, the DNA probe forms a stable DNA:RNA hybrid. A selection reagent inactivates unhybridized probe and the labeled DNA:RNA hybrids are detected in a luminometer.

(1) Label a sufficient number of probe reagent tubes to test the number of culture isolates and a positive (*C. jejuni* ATCC 33560) and negative (*C. fetus* ATCC 27374) control.

(2) Pipette 50 µl of lysis reagent into all tubes.

(3) Transfer a 1-µl loopful of cells or several small colonies (<48 h old) to the tubes and twirl the loop to remove the cells. Test a positively and negatively reacting organism with each run.

(4) Pipette 50 µl of hybridization buffer into all tubes and mix by vortexing.

(5) Incubate tubes for 15 min at 60°C in a water bath or dry-heat block.

(6) Remove and cool the tubes. Pipette 300 µl of selection reagent to each tube and mix by vortexing.

(7) Incubate tubes for 5 min at 60°C in a water bath or dry-heat block.

(8) Cool the tubes to room temperature and read results in the luminometer within 30 min. Cutoff values for positive and negative signals are established by the manufacturer for the type of luminometer used.

■ NOTE: The AccuProbe assay was compared to standard culture and biochemical identification using 301 isolates of thermophilic *Campylobacter* species (*C. jejuni*, *C. coli*, and *C. lari*), 41 isolates of other *Campylobacter* spp., and 300 other bacterial cultures from 53 genera at three clinical sites. The sensitivities and specificities were 100 and 99.1%, 100 and 100%, and 100 and 100%, respectively. One false positive was detected with *C. hyoilealis* (product insert).

e. Oxoid Dryspot *Campylobacter* test (latex agglutination), product code DR0150

■ NOTE: The Oxoid Dryspot *Campylobacter* test is a latex agglutination test for the identification of enteropathogenic campylobacters from solid culture media. *C. jejuni*, *C. lari*, *C. coli*, *C. fetus*, and *C. upsaliensis* will give positive results. Isolates of other *Campylobacter* spp. such as *C. fetus* subsp. *fetus* will give variable results.

V. PROCEDURE (continued)

- (1) Test 48-h colonies with morphology suggestive of *Campylobacter* spp. from *Campylobacter* selective media.
- (2) Remove the reagents from the refrigerator and allow to reach room temperature.
- (3) Label a sufficient number of reagent tubes to test the number of culture isolates (and a positive and negative control, which should be tested with each new lot and every 6 months thereafter).
- (4) Add 1 drop of extraction reagent 1 into the extraction tube.
- (5) Remove sufficient suspect growth to fill the internal diameter of the sterile loop.
- (6) Thoroughly suspend the cells in the drop of reagent 1. Allow the loop to stand in reagent 1 for 3 min. Do not discard the loop.
- (7) Add 2 drops of extraction reagent 2 to the extract. Mix using the retained loop.
- (8) Using a paddle pipette, place 1 drop (50 µl) of the neutralized extract onto the test circle and 1 drop onto the control circle.
- (9) Using the flat end of the paddle pastette, mix the extract into the dry control reagent spot until completely resuspended. Spread to cover the reaction area. Use the same pastette to repeat this procedure for the test reagent.
- (10) Pick up and rock the card for 3 min. Look for agglutination under normal lighting conditions. Do not use a magnifying glass.
- (11) Interpretation

A result is positive if agglutination of the latex particles occurs within 3 min; this indicates the presence of *Campylobacter* spp. A negative result is obtained if no agglutination occurs and a smooth blue suspension remains in the test area after 3 min of mixing. This indicates that the isolate is not *C. jejuni*, *C. coli*, *C. upsaliensis*, or *C. lari*. Reactions occurring after 3 min should be ignored. The test is uninterpretable if the control reagent shows agglutination. This indicates that the culture causes autoagglutination.
- (12) The performance of the Oxoid Dryspot *Campylobacter* test was evaluated at one clinical center in Finland. The sensitivity and specificity in the study were 100 and 100%, respectively. In addition, a total of 93 stock cultures and clinical isolates were tested at a clinical center in the United Kingdom. Strains were cultured on Columbia blood agar and/or modified CCDA and CAT (charcoal-based medium containing cefoperazone, amphotericin, and teicoplanin) agars at 37 or 42°C, under microaerobic conditions for 48 to 72 h. The assay detected all *C. jejuni*, *C. coli*, *C. lari*, *C. upsaliensis*, *C. hyoilectinalis*, and *C. mucosalis* isolates tested, six of eight *C. fetus* subsp. *fetus* isolates, and one of two *H. pylori* isolates. Other species of *Campylobacter*, *Arcobacter*, and *Helicobacter* as well as 23 other genera of organisms failed to react in the kit.

POSTANALYTICAL CONSIDERATIONS**VI. REPORTING RESULTS****A. Direct smears**

1. If Gram stain of fecal specimens demonstrates the presence of gull wing-shaped, curved, gram-negative rods, report as “Presumptive *Campylobacter* species.”
2. If wet mount of stool demonstrates darting motility, report as “Presumptive *Campylobacter* species.”

B. Culture

1. All *Campylobacter* and *Helicobacter* strains are oxidase positive, vibrio-like, or spiral gram-negative rods that have darting motility.
2. Report “Presumptive *Campylobacter* species” if the following criteria are met.
 - a. Growth has typical colonial morphology on selective media in a microaerobic environment incubated at 42°C.
 - b. Curved, gram-negative rods are demonstrated on Gram stain from colony.
 - c. Organism exhibits darting motility.
 - d. Organism is oxidase positive and catalase positive.
3. Use the biochemical tests listed in Table 3.8.2–4 and the flowchart in Fig. 3.8.2–1 to differentiate *Campylobacter* and *Helicobacter* species of human importance. Report *C. jejuni*, *C. jejuni* subsp. *doylei*, *C. jejuni/coli*, *C. lari*, or *Helicobacter pullorum* based on the flowchart in Fig. 3.8.2–1.
4. If isolate is not identified by Fig. 3.8.2–1, report species as denoted by biochemical characterization in Table 3.8.2–4.
5. If unable to identify isolates using the above schemes or to confirm identification, submit to a reference laboratory. Report “*Campylobacter*-like organism; submitted to reference laboratory for definitive identification.”
■ NOTE: For definitive identification, cellular fatty acid analysis and/or molecular analysis may be required.

C. Susceptibility testing

1. Standardized antimicrobial susceptibility testing methods for testing the susceptibility of *Campylobacter* isolates, with the exception of the agar dilution method, are not available.
2. However, the lack of a zone around either erythromycin or ciprofloxacin disks indicates that the isolate is likely resistant to the antimicrobial agent.
■ NOTE: In over 200 disk diffusion tests with erythromycin and ciprofloxacin, there was consistently a bimodal distribution of results, with most results either at ≥25 mm or showing no zone (6 mm). More recently, zone sizes have been found to be smaller, but the bimodal distribution is still valid. No *Campylobacter* isolates have been found that are susceptible to NA (large zone) but resistant to ciprofloxacin (no zone) (R. Nadarajah, personal communication).
3. If there is no zone around the ciprofloxacin or erythromycin disk, enter the following: “By a nonstandard susceptibility test method, isolate is resistant to [drug].”
4. Do not report any isolate as susceptible, since there are no standardized interpretive criteria for *Campylobacter*.

D. Notification

1. Notify caregiver of positive findings.
2. Report *Campylobacter* in fecal cultures to the local health department, per local reportable-disease regulations.

VII. INTERPRETATION

- A. Treatment with hydration and electrolyte balance is the cornerstone of therapy for *C. jejuni*. In cases with high fever, bloody stools, and symptoms lasting greater than 1 week, antimicrobial treatment is indicated and can reduce the symptoms and severity as well as reduce the likelihood of complications, such as Guillain-Barré syndrome. Because of these complications, resistance to quinolones needs to be communicated to the physician.
- B. *C. jejuni* is a major cause of bloody diarrhea that can have serious sequelae. Isolation of other species in fecal cultures, especially *C. upsaliensis*, *Arcobacter* spp., and *H. pullorum*, is generally associated with milder diseases.
- C. Because campylobacters and helicobacters are biochemically inert bacteria, few phenotypic tests are available in the clinical laboratory to group them by species. Most clinical laboratories are capable of characterizing the common species that are of medical importance.

VIII. LIMITATIONS

- A. Methods presented here are designed to recover and identify only the most common pathogenic strains of *Campylobacter*, with isolation and definitive identification of *C. jejuni*, the most common species. The optimal methods to recover campylobacters from clinical specimens have yet to be determined. While confidence can be placed in routine methods for detection of the most common agents in diarrhea (*C. jejuni* and *C. coli*), recovery procedures for other agents are not clearly defined.
- B. For recovery of campylobacters from nonfecal specimens, it is essential that there be communication between the clinician and the laboratory. It is impractical and unreasonable for a laboratory to routinely plate specimens under multiple atmospheric conditions and at different temperatures. An understanding of the critical growth requirements is necessary to establish optimal conditions for recovery of these organisms in clinical specimens.
- C. If *C. jejuni* is resistant to fluoroquinolones, it will be resistant to NA, and the disk will not be useful for identification.
- D. Other strains of *Helicobacter* besides *H. pylori* produce urease, including “*Flexispira rappini*”; these isolates are best identified with molecular assays (24).

REFERENCES

1. Allos, B. M. 2001. *Campylobacter jejuni* infections: update on emerging issues and trends. *Clin. Infect. Dis.* **32**:1201–1206.
2. Barrett, T. J., C. M. Patton, and G. K. Morris. 1988. Differentiation of *Campylobacter* species using phenotypic characterization. *Lab. Med.* **19**:96–102.
3. Clinical and Laboratory Standards Institute. 2004. *Quality Assurance for Commercially Prepared Microbiological Culture Media*, 3rd ed. Approved Standard M22-A3. Clinical and Laboratory Standards Institute, Wayne, Pa.
4. Endtz, H. P., G. J. Ruijs, A. H. Zwijnderman, T. van der Reijden, M. Biever, and R. P. Mouton. 1991. Comparison of six media, including semisolid agar, for the isolation of various *Campylobacter* species from stool specimens. *J. Clin. Microbiol.* **29**:1007–1010.
5. Engberg, J., S. L. On, C. S. Harrington, and P. Gerner-Smidt. 2000. Prevalence of *Campylobacter*, *Arcobacter*, *Helicobacter*, and *Sutterella* spp. in human fecal samples as estimated by a reevaluation of isolation methods for campylobacters. *J. Clin. Microbiol.* **38**:286–291.
6. Fox, J. G., C. C. Chien, F. E. Dewhurst, B. J. Paster, Z. Shen, P. L. Melito, D. L. Woodward, and F. G. Rogers. 2000. *Helicobacter canadensis* sp. nov. isolated from humans with diarrhea as an example of an emerging pathogen. *J. Clin. Microbiol.* **38**:2546–2549.
7. Friedman, C. R., J. Neimann, H. C. Wegeener, and R. V. Tauxe. 2000. Epidemiology of *Campylobacter jejuni* infections in the United States and other industrialized nations, p. 121–138. In I. Nachamkin and M. J. Blaser (ed.), *Campylobacter*, 2nd ed. ASM Press, Washington, D.C.
8. Hindiyeh, M., S. Jense, S. Hohmann, H. Bennett, C. Edwards, W. Aldeen, A. Croft, J. Daly, S. Mottice, and K. C. Carroll. 2000. Rapid detection of *Campylobacter jejuni* in stool specimens by enzyme immunoassay and surveillance for *Campylobacter upsaliensis* in the greater Salt Lake City area. *J. Clin. Microbiol.* **38**:3076–3079.
9. Hutchinson, D. N., and F. J. Bolton. 1984. Improved blood free selective medium for the isolation of *Campylobacter jejuni* from faecal specimens. *J. Clin. Pathol.* **37**:956–957.

REFERENCES (continued)

10. Lambert, M. A., C. M. Patton, T. J. Barrett, and C. W. Moss. 1987. Differentiation of *Campylobacter* and *Campylobacter*-like organisms by cellular fatty acid composition. *J. Clin. Microbiol.* **25**:706–713.
11. Luechtfeld, N. W., W. L. Wang, M. J. Blaser, and L. B. Reller. 1981. Evaluation of transport and storage techniques for isolation of *Campylobacter fetus* subsp. *jejuni* from turkey cecal specimens. *J. Clin. Microbiol.* **12**:438–443.
12. Megraud, F., D. Chevrier, N. Desplaces, A. Sedallian, and J. L. Guesdon. 1988. Urease-positive thermophilic *Campylobacter* (*Campylobacter laridis* variant) isolated from an appendix and from human feces. *J. Clin. Microbiol.* **26**:1050–1051.
13. Morris, G. K., M. R. el Sherbeeny, C. M. Patton, H. Kodaka, G. L. Lombard, P. Edmonds, D. G. Hollis, and D. J. Brenner. 1985. Comparison of four hippurate hydrolysis methods for identification of thermophilic *Campylobacter* spp. *J. Clin. Microbiol.* **22**:714–718.
14. Morris, G. K., and C. M. Patton. 1985. *Campylobacter*, p. 302–308. In E. H. Lennette, A. Balows, W. J. Hausler, Jr., and H. J. Shadomy (ed.), *Manual of Clinical Microbiology*, 4th ed. American Society for Microbiology, Washington, D.C.
15. Nachamkin, I., H. Ung, and M. Li. 2002. Increasing fluoroquinolone resistance in *Campylobacter jejuni*, Pennsylvania, USA, 1982–2001. *Emerg. Infect. Dis.* **8**:1501–1503.
16. On, S. L. 1996. Identification methods for campylobacters, helicobacters, and related organisms. *Clin. Microbiol. Rev.* **9**:405–422.
17. Owen, R. J., S. R. Martin, and P. Borman. 1985. Rapid urea hydrolysis by gastric campylobacters. *Lancet* **1**:111.
18. Park, C. H., D. L. Hixon, A. S. Polhemus, C. B. Ferguson, S. L. Hall, C. C. Risheim, and C. B. Cook. 1983. A rapid diagnosis of *Campylobacter* enteritis by direct smear examination. *Am. J. Clin. Pathol.* **80**:388–390.
19. Popovic-Uroic, T., C. M. Patton, M. A. Nicholson, and J. A. Kiehlbauch. 1990. Evaluation of the indoxyl acetate hydrolysis test for rapid differentiation of *Campylobacter*, *Helicobacter*, and *Wolinella* species. *J. Clin. Microbiol.* **28**:2335–2339.
20. Sazie, E. S. M., and A. E. Titus. 1982. Rapid diagnosis of *Campylobacter* enteritis. *Ann. Intern. Med.* **96**:62–63.
21. Tolcinc, R., M. M. LaSalvia, B. A. Kirkley, E. A. Vetter, F. R. Cockerill III, and G. W. Procop. 2000. Evaluation of the Alexon-Trend ProSpecT *Campylobacter* microplate assay. *J. Clin. Microbiol.* **38**:3853–3855.
22. Vandamme, P., E. Falsen, R. Rossau, B. Hoste, P. Segers, R. Tytgat, and J. De Ley. 1991. Revision of *Campylobacter*, *Helicobacter*, and *Wolinella* taxonomy: emendation of generic descriptions and proposal of *Arcobacter* gen. nov. *Int. J. Syst. Bacteriol.* **41**:88–103.
23. Vandamme, P., L. J. Van Doorn, S. T. al Rashid, W. G. Quint, J. van der Plas, V. L. Chan, and S. L. On. 1997. *Campylobacter hyoilei* Alderton et al. 1995 and *Campylobacter coli* Veron and Chatelain 1973 are subjective synonyms. *Int. J. Syst. Bacteriol.* **47**:1055–1060.
24. Weir, S., B. Cuccherini, A. M. Whitney, M. L. Ray, J. P. MacGregor, A. Steigerwalt, M. I. Daneshvar, R. Weyant, B. Wray, J. Steele, W. Strober, and V. J. Gill. 1999. Recurrent bacteremia caused by a “Flexispira”-like organism in a patient with X-linked (Bruton’s) agammaglobulinemia. *J. Clin. Microbiol.* **37**:2439–2445.

SUPPLEMENTAL READING

- Nachamkin, I. 2003. *Campylobacter* and *Arco-bacter*, p. 902–914. In P. R. Murray, E. J. Baron, J. H. Jorgensen, M. A. Pfaller, and R. H. Yolken (ed.), *Manual of Clinical Microbiology*, 8th ed. ASM Press, Washington, D.C.
- Nachamkin, I., and M. J. Blaser (ed.). 2000. *Campylobacter*, 2nd ed. ASM Press, Washington, D.C.
- Penner, J. L. 1988. The genus *Campylobacter*: a decade of progress. *Clin. Microbiol. Rev.* **1**:157–172.
- Versalovic, J., and J. G. Fox. 2003. *Helicobacter*, p. 915–928. In P. R. Murray, E. J. Baron, J. H. Jorgensen, M. A. Pfaller, and R. H. Yolken (ed.), *Manual of Clinical Microbiology*, 8th ed. ASM Press, Washington, D.C.

APPENDIX 3.8.2-1

Direct Detection of *Campylobacter* by EIA Method (ProSpecT)

I. PRINCIPLE

Diagnosis of campylobacteriosis by isolation of the organism can take 2 days to a week. Direct detection of the *Campylobacter* surface antigen (SA) from fecal specimens in an EIA can be done the same day the specimen is received. In this test two molecules with molecular masses of 15 and 66 kDa are detected. Cross-reactivity studies indicate that the antigens are shared by *C. jejuni* and *C. coli*.

The test can be performed directly on extracts of fecal specimens or fecal enriched broth cultures. Diluted specimens are added to breakaway microplate wells on which rabbit polyclonal anti-*Campylobacter* SA antibody is bound. If antigen is present, it is captured by the bound antibody. When an enzyme conjugate (polyclonal rabbit anti-

APPENDIX 3.8.2-1 (continued)

Campylobacter SA labeled with horseradish peroxidase enzyme) is added, captured *Campylobacter*-specific antigen binds the enzyme conjugate to the well. After the substrate for the enzyme is added, it is converted to a colored reaction product. Color development can be detected visually or spectrophotometrically. In a negative reaction, there is no *Campylobacter* SA or an insufficient amount of antigen present to bind the enzyme conjugate to the well and no colored reaction product develops.

II. SPECIMEN COLLECTION AND HANDLING**A. Collection of direct stool (feces)**

1. Unpreserved stool specimens, specimens in Cary-Blair medium, or specimens diluted in the ProSpecT bacterial specimen diluent can be stored at 2 to 8°C for up to 72 h, although this storage can be extended to up to 7 days if in Cary-Blair medium.
2. If testing is to be delayed, store frozen at -20°C or lower immediately after collection.
3. Optimal specimen results will be obtained if the specimen is tested immediately upon receipt in the laboratory.

B. Specimen preparation for assay**1. Direct method**

- Dilute unpreserved specimens approximately 1:3 by mixing 1 part specimen with 2 parts bacterial specimen diluent as follows.
- a. Add 0.6 ml of bacterial specimen diluent to a clean plastic or glass disposable tube.
 - b. Thoroughly mix stool.
 - c. Using a transfer pipette, remove approximately 0.3 ml of specimen (third mark from the tip of the pipette). Expel sample into bacterial specimen diluent and mix by drawing up and down once. Leave transfer pipettes in the tubes.
 - d. For specimens that will not pipette, use an applicator stick to add 0.3 g (~6 mm in diameter) of specimen. Vigorously stir specimen into bacterial specimen diluent. Add a transfer pipette to the tube and mix by drawing up and down once. Leave transfer pipettes in the tubes.

2. For specimens in Cary-Blair transport, mix well and transfer to the microplate well.

3. Broth method

- a. Inoculate 150 µl or 3 drops of fresh stool or stool in Cary-Blair transport medium into 5 ml of GN broth (Table 3.8.1-1).
- b. Incubate at 35 ± 2°C under ambient atmospheric conditions for 18 to 24 h.
- c. Add 0.6 ml of bacterial specimen diluent to a clean 12- by 75-mm tube.
- d. Transfer 0.3 ml of broth culture into 0.6 ml of bacterial specimen diluent using a transfer pipette. Leave the transfer pipette in the tube.

III. MATERIALS**A. Reagents**

1. The ProSpecT *Campylobacter* microplate assay (48- or 96-test microplate assay) catalog no. is 760-96 (Remel, Inc.).
 - a. 100 ml of bacterial specimen diluent, containing buffered solution with rabbit serum with 0.02% thimerosal
 - b. Microwell eight-well strips coated with rabbit polyclonal anti-*Campylobacter* SA antibody
 - c. Positive control containing *C. jejuni* culture supernatant suspended in negative control with fetal bovine serum and 0.02% thimerosal
 - d. Low positive control containing *C. jejuni* culture supernatant suspended in negative control with fetal bovine serum and 0.02% thimerosal
 - e. Negative control containing human fecal material with rabbit serum and 0.02% thimerosal
 - f. Enzyme conjugate: horseradish peroxidase-labeled rabbit polyclonal anti-*Campylobacter* SA and 0.01% thimerosal
 - g. Wash buffer: 10× concentrated buffered solution with 0.1% thimerosal
 - h. Color substrate in buffer
 - i. Stop solution containing 0.5 N hydrochloric acid (corrosive)
2. Store reagents at 2 to 8°C until use.

APPENDIX 3.8.2-1 (continued)

3. Preparation of wash buffer
 - a. Dilute 10× wash buffer concentrate to 1× by adding 1 part concentrate to 9 parts distilled or deionized water.
 - b. Label diluted wash buffer with 1-month expiration date, and store at 2 to 8°C.

B. Materials

1. Disposable applicator sticks
2. Plastic or glass disposable tubes for specimen preparation, ~1 to 2-ml capacity
3. 1- to 10-ml pipettes
4. Wash bottle or dispenser for wash buffer or microwell plate washer
5. Deionized or distilled water
6. Plate reader capable of reading at 450 nm or 450 and 630 nm (optional)
7. Vortex mixer with microplate adapter or shaker (optional)

IV. QUALITY CONTROL

- A. Test the positive and negative controls each time the test is performed.
- B. Optionally, the low positive control may be run when an indeterminate or very low positive result is repeated. The low positive control may be included in the repeat run as an indication of the performance of the assay near the cutoff level.
- C. The optical density (OD) of the negative control should be <0.100 at 450 nm or <0.070 at 450 and 630 nm, or colorless when read visually. If yellow color equal to 1+ or greater on the procedure card is present in the negative control, the test should be repeated with careful attention to the wash procedure.
- D. The OD of the positive control should be >0.500 at 450 nm or 450 and 630 nm. Visually the intensity of color in the positive control should be equal to or greater than the 2+ reaction on the procedure card.

V. PROCEDURE

- A. Allow all reagents and specimens to reach room temperature (20 to 25°C) before use.
- B. Open the foil pouch, remove the required number of microplate strip wells, and place into a microplate strip holder. Use one well for the negative control and one well for the positive control. If using fewer than eight wells, break off the required number of wells from the strips and return the unused microwells to the foil pouch.
Reseal pouch tightly to exclude moisture and return to the refrigerator.
- C. Add 4 drops of negative control to the first well. Add 4 drops of positive control to a second well. (Optionally, add 4 drops of low positive control to a third well.)
- D. Using a transfer pipette, add 4 drops of diluted specimen or enriched broth culture, or 4 drops of specimen in transport medium per well. *Note:* Place the opening of the transfer pipette just inside the well to avoid splashing into adjacent wells.
- E. Cover the microplate and incubate at room temperature (20 to 25°C) for 60 min. Begin timing after the addition of the last specimen.
- F. Shake out or aspirate the contents of the wells. Wash by completely filling each well with diluted wash buffer (350 to 400 µl/well) a total of three times. After the last wash, remove all fluid from the wells.
- G. Add 4 drops (200 µl) of enzyme conjugate to each well.
- H. Cover the microplate and incubate at room temperature for 30 min.
- I. Decant or aspirate. Wash each well five times.
- J. Add 4 drops (200 µl) of color substrate to each well.
- K. Cover the microplate and incubate at room temperature for 10 min.
- L. Add 1 drop (50 µl) of stop solution to each well. Gently tap or vortex the wells until the yellow color is uniform. Read reactions within 10 min after adding stop solution.
- M. Read visually or spectrophotometrically at 450 nm (single wavelength) and/or 450 and 630 to 650 (dual wavelength).

VI. INTERPRETATION

- A. Interpretation of visual results: refer to the procedure card for color interpretations.
 1. Negative: a colorless reaction is a negative result and indicates that no *Campylobacter* SA or an undetectable level of *Campylobacter* SA is present in the sample tested.
 2. Indeterminate: if faint yellow color that is less than the 1+ reaction develops, the test is indeterminate. Tests with indeterminate results should be repeated.

APPENDIX 3.8.2-1 (continued)

3. Positive: if yellow color of at least 1+ intensity develops in the test wells, the sample contains *Campylobacter* SA and the test is positive.
- B. Read spectrophotometric results at the single (450 nm) and/or dual (450 and 630 to 650 nm) wavelength. Tests with indeterminate results should be repeated.

Single wavelength		
Transport media	<u>Fresh stool</u>	
Negative: OD < 0.100	OD < 0.130	
Indeterminate: OD = 0.100 to 0.130	OD = 0.130 to 0.170	
Positive: OD > 0.130	OD > 0.170	
Dual wavelength		
Transport media	<u>Fresh stool</u>	
Negative: OD < 0.070	OD < 0.100	
Indeterminate: OD = 0.070 to 0.100	OD = 0.100 to 0.140	
Positive: OD > 0.100	OD > 0.140	

VII. LIMITATIONS

- A. The ProSpecT *Campylobacter* microplate assay was evaluated in three studies and was found to have sensitivities of 80, 89, and 96% (1, 2, 3). Specificities were 99%.
- B. The overall performance of the ProSpecT *Campylobacter* microplate assay for broth-enriched culture was a sensitivity of 90% and specificity of 100%, as indicated in the package insert.
- C. The test detects the presence of *Campylobacter* SA in diarrheal stools and cultures. Correlation between the amount of antigen in a sample and clinical presentation has not been established.
- D. A negative test result does not exclude the possibility of the presence of *Campylobacter* and may occur when the antigen level in the sample is below the detection level of the test.
- E. The ProSpecT *Campylobacter* microplate assay does not differentiate *C. jejuni* and *C. coli*, and there are other serotypes and subspecies that may or may not be detected.
- F. It is not known whether *C. upsaliensis*, *C. hyoilealis*, or *C. helveticus* cross-reacts.

References

1. Endtz, H. P., C. W. Ang, N. van den Braak, A. Luijendijk, B. C. Jacobs, P. de Man, J. M. van Duin, A. van Belkum, and H. A. Verbrugh. 2000. Evaluation of a new commercial immunoassay for rapid detection of *Campylobacter jejuni* in stool samples. *Eur. J. Clin. Microbiol. Infect. Dis.* **19**:794–797.
2. Hindiyeh, M., S. Jense, S. Hohmann, H. Bennett, C. Edwards, W. Aldeen, A. Croft, J. Daly, S. Mottice, and K. C. Carroll. 2000. Rapid detection of *Campylobacter jejuni* in stool specimens by an enzyme immunoassay and surveillance for *Campylobacter upsaliensis* in the greater Salt Lake City area. *J. Clin. Microbiol.* **38**:3076–3079.
3. Tolcin, R., M. M. LaSalvia, B. A. Kirkley, E. A. Vetter, F. R. Cockerill III, and G. W. Procop. 2000. Evaluation of the Alexon-Trend ProSpecT *Campylobacter* microplate assay. *J. Clin. Microbiol.* **38**:3853–3855.

3.8.3

Clostridium difficile Toxin Detection

[Updated November 2006]

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Clostridium difficile is an opportunistic pathogen present in healthy adults and children and is responsible for 300,000 to 3,000,000 cases of diarrhea and colitis in hospitalized patients in the United States every year (12). When the intestinal microbiota is altered, toxigenic *C. difficile* flourishes and toxin production increases. *C. difficile* toxin is responsible for 25% of antimicrobial agent-associated diarrhea cases and almost all cases of pseudomembranous colitis (11). Patients with the latter present with pseudomembranous nodules or plaques in the distal and sigmoid colon and rectum, which lead to colonic dilation (toxic megacolon) or even perforation. If unrecognized or untreated, it can be fatal. The definitive diagnosis of pseudomembranous colitis is made by the endoscopic detection of pseudomembranes or microabscesses.

For hospitalized patients, *C. difficile*-associated diarrhea (CDAD) correlates with antimicrobial therapy within the last 30 days and either three unformed stools over a 24-h period or abdominal pain (6). Although clindamycin and cephalosporin usage is highly associated with the disease, any antimicrobial agent can precipitate toxin production, as can various anti-cancer chemotherapeutic agents and diseases, such as AIDS. Generally, the implicated antimicrobial agent is discontinued, and treatment with oral vancomycin or metronidazole may be needed. The latter agent is preferred to prevent the emergence of vancomycin-resistant enterococci.

Nosocomial spread of *C. difficile* can be a problem, particularly in hospitals with

neutropenic patients or long-term-care patients. The Society for Healthcare Epidemiology of America (SHEA) and America College of Gastroenterology have issued separate guidelines for prevention and control of *C. difficile* disease (3, 4). *C. difficile* produces two toxins, enterotoxin (toxin A) and cytotoxin (toxin B). Toxin A causes extensive tissue damage (lesions) to the mucosal wall of the intestine. Toxin B is a highly potent cytotoxin, with as little as 1 pg of the toxin causing rounding of tissue culture cells (9). The toxins are very unstable, even at refrigerator temperatures and in the frozen state. Several immunologic tests are available to detect the toxin, using antibody to the toxin. Culture for the organism is very sensitive but not specific, since it detects colonization with the organism, even when no toxin is produced (5). In addition, it takes several days for a positive result. Hence, it is used only to collect isolates for an epidemiologic investigation (see procedure 3.8.1 for culture methods). A latex agglutination test detects glutamate dehydrogenase, a nontoxin protein associated with *C. difficile* and other anaerobes that may correlate with CDAD. This test will yield false-positive results with non-toxin-producing isolates of *C. difficile*, *Clostridium botulinum*, *Clostridium sporogenes*, *Peptostreptococcus anaerobius*, and *Bacteroides caccharolyticus* (10).

The reference method for laboratory diagnosis of CDAD has been detection of toxin B by cytotoxin (tissue culture) assay. Yet the sensitivity of this "gold standard" method has recently been reevaluated, and in some studies, it is reported to be about

85 to 90% (15) compared to cytotoxin assay of broth cultures from the organism isolated from anaerobic fecal cultures on prereduced selective medium (procedure 3.8.1). EIAs, for either toxin A or toxins A and B are less sensitive than tissue culture cytotoxicity (generally 70 to 82% sensitive [13, 15]) but may be more practical, because of their rapid turnaround time and the instability of the toxins, especially if a tissue culture assay must be sent to a reference laboratory. Many commercial tests detect toxin A, which is present in greater concentration. Some also detect toxin B, and a third type detects toxin A and glutamate dehydrogenase (Triage; Biosite Diagnostics, San Diego, Calif.). Tests that detect only toxin A may miss some isolates that produce toxin B only (2, 11). However, since toxin B is present in lower quantities, tests that detect both toxin A and toxin B may still not be able to detect low levels of toxin B in stool specimens and may not have much advantage over those tests that detect only toxin A. The Triage is less likely to miss a positive and has a reported sensitivity of 97.5% (package insert) to 89% compared to cytotoxin assay (15). However, the specificity of the test is 89%. Thus, those results that are toxin A negative and glutamate dehydrogenase positive should be confirmed by a toxin B assay or reported with a qualification that they may not be positive for the toxin (8). Since procedural methods for the immunologic tests are available in the package inserts and the options, sensitivities, and specificities are discussed elsewhere (1, 15), this procedure presents only the cytotoxin assay. However, the speci-

men collection, reporting, and some limitations apply to all methods.

Cytotoxin B tissue culture assay is based on the principle that a mixture of a bacterial-cell-free stool filtrate containing

C. difficile toxin will show cytopathic effect (CPE) (rounding of the cells) in a susceptible cell line. The filtrate of *C. difficile* toxin mixed with *C. difficile* antitoxin will

not produce a CPE. The neutralization of the toxin confirms the presence of *C. difficile* cytotoxin in the patient's stool sample.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING

- A. Collect stool specimens in leakproof container, and transport and store without preservative at 2 to 8°C until processing (within 24 h of collection).
- B. Also acceptable are lumen contents and surgical or autopsy samples of the large bowel.
- C. Rejection criteria
 - NOTE:** The SHEA recommends that testing not be performed on asymptomatic patients, even for test of cure (4, 5, 6).
 - 1. A rectal swab will not provide enough specimen for the test and therefore is unacceptable.
 - 2. Reject stools that are not liquid or soft.
 - 3. Request repeat collection if there is not enough specimen for test; 10 to 20 ml of watery, diarrheal stool is preferred, or a minimum of 3 ml or 3 g is required.
 - 4. Request recollection if test will not be performed within 24 h of receipt.
 - 5. Meconium may interfere with the assay.
 - 6. Discourage testing specimens from infants less than 1 year of age (reference 6 and SHEA guidelines). Include comment on report indicating lack of specificity of test for this population.
 - NOTE:** Infants have been shown to be asymptomatic carriers, with colonization rates as high as 50% (7, 16, 17).
 - 7. Limit testing of stools from cystic fibrosis patients (4), because these patients have been shown to have colonization rates as high as 32% (14).
 - 8. Patients with positive tests should *not* have repeat testing for cure, unless they again become symptomatic after completion of therapy (6).
 - NOTE:** In one study, limiting the number of stool samples processed for *C. difficile* toxin to one per 72 h would have reduced the number of duplicates by 40%, without adversely affecting the identification of toxin-positive patients (M. LaRocco, personal communication).

III. MATERIALS

- A. Minimum essential medium (MEM) with 2% fetal bovine serum (FBS), gentamicin (50 µg/ml), vancomycin (500 µg/ml), and nystatin (10,000 U/ml) (*see* procedure 10.3 for details of preparation of tissue cultures and media)
- B. 96-well flat-bottomed microtiter plate with monolayer of diploid fibroblasts (e.g., human fetal foreskin, MRC-5, Chinese hamster ovary K1 cells) and 0.1 ml of MEM per well
 1. Preparation of cell monolayer
 - a. Make a suspension of cells at a concentration of 4×10^4 cells per ml in growth medium (90% MEM plus 10% FBS).
 - b. Inoculate 100 µl of suspension into each well.
 - c. Incubate at 35°C aerobically with 5% CO₂ for several days until a monolayer forms.
 - d. Aspirate growth medium and replace with 100 µl of MEM with 2% FBS.
 - e. To avoid drying of outer wells, add 200 µl of MEM with 2% FBS to perimeter wells and do not use them for the assay if they do not look healthy.
- 2. Store at 35°C aerobically with 5% CO₂.

III. MATERIALS (continued)

3. Use trays up to 3 weeks after preparation.
- **NOTE:** The Bartels (Issaquan, Wash.) cytotoxicity assay for *C. difficile* toxin utilizes a microtiter tray containing tissue culture cells, thus allowing laboratories not equipped for standard tissue culture to use the assay.
- C. *C. difficile* culture filtrate, lyophilized (Techlab Inc., Blacksburg, Va.)
 1. Contains toxins A and B
 2. Add 1 ml of sterile distilled water to vial.
 3. Aliquot small volumes (50 to 100 µl) of the reconstituted filtrate in an airtight container at -20°C.
- D. *C. difficile* antitoxin, lyophilized (Techlab Inc.)
 1. Add 3 ml of sterile distilled water for stock.
 2. Aliquot small volumes (50 to 100 µl) of the reconstituted antitoxin in an airtight container at -20°C.
 3. Place a 6-month expiration date on reconstituted antitoxin.
 4. For working dilution, dilute stock 1:25 with 98% MEM.
 - a. Add 0.1 ml of stock to 2.4 ml of MEM.
 - b. Prepare fresh every 2 weeks.
 - c. Store at 2 to 8°C.
 5. Determine the titer of each new lot of both toxin and antitoxin before use.
 - a. Make 10-fold dilutions from 10⁻¹ to 10⁻⁵ in 98% MEM.
- b. Remove MEM from right wells of double rows of microtiter plate.
- c. Inoculate 0.1 ml of working dilution of antitoxin into each right well.
- d. Inoculate 0.1 ml of culture filtrate into the right and left wells.
- e. Incubate in 3 to 5% CO₂ at 33 to 37°C for 48 h.
- f. The titer is the highest dilution showing typical 4+ toxin CPE and no CPE in the tube with antitoxin. The working dilution is 10 times this dilution. (If the highest dilution showing 4+ CPE is 10⁻⁵ the working dilution is 10⁻⁴.)
- E. Materials required but not provided
 1. Pipettors that dispense 100 µl
 2. Powderless disposable latex or polypropylene gloves
 3. 1-, 5-, and 10-ml sterile pipettes
 4. *Refrigerated* centrifuge, capable of centrifugation at ≥12,000 × g
 5. 15-ml round-bottomed polypropylene or other appropriate centrifuge tubes for specimen dilution and centrifugation
 6. Syringe, 3 ml
 7. Repeating pipetting device
 8. Millipore 0.45-µm-pore-size filters
 9. Incubator with 5% CO₂
 10. Inverted microscope

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

- A. Each time the test is run, even if on the same tray, set up the following controls after aspirating the maintenance medium (*see* Fig. 3.8.3-1).
 1. 100 µl of toxin plus 100 µl of MEM
 2. 100 µl of toxin plus 100 µl of antitoxin
- B. In addition, set up the following controls once per plate after aspirating the maintenance medium.
 1. 100 µl of antitoxin plus 100 µl of MEM
 2. 200 µl of MEM
- C. Expected results
 1. Well with toxin shows typical CPE within 12 to 18 h. The cells round up and appear evenly throughout the cell sheet.
 2. Well with toxin plus antitoxin shows no CPE.
 3. Wells with MEM and wells with antitoxin alone show no CPE.

	Day 1						Day 2					
A/1	2	3	4	5	6	7	8	9	10	11	12	
B	Toxin	Toxin + A	Patient D 1:20	Patient D 1:20 + A	Toxin	Toxin + A						
C	MEM	A	Patient D 1:100	Patient D 1:100 + A	Patient F 1:20	Patient F 1:20 + A						
D	Patient A 1:20	Patient A 1:20 + A	Patient E 1:20	Patient E 1:20 + A	Patient F 1:100	Patient F 1:100 + A						
E	Patient A 1:100	Patient A 1:100 + A	Patient E 1:100	Patient E 1:100 + A	Patient G 1:20	Patient G 1:20 + A						
F	Patient B 1:20	Patient B 1:20 + A			Patient G 1:100	Patient G 1:100 + A						
G	Patient B 1:100	Patient B 1:100 + A										
H												

Figure 3.8.3–1 Example of worksheet diagram to accompany a 96-well microtiter tray previously inoculated with a monolayer of fibroblasts and subsequently inoculated with patient specimen, controls, toxin, and antitoxin. Day 1 is the first date the tray is used. Day 2 illustrates the controls omitted on subsequent days; i.e., MEM and antitoxin control wells are not needed. Patient stool specimens in dilutions of 1:20 and 1:100 are indicated as patient A, B, C, D, and E, inoculated on day 1, and patients F and G, inoculated on day 2. Note that outer wells are not used. A, antitoxin.

IV. QUALITY CONTROL (continued)

D. Troubleshooting

Problem

Positive control shows no CPE.

1. Positive control has deteriorated. Dilute new aliquot.

2. Cells insensitive—rerun test with another cell lot.

Toxin-antitoxin well shows CPE.

1. Antitoxin deteriorated. Dilute and use frozen aliquot of the same lot.

2. Dilute and use different lot of antitoxin.

3. Cell sheet contaminated. Try new lot of cells or reagents.

V. PROCEDURE



Observe standard precautions.

A. Preparation of stool sample

1. Add 3 ml of 98% MEM to a 15-ml centrifuge tube.
2. Add enough stool sample to bring the level of medium up to the 5-ml mark (2:5 dilution).
3. Vortex at the highest speed.
4. Centrifuge at $12,000 \times g$ for 15 min in refrigerated centrifuge.
5. Filter the supernatant into a tube using a 0.45-μm-pore-size filter attached to a 3-ml syringe.
6. Add 100 μl of supernatant to 300 μl of 98% MEM in a 12- by 75-mm tube (final dilution, 1:10).
7. Add 100 μl of the 1:10 dilution to 400 μl of 98% MEM in a 23- by 75-mm tube (final dilution, 1:50)

V. PROCEDURE (continued)

- B. Inoculation (see Fig. 3.8.3-1)
1. Remove medium from right wells of double rows.
 2. Add 100 µl of working dilution antitoxin to each right well immediately after medium is removed but before adding patient's specimen.
 3. Add 100 µl of the 1:50 dilution of patient's specimen to the left and right wells of the double rows (1:100 dilution).
 4. Add 100 µl of the 1:10 dilution of patient's specimen to the left and right wells of the double rows (1:20 dilution).
 5. The same pipette tip can be used for pipetting both dilutions of the same specimen if the higher dilution is inoculated first.
 6. Save dilutions and stool at 4°C for further testing.
 7. Inoculate toxin and toxin-antitoxin controls.
- C. Incubate the tray for a minimum of 12 to 18 h at 33 to 35°C with 3 to 5% CO₂.
- D. Read for CPE at 24 and 48 h.

POSTANALYTICAL CONSIDERATIONS**VI. INTERPRETATION**

- A. If stool filtrate and stool filtrate plus antitoxin are both negative, this is interpreted as negative for *C. difficile* toxin.
- B. If stool filtrate is positive and stool filtrate plus antitoxin is negative at the same dilution, this is interpreted as positive for *C. difficile* toxin.
- C. If stool filtrate and stool filtrate plus antitoxin are both positive, this is an equivocal result.
1. Wells with 1:20 dilution stool extract plus antitoxin can show breakthrough CPE in a strongly positive specimen. In this case a definite reduction (50%) in CPE of the 1:100 dilution stool extract plus antitoxin compared to the 1:20 dilution plus antitoxin well suggests presence of *C. difficile* toxin.
 - a. If the well with the 1:100 dilution plus antitoxin also shows breakthrough CPE, set up further dilutions of 1:200, 1:400, etc., with and without the antitoxin.
 - b. Use the 1:20 dilution extract to make further dilutions.
 - c. Determine the highest dilution of stool extract plus antitoxin that shows no CPE. If the stool extract at that dilution shows CPE, the specimen is positive for *C. difficile* toxin.
 2. If CPE shows in stool extract-plus-antitoxin well of the highest dilution of stool extract that shows CPE, the CPE is not due to *C. difficile* toxin. Request another sample, since the results cannot be interpreted.
 3. If the cell sheet is deteriorating nonspecifically, request another sample, since these results cannot be interpreted.

VII. REPORTING RESULTS

- A. Report positive results as “*C. difficile* toxin positive.”
 1. Notify the caregiver who ordered the test.
 2. Report positives as soon as neutralized CPE is observed.
- B. Report preliminary negative results as “No *C. difficile* toxin detected at 24 h; final reading at 48 h.”
- C. Report final negative results as “No *C. difficile* toxin detected.”

VII. REPORTING RESULTS

(continued)

- D. For equivocal results (cells show rounding that is not neutralized by antitoxin), questionable weak results, or specimens that are toxic to the cell sheet (cell sheet falls off sides of well), report “Specimen result not interpretable. Suggest repeat sample.”
 - 1. Notify caregiver to submit another specimen if diarrhea persists.
 - 2. Communicate this result as soon as possible to avoid delays in obtaining a repeat specimen. It is likely that if the patient has *C. difficile* toxin, the repeat specimen will be a strong positive.

VIII. LIMITATIONS

- A. The etiology of diarrhea caused by microorganisms other than *C. difficile* will not be established with this assay.
- B. Levels of toxin can be low. A negative result alone may not rule out the possibility of CDAD.
- C. Always evaluate assay result along with clinical signs and patient history.
- D. Certain isolates of *Clostridium sordellii* produce the same type of rounding on tissue culture cells as toxigenic *C. difficile*, due to the similarities of the toxins. *C. sordellii* has not been detected in patients with antimicrobial agent-associated diarrhea and colitis. It is also unlikely that *C. sordellii* will be present in human fecal specimens.
- E. False-positive results are associated with misinterpretation of CPE and its neutralization.
- F. Lack of homogeneity in a stool sample may lead to incorrect results. Thorough mixing of stool specimens is essential to avoid this problem.
- G. Stool specimens that appear to have large amounts of fat, mucus, or blood present are more likely to give toxic results.
- H. Healthy newborns are frequently toxin positive.

REFERENCES

1. Allen, S. A., C. L. Emery, and J. A. Siders. 2002. Anaerobic bacteriology, p. 69–76. In A. L. Truant (ed.), *Manual of Commercial Methods in Microbiology*. ASM Press, Washington, D.C.
2. Depitre, C., M. Delmee, V. Avesani, R. L'Haridon, A. Roels, M. Popoff, and G. Corthier. 1993. Serogroup F strains of *Clostridium difficile* produce toxin B but not toxin A. *J. Med. Microbiol.* **38**:434–441.
3. Fekety, R. 1997. Guidelines for the diagnosis and management of *Clostridium difficile*-associated diarrhea and colitis. American College of Gastroenterology, Practice Parameters Committee. *Am. J. Gastroenterol.* **92**:739–750.
4. Gerding, D. N., S. Johnson, L. R. Peterson, M. E. Mulligan, and J. Silva, Jr. 1995. *Clostridium difficile*-associated diarrhea and colitis. *Infect. Control Hosp. Epidemiol.* **16**:459–477.
5. Gerding, D. N., M. M. Olson, L. R. Peterson, D. G. Teasley, R. L. Gebhard, M. L. Schwartz, and J. T. Lee, Jr. 1986. *Clostridium difficile*-associated diarrhea and colitis in adults. A prospective case-controlled epidemiologic study. *Arch. Intern. Med.* **146**:95–100.
6. Johnson, S., and D. N. Gerding. 1998. *Clostridium difficile*-associated diarrhea: a review. *Clin. Infect. Dis.* **26**:1027–1034.
7. Kader, H. A., D. A. Piccoli, A. F. Jawad, K. L. McGowan, and E. S. Maller. 1998. Single toxin detection is inadequate to diagnose *Clostridium difficile* diarrhea in pediatric patients. *Gastroenterology* **115**:1329–1334.
8. Landry, M. L., J. Topal, D. Ferguson, D. Giudetti, and Y. Tang. 2001. Evaluation of Biosite Triage *Clostridium difficile* panel for rapid detection of *Clostridium difficile* in stool samples. *J. Clin. Microbiol.* **39**:1855–1858.
9. Lyerly, D., H. Krivan, and T. Wilkins. 1988. *Clostridium difficile*: its diseases and toxins. *Clin. Microbiol. Rev.* **1**:1–18.
10. Lyerly, D. M., D. W. Ball, J. Toth, and T. D. Wilkins. 1988. Characterization of cross-reactive proteins detected by Culturette brand rapid latex test for *Clostridium difficile*. *J. Clin. Microbiol.* **26**:397–400.
11. Moncrief, J. S., L. Zheng, L. M. Neville, and D. M. Lyerly. 2000. Genetic characterization of toxin A-negative, toxin B-positive *Clostridium difficile* isolates by PCR. *J. Clin. Microbiol.* **38**:3072–3075.
12. Mylonakis, E., E. T. Ryan, and S. B. Calderwood. 2001. *Clostridium difficile*-associated diarrhea: a review. *Arch. Intern. Med.* **161**:525–533.

REFERENCES (continued)

13. O'Connor, D., P. Hynes, M. Cormican, E. Collins, G. Corbett-Feeney, and M. Cassidy. 2001. Evaluation of methods for detection of toxins in specimens of feces submitted for diagnosis of *Clostridium difficile*-associated diarrhea. *J. Clin. Microbiol.* **39**:2846–2849.
14. Peach, S. L., S. P. Borriello, H. Gaya, F. E. Barclay, and A. R. Welch. 1986. Asymptomatic carriage of *Clostridium difficile* in patients with cystic fibrosis. *J. Clin. Pathol.* **39**:1013–1018.
15. Turgeon, D. K., T. J. Novicki, J. Quick, L. Carlson, P. Miller, B. Ulness, A. Cent, R. Ashley, A. Larson, M. Coyle, A. P. Limaye, B. T. Cookson, and T. R. Fritsche. 2003. Six rapid tests for direct detection of *Clostridium difficile* and its toxins in fecal samples compared with the fibroblast cytotoxicity assay. *J. Clin. Microbiol.* **41**:667–670.
16. Viscidi, R., S. Willey, and J. G. Bartlett. 1981. Isolation rates and toxigenic potential of *Clostridium difficile* isolates from various populations. *Gastroenterology* **81**:5–9.
17. Wilson, K. H. 1993. The microbiology of *Clostridium difficile*. *Clin. Infect. Dis.* **16**(suppl. 4):S214–S218.

3.8.4

Helicobacter pylori Cultures

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

In 1984, a campylobacter-like organism, *Helicobacter pylori*, was reported to be present in high numbers in the deep mucus layer of the gastric surface epithelium in patients with chronic active gastritis (10). Since its discovery, *H. pylori* has been estimated to be present in 50% of the population (9, 10), and it is known to be associated with an increased risk for the development of peptic ulcer disease, gastric adenocarcinoma, and gastric non-Hodgkin's B-cell lymphomas (mucosa-associated lymphoid tissue) (2). While *H. pylori* is the primary agent of disease, there are increasing reports of evidence of other spiral organisms (e.g., *Helicobacter heilmannii*) present as part of the human microbiota (1, 2, 13), which are often uncultivable but may be responsible for gastritis.

There are a number of tests available to diagnose *H. pylori* infection which can be categorized into invasive (requiring endoscopy and biopsy) and noninvasive. The most common noninvasive test is serology to determine the presence of antibodies to the organism (see procedure 11.9). Sero-

logic testing, if positive, for a symptomatic patient who has not been previously treated supports the diagnosis of *H. pylori*. Caution should be exercised in interpretation in areas of high prevalence of *H. pylori*, as antibodies generally remain for life. These tests have been reviewed by Laheij et al. (5) and are not discussed further here. Another noninvasive test is the urea breath test (3), which is an accurate means of identifying the presence of *H. pylori* infection before and after antimicrobial therapy. The test is labor-intensive and requires expensive instrumentation for analysis (scintillation counter or mass spectrometer), yet for the patient it is simple, rapid, and relatively inexpensive. A stool EIA antigen test (Appendix 3.8.4-1) is also a sensitive noninvasive test that has been shown to be a good method for initial diagnosis and for monitoring eradication after antimicrobial therapy (12). However, the cost of this assay is high.

There are three tests available to diagnose the disease invasively from gastric biopsy specimens. Because of their ease of use, rapidity, and cost-effectiveness, rapid

urease biopsy tests are the most common test. Several commercial tests are approved for performance by nonlaboratory scientists ("waived" tests) in the gastroenterologists' offices, with sensitivities of 89 to 98% and specificities of 93 to 98% (3, 4). Other tests include histologic examination and culture. Culture is demanding, requires special conditions of transport and incubation, and does not provide a result in a timely manner, making it useful only in selected cases.

H. pylori organisms appear in gull wing formation on Gram stain, although after culture they may form a U shape. With age or after prolonged exposure to ambient air, the organisms may appear as coccoidal forms. Unlike most other campylobacters, *H. pylori* organisms possess abundant amounts of urease. They produce superoxide dismutase and catalase in much greater amounts than other campylobacters. The presence of gull wing or U-shaped gram-negative organisms from a gastric specimen that are microaerophilic and urease, catalase, and oxidase positive makes the diagnosis of *H. pylori*.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING

A. Specimen collection

1. Obtain biopsy specimens.
 - a. Pass an endoscope orally.
 - b. Obtain specimens through a channel in the endoscope by using one of the following procedures.
 - (1) Using biopsy forceps, obtain samples from the stomach or duodenum.
 - (2) Using a sheathed brush, brush suspicious areas several times to obtain adequate cellular material.
 - c. If a gastric ulcer is seen, obtain biopsy samples from the base, the surrounding gastric mucosa, and each of the four quadrants of the margin.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING (continued)

2. Transport
 - a. Urease test
 - (1) Prewarm agar.
 - (2) Inoculate several biopsy samples by pushing into the urea agar, or inoculate into broth or other urea test (6).
 - (3) Seal tube and incubate at 25°C or, preferably, at 35°C until submitted to laboratory (7) or agar turns pink.
 - b. Submit biopsy samples within 3 h in 1 ml of modified Cary-Blair transport medium or place in saline on ice.
NOTE: Prompt submission of biopsy specimens to the laboratory for homogenization and urease testing has a higher yield than direct inoculation.
 - c. Label all tubes and specimens with date and time of collection.
3. Optional: collect a stool specimen for direct antigen test. Store at 4°C for up to 72 h or at -20°C until tested. Do not freeze-thaw more than two times.

B. Rejection criteria

1. Do not accept specimens in formalin for culture.
2. For specimens not cultured within 3 h, document in the final report that specimen quality may have been compromised.
3. Gastric washes are not acceptable specimens for culture.

III. MATERIALS

- A. Urease tests—choose from the following or use other commercially available sensitive test for urease detection (8).
 1. Pyloritek urease test (Bard, Inc., Billerica, Mass.)
 2. Rapid selective urea agar (Hardy Diagnostics; Remel, Inc.) (procedure 3.17.48)
 3. CLO test (Ballard Medical Products, Draper, Utah)
 4. HPFAST (GI Supply, Camp Hill, Pa.)
 5. 0.5 ml of Christensen's urea solution without agar (procedure 3.17.48)
- B. Optional: Premier Platinum HpSA direct antigen test (Meridian Diagnostics, Cincinnati, Ohio). See Appendix 3.8.4–1.
- C. Media for culture—for best results, choose one selective and one nonselective medium.
 1. Nonselective media: BAP, BHI with 7% horse blood, brucella agar, or CHOC
2. Selective media: Modified Thayer-Martin medium, Campy-CVA, or Columbia agar with 10% egg yolk emulsion, 1% Vitox, and 40 mg of 2,3,5-triphenyltetrazolium chloride per liter with cefsulodin, trimethoprim, vancomycin, and amphotericin B (CEYE)
NOTE: CHOC and CEYE were reported in one study to provide the best combination (11).

D. Gram stain reagents

E. Other supplies

1. Microaerobic environment (see Table 3.8.2–3 in procedure 3.8.2)
2. Catalase (procedure 3.17.10) and oxidase (procedure 3.17.39) reagents

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

- A. Verify that media meet expiration date and QC parameters per current NCCLS M22 document. See procedures 14.2 and 3.3.1 for further procedures, especially for in-house-prepared media.
- B. Check plate media for ability to support growth of *H. pylori* ATCC 43504.

IV. QUALITY CONTROL (continued)

C. Urea direct tests

1. Examine each lot visually to determine that no reaction has occurred and that the test is yellow or straw colored.
2. Prior to each use, make sure there are no signs of contamination or deterioration, (shrinking, cracking, or discoloration) and that the tube is yellow. Discard any defective tubes and obtain a new one.
3. Check each lot prior to putting it into use with a positively and negatively reacting organism.

Test organism	Result
<i>Proteus mirabilis</i> ATCC 12453	Positive; color change from the original yellow to pink-red, slower reaction time than <i>H. pylori</i>
<i>Escherichia coli</i> ATCC 25922	Negative; no color change observed

4. For proficiency, QC, and validation of direct urease test, obtain urease tablets (Kimberly Clark, Draper, Utah). As a positive patient control, insert the tablet into a negative test, as you would a biopsy sample. After 5 min, inspect for a positive color change. If the test remains negative, take corrective action.

V. PROCEDURE



Observe standard precautions.

A. Inoculation

1. Gently homogenize the tissue (refer to procedure 3.13.1 for details on grinding).
- NOTE:** This method of processing results in heavier yields of organisms compared to either mincing the specimen or direct plating of the specimen.
2. Place several pieces of biopsy material into one of the urea test media and incubate at 35°C under aerobic conditions.
3. Inoculate plate media and incubate under microaerobic conditions at 35°C. When using an airtight jar, add a moistened gauze pad in the bottom.
4. Inoculate additional CHOC plate at 35°C under aerobic conditions in 5% CO₂.

B. Direct tests

1. Examine the urea test for a pink to magenta color change at 30 min, 4 h, and 24 h, depending on the manufacturer's instructions for incubation and reading.
2. Gram stain and examine for gull wing-shaped, gram-negative rods.

C. Culture methods (also see *Campylobacter* procedure [3.8.2])

1. Incubate plates at 35 to 37°C for 3 to 5 days. Some strains require as long as 7 days for growth on primary isolation.
2. Examination of culture media
 - a. Colonies of *H. pylori* appear as small, gray, and translucent. Some strains demonstrate weak beta-hemolysis with growth on the microaerobic plates but *not* on the aerobically incubated plate.
 - b. Gram stain suspicious colonies. *H. pylori* organisms from solid media appear as slightly curved gram-negative rods.
 - c. Perform oxidase, catalase, and urea tests from colonies. *H. pylori* is positive in all three tests.

POSTANALYTICAL CONSIDERATIONS**VI. REPORTING RESULTS**

- A. Report direct results.
 - 1. Report results of urea test as “positive” if any pink to magenta color is observed or “negative” if there is no color change in 24 h.
 - 2. Report results of stool direct antigen test as “positive” or “negative for *Helicobacter pylori* antigen” per criteria in package insert.
 - 3. If the direct urease is positive (pink) and “gull wings” are seen on Gram stain, a preliminary culture report can be sent as “Probable *Helicobacter pylori* present.”
- B. Report culture as “*Helicobacter pylori*” if the following are true.
 - 1. The colony Gram stain demonstrates curved, gram-negative rods.
 - 2. Oxidase and catalase reactions are positive.
 - 3. Urease reaction is positive (usually within several minutes).
- C. Refer to procedure 3.3 for general criteria for reporting.

VII. INTERPRETATION

- A. The presence of a urease-positive curved rod in a gastric specimen is indicative of the presence of *H. pylori*, although there are reports of other urea-positive curved rods in gastric specimens (13).
- B. The presence of *H. pylori* indicates the need for aggressive treatment of the infection to eradicate the organism.
- C. There are a number of current protocols for therapy of *H. pylori*. In general, they consist of agents to decrease acidity to allow healing and multiple antimicrobial agents to eradicate the organism. Common regimens include amoxicillin, clarithromycin, and omeprazole (triple therapy) and bismuth, tetracycline, metronidazole and a proton pump inhibitor (quadruple therapy) (9). In addition, pharmaceutical companies have packaged antihelicobacter therapy in convenient dosing formats that include “PrevPak” and “Helidac.” In >90% of treated patients, there is healing of the gastric inflammation, prevention and healing of peptic ulcers, and loss of the symptoms of gastritis (1, 4).
- D. Perform antimicrobial susceptibility testing in cases of treatment failures.

VIII. LIMITATIONS

- A. No one test has 100% sensitivity in detecting *H. pylori*, and several tests, including serologic assays and tests described in this procedure, may be necessary.
- B. False-negative urease tests may occur when very low numbers of *H. pylori* organisms are present in the stomach or when the bacterium has a patchy distribution. Testing multiple biopsy samples will increase the likelihood of a positive test result.
- C. The urease test and culture are less sensitive if the patient has recently taken antimicrobial agents or bismuth. Suppression of *H. pylori* by these agents makes the organism difficult to detect by any means. Regrowth of *H. pylori* may be patchy in the first 3 weeks after treatment.
- D. After treatment, the stool antigen test and the urea breath test are the most accurate to monitor therapy and cure.
- E. Since there are now a number of urease-positive spiral organisms, the preliminary testing described in this procedure cannot be considered definitive without further molecular testing (1, 13).

REFERENCES

1. Blaser, M. J. 1998. Helicobacters are indigenous to the human stomach: duodenal ulceration is due to changes in gastric microecology in the modern era. *Gut* **43**:721–727.
2. Blaser, M. J. 1999. Hypothesis: the changing relationships of *Helicobacter pylori* and humans: implications for health and disease. *J. Infect. Dis.* **179**:1523–1530.
3. Chey, W. D. 2000. Accurate diagnosis of *Helicobacter pylori*. 14C-urea breath test. *Gastroenterol. Clin. N. Am.* **29**:895–902.
4. Dunn, B. E., H. Cohen, and M. J. Blaser. 1997. *Helicobacter pylori*. *Clin. Microbiol. Rev.* **10**:720–741.
5. Laheij, R. J. F., H. Straatman, J. B. M. J. Jansen, and A. L. M. Verbeek. 1998. Evaluation of commercially available *Helicobacter pylori* serology kits: a review. *J. Clin. Microbiol.* **36**:2803–2809.
6. Laine, L., D. Chung, C. Stein, I. El-Beblawi, V. Sharma, and P. Chandra-Soma. 1996. The influence of size or number of biopsies on rapid urease test results: a prospective evaluation. *Gastrointest. Endosc.* **43**:49–53.
7. Laine, L., R. Estrada, D. N. Lewin, and H. Cohen. 1996. The influence of warming on rapid urease test results—a prospective evaluation. *Gastrointest. Endosc.* **44**:429–432.
8. Laine, L., D. Lewin, W. Naritoku, R. Estrada, and H. Cohen. 1996. Prospective comparison of commercially available rapid urease tests for the diagnosis of *Helicobacter pylori*. *Gastrointest. Endosc.* **44**:523–526.
9. Leung, W. K., and D. Y. Graham. 2000. Clarithromycin for *Helicobacter pylori* infection. *Exp. Opin. Pharmacother.* **1**:507–514.
10. Marshall, B. J., and J. R. Warren. 1984. Unidentified curved bacilli in the stomach of patients with gastritis and peptic ulceration. *Lancet* **i**:1311–1315.
11. Piccolomini, R., G. Di Bonaventura, D. Festi, G. Catamo, F. Laterza, and M. Neri. 1997. Optimal combination of media for primary isolation of *Helicobacter pylori* from gastric biopsy specimens. *J. Clin. Microbiol.* **35**:1541–1544.
12. Vaira, D., P. Malfertheiner, F. Mégraud, A. T. R. Axon, M. Deltenre, A. M. Hirschl, G. Gasbarrini, C. O'Morain, J. M. Pajares Garcia, M. Quina, G. N. J. Tytgat, and the HpSA European Study Group. 1999. Diagnosis of *Helicobacter pylori* infection with a new non-invasive antigen-based assay. *Lancet* **354**:30–33.
13. Weir, S., B. Cuccherini, A. M. Whitney, M. L. Ray, J. P. MacGregor, A. Steigerwalt, M. I. Daneshvar, R. Weyant, B. Wray, J. Steele, W. Strober, and V. J. Gill. 1999. Recurrent bacteremia caused by a “Flexispira”-like organism in a patient with X-linked (Bruton's) agammaglobulinemia. *J. Clin. Microbiol.* **37**:2439–2445.

APPENDIX 3.8.4-1
***Helicobacter pylori* Antigen Assay (HpSA)**
I. PRINCIPLE

A microplate EIA is commercially available for the qualitative detection of *Helicobacter pylori* antigens in human stool (HpSA) by a noninvasive method (1). The test can identify current infections and can be used to confirm the eradication of the microorganism after the end of the therapy. Unlike serologic tests (procedure 11.9), which are unable to differentiate between current and past infections, the HpSA test detects current infection only. The HpSA EIA is a standard microtiter well assay using polyclonal antibodies.

II. SPECIMENS

Collect a stool specimen in an empty container.

- A. Store for up to 3 days at 2 to 8°C before testing.
- B. If testing cannot be performed within this time frame, store specimens frozen upon receipt at –20 to –80°C.

III. MATERIALS

Premier Platinum HpSA, catalog no. 601348—48 tests (Meridian Diagnostics, Cincinnati, Ohio).

The kit contains antibody-coated microwells, a positive control and negative control, sample diluent, wash buffer, enzyme conjugate, substrate, stop solution, transfer pipettes, strip holder, strip sealer, and wooden stick applicators. Store kit at 2 to 8°C.

IV. QUALITY CONTROL

Test the positive and negative controls upon receipt of new lots and each time patient testing is performed.

V. PROCEDURE

- A. Emulsify 5- to 6-mm-diameter portion of stool into 200 µl of sample diluent in a test tube.
- B. Transfer 50 µl of diluted stools and controls in the appropriate wells in the microtiter plate.

APPENDIX 3.8.4–1 (continued)

- C. Add 1 drop of enzyme conjugate to each well, seal, and incubate at room temperature for 1 h.
 - D. Wash five times with the wash buffer.
 - E. Add 2 drops of substrate to all wells and incubate at room temperature for 10 min.
 - F. Add 1 drop of stop solution and read at dual wavelength (450 and 630 nm).
- VI. INTERPRETATION
- Cutoff values (absorbance at 450 nm) for HpSA test are ≥ 0.160 for positive results, 0.159 to 0.140 for indeterminate results, and < 0.140 for negative results.
- VII. LIMITATIONS
- A. At present no single test can be relied upon to detect definitely colonization by *H. pylori*, and a combination of two is recommended if this is feasible.
 - B. In a large prospective evaluation comparing the HpSA test to invasive tests, the sensitivity and the specificity of the HpSA were 94 and 92%, respectively, compared to biopsy-based tests (3).
 - C. In an evaluation of eradication (4) on day 35 after antimicrobial therapy, the urea breath test had a sensitivity of 94% and a specificity of 100%. The stool antigen test had a sensitivity of 94% and a specificity of 97%. On day 7 after treatment, the stool antigen test was predictive of eradication (positive predictive value, 100%; negative predictive value, 91%).
 - D. The test has been shown to be accurate for pediatrics patients, for whom serology tests are less sensitive (2).

References

1. Evangelista, A. T., A. L. Truant, and P. Bourbeau. 2002. Rapid systems and instruments for the identification of bacteria, p. 38–39. In A. L. Truant (ed.), *Manual of Commercial Methods in Microbiology*. ASM Press, Washington, D.C.
2. Oderda, G., A. Rapa, B. Ronchi, P. Lerro, M. Pastore, A. Staiano, G. L. dè Angelis, and P. Strisciuglio. 2000. Detection of *Helicobacter pylori* in stool specimens by non-invasive antigen enzyme immunoassay in children: multicentre Italian study. *BMJ* **320**:347–348.
3. Vaira, D., P. Malfertheiner, F. Mègraud, A. Axon, M. Deltandre, A. M. Hirschl, G. Gasbarrini, C. O'Morain, J. M. Pajares, M. Quina, G. N. J. Tytgat, and the HpSA European Study Group. 1999. Diagnosis of *Helicobacter pylori* infection with a new non-invasive antigen-based assay. *Lancet* **354**:30–33.
4. Vaira, D., N. Vakil, M. Menegatti, B. van Hoff, C. Ricci, L. Gatta, G. Gasbarrini, and M. Quina. 2002. The stool antigen test for detection of *Helicobacter pylori* after eradication therapy. *Ann. Intern. Med.* **136**:280–287.

3.8.5

Screen for Vancomycin-Resistant Enterococci in Fecal Cultures

[Updated March 2007]

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Since the first report of enterococci resistant to high concentrations of glycopeptide antimicrobial agents in 1988, a sudden and widespread increase in the incidence of infection and colonization with vancomycin-resistant enterococci (VRE) has occurred over the last two decades (6). Specific classes of antimicrobial agents appear to be responsible for the spread, including extended-spectrum cephalosporins and drugs with activity against anaerobic bacteria. The use of vancomycin to treat *Clostridium difficile* colitis has also been implicated (6). Transmission of VRE can be through patient-to-patient contact, health care workers, and contaminated environmental surfaces and patient care equipment. Control methods include routine screening for vancomycin resistance among clinical isolates, active surveillance for VRE in intensive care units, contact isolation to minimize person-to-person transmission, rigorous decontamination of patient contact areas, and restriction of the use of vancomycin and other antimicrobial agents (1, 2). Patients who are colonized with VRE should be identified so that contact isolation can be instituted. Screening for VRE may facilitate removal of patients from isolation if three consecutive fecal cultures are negative for VRE. This procedure describes a method to screen for fecal carriage of VRE.

Various types of vancomycin resistance have been genotypically characterized, including *vanA*, *vanB*, *vanC*, *vanD*, and *vanE*. *vanA* and *vanD* are most often seen in *Enterococcus faecium*. *vanB* and *vanE* are mostly seen in *Enterococcus faecalis*. The mechanism of resistance of *vanA* and *vanB* is a plasmid-mediated transposon capable of altering the dipeptide terminus of peptidoglycan from a D-alanyl-D-alanine to the depsipeptide D-alanyl-D-lactate, which then binds vancomycin with a very low affinity. The MICs of vancomycin for enterococci with the *vanA* or *vanB* genotype are typically greater than 32 µg/ml. It is these genotypes that have been associated with spread of resistance within hospitals and nursing homes. Phenotypically, the definition of VRE includes those *E. faecalis*, *E. faecium*, and *Enterococcus raffinosus* organisms that are resistant to vancomycin with MICs usually ≥ 32 µg/ml (3). It does not include motile enterococci, *Enterococcus gallinarum*, and *Enterococcus casseliflavus*, which have intrinsic low-level vancomycin resistance mediated by *vanC*. These species do not account for the spread of vancomycin resistance and are generally susceptible to other agents, such as ampicillin and aminoglycosides. Al-

though most commercially available identification systems adequately differentiate *E. faecalis* from other species of enterococci, additional tests, including motility, are required to distinguish the motile enterococci, *E. gallinarum*, and *E. casseliflavus* from *E. faecium* and *E. raffinosus* (2). Such identification to the species level is necessary to limit the reporting of VRE to only those strains that are of epidemiologic concern.

Screening for fecal carriage of VRE is accomplished by fecal culture on the selective and differential medium bile-esculin-azide (BEA) broth and agar with vancomycin. Esculin is included to detect esculin-hydrolyzing enterococci, ferric citrate to provide ferric ions, bile salts to inhibit gram-positive bacteria other than enterococci, and sodium azide to inhibit gram-negative bacteria. Esculin forms glucose and esculetin when hydrolyzed by enterococci. Esculetin reacts with the ferric ions to produce dark brown to black color development in the medium, allowing recognition of the enterococci (4). The vancomycin concentration ranges from 6 to 10 µg/ml depending on the manufacturer; sensitivity and specificity will vary with the concentration used (7). Also see procedures 5.6 and 13.17.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING

3.8.5.1

A. Specimen collection

1. Collect fecal specimen and submit immediately or place in VRE broth.
 - **NOTE:** The yield from feces has been shown to be greater than that from perirectal swabs (5).
2. Swabs of wounds or urine may also be submitted.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING

(continued)

- B. Generally submit three specimens from different days to rule out the presence of VRE in a patient who was previously positive.
- C. Rejection criterion
Reject specimens if VRE were isolated from the patient in other cultures collected in the previous 2 weeks.

III. MATERIALS

- A. Media
 - 1. Agar
 - a. BEA agar with 6 µg of vancomycin per ml (Remel, Inc.) or
 - b. BEA agar with 10 µg of vancomycin per ml (Hardy Diagnostics) or
 - c. Vancomycin-supplemented Enterococcosel agar (BD Diagnostic Systems) with either 6 or 8 µg of vancomycin per ml
 - NOTE:** With 10 µg of vancomycin per ml, BEA agar with vancomycin is less likely to support the growth of *E. casseliflavus* and *E. gallinarum*, owing to their low-level resistance. True VRE may be inhibited as well.
 - 2. VRE broth containing BEA broth with vancomycin
 - NOTE:** Use of both broths in conjunction with agar culture has been shown to increase the detection of VRE by 50% but also increases the yield of motile enterococci (5).
- 3. BAP
- 4. Store media at 2 to 8°C. Allow media to come to room temperature before use.
- B. Gram stain reagents
- C. Other reagents
 - 1. Pyrrolidonyl-β-naphthylamide (PYR) (procedure 3.17.41)
 - 2. Catalase (procedure 3.17.10)
 - 3. 0.5 ml of BHI broth for motility (procedure 3.17.31)
 - 4. Methyl glucopyranoside MGP broth (procedure 3.17.30) (optional)

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

- A. Verify that media meet expiration date and QC parameters per current Clinical and Laboratory Standards Institute (CLSI; formerly NCCLS) M22 document. See procedures 14.2 and 3.3.1 for further QC procedures, especially for in-house-prepared media.
- B. BEA broth or agar with vancomycin should appear clear and light amber.
- C. Subculture the following QC strains to VRE broth and agar containing vancomycin prior to use, and incubate at 35°C aerobically for 18 to 24 h. Verify that they produce the desired reactions listed. Repeat QC of agar containing vancomycin at weekly intervals with both enterococcal species (3).

Test organism	Result
<i>Enterococcus faecalis</i> ATCC 51299	Growth with blackening in medium surrounding colony or blackening and turbidity of broth
<i>Enterococcus faecalis</i> ATCC 29212	Inhibition (clear broth or no growth on agar)
<i>Escherichia coli</i> ATCC 25922	Inhibition (clear broth or no growth on agar)

V. PROCEDURE



Observe standard precautions.

A. Inoculation

1. Process specimen not in VRE broth as soon as received.

NOTE: Use of biosafety cabinet will avoid contamination of the culture or specimen as well as protect laboratory processing personnel.

2. Inoculate first quadrant of vancomycin-containing BEA agar heavily with stool or swab; with a loop, streak in three other quadrants, as illustrated in Fig. 3.3.1–1.

3. Inoculate VRE broth with a small amount of fecal specimen.

B. Incubate plate and broth at 35°C aerobically for up to 72 h.

C. Check agar plates daily.

1. If brown-black color is diffused in medium, then consider it to be bile-esculin positive. Using a stereoscope, check for different morphotypes. With a plate marker, number the colonies, and Gram stain each.

2. Subculture only gram-positive cocci to BAP.

3. Incubate subculture at 35°C for 24 h.

D. Subculture turbid broth, if black, to vancomycin-containing BEA agar. Incubate for 24 h and subculture black colonies to BAP if they are gram-positive cocci on Gram stain.

E. Perform catalase and PYR tests on suspected colonies from BAP. Because of exposure to vancomycin, the PYR test might be negative; it should turn positive on a second subculture.

F. Lightly inoculate each different morphology of catalase-negative, PYR-positive, gram-positive cocci to 0.5 ml of BHI and incubate at 30°C for 2 h. Observe microscopically at $\times 400$ for motile cocci.

1. Nonmotile organisms are considered to be VRE.

2. Optionally, inoculate MGP broth. VRE do *not* produce a yellow color.

3. A rapid D-xylose tablet test also differentiates *E. gallinarum/E. casseliflavus* from *E. faecium/E. faecalis* (8).

4. Further species identification and antimicrobial susceptibility testing (AST) are necessary only if patient has not had a prior positive result (*see* Table 3.18.1–3).

5. Notify caregiver of positive culture findings.

6. Hold positive culture plates for at least 7 days.

POSTANALYTICAL CONSIDERATIONS

VI. REPORTING RESULTS

- A. If no VRE are present, report “No vancomycin-resistant *Enterococcus* organisms isolated.”
- B. If culture is positive for VRE, report “Vancomycin-resistant *Enterococcus* present.”
- C. Document notification to caregiver of positive findings.

VII. INTERPRETATION

- A. The presence of VRE in fecal specimens is an indication of carriage or colonization with the organism but does not indicate infection, since enterococci are a part of the normal intestinal microbiota.
- B. It is recommended that VRE be confirmed by a CLSI MIC method (3) if the patient has not previously been positive for VRE.

VIII. LIMITATIONS

- A. Organisms other than enterococci (*Pediococcus*, *Leuconostoc*, and *Weissella*) may grow on these media and produce a positive reaction for esculin. These genera are PYR negative.
- B. False-negative results can be caused by low numbers of organisms or prior antimicrobial treatment.

REFERENCES

1. Centers for Disease Control and Prevention. 1994. Preventing the spread of vancomycin resistance—report from the Hospital Infection Control Practices Advisory Committee. *Fed. Regist.* **59**:25758–25763.
2. Centers for Disease Control and Prevention. 1995. Recommendations for preventing the spread of vancomycin resistance: recommendations of the Hospital Infection Control Practices Advisory Committee (HICPAC). *Morb. Mortal. Wkly. Rep.* **44**(RR-12):1–13.
3. Clinical and Laboratory Standards Institute. 2006. *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically*, 7th ed. Approved standard M7-A7. Clinical and Laboratory Standards Institute, Wayne, Pa.
4. Difco Laboratories. 1984. *Difco Manual*, 10th ed., p. 129–131. Difco Laboratories, Detroit, Mich.
5. Edberg, C. E., C. J. Hardalo, C. Kontnick, and S. Campbell. 1994. Rapid detection of vancomycin-resistant enterococci. *J. Clin. Microbiol.* **32**:2182–2184.
6. Gold, H. S. 2001. Vancomycin-resistant enterococci: mechanisms and clinical observations. *Clin. Infect. Dis.* **33**:210–219.
7. Ieven, M., E. Vercauteren, P. Deschêne maeker, F. van Laer, and H. Goossens. 1999. Comparison of direct plating and broth enrichment culture for the detection of intestinal colonization by glycopeptide-resistant enterococci among hospitalized patients. *J. Clin. Microbiol.* **37**:1436–1440.
8. Willey, B. M., R. N. Jones, A. McGeer, W. Witte, G. French, R. B. Roberts, S. G. Jenkins, H. Nadler, and D. E. Low. 1999. Practical approach to the identification of clinically relevant *Enterococcus* species. *Diagn. Microbiol. Infect. Dis.* **34**:165–171.

SUPPLEMENTAL READING

- Boyce, J. M. 1997. Vancomycin-resistant enterococcus: detection, epidemiology and control measures. *Infect. Dis. Clin. N. Am.* **11**:367–368.

3.9.1

Guidelines for Performance of Genital Cultures

[Updated March 2007]

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Specimens from genital sites are sent to the clinical microbiology laboratory for detection of microorganisms from females presenting with clinical syndromes such as cervicitis, vulvovaginitis, urethritis, bacterial vaginosis (BV), salpingitis (pelvic inflammatory disease [PID]), endometritis, or genital ulcers and from males exhibiting urethritis, epididymitis, prostatitis, or genital ulcers (Tables 3.9.1–1 to 3.9.1–3) (3). Specimens are also submitted from pregnant females to diagnose the presence of organisms that may cause disease in the neonate. Less commonly, specimens are sent from children and postmenopausal women (12, 13, 17, 20, 21).

The syndromes that are associated with female and male genital tract infections are listed in Tables 3.9.1–1 to 3.9.1–3, with the major pathogens for each syndrome indicated. For surgically collected specimens and those from normally sterile sites, a routine wound and abscess culture will detect most bacterial pathogens, as long as selective media for *Neisseria gonorrhoeae* are included. Anaerobic cultures are often indicated for these specimens (10), and mycoplasma cultures may be appropriate in selected cases (procedure 3.15).

The human vagina is lined with 25 layers of epithelium cells. Many indigenous microorganisms colonize these surfaces. Accurate diagnosis of genital infections from the male and female genitalia depends on the separation of microbial pathogens from the normal genital microbiota. The microorganisms colonizing the female genital tract have been studied extensively and include lactobacilli, *Corynebacterium* spp., *Gardnerella vaginalis*,

coagulase-negative staphylococci, *Staphylococcus aureus*, *Streptococcus agalactiae*, *Enterococcus* spp., *Escherichia coli*, anaerobes, and yeasts (14). For prepubescent females, diphtheroids and coagulase-negative staphylococci predominate; lactobacilli predominate in the adult female. Postmenopausal women are generally colonized with fewer lactobacilli but have a greater number of *Enterobacteriaceae* than premenopausal women. They also lack the presence of yeasts and mycoplasmas. Many adult female genital tract infections arise from endogenous microorganisms, the pathogenicity of which has been activated by host factors and other microorganisms. Various viruses, including herpes simplex virus (HSV), human papillomavirus (HPV), and human immunodeficiency virus, may also influence the receptivity of the host surface to microorganisms. The male urethra normally contains relatively few skin microbiota, consisting of coagulase-negative staphylococci, micrococci, *Corynebacterium* spp., and viridans group streptococci.

Because the agents of disease have diverse culture and detection requirements and selective media are often needed, a “routine” genital culture, with the intent to “detect what is there,” rarely has an indication. Occasionally such specimens are appropriate for prepubescent or postmenopausal women. Otherwise, laboratories should provide a special requisition, such as in Appendix 3.9.1–1, to be used to request the appropriate tests in the outpatient setting. For certain pathogens, such as *N. gonorrhoeae*, *Haemophilus ducreyi*, or *S. agalactiae* (group B streptococcus [GBS]), cultures are ordered specifically

for those pathogens, with proper collection and selection media. Because of the special nature of cultures for these pathogens, procedures to detect them are listed separately in this handbook following this procedure.

To diagnose vulvovaginitis and BV, routine bacterial cultures are not helpful (1, 22). In Table 3.9.1–4, the recommended tests are indicated to make these diagnoses. The Gram stain is useful to diagnose BV using the Nugent scoring system (16) (Appendix 3.2.1–3). A wet mount and a yeast culture and *Trichomonas* culture (see procedure 9.9.4) are the recommended tests to diagnose vaginitis. It should be noted that by performing only a wet mount, without yeast or *Trichomonas* culture, 50% of either of these agents of vaginitis will be missed (3). Alternatively, a sensitive DNA probe assay is available that combines the detection of yeasts, *Trichomonas*, and *G. vaginalis* as a marker for BV (4).

For primary syphilis, a dark-field exam is useful but is rarely performed (Appendix 3.2.3–1). *Calymmatobacterium granulomatis* is detected by demonstration of Donovan bodies (intracytoplasmic cysts of enlarged mononuclear cells containing 10 or more deeply staining pleomorphic rods). These bodies are usually stained either by the Giemsa method or by silver stains of formalin-fixed biopsy material. (See procedure 9.8.5 for details of Giemsa stain preparation.) *Chlamydia* and herpes viral cultures are also important diagnostic tools in the evaluation of sexually transmitted diseases.

For the recognition of toxic shock syndrome (TSS), isolation of *S. aureus* is dif-

Table 3.9.1–1 Female genital infections that are sexually transmitted

Clinical syndrome	Location of infection	Clinical symptoms/signs	Primary pathogen(s)	Specimen(s) collected
Bartholinitis	Bartholin gland	Pain, induration, and redness of gland area	<i>Chlamydia trachomatis</i> <i>Neisseria gonorrhoeae</i> <i>Staphylococcus aureus</i> Enteric bacteria (e.g., <i>Escherichia coli</i> , <i>Proteus mirabilis</i> , and other enteric GNB ^a) plus anaerobes <i>Ureaplasma urealyticum</i>	Aspirate of gland Swab of abscess pus
Cervicitis	Endocervical canal	Mucopurulent discharge, pain with movement of the cervix (dyspareunia). HSV primarily causes blisters and painful ulcers of the external cervical os.	<i>Chlamydia trachomatis</i> <i>Neisseria gonorrhoeae</i> HSV	Endocervical swab Ulcer swab for viral culture
Genital ulcer with inguinal lymphadenopathy	Skin and soft tissues of the genital area and inguinal lymph nodes	Genital ulcer(s) in syphilis, LGV, ^b and granuloma inguinale (donovanosis) are usually painless. Chancroid ulcer is typically painful, with undermined edges. Inguinal lymphadenopathy may occur on one or both sides.	<i>Calymmatobacterium granulomatis</i> (donovanosis) <i>Chlamydia trachomatis</i> —LGV serovars <i>Haemophilus ducreyi</i> (chancroid) <i>Treponema pallidum</i> (syphilis)	Ulcer scrapings Inguinal lymph node aspirate Dark-field exam Syphilis serology LGV serology
Endometritis ^c	Endometrial lining of the uterus	Fever, leukocytosis, pelvic pain, cramps, abnormal bloody discharge or spotting	<i>Actinomyces</i> spp. <i>Chlamydia trachomatis</i> Enteric bacteria (e.g., <i>Escherichia coli</i> , <i>Proteus mirabilis</i> , and other enteric GNB) plus anaerobes <i>Neisseria gonorrhoeae</i> <i>Streptococcus pyogenes</i>	Transvaginal aspirate or biopsy sample of endometrium
Salpingitis (PID)	Ascending infection of the fallopian tubes and peritoneal cavity, with the formation of abscesses	Fever, leukocytosis, lower abdominal and pelvic pain associated with discharge	<i>Chlamydia trachomatis</i> <i>Neisseria gonorrhoeae</i> Enteric bacteria (e.g., <i>Escherichia coli</i> , <i>Proteus mirabilis</i> , and other enteric GNB) plus anaerobes <i>Staphylococcus aureus</i> <i>Streptococcus pyogenes</i>	Culdocentesis Laparoscopy sample of fallopian tube and pelvic abscesses
Skenitis	Skene's gland	Pain, induration, and redness in the gland area	<i>Neisseria gonorrhoeae</i> <i>Staphylococcus aureus</i>	Aspirate of gland Swab of abscess pus
Urethritis, urethral syndrome	Urethra	Dysuria with initiation of urination, urethral discharge, and pain. Cystitis should be ruled out by doing a urine culture.	<i>Chlamydia trachomatis</i> <i>Neisseria gonorrhoeae</i> <i>Mycoplasma genitalium</i> <i>Ureaplasma urealyticum</i>	Urethral swab Urine culture
Vaginosis	Vagina	Vaginal discharge that may be fishy or foul smelling, pruritus, terminal dysuria, painful intercourse	<i>Candida</i> spp. <i>Gardnerella vaginalis</i> <i>Mobiluncus</i> <i>Trichomonas vaginalis</i>	Vaginal swab

(continued)

Table 3.9.1–1 Female genital infections that are sexually transmitted (*continued*)

Clinical syndrome	Location of infection	Clinical symptoms/signs	Primary pathogen(s)	Specimen(s) collected
Vulvovaginitis	Vagina and vulva	Pruritus, redness of the skin, white curd-like discharge, macerate skin, and terminal dysuria	<i>Candida</i> spp. <i>Staphylococcus aureus</i> ^d	Vaginal swab Vulval swab
Vulvovaginal, perineal ulcers	Vagina, vulva, perineum	Painful blisters on an erythematous base, ulcers	HSV	Ulcer swab for viral culture

^a GNB, gram-negative bacillus.^b LGV, lymphogranuloma venereum.^c Associated with use of intrauterine device.^d In wounds, TSS, and tampon-associated ulcerations.**Table 3.9.1–2** Female genital infection clinical syndromes associated with intrapartum, postpartum, and postabortal infections

Clinical syndrome	Location of infection	Clinical symptoms and signs	Primary pathogen(s)	Specimen(s) collected
Intra-amniotic infection syndrome	Uterus and its contents during pregnancy, including the amniotic membrane and fluid	Fever, leukocytosis, abdominal pain, uterine tenderness, premature rupture of membranes, bacteremia. May cause premature labor and delivery.	Enteric bacteria (e.g., <i>Escherichia coli</i> , <i>Proteus mirabilis</i> , and other enteric GNB ^a) plus anaerobes <i>Listeria monocytogenes</i> <i>Neisseria gonorrhoeae</i> <i>Streptococcus agalactiae</i> <i>Streptococcus pyogenes</i>	Amniotic fluid Amniotic tissue Blood cultures
Postpartum endometritis, endomyometritis, endoparametritis	Endometrium and uterine muscle	Fever, leukocytosis, abdominal pain, uterine tenderness, foul-smelling lochia/discharge, bacteremia	<i>Chlamydia trachomatis</i> Enteric bacteria (e.g., <i>Escherichia coli</i> , <i>Proteus mirabilis</i> , and other enteric GNB) plus anaerobes <i>Gardnerella vaginalis</i> <i>Streptococcus agalactiae</i> <i>Streptococcus pyogenes</i>	Transvaginal aspirate of endometrium Endometrial tissue Blood cultures
Puerperal sepsis/ septic abortion	Endometrium and uterine muscle and pelvis	Fever, leukocytosis, abdominal pain, uterine tenderness, foul-smelling lochia/discharge, hypotension, bacteremia	<i>Clostridium perfringens</i> and other anaerobes Enteric bacteria (e.g., <i>Escherichia coli</i> , <i>Proteus mirabilis</i> , and other enteric GNB) <i>Streptococcus agalactiae</i> <i>Streptococcus pyogenes</i>	Transvaginal aspirate of endometrium Endometrial tissue Blood cultures
Septic pelvic thrombophlebitis	Deep pelvic veins	Refractory postpartum fever and leukocytosis with no other obvious focus of infection. CT ^b scan shows uterine vein thrombosis.	Enteric bacteria (e.g., <i>Escherichia coli</i> , <i>Proteus mirabilis</i> , and other enteric GNB) plus anaerobes <i>Streptococcus agalactiae</i> <i>Streptococcus pyogenes</i>	Blood cultures

^a GNB, gram-negative bacillus.^b CT, computed tomography.

ficult and not sufficient for the diagnosis. Further characterization of the strain is necessary to confirm the diagnosis. Two commercial kits are available for testing, which is usually done in reference laboratories (Toxic Shock ELISA [Toxin

Technologies, Inc., Madison, Wis.] and TST-RPLA reverse passive latex agglutination [Oxoid, Columbia, Md.]). Alternatively, testing acute- and convalescent-phase sera for antibodies to the exotoxin (TSST-1) in a reference laboratory can be

helpful. Most patients with TSS lack antibodies at the onset of infection but then produce them in response to the infection. Greater than 90% of women have antibodies to the exotoxin.

Table 3.9.1–3 Male genital infections

Clinical syndrome	Location of infection	Clinical symptoms and signs	Primary pathogen(s)	Specimen(s) collected
Balanoposthitis	Head of penis and foreskin	Pain, swelling, exudates and pus under foreskin	Enteric bacteria (e.g., <i>Escherichia coli</i> and other <i>Enterobacteriaceae</i>) plus anaerobes <i>Streptococcus pyogenes</i> <i>Staphylococcus aureus</i>	Swab from under foreskin
Epididymitis	Epididymus	Pain, swelling, tenderness of scrotum and epididymus	<i>Chlamydia trachomatis</i> Enteric bacteria (e.g., <i>Escherichia coli</i> and other <i>Enterobacteriaceae</i> , <i>Enterococcus</i> spp., <i>Pseudomonas</i> spp.) <i>Mycobacterium tuberculosis</i> <i>Neisseria gonorrhoeae</i>	Urethral swab Urine culture Epididymus tissue
Orchitis	Testes	Pain, swelling, tenderness of scrotum	<i>Chlamydia trachomatis</i> Enteric bacteria (e.g., <i>Escherichia coli</i> and other <i>Enterobacteriaceae</i> , <i>Enterococcus</i> spp., <i>Pseudomonas</i> spp.) <i>Mycobacterium tuberculosis</i> <i>Neisseria gonorrhoeae</i>	Urethral swab Urine culture Testicular tissue
Prostatic abscess	Prostate gland	Dysuria, frequency, decreased urinary stream or obstruction, passing air on urination if vesiculointestinal fistula	Enteric bacteria (e.g., <i>Escherichia coli</i> and other <i>Enterobacteriaceae</i> , <i>Enterococcus</i> spp., <i>Pseudomonas</i> spp.) <i>Staphylococcus aureus</i>	Swab abscess Abscess fluid/pus
Prostatitis	Prostate gland	Dysuria, frequency, decreased urinary stream or obstruction	Enteric bacteria (e.g., <i>Escherichia coli</i> and other <i>Enterobacteriaceae</i> , <i>Enterococcus</i> spp., <i>Pseudomonas</i> spp.) <i>Neisseria gonorrhoeae</i>	Urine culture ^a Urethral swab Prostatic fluid
Urethritis	Urethra	Dysuria, mucopurulent discharge, urethral Gram stain shows >4 PMNs per oil immersion field	<i>Chlamydia trachomatis</i> <i>Neisseria gonorrhoeae</i> HSV <i>Mycoplasma genitalium</i> <i>Ureaplasma urealyticum</i> <i>Trichomonas vaginalis</i>	Urethral swab DFA ^b /viral culture Wet mount of urethral secretions

^a See Table 3.9.1–5 and Appendix 3.9.1–2.^b DFA, direct fluorescent antibody.**Table 3.9.1–4** Diagnostic characteristics for vaginitis and vaginosis^a

Disease or condition	pH	Observation on wet mount	Amine odor ^b	Other test for diagnosis ^c
None (normal)	4–4.5	Long rod form bacteria	Absent	
Atrophic	>6		Absent	
Candidiasis	4–4.5	Pseudohyphae or budding yeasts	Absent	Yeast culture
BV	>4.5	Clue cells	Present	Gram stain
Trichomoniasis	5–6	Flagellated parasites ^d	Usually present	<i>Trichomonas</i> culture

^a Data for table extrapolated from Sobel (22).^b A drop of 10% KOH is added to vaginal fluid and sniffed for release of fishy or amine odor.^c These tests are recommended for increased sensitivity and might be offered as a panel by laboratory.^d Caution: occasional contaminants from the environment, such as *Colpoda*, while amoeboid in motion, may be seen.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING

NOTE: Refer to procedure 3.3.1 for additional details. The following can be copied for preparation of a specimen collection instruction manual for physicians and other caregivers. It is the responsibility of the laboratory director or designee to educate physicians and caregivers on proper specimen collection. In many cases the procedures to collect the specimens listed below should be performed by qualified physicians with appropriate training.

A. Specimen collection (*see Appendix 3.9.1–1 and Table 3.9.1–4 for options for outpatient testing*)

1. Female specimens

a. Amniotic fluid

- (1) Aspirate fluid by catheter at cesarean section or at amniocentesis.
- (2) Order body fluid culture (procedure 3.5).

b. Bartholin cyst

- (1) Decontaminate the skin with povidone-iodine, 3% chloroxylenol, and 3% cocamidopropyl PG-dimonium chloride phosphate (Techni-Care, Care-Tech, St. Louis, Mo.) or other surgical disinfectant, and aspirate material from the duct(s).

NOTE: Bartholin glands are small mucus-secreting glands located beneath the posterior portion of the labia majora.

- (2) Order aerobic and anaerobic wound/abscess cultures (procedure 3.13.1).

c. Cervical

- (1) Clear away vaginal mucus and exudate with large swab. Moisten speculum with warm water, not lubricants, which can be antibacterial. Using a small swab (not cotton or wood shaft) inserted through a speculum, sample endocervical canal. Avoid the vaginal walls during collection.
- (2) See Appendix 3.9.1–1 and Table 3.9.1–4 for ordering options.

d. Culdocentesis

- (1) After cleaning the vaginal wall with surgical disinfectant, such as povidone-iodine or 3% chloroxylenol and 3% cocamidopropyl PG-dimonium chloride phosphate, perform transvaginal puncture of the cul-de-sac to aspirate fluid.

NOTE: The cul-de-sac is the pouch between the anterior wall of the rectum and the posterior wall of the uterus. Collection is often done to diagnose PID without more invasive laparoscopy; however, results may not correlate with more invasive testing.

- (2) Order aerobic and anaerobic wound/abscess cultures.

e. Endometrium

- (1) Insert endometrial suction curette or catheter-protected Dacron swab through the cervical os and transfer beyond the cervical opening into the uterine cavity. Collect sample from within the cavity.

- (2) Order aerobic and anaerobic wound/abscess cultures (10).

f. Fallopian tubes and pelvic cavity

- (1) Collection: obtain aspirates and biopsy samples during laparoscopy. Also sample the pelvic peritoneum. Biopsies often yield better diagnostic specimens.

- (2) Order aerobic and anaerobic wound/abscess cultures (10).



Observe standard precautions.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING (continued)

- g. Skene's glands**
 - (1) Decontaminate the skin with surgical disinfectant, and aspirate material from the gland(s).
 - ▣ **NOTE:** Skene's glands are paraurethral glands located at both sides of the outer end of the urethra.
 - (2) Order aerobic and *N. gonorrhoeae* culture.
- h. Vagina**
 - (1) Collect fluid from the vagina with sterile pipette or Dacron swab. Successful self-collection of vaginal swabs can be done (18).
 - (2) See Appendix 3.9.1–1 and Table 3.9.1–4 for ordering options.
- i. Vulva**
 - (1) Collect only if pain, erythema, or edema is present.
 - (2) Clean the surface of the lesion with 0.85% NaCl and collect by one of the methods below.
 - (a) Sample exudate or area of erythema with swab for yeast culture.
 - (b) If there is a vesicle present, collect for HSV culture.
 - i. Unroof vesicle.
 - ii. Collect fluid with a sterile swab *or*
 - iii. Aspirate vesicular fluid with a 26- to 27-gauge needle and syringe.
 - iv. Then scrape the base of the vesicle with a sterile scalpel blade, and collect specimen with a Dacron swab by vigorously rubbing the base of the vesicle.
 - (c) If there is a crust on the lesion, gently remove it.
 - i. Moisten swab with saline and collect specimen by vigorously rubbing the base of the lesion for *H. ducreyi* culture.
 - ii. Alternatively, gently abrade the lesion with a sterile scalpel or needle until serous fluid emerges. (Try to avoid bleeding.) Irrigate with saline.
 - (i) For *H. ducreyi* culture, rub the base vigorously with a sterile swab or aspirate fluid with flamed smoothed Pasteur pipette or needle and syringe.
 - (ii) For *Treponema pallidum*, wipe away fluid, blood, and debris with sterile gauze. Apply gentle pressure to the base of the lesion until clear fluid is expressed. Touch a slide to the fluid, and cover the fluid on the slide with a coverslip. If no exudate is present, add a drop of saline to the lesion or insert a needle and syringe at the lesion base, aspirate, and then draw a drop of saline into the needle. Express the material onto a slide (*see* Appendix 3.2.3–1).
 - (3) Order *T. pallidum* dark-field microscopy, *H. ducreyi* culture, or HSV culture or request yeast culture for most cases showing only erythema or edema.
- 2. Male specimens**
 - a. Epididymis or testicular fluid**
 - ▣ **NOTE:** The specimen of choice for diagnosis of infected epididymis is urethral culture. If that does not yield a diagnosis, collect first-voided and midstream urine, and compare the yield from smear and culture of each specimen. Collect testicular fluid only if the diagnosis cannot be made otherwise.
 - (1) Disinfect skin surface with surgical disinfectant. Use a needle and syringe to aspirate material from the epididymis or testicles.

**II. SPECIMEN COLLECTION,
TRANSPORT, AND HANDLING**
(continued)

- (2) Choose from the following tests.
- Routine aerobic wound and abscess culture for bacteria, most commonly members of the family *Enterobacteriaceae* or pseudomonads and generally encountered in men over 35 years of age.
 - Mycobacterium tuberculosis*, generally occurring after involvement of the prostate or seminal vesicles.
 - Chlamydia trachomatis* and *N. gonorrhoeae* culture or probe test.
- b. Penile lesion or vesicle**
- Clean the surface of the lesion with 0.85% NaCl and collect by one of the methods below.
 - Unroof vesicle
 - Collect fluid with a sterile swab *or*
 - Aspirate vesicular fluid with a 26- to 27-gauge needle and syringe.
 - Then scrape the base of the vesicle with a sterile scalpel blade, and collect specimen with a Dacron swab by vigorously rubbing the base of the vesicle.
 - If there is a crust on the lesion, gently remove it.
 - Moisten swab with saline and collect specimen by vigorously rubbing the base of the lesion for *H. ducreyi* culture.
 - Alternatively, gently abrade the lesion with a sterile scalpel or needle until serous fluid emerges. (Try to avoid bleeding.) Irrigate with saline.
 - For *H. ducreyi* culture, rub the base vigorously with a sterile swab or aspirate fluid with flamed smoothed Pasteur pipette or needle and syringe.
 - For *T. pallidum*, wipe away fluid, blood, and debris with sterile gauze. Apply gentle pressure to the base of the lesion until clear fluid is expressed. Touch a slide to the fluid, and cover the fluid on the slide with a coverslip. If no exudate is present, add a drop of saline to the lesion or insert a needle and syringe at the lesion base, aspirate, and then draw a drop of saline into the needle. Express the material on to a slide (*see Appendix 3.2.3–1*).
- (2) Order *T. pallidum* dark-field microscopy, *H. ducreyi* culture, or HSV culture.
- c. Prostate**
- After the patient urinates, perform a digital massage through the rectum.
 - Have patient pass prostatic secretions in the urethra by urinating into a cup. Alternatively, pass the urethral genital wire swab or a bacteriological loop several centimeters into the urethra.
 - Sequential urine cultures may be used to diagnose the location of a lower urinary tract infection in men (23). Table 3.9.1–5 outlines the various portions of the urine sample that are quantitatively cultured (*see procedure in Appendix 3.9.1–2*) (23). The different urine samples and the prostatic massage specimen should be clearly labeled by their clinical designations (e.g., VBS no. 1, etc.). A urethral swab may also be collected for detection of *N. gonorrhoeae* and *Chlamydia trachomatis* and other urethritis primary pathogens (Table 3.9.1–3).

Table 3.9.1–5 Specimens collected to diagnose the location of a lower urinary tract infection in men^a

Specimen	Clinical designation	Description
Voided bladder urine no. 1	VBS no. 1	Initial 5–10 ml of urinary stream
Voided bladder urine no. 2	VBS no. 2	Midstream urine specimen
Expressed prostatic specimen	EPS	Secretions expressed from the prostate during prostatic massage
Voided bladder urine no. 3	VBS no. 3	First 5–10 ml of urinary stream collected immediately after prostatic massage

^a Data adapted with permission from T. Stamey. 1980. *Pathogenesis and Treatment of Urinary Tract Infections*. Williams & Wilkins, Baltimore, Md.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING (continued)

3. Male or female cultures
 - a. Rectal cultures
 - (1) Insert swab past anal sphincter, move swab from side to side, allow 10 to 30 s for absorption, and withdraw.
 - (2) If contaminated with feces, recollect.
 - (3) Order *N. gonorrhoeae* culture.
 - b. Throat cultures
 - (1) Depress tongue gently with tongue depressor.
 - (2) Extend one or two sterile swabs (one for antigen test and one for culture) between the tonsillar pillars and behind the uvula, avoiding the tongue, inner cheeks, and uvula.
 - (3) Sweep the swabs back and forth across the posterior pharynx, tonsillar areas, and any inflamed or ulcerated areas to obtain sample.
 - (4) Order *N. gonorrhoeae* culture.
 - c. Urethral discharge
 - (1) Express exudate onto swab from distal urethra.
 - (2) If there is no exudate, collect 1 h after urination. Wipe area clean, insert a urethrogenital swab 2 to 4 cm into the endourethra, gently rotate the swab, leave it in place for 1 to 2 s, and withdraw it.
 - (3) Order *N. gonorrhoeae* and *Chlamydia* culture or probe.
 4. Abscess material (e.g., bubo, lymph node, etc.)
 - a. Disinfect skin with surgical disinfectant.
 - b. Aspirate the lesion with needle and syringe (refer to Specimen Collection, Transport, and Handling in procedure 3.13.1 for other details).
 - c. Order Gram stain, aerobic and anaerobic wound and abscess cultures, and, if indicated from a lymph node, *Chlamydia* or *H. ducreyi* culture.
- B. Specimen transport**
1. Transport medium
 - a. For transport for specific organisms, refer to the separate procedures. Otherwise, submit swab in Amies transport tube.
 - b. Place immediately on ice or in the refrigerator until and during transport.

NOTE: Previous literature indicates that *N. gonorrhoeae* does not survive well at refrigeration temperatures, but recent studies indicate otherwise (see procedure 3.9.3).
 2. Label specimens and accompanying requisition with patient name, hospital medical record number, room number or clinic location, other patient demographics, and date, time, and site of collection.
 3. Indicate the pathogens sought on requisition or computer entry. Do not order “routine culture” from sexually active patients. These are rarely indicated and are performed mostly from prepubescent or postmenopausal females.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING (continued)

C. Rejection criteria

1. Do not accept vaginal swabs from women in childbearing years for “routine genital culture.” Using a form similar to that in Appendix 3.9.1–1, require that the disease or agent sought be ordered specifically.
2. Reject specimens not received in transport medium, since the agents of genital infections lose viability easily.

III. MATERIALS

A. Media

1. CHOC
 2. Thayer-Martin (TM) or similar selective medium for *N. gonorrhoeae*
 3. BAP
 4. MAC or EMB
 5. Columbia colistin-nalidixic acid agar (CNA)
 6. Selective medium for yeasts (by separate request only).
- NOTE:** For invasively collected specimens, refer to other procedures in this handbook for culture, including anaerobic cultures.
- ### B. Identification methods
1. Gram stain (procedure 3.2.1)
 2. Catalase test (procedure 3.17.10)
 3. Oxidase test (procedure 3.17.39)

4. Indole test (procedure 3.17.23)

5. Aminolevulinic acid (ALA) test (procedure 3.17.3)
6. Pyrrolidonyl-β-naphthylamide (PYR) (procedure 3.17.41)
7. Sodium polyanethol sulfonate (SPS) disks (procedure 3.17.45)
8. Identification kits for gram-negative bacteria
9. Other tests as indicated in procedures 3.3.2, 3.18.1, and 3.18.2

C. Other supplies

1. Incubator at 35°C, with 5% CO₂ or ambient air
2. Self-contained CO₂-generating system for culture incubation if incubator is ambient air

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

- A. Verify that media meet expiration date and QC parameters per current Clinical and Laboratory Standards Institute (CLSI; formerly NCCLS) M22 document. See procedures 14.2 and 3.3.1 for further procedures, especially for in-house-prepared media.
- B. See procedures 3.3.1 and 3.9.3 for additional QC of CHOC and TM, respectively.

V. PROCEDURE



Observe standard precautions.

A. Inoculation

- NOTE:** Use of a biosafety cabinet will avoid contamination of cultures as well as protect laboratory processing personnel.
1. If the specimen is not received on plates, inoculate plates from the swab in transport medium.
 2. See Appendix 3.9.1–1 for tests for reference to specific organism procedures. For specimens from wounds, abscesses, and normally sterile sites, refer to the wound and abscess procedure (3.13.1) or body fluid culture procedure (3.5).
 3. For cervical, vaginal, or other noninvasive genital source specimens submitted for unusual culture requests, inoculate the first five media listed above.

B. Direct smear

1. Prepare a Gram stain from the swab after plate inoculation.
2. Stain slide (procedure 3.2.1) and, for women in childbearing years, observe for evidence of BV from vaginal specimens (Appendix 3.2.1–3), for yeasts, and for evidence of other bacteria associated with WBCs.

V. PROCEDURE (continued)**C. Incubation**

1. Incubate plates at 35°C in 5% CO₂, or use other CO₂-generating system to provide the proper atmosphere and moisture.
2. Observe for growth after 18 to 24 h of incubation. Hold negative plates for up to 72 h.

D. Culture examination

1. Observe plates after 24 h for growth of abnormal microbiota.
2. Correlate growth with Gram stain result to determine the extent of workup.
3. Identify the following pathogens if present, using identification kits or methods described in Table 3.3.2–5.

- a. Examine non-lactose-fermenting, gram-negative rods for *Shigella* spp. or other enteric pathogens, especially from pediatric patients (15).
☒ **NOTE:** Do not examine cervical or vaginal specimens for other *Enterobacteriaceae*, as these microorganisms are normally found in the female genital tract.
- b. *Streptococcus pyogenes* (20)
- c. *S. agalactiae* (procedure 3.9.2)
- d. *Listeria monocytogenes*
- e. *N. gonorrhoeae* (procedure 3.9.3)
- f. *Candida albicans*. Mention other yeasts. *Candida glabrata* has been implicated as a cause of vulvovaginitis (22).

4. Identify the following only if the specimen was invasively collected or there is heavy growth and they are the predominant microorganism in the culture.

- a. *Haemophilus* spp. (6, 11, 24)
- b. Gram-negative rods
 - (1) Enteric gram-negative rods (exception: *Enterobacteriaceae* are part of the normal microbiota of the vagina and should not be reported).
 - (2) *Pseudomonas* spp. and other non-glucose-fermenting, gram-negative rods
 - (3) *Pasteurella bettiae* (CDC group HB-5). See Table 3.9.1–6.
☒ **NOTE:** *P. bettiae* has been associated with genital infections, especially in neonates. It is an indole-positive gram-negative rod, but unlike *E. coli*, it is catalase negative and oxidase variable and does not grow or grows as pinpoint colonies on MAC (2, 25).

- (4) *Capnocytophaga* spp.

☒ **NOTE:** This group of organisms have been associated with genital infections. They are catalase-negative, oxidase-negative glucose-fermenting gram-negative rods that do not grow on MAC. The glucose reaction may not be seen on triple sugar iron agar (TSI) or Kligler's iron agar. They are generally esculin positive (procedure 3.17.5).

- (5) *Campylobacter fetus* (see procedure 3.8.2)

- c. *S. aureus*
- d. *Streptococcus pneumoniae*
- e. *Neisseria meningitidis*
- f. *G. vaginalis*

- (1) For vaginal specimens, do *not* use selective medium to isolate this organism, because the importance of its isolation is determined by the quantity compared to that of lactobacilli in the culture (1, 5). *G. vaginalis* grows well on both CNA and CHOC.

- (2) When present in quantities less than the other normal microbiota, it should be included as part of normal vaginal microbiota. However, for children report its presence regardless of the quantity present.

Table 3.9.1–6 Biochemical reactions of *Haemophilus* and other fastidious gram-negative coccobacilli found in genital microbiota^a

Test	<i>H. influenzae/haemolyticus</i> ^b	<i>H. parahaemolyticus/paraphrophilus/parainfluenzae</i> ^b	<i>H. ducreyi</i>	<i>Capnocytophaga</i> DF-1 and DF-3 ^c	<i>P. bettiae</i> (HB-5)	<i>C. fetus</i>	<i>Kingella denitrificans</i> ^d	<i>Kingella kingae</i>
Catalase	+	V	—	—	—	+	—	—
Oxidase	V	V	+	—	V	+	+	+
Indole	V	V	—	V	+	—	—	—
Motility	—	—	—	—	—	+	—	—
Nitrate with gas ^e	NA	NA	NA	V	+/-	+	+/V	-/V
Urease	V	V	—	—	—	—	—	—
Growth on BAP at 35°C	Satellite	Satellite	—	+	+	V	+	+; beta
ALA	—	+	—	+	NA	NA	NA	NA
TSI ^f	NA	NA	NA	Acid slant and butt	Acid butt only	—	—	Rare acid in slant and butt

^a Strains in this table are characterized by no growth on MAC, with the exception of a rare *P. bettiae* strain. NA, not applicable; V, variable. This table is prepared from biochemical tables from Weyant et al. (25).

^b Separate by hemolysis on horse or rabbit blood, if desired; however, *H. haemolyticus*, *H. paraphrophilus*, and *H. parahaemolyticus* generally are not found in the genital tract.

^c *Capnocytophaga* spp. (DF-1) are rods with tapered ends and frequently have a yellow pigment; DF-3 is a coccobacillus. They both may hydrolyze esculin.

^d Not generally a pathogen but mimics *N. gonorrhoeae*; it will grow on BAP, is nitrate positive, and is a coccobacillus.

^e The nitrate reaction is first; if the gas reaction is known, it is listed second, preceded by a slash.

^f May not be able to demonstrate reactions without addition of rabbit serum, if organism is fastidious.

V. PROCEDURE (continued)

- (3) If it is the predominant microorganism from the female vaginal tract and is isolated in 3 to 4 + quantities (third or fourth quadrant on the plate), report its presence.
- (4) Identification
 - (a) Colonies appear pinpoint and transparent, with no greening of the agar
 - (b) Gram-variable to gram-negative small, pleomorphic coccobacilli that do not elongate into filaments or chains
 - (c) Catalase negative. To confirm the lack of catalase enzyme, streak colony on CHOC plate and add a dot of viridans group streptococci (*Streptococcus sanguis* ATCC 35557). A clear zone of inhibition around a dot of viridans group streptococci confirms the lack of catalase (19).
 - (d) SPS sensitive (1, 19) or hippurate positive (procedure 3.17.21) or beta-hemolytic on human blood agar

■ **NOTE:** The API CORYNE strip accurately identifies this microorganism (9). It is not necessary to confirm the identification with tests other than colony morphology, catalase, and smear, if the direct Gram stain is consistent with diagnosis of BV. Aroutcheva et al. (1) report that the strains isolated from cases of BV were more likely to have a negative hippurate or lipase test reaction than those from patients without BV. They also showed that the isolation of 3 or 4+ *G. vaginalis* from culture correlated well with BV, although 26% of the cases diagnosed by Gram stain were missed on culture using selective human blood-Tween bilayer media, which is reported to be superior to V agar (1).

V. PROCEDURE (continued)**E. Susceptibility testing**

1. Antimicrobial susceptibility testing should be performed on all aerobic bacterial isolates from pelvic or amniotic fluid or tissue specimens that are considered to be primary pathogens.
2. Anaerobes isolated from pelvic or amniotic fluid or tissue specimens that are considered to be primary pathogens should have antibiotic susceptibility testing done according to CLSI (formerly NCCLS) guidelines (7).
3. *N. gonorrhoeae* isolated from any genital site should be referred for antimicrobial susceptibility testing (8).
4. Fastidious gram-negative coccobacilli or rods that do not grow on MAC or EMB should have a beta-lactamase test.

F. Hold positive culture plates for at least 7 days should further testing be indicated.

POSTANALYTICAL CONSIDERATIONS**VI. REPORTING RESULTS**

- A. From surgical specimens and those from normally sterile sites, report the pathogens isolated. For cultures with mixed microbiota, grouping of pathogens may be indicated, e.g., "Mixed enteric rods or mixed anaerobes present."
- B. If no pathogens are isolated but normal microbiota is present, report as "Normal genital microbiota isolated" for vaginal and cervical specimens and as "Normal cutaneous microbiota isolated" for male urethral specimens.
 1. See item I of this procedure for list of organisms in the normal microbiota of male and female genitalia.
 2. Do not list genera and species of normal microbiota individually.
 3. If specific pathogens are requested, report "No [pathogen name] isolated."
- C. Positive reporting
 1. Quantitate pathogens (Table 3.3.2-2).
 2. Notify physician of pathogens of serious significance in pregnant females (e.g., *L. monocytogenes*) or communicable diseases (e.g., *N. gonorrhoeae*).
 3. Notify the local health department of isolation of agents of reportable diseases.

VII. INTERPRETATION

- A. The presence of any microorganism from a normally sterile site is generally considered significant.
- B. Isolation of sexually transmitted disease pathogens (e.g., *N. gonorrhoeae* and *H. ducreyi*) is considered clinically significant from any genital site. Isolation of certain organisms such as *S. pyogenes* and *Clostridium perfringens* from soft tissue as well as endometrial tissue or transvaginal fluid samples should also be considered clinically significant.
- C. The presence of other pathogens may or may not be a cause of disease and must be evaluated with consideration of relative amounts and symptoms or other conditions (e.g., pregnancy) in the patient.
- D. Due to the increasing resistance of many primary pathogens to commonly used agents, antimicrobial susceptibility testing may have to be performed on clinically significant aerobic and anaerobic isolates as outlined under item V.E.

VIII. LIMITATIONS

- A. Many agents of disease are difficult to culture, and the lack of isolation may not indicate that the pathogen is not the cause of disease.
- B. Communication between the laboratory and the physician is necessary to provide the appropriate cultures for the disease present, since agents such as *Ureaplasma* and *H. ducreyi* may not grow on routine laboratory media.
- C. Unless selective media and incubation are used, a routine genital culture will not detect carriage of GBS in all cases.
- D. Because of the difficulty in evaluating the significance of *G. vaginalis* in culture, unless it is clearly predominant and numerous, BV is best diagnosed by Gram stain.
- E. The presence of fastidious gram-negative rods in genital specimens may or may not indicate infection. Cases have been reported, but they are infrequent (2, 6, 11, 24).

REFERENCES

1. Aroutcheva, A. A., J. A. Simoes, K. Behbakht, and S. Faro. 2001. *Gardnerella vaginalis* isolated from patients with bacterial vaginosis and from patients with healthy vaginal ecosystems. *Clin. Infect. Dis.* **33**:1022–1027.
2. Baddour, L. M., M. S. Gelfand, R. E. Weaver, T. C. Woods, M. Altweig, L. W. Mayer, R. A. Kelly, and D. J. Brenner. 1989. CDC group HB-5 as a cause of genitourinary infections in adults. *J. Clin. Microbiol.* **27**:801–805.
3. Baron, E. J., G. H. Cassell, D. A. Eschenbach, J. R. Greenwood, S. M. Harvey, N. E. Madinger, E. M. Paterson, and K. B. Waites. 1993. Cumitech 17A, *Laboratory Diagnosis of Female Genital Tract Infections*. Coordinating ed., E. J. Baron. American Society for Microbiology, Washington, D.C.
4. Brown, H. L., D. D. Fuller, T. E. Davis, J. R. Schwebke, and S. L. Hillier. 2001. Evaluation of the Affirm Ambient Temperature Transport System for the detection and identification of *Trichomonas vaginalis*, *Gardnerella vaginalis*, and *Candida* species from vaginal fluid specimens. *J. Clin. Microbiol.* **39**:3197–3199.
5. Catlin, B. W. 1992. *Gardnerella vaginalis*: characteristics, clinical considerations, and controversies. *Clin. Microbiol. Rev.* **5**:213–237.
6. Chowdhury, M. N. H., and S. S. Parek. 1983. Urethritis associated with *Haemophilus parainfluenzae*: a case report. *Sex. Transm. Dis.* **10**:45–46.
7. Clinical and Laboratory Standards Institute. 2004. *Methods for Antimicrobial Susceptibility Testing of Anaerobic Bacteria*, 6th ed. Clinical and Laboratory Standards Institute, Wayne, Pa.
8. Clinical and Laboratory Standards Institute. 2006. *Performance Standards for Antimicrobial Susceptibility Testing*, M100-S16, 16th supplement. Clinical and Laboratory Standards Institute, Wayne, Pa.
9. Gavin, S. E., R. B. Leonard, A. M. Briselden, and M. B. Coyle. 1992. Evaluation of the rapid CORYNE identification system for *Corynebacterium* species and other coryneforms. *J. Clin. Microbiol.* **30**:1692–1695.
10. Hager, W. D., and B. Majudar. 1979. Pelvic actinomycosis in women using intrauterine contraceptive devices. *Am. J. Gynecol.* **133**:60–63.
11. Hall, G. D., and J. A. Washington. 1983. *Haemophilus influenzae* in genitourinary tract infections. *Diagn. Microbiol. Infect. Dis.* **1**:65–70.
12. Hammerschlag, M. R., S. Alpert, I. Rosmar, P. Thurston, D. Semine, D. McComb, and W. M. McCormack. 1978. Microbiology of the vagina in children: normal and potentially pathogenic organisms. *Pediatrics* **62**:57–62.
13. Heller, R. H., J. M. Joseph, and H. J. Davis. 1969. Vulvovaginitis in the premenarcheal child. *J. Pediatr.* **74**:370–377.
14. Larsen, B., and G. R. G. Monif. 2001. Understanding the bacterial flora of the female genital tract. *Clin. Infect. Dis.* **32**:e69–e71.
15. Murphy, T. V., and J. D. Nelson. 1979. *Shigella* vaginitis: report of 38 patients and review of the literature. *Pediatrics* **63**:511–516.
16. Nugent, R. P., M. A. Krohn, and S. L. Hillier. 1991. Reliability of diagnosing bacterial vaginosis is improved by a standardized method of Gram stain interpretation. *J. Clin. Microbiol.* **29**:297–301.
17. Osborne, N. G., R. C. Wright, and L. Grubin. 1979. Genital bacteriology: a comparative study of premenopausal women with postmenopausal women. *Am. J. Obstet. Gynecol.* **135**:195–198.
18. Polaneczky, M., C. Quigley, L. Pollock, D. Dulko, and S. S. Whitkin. 1998. Use of self-collected vaginal specimens for detection of *Chlamydia trachomatis* infection. *Obstet. Gynecol.* **9**:375–378.
19. Reimer, L. G., and L. B. Reller. 1985. Use of a sodium polyanetholesulfate disk for the identification of *Gardnerella vaginalis*. *J. Clin. Microbiol.* **21**:146–149.
20. Schwartz, R. H., R. L. Wientzen, and R. G. Barsanti. 1982. Vulvovaginitis in prepubertal girls: the importance of group A streptococcus. *South. Med. J.* **75**:446–447.
21. Singleton, A. F. 1980. Vaginal discharge in children and adolescents. *Clin. Pediatr.* **19**:799–805.

REFERENCES (continued)

22. **Sobel, J. D.** 1997. Vaginitis. *N. Engl. J. Med.* **337**:1896–1903.
23. **Stamey, T.** 1980. *Pathogenesis and Treatment of Urinary Tract Infections*. Williams & Wilkins, Baltimore, Md.
24. **Sturm, A. W.** 1986. *Haemophilus influenzae* and *Haemophilus parainfluenzae* in nongonococcal urethritis. *J. Infect. Dis.* **153**:165–167.
25. **Weyant, R. S., C. W. Moss, R. E. Weaver, D. G. Hollis, J. G. Jordan, E. C. Cook, and M. I. Daneshvar.** 1995. *Identification of Unusual Pathogenic Gram-Negative Aerobic and Facultatively Anaerobic Bacteria*, 2nd ed. Williams & Wilkins, Baltimore, Md.

SUPPLEMENTAL READING

- Casey, B. M., and S. M. Cox.** 1997. Chorioamnionitis and endometritis. *Infect. Dis. Clin. N. Am.* **11**:203–222.
- Centers for Disease Control and Prevention.** 2002. Sexually transmitted diseases treatment guidelines. *Morb. Mortal. Wkly. Rep.* **51**:1–80.
- Hillier, S. L., and R. J. Lau.** 1997. Vaginal microflora in postmenopausal women who have not received estrogen replacement therapy. *Clin. Infect. Dis.* **25**(Suppl. 2):S123–S126.
- Holmes, K. K., P. A. Mardh, P. F. Sparling, P. J. Wiesner, W. Cates, Jr., S. M. Lemon, and W. E. Stamm.** 1990. *Sexually Transmitted Diseases*, 2nd ed. McGraw Hill Book Co., New York, N.Y.
- Kent, H. L.** 1991. Epidemiology of vaginitis. *Am. J. Obstet. Gynecol.* **165**:1168–1176.
- Landers, D. V., and R. L. Sweet.** 1986. Upper genital tract infections, p. 187–207. In R. P. Galask and B. Larsen (ed.), *Infectious Diseases in the Female Patient*. Springer-Verlag, New York, N.Y.
- Larsen, B.** 1986. Intrauterine bacterial infections, p. 141–162. In R. P. Galask and B. Larsen (ed.), *Infectious Diseases in the Female Patient*. Springer-Verlag, New York, N.Y.
- Lawson, M. A., and M. J. Blythe.** 1999. Pelvic inflammatory disease in adolescents. *Pediatr. Clin. N. Am.* **46**:767–782.

APPENDIX 3.9.1-1**Sample Request Form for Submission of Specimens for Diagnosis of Genital Infections for Women in Childbearing Years and Sexually Active Adults**

- Bacterial vaginosis:** Prepare a smear of vaginal discharge. Place slide in the slide holder and patient label on outside of holder.
- Test:* Gram stain (procedure 3.2.1).
- Candidiasis:** Prepare specimen for viewing by placing swab moistened with vaginal fluid (add small amount of saline if necessary) onto glass slide or submit swab in transport medium.
- Test:* wet mount for yeast (procedure 3.2.3) and yeast culture.
- Chancroid:** Submit aspirate and scraping of base of ulcer in transport medium. Contact laboratory prior to collection to arrange for appropriate transport.
- Test:* *Haemophilus ducreyi* culture (procedure 3.9.4).
- Chlamydia infection:** Submit cervical (females) or urethral (males) swab. First voided 20 ml of urine may be sent for nucleic acid amplification tests. Test can be done by probe technology if submitted in appropriate transport tube, but this is not accepted for abuse cases.
- Test:* chlamydia culture probe test (see procedure 10.6 and Table 12.1–2, respectively).
- Trichomonas infection:** Submit *vaginal* swab in InPouch after swirling swab in top portion of broth, or submit swab in charcoal transport tube.
- Test:* *Trichomonas* wet mount and culture (see procedures 3.2.3, 9.6.6, and 9.9.4).
- Gonorrhea:** Submit swab from cervix (females) or urethra (males) or rectum or throat in charcoal transport medium within 3 h of collection. First voided 20 ml of urine may be sent for nucleic acid amplification tests only. Test can be done by probe technology if submitted in appropriate transport tube, but this is not accepted for abuse cases or from throat or rectum.
- Test:* GC culture probe test (procedure 3.9.3 and Table 12.1–2, respectively).
- Herpes infection:** Submit aspirate and scraping of base of vesicle.
- Test:* herpes culture (see procedure 10.5). *Note:* Herpes serology can be used to document past infection.
- Genital mycoplasma infection:** Vaginal or cervical (females) or urethral (males) swab for culture. Other more invasive specimens are preferred _____. This test should only be ordered for symptomatic patients and not as a screening test.
- Test:* *Ureaplasma* culture (procedure 3.15).
- Prevention of neonatal group B streptococcal (GBS) disease:** Submit swab from *vaginal* and anal area in tube. Collection should be at 35 to 37 weeks gestation. Testing for GBS early in pregnancy is not recommended. Laboratory will enrich for organism in broth culture followed by plate culture.
- Test:* GBS culture (procedure 3.9.2).
- Syphilis:** *Note:* Diagnosis is generally done by serologic means. Collect lesion scraping and deliver to laboratory immediately.
- Test:* dark-field exam.
- Other:** State symptoms and organisms sought. _____

APPENDIX 3.9.1–2

Quantitative Cultures of Sequential Urine and Prostate Secretion Specimens in the Localization of Lower Urinary Tract Infection**I. PROCEDURE**

- A. Urine cultures and expressed prostatic secretions are collected and labeled using the correct clinical designation as outlined in Table 3.9.1–5.
 1. VBS no. 1 is the initial 5 to 10 ml of the urinary stream.
 2. VBS no. 2 is the midstream portion of the urinary stream.
 3. EPS are secretions expressed from the prostate by digital massage.
 4. VBS no. 3 is the first 5 to 10 ml of the urinary stream voided immediately after prostatic massage.
- B. Each specimen is placed in a urine collection tube containing boric acid preservative and transport to the laboratory within 2 to 4 h after collection.
- C. VBS no. 1, 2, and 3 and EPS are quantitatively cultured using a 0.01 loop (10 µl) and plated onto a blood agar (BA) and MAC plate.
- D. Colony counts for each primary urinary tract infection pathogen are determined by the following formula: colonies on the BA plate $\times 10^2 = \text{___ CFU/ml}$.
- E. Colony counts should be rounded and reported as a single-digit number $\times 10^6 = \text{___ CFU/ml}$.
- F. Primary pathogens that should be identified include *Enterobacteriaceae*; *Pseudomonas aeruginosa*; *Enterococcus* spp.; *Staphylococcus aureus*; group A, B, C, and G streptococci; and *Candida* spp.
- G. Coagulase-negative staphylococci, *Corynebacterium* spp., and other potential skin microbiota should only be worked up if isolated pure or in the same amount as the primary pathogens.
- H. Antibiotic susceptibility testing should be done on all clinically significant isolates. If the same organism is isolated from more than one sample in the sequence, then the isolate from only one of the samples needs to have antibiotic susceptibility testing performed provided the morphotypes are biochemically similar in all of the samples.

II. CLINICAL SIGNIFICANCE

Diagnosis of bacterial prostatitis by the sequential localization testing requires that the colony count in the midstream urine (VBS no. 2) be increased by at least 10-fold (1 log unit) in the postprostatic massage urine (VBS no. 3) or EPS compared with the colony count in the first void urine (VBS no. 1). Chronic bacterial prostatitis patients have only low numbers of organisms in the gland itself. Quantitative culture of EPS may be particularly important for these men. EPS should also be examined microscopically to look for pus and large lipid-laden macrophages that are characteristic of the prostatic inflammatory response.

References

1. Kreiger, J. N. 2005. Prostatitis, epididymitis, and orchitis, p. 1381–1386. In G. L. Mandell, J. E. Bennett, and R. Dolin (ed.), *Principles and Practice of Infectious Diseases*, 6th ed. Elsevier Churchill Livingstone, Philadelphia, Pa.
2. Stamey, T. 1980. *Pathogenesis and Treatment of Urinary Tract Infections*. Williams & Wilkins, Baltimore, Md.
3. Thin, R. N. 1997. Diagnosis of chronic prostatitis: do urologists use the four-glass test? *Urology* 55:403–407.

3.9.2

Group B Streptococcus Cultures

[Updated March 2007]

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Group B streptococcus (GBS) (*Streptococcus agalactiae*) has been recognized as the leading infectious cause of perinatal morbidity and mortality in the United States. In pregnant women, it is associated with asymptomatic bacteriuria, urinary tract infection, and amnionitis, and in women who have recently delivered, it causes endometritis and wound infection. Early-onset neonatal disease (during the first week of life) results from transmission of GBS during labor or delivery from mother to infant; late-onset disease (from 1 to 3 months after birth) is thought to be acquired in the nursery. Both are characterized by septicemia, pneumonia, or meningitis and can result in death or permanent neurological sequelae.

In 1986 Boyer and Gotoff (6), in a carefully controlled study, demonstrated that intrapartum ampicillin prophylaxis can prevent early-onset neonatal GBS disease in women with positive prenatal cultures for GBS and certain perinatal risk factors. In 1992 the American Academy of Pediatrics issued guidelines for prevention of neonatal GBS disease (1), which included prenatal cultures at 26 to 28 weeks' gestation. In 1996 and 1997, the American Academy of Pediatrics (2), the American College of Obstetricians and Gynecolo-

gists (3), and the CDC (7, 8) issued guidelines that included a combination of treating all women in risk groups and/or culturing at 35 to 37 weeks' gestation to identify carriers for whom treatment would most likely prevent disease. The rationale for this change in the timing of culturing was that at any given time 20% of women are colonized with GBS, but it is colonization at the time of delivery that is associated with disease. By the end of 1998, it was estimated that in the United States in that year alone, 3,900 cases of GBS disease in neonates and 200 deaths from early-onset GBS disease were prevented by the guidelines (18). In 2002 (9), the CDC issued new guidelines based on 6 years of experience, stating the need for doing culture and providing more exact details for the culture techniques. A preincubation step in a selective broth containing antimicrobials was included as part of the guidelines, because it is the most sensitive culture method. A commercial test for rapid molecular detection of GBS has recently been given clearance by the FDA for prenatal testing at 35 to 37 weeks, or during labor and delivery. The GeneOhm IDI-Strep B assay (Somagen Diagnostics) uses a reverse transcription (RT)-PCR method to detect GBS directly from

vaginal-rectal swabs within 1 h after receipt by the laboratory. The IDI-Strep B assay has been shown to be highly sensitive and specific compared to prenatal GBS cultures or empirical treatment of women based on risk factors alone at the time of delivery (11). Commercial availability of this assay has the potential to further decrease the rates of neonatal GBS disease.

GBS disease is increasing in nonpregnant adults, especially the elderly and those with significant underlying disease (14). Diabetes mellitus, neurological impairment, and cirrhosis appear to be risk factors. Skin, soft tissue infections, pneumonia, and urosepsis are common presentations, although meningitis and endocarditis are reported. Disease is frequently nosocomial, possibly related to catheter placement (14). For diagnosis of GBS disease in nonpregnant adults and in neonates, routine culture of the symptomatic body site (e.g., blood, CSF, and amniotic and joint fluid cultures) will detect this pathogen along with the other potential pathogens which can be isolated from those cultures. This procedure addresses only the perinatal screening culturing where the physician specifically orders culture for GBS to detect colonization.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING



Observe standard precautions.

NOTE: Refer to procedure 3.3.1 for additional details. The following can be copied for preparation of a specimen collection instruction manual for physicians and other caregivers. It is the responsibility of the laboratory director or designee to educate physicians and caregivers on proper specimen collection.

- A. Collect specimen at 35 to 37 weeks' gestation. Using a single swab or two separate swabs, swab the distal vagina (vaginal introitus), followed by the rectum (i.e., insert swab through the anal sphincter).

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING

(continued)

- NOTE:** The CDC recommends that women be empirically treated on the basis of risk factors if GBS test results are not available at the time of delivery. However, a vaginal-rectal swab should also be collected and submitted for culture to determine the GBS status of the mother at the time of delivery because this result is clinically important in diagnosing subsequent disease in the neonate. Alternatively, the IDI-Strep B RT-PCR assay may be performed STAT during labor and delivery to enable antibiotic therapy to be given on the basis of a positive rapid test result. *Rapid GBS antigen tests should not be performed during labor and delivery or in place of prenatal cultures because they are not sensitive enough to be used to direct antibiotic treatment (9).*
- B. If symptoms of sepsis or amnionitis are present, also submit amniotic fluid (procedure 3.5), blood, or urine for culture.
 - C. Specimen transport
 1. Submit one or both swabs to the laboratory in a *single* tube of nonnutritive transport medium (Amies or Stuart's). It is not necessary to submit anal and vaginal swabs separately.
 2. Label specimens and accompanying requisition with demographic information on the patient (name, identifying number, age, and diagnosis), the date and site of collection, and the week of gestation.
 3. Store at 25°C or refrigerate at 4°C.
 4. Order culture for GBS; if the patient is penicillin allergic, also order testing for susceptibility to clindamycin and erythromycin.
 5. Submit to the laboratory within 4 days of collection.
 - D. Rejection criteria

■ NOTE: According to an alert from the CDC on 24 March 1997, gravely erroneous information and potentially fatal misdiagnoses can result from the use of direct antigen testing to detect GBS directly in urine of pregnant women or infants.

 1. If a direct antigen assay is performed on CSF, always perform a culture for confirmation of viable organisms.
 2. Do not accept urine or vaginal swabs for direct antigen assay.
 3. For the neonate, screening cultures (e.g., axilla, throat, etc.) and urinary antigen tests are not recommended because of their lack of sensitivity and specificity for diagnosis.

■ NOTE: Collect blood, CSF, and respiratory secretions to diagnose disease in symptomatic neonates.

III. MATERIALS

- | | |
|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| A. Media <ol style="list-style-type: none"> 1. Selective streptococcal broth <ol style="list-style-type: none"> a. LIM broth: Todd-Hewitt broth with 10 µg of colistin per ml and 15 µg of nalidixic acid per ml (available from most medium vendors) b. Trans-Vag broth with 5% defibrinated sheep blood: Todd-Hewitt broth with 8 µg of gentamicin per ml and 15 µg of nalidixic acid per ml (Remel, Inc.). <p>■ NOTE: Fackrell and Dick (13) reported that LIM broth supported growth of GBS better than Trans-Vag broth but was equivalent to Trans-Vag broth with blood.</p> | <ol style="list-style-type: none"> 2. Agar media <ol style="list-style-type: none"> a. BAP b. NNA: BAP with 30 µg of neomycin per ml and 15 µg of nalidixic acid per ml (BD Diagnostic Systems) c. Selective streptococcal agar: BAP with neomycin and polymyxin B (Remel, Inc.; Hardy Diagnostics) or colistin and oxolinic acid (Remel, Inc.). d. Columbia colistin-nalidixic acid agar (CNA) |
|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
- B. Identification methods**
1. Gram stain (procedure 3.2.1)
 2. Catalase test (procedure 3.17.10)
 3. One of the following

III. MATERIALS (continued)

- a. *Staphylococcus aureus* ATCC 25923 for CAMP test (procedure 3.17.8)
- b. Rapid hippurate (procedure 3.17.21)
- c. AccuProbe GBS RNA probe assay (Gen-Probe Inc., San Diego, Calif.)
- d. Latex or coagglutination serologic test for streptococcal grouping (see procedure 3.11.8 for options)
- 4. Other biochemical tests
 - a. Pyrrolidonyl- β -naphthylamide (PYR) (procedure 3.17.41)
 - b. Bile-esculin (procedure 3.17.5)

C. Other supplies/equipment

- 1. Incubator at 35°C with 5% CO₂ or other CO₂-generating system
- 2. Disposable cards for serologic tests

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

- A. Verify that media meet expiration date and QC parameters per current Clinical and Laboratory Standards Institute (formerly NCCLS) M22 document. See procedures 14.2 and 3.3.1 for further procedures, especially for in-house-prepared media.
- B. Test each lot of BAP with a known GBS to verify the ability of the media to produce a positive CAMP reaction (procedure 3.17.8), or obtain documentation from medium vendor that each lot is checked for the CAMP reaction.
- C. Refer to the individual biochemical test procedures for further QC requirements.
- D. Perform QC with each lot of both selective streptococcal broth and agar that contain antimicrobial agents (excluding CNA). Incubate aerobically overnight at 35°C.

Test organism	Result(s)
<i>Streptococcus agalactiae</i> ATCC 12386	Broth: growth; beta-hemolytic colonies upon subculture to sheep blood agar. Agar: growth of beta-hemolytic colonies.
<i>Proteus mirabilis</i> ATCC 12453	Broth or agar: partial to complete inhibition.
<i>Streptococcus pyogenes</i> ATCC 19615	Agar: test if agar is used to test for group A streptococcus. Growth of beta-hemolytic colonies.

■ NOTE: NCCLS has proposed elimination of user QC of LIM broth and some selective streptococcal agars (16) purchased from commercial vendors. Consult with current regulatory agencies prior to discontinuation of user QC.

V. PROCEDURE



Observe standard precautions.

A. Inoculation

1. Inoculate broth from the swab.
2. Optional, in addition to the broth inoculation: inoculate BAP, CNA, or selective streptococcal agar with swab immediately upon receipt.

■ NOTE: Dunne and Holland-Staley (12) have shown that the addition of a selective direct plate to the broth culture increased detection by 15%, because *Enterococcus faecalis* can overgrow in the broth cultures and inhibit the growth of *S. agalactiae*.

B. Incubation

1. Incubate selective direct plate at 35 to 37°C in 5% CO₂ (preferred) or ambient air. Alternatively, use other CO₂-generating system to provide the 5% CO₂.
2. Incubate broth at 35 to 37°C in ambient air or in 5% CO₂.
3. Subculture the broth after 18 to 24 h of incubation to BAP, if GBS have not been isolated on the direct plate.
4. Incubate subculture plate at 35 to 37°C in ambient air or 5% CO₂ for 48 h.

V. PROCEDURE (continued)

5. Optionally, in addition to subculture, test the broth after 18 to 24 h of incubation with either of the following.
 - a. A streptococcal grouping test that includes an extraction step; this method is reported to be more sensitive than plate culture (17).
 - b. AccuProbe GBS RNA probe assay or PCR method (4, 5) (Table 12.1.4)
- C. Culture examination
 1. Observe plates at 24 and 48 h for large, gray, translucent colonies with a small zone of beta-hemolysis or no zone of hemolysis.
 2. Perform catalase test.
 3. Perform Gram stain if catalase negative.
 4. Identify gram-positive cocci in pairs and chains as *S. agalactiae* if they are catalase negative and demonstrate one of the following reactions.
 - a. Positive CAMP test
 - b. Positive with group B streptococcal grouping antisera
 - c. Positive GenProbe AccuProbe GBS test
 - d. Positive rapid hippurate
 - (1) If strain is nonhemolytic, a second test from the above list must be used for confirmation.
 - (2) If strain is hemolytic, perform PYR test (procedure 3.17.41). Enterococci are PYR positive but can be hippurate positive and hemolytic.
 5. If the isolate is hemolytic and not identified as *S. agalactiae*, perform PYR and esculin or bile-esculin test. *S. pyogenes* is PYR positive, hemolytic, and esculin negative. Enterococci can be hemolytic and PYR positive but are esculin positive.
 - D. Perform disk diffusion testing for susceptibility to clindamycin and erythromycin, on request for penicillin-allergic patients (9) (see procedure 5.1). Place disks 15 to 26 mm apart.
 - E. If antimicrobial susceptibility testing (AST) is not performed, hold positive culture plates for several (3 to 7) days should AST be requested at a later date.
 - F. Perform the IDI-Strep B RT-PCR assay directly from vaginal-rectal swabs using Cepheid's SmartCycler instrument according to the manufacturer's instructions (Somagen Diagnostics).

POSTANALYTICAL CONSIDERATIONS**VI. REPORTING RESULTS**

- A. If the culture is negative, report "No group B streptococci isolated."
- B. Positive reporting
 1. Report the presence of *S. agalactiae* (group B) or *S. pyogenes* (group A) as soon as preliminary tests are completed.
 2. Do not report any enumeration.
 3. If the patient has been admitted to the hospital, notify the physician of positive results. For other patients, ensure that the results are easily available to the physician when the patient goes into labor.
 NOTE: A policy that may be useful for laboratories that lack a readily accessible computer system is to notify the patients themselves to be sure they tell their physicians when they go into labor.
 4. Document notification of physician.
 5. Include in the report the following: "If patient is penicillin allergic, contact laboratory for alternative susceptibility testing."
 - a. Report a zone size of ≥ 21 mm with the erythromycin disk as susceptible to erythromycin (10).
 - b. Report a zone size of ≥ 19 mm with the clindamycin disk (10) and no inhibition or flattening with the erythromycin disk as susceptible to clindamycin.

VII. INTERPRETATION

- A. A positive culture indicates colonization with the organism, which may or may not indicate infection.
- B. Urinary tract infections with *S. agalactiae* should be treated.
- C. Virtually 100% of *S. agalactiae* strains are susceptible to penicillin (the drug of choice to treat colonization), negating the need for testing. If testing is performed, resistance should be confirmed in a reference laboratory (10).
- D. For penicillin-allergic women, erythromycin or clindamycin is used. Approximately 6 to 15% of strains are resistant to clindamycin, and 20% are resistant to erythromycin (15).

VIII. LIMITATIONS

- A. Although RT-PCR-based methods for the detection of GBS have been shown to be more rapid and sensitive than culture techniques, a major disadvantage of this technology is that the isolate is not retrieved in order to perform AST. Due to the concern about increasing rate of GBS resistance to both clindamycin and erythromycin, a subsequent culture may need to be performed so that AST can be done, particularly if there is treatment failure while the patient is on one of these antimicrobials.
- B. False-negative cultures can result from contamination of the specimen with genital microbiota, especially *E. faecalis*, or from the inability to recognize non-hemolytic colonies.
- C. False-positive results can be caused by misinterpretation of the confirmatory tests. Occasionally enterococci can have a positive hippurate test result, but it usually is not rapid.
- D. *Listeria* colonies can be confused with *S. agalactiae*, if the Gram stain or catalase test is omitted. *Listeria* organisms are *catalase positive*, gram-positive rods that are hemolytic and CAMP and hippurate positive, but these are rarely seen in vaginal specimens.
- E. For neonates with sepsis, blood and CSF cultures should be performed to diagnose GBS disease along with other microorganisms responsible for sepsis, such as *Listeria monocytogenes*.
- F. While the methods presented here are designed to detect GBS colonization in pregnant females, disease in nonpregnant adults is increasing (8). The microbiologist must be aware of the organism's significance and report it when present in clinically significant specimens.

REFERENCES

1. American Academy of Pediatrics. 1992. Guidelines for prevention of group B streptococcal infection by chemoprophylaxis. *Pediatrics* **90**:775–778.
2. American Academy of Pediatrics and COID/COFN. 1997. Revised guidelines for prevention of early-onset group B streptococcal (GBS) infection. *Pediatrics* **99**:489–496.
3. American College of Obstetricians and Gynecologists. 1996. Prevention of early-onset group B streptococcal disease in newborns. *ACOG Comm. Opin.* **173**:1–8.
4. Bergeron, M. G., D. Ke, C. Menard, F. J. Francois, M. Gagnon, M. Bernier, M. Ouellette, P. H. Roy, S. Marcoux, and W. D. Fraser. 2000. Rapid detection of group B streptococci in pregnant women at delivery. *N. Engl. J. Med.* **343**:175–179.
5. Bourbeau, P. P., B. J. Heiter, and M. Figdore. 1997. Use of Gen-Probe AccuProbe group B streptococcus test to detect group B streptococci in broth cultures of vaginal-anorectal specimens from pregnant women: comparison with traditional culture method. *J. Clin. Microbiol.* **35**:144–147.
6. Boyer, K. M., and S. P. Gotoff. 1986. Prevention of early-onset group B streptococcal disease with selective intrapartum chemoprophylaxis. *N. Engl. J. Med.* **314**:1665–1669.
7. Centers for Disease Control and Prevention. 1996. Prevention of perinatal group B streptococcal disease: a public health perspective. *Morb. Mortal. Wkly. Rep.* **45**:1–24.

REFERENCES (continued)

8. **Centers for Disease Control and Prevention.** 1997. Adoption of hospital policies for prevention of perinatal group B streptococcal disease—United States. *Morb. Mortal. Wkly. Rep.* **47**:665–670.
9. **Centers for Disease Control and Prevention.** 2002. Prevention of perinatal group B streptococcal disease: revised guidelines from CDC. Recommendations and Reports. *Morb. Mortal. Wkly. Rep.* **51**:1–24.
10. **Clinical and Laboratory Standards Institute.** 2006. *Performance Standards for Antimicrobial Susceptibility Testing*. Sixteenth informational supplement. Approved standard M100-S16. Clinical and Laboratory Standards Institute, Wayne, Pa.
11. **Davies, H. D., M. A. Miller, S. Faro, D. Gregson, S. C. Kehl, and J. A. Jordan.** 2004. Multicenter study of a rapid molecular-based assay for the diagnosis of group B Streptococcus colonization in pregnant women. *Clin. Infect. Dis.* **39**:1129–1135.
12. **Dunne, W. M., Jr., and C. Holland-Staley.** 1998. Comparison of NNA agar culture and selective broth culture for detection of group B streptococcal colonization in women. *J. Clin. Microbiol.* **36**:2298–2300.
13. **Fackrell, K. C., and N. K. Dick.** 2001. Comparison of LIM broth and Todd-Hewitt broth with gentamicin and nalidixic acid for recovery of group B streptococci, abstr. C-107. *Abstr. 101st Gen. Meet. Am. Soc. Microbiol.* American Society for Microbiology, Washington, D.C.
14. **Farley, M. M.** 2001. Group B streptococcal disease in nonpregnant adults. *Clin. Infect. Dis.* **33**:556–561.
15. **Lin, F. C., P. H. Azimi, L. E. Weisman, J. B. Philips III, J. Regan, P. Clark, G. G. Rhoads, J. Clemens, J. Troendle, E. Pratt, R. A. Brenner, and V. Gill.** 2000. Antibiotic susceptibility profiles for group B streptococci isolated from neonates, 1995–1998. *Clin. Infect. Dis.* **31**:76–79.
16. **NCCLS.** 2003. *Quality Control for Commercially Prepared Microbiological Culture Media*, 2nd ed. Proposed standard M22-P2. NCCLS, Wayne, Pa.
17. **Park, C. H., N. M. Vandel, D. K. Ruprai, E. A. Martin, K. M. Gates, and D. Coker.** 2001. Detection of group B streptococcal colonization in pregnant women using direct latex agglutination testing of selective broth. *J. Clin. Microbiol.* **39**:408–409.
18. **Schrag, S. J., S. Zywicki, M. M. Farley, A. L. Reingold, L. H. Harrison, L. B. Lefkowitz, J. L. Hadler, R. Danila, P. R. Cieslak, and A. Schuchat.** 2000. Group B streptococcal disease in the era of intrapartum antibiotic prophylaxis. *N. Engl. J. Med.* **342**:15–20.

SUPPLEMENTAL READING

- Schuchat, A.** 1998. Epidemiology of group B streptococcal disease in the United States: shifting paradigms. *Clin. Microbiol. Rev.* **11**:497–513.

3.9.3

Neisseria gonorrhoeae Cultures

[Updated March 2007]

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

The fastidious and fragile nature of *Neisseria gonorrhoeae* requires careful consideration of proper methods of specimen collection and transport. A good selective medium is required for reliable isolation of gonococci from patient specimens, especially those that might contain a variety of microorganisms, including saprophytic *Neisseria* spp. Methods for the identification of *N. gonorrhoeae* from extragenital sites and cultures taken from children must be chosen and performed with the utmost care, since a positive report can have far-reaching psychosocial and medico-legal implications. In the United States, culture identification of *N. gonorrhoeae* is the only definitive method of diagnosis from a legal standpoint.

In women, the endocervix is the primary site of infection, but the organism can also be recovered from the urethra, rectum, oropharynx, conjunctiva, and the ducts of Bartholin's glands. A vaginal swab is not considered optimal for the recovery of gonococci from women but can be a valuable specimen for the diagnosis of gonorrhea in preteen-aged girls. As-

cending genital infection in women can lead to pelvic inflammatory disease and, occasionally, perihepatitis. Transmission of *N. gonorrhoeae* from mother to newborn will often present as conjunctivitis (ophthalmia neonatorum).

The urethra is the primary site of infection in men, but extragenital sites, including the rectum, oropharynx, conjunctiva, and epididymis, can act as sources of *N. gonorrhoeae*, in addition to first-voided urine. Exogenous cultures of the rectum, conjunctiva, and pharynx for *N. gonorrhoeae* are performed only when specifically requested and samples are procured by the clinician. Such a request is not uncommon, however, as part of the overall diagnostic evaluation for gonorrhea.

Culture-independent nucleic acid probe hybridization and amplification assays for the detection of *N. gonorrhoeae* from urethral and cervical specimens are currently available that circumvent problems associated with specimen transport and loss of organism viability inherent in culture-based methods. These assays in-

clude the PCR AMPLICOR CT/NG assay and the PCR COBAS AMPLICOR CT/NG assay (Roche Molecular Diagnostics), the APTIMA COMBO 2 assay (Gen-Probe, Inc.), the BD ProbeTecET system (BD Diagnostic Systems), and the ligase chain reaction assay (Abbott Diagnostics). The two PCR assays are performed similarly except that the COBAS instrument automates the amplification and detection steps. All of these assays have been given clearance by the FDA for combined testing for *Chlamydia trachomatis* and *N. gonorrhoeae* in endocervical swabs, male urethral swabs, and female and male urine samples, except the two AMPLICOR assays, which have not been approved for female *N. gonorrhoeae* testing on urine and testing of urethral swabs from asymptomatic males. All of these assays have comparable performance for the detection of genital *C. trachomatis* and *N. gonorrhoeae* infection. (See section 12 for further information on these tests and reference 19 for evaluation of different systems for detection of *C. trachomatis*.)

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING

A. Specimen collection

1. Cervical
 - a. Do not use lubricant during procedure.
 - b. Wipe the cervix clean of vaginal secretion and mucus.
 - c. Rotate a sterile swab, and obtain exudate from the endocervical glands.
 - d. If no exudate is seen, insert a sterile swab into the endocervical canal, and rotate the swab.
2. Vaginal
 - a. Insert a sterile swab into the vagina.
 - b. Collect discharge or vaginal secretions from the mucosa high in the vaginal canal.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING (continued)

- **NOTE:** Vaginal specimens are not considered optimal for the diagnosis of gonorrhea in women and should be reserved only for the evaluation of preteen-aged girls with suspected sexually transmitted diseases due to presumed sexual abuse.
3. Urethral
 - a. Collect specimen 1 to 2 h or more after patient has urinated.
 - b. Female: stimulate discharge by gently massaging the urethra against the pubic symphysis through the vagina.
 - (1) Collect the discharge with a sterile swab.
 - (2) If discharge cannot be obtained, wash external urethra with Betadine soap and rinse with water. Insert a urethrogenital swab 2 to 4 cm into the endourethra, gently rotate the swab, leave it in place for 1 to 2 s, and withdraw it.
 - c. Male: insert a thin urethrogenital swab 2 to 4 cm into the endourethra, gently rotate it, leave it in place for 1 to 2 s, and withdraw it.
 4. Epididymis
Use a needle and syringe to aspirate material from the epididymis.
 5. Pharyngeal
 - a. Depress tongue gently with tongue depressor.
 - b. Extend sterile swab between the tonsillar pillars and behind the uvula. Avoid touching other surfaces of the mouth.
 - c. Sweep the swab back and forth across the posterior pharynx, tonsillar areas, and any inflamed or ulcerated areas to obtain sample.
 6. Rectal
 - a. Pass the tip of a sterile swab approximately 2 cm beyond the anal sphincter.
 - b. Carefully rotate the swab to sample the anal crypts, and withdraw it.
 7. Urine
 - a. Do not collect urine for culture of *N. gonorrhoeae*.
■ **NOTE:** Urine cultures provide reasonable sensitivity for men with gonorrhea provided a first morning-voided specimen is obtained and plated on appropriate selective and nonselective culture media. Urine culture is not a sensitive alternative for the diagnosis of gonorrhea in women.
 - b. Collect no more than 20 ml of first morning-voided urine for nucleic acid testing.
■ **NOTE:** In contrast to routine urine cultures, the first part of the voided urine specimen, rather than the midstream urine, is collected for nucleic acid testing. The use of urine for the diagnosis of *N. gonorrhoeae* and/or *C. trachomatis* infection by nucleic acid amplification assays has been successfully adapted for several commercially available diagnostic kits. However, for females the sensitivity of the urine compared to an endocervical swab is generally 10% lower. Currently, the AMPLICOR CT/NG assay (Roche Molecular Diagnostics) is not FDA cleared for use with female urine samples (19).
 8. Conjunctivae
 - a. Collect purulent material on a swab.
 - (1) Roll sterile swab over the conjunctiva before topical medications are applied.
 - (2) Culture both eyes with separate swabs.
 - b. Alternatively, obtain material with a sterile spatula and inoculate directly onto culture media.
- B. Specimen transport**
1. Direct inoculation
 - a. Inoculate specimen directly onto the surface of nonselective and selective medium plates. Roll the swab across one quadrant of the plate and streak for isolation, or roll swabs across the surface of the plate in a “Z” or “N”

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING (continued)

- figure. Plates are cross-streaked for isolation when received by the laboratory.
- b. Place inoculated media in a holding jar or other system with a suitable CO₂-enriched (3 to 5%) atmosphere.
 - c. Transport systems that provide growth medium and an atmosphere-generating tablet in a self-contained transport pack can be substituted for direct medium inoculation and the holding-jar method. These include the following.
 - (1) JEMBEC (BD Diagnostic Systems)
 - (2) InTray GC (BioMed Diagnostics, San Jose, Calif.)
 - (3) Gono-Pak (BD Diagnostic Systems)
 2. If direct medium inoculation is not practical, collect specimens on one of the following swab-based transport systems.
 - a. Difco Cultureswab (BD Diagnostic Systems)
 - b. Copan Venturi Transystem (Copan Italia, Bovezza, Italy)
 - c. BD Culturette EZ (BD Diagnostic Systems)
 - d. Starplex Starswab (Starplex Scientific, Inc., Etobicoke, Ontario, Canada)

■ NOTE: The performance of each of these swab-based transport systems is comparable if specimens are submitted and cultured within 6 h of collection and either held at ambient temperature or refrigerated. However, recovery rates vary considerably if delivery is delayed (1, 14, 21). At 24 h, the Copan system held at either room temperature or 4°C and the Difco system held at refrigeration temperatures provide recovery rates of 96% (1). The addition of charcoal to Amies transport medium does not appear to increase recovery rates (21).

 - 3. For Gram stain of male urethral discharge or conjunctival exudate, transfer some of the specimen to a slide and spread in a thin film. Allow to air dry.

■ NOTE: Preparation of the smear at the time of specimen collection will avoid deterioration of the specimen, which results in false-negative smear reports. For children, a smear must be accompanied by a culture, since smears lack the specificity for accurate *N. gonorrhoeae* diagnosis.
 - 4. For molecular detection assays, use the manufacturer's product-specific collection devices. These devices cannot be used when culture has been requested.

■ NOTE: While cultures must be performed in all medico-legal cases and with specimens from prepubescent children, nucleic acid tests can be performed in addition to culture.
 - 5. Once collected, submit specimens for culture to laboratory as soon as possible. If 24-h laboratory coverage is not provided, alert the laboratory that a specimen is in transit.
 - 6. Store at ambient or refrigeration temperature after collection and during transport (1, 24).
 - 7. Ensure that specimens are labeled with demographic information on the patient and the site and time of collection.
 - 8. Record the patient diagnosis for improved processing of specimen.
- C. Rejection criteria**
1. Reject specimens on swabs in which significant desiccation has occurred or when instructions for correct use of the transport system have not been followed.
 2. Consider rejection of specimens if significant delay (e.g., >24 h) in transportation and plating occurs. However, a more practical approach might include a provision for culture in all cases of delayed transportation with an accompanying comment for cultures negative for *N. gonorrhoeae* (see item VI below). Accompany such a provision with active education and revision of policies to improve transport.

III. MATERIALS

A. Media

■ NOTE: For maximum recovery, culture media for isolation of *N. gonorrhoeae* should include both selective and nonselective agar formulations capable of supporting the growth of the organism. Selective media are designed to prevent overgrowth of contaminating normal microbiota. The addition of a nonselective medium such as CHOC allows for the growth of a small percentage of gonococci (approximately 2%) that are inhibited in the presence of vancomycin at concentrations of 4 µg/ml. Formulations such as GC-Lect reduce the concentration of vancomycin to 2 µg/ml (4, 13, 15, 16, 22, 24, 25). In selective medium formulations, anisomycin has a longer shelf life than nystatin to inhibit *Candida*. Trimethoprim is added to inhibit the growth of *Proteus* species. (For vendor information, see procedure 3.1.)

1. CHOC (omit for nasopharyngeal and rectal sites)
2. One of the following selective media
 - a. Modified Thayer-Martin medium (contains vancomycin, colistin, nystatin, and trimethoprim) is available from most medium manufacturers.
 - b. Martin-Lewis medium (contains vancomycin, colistin, and anisomycin) is available from most medium manufacturers. The vancomycin concentration is higher (400 µg/100 ml) in Martin-Lewis than in Thayer-Martin medium (300 µg/100 ml).
 - c. Thayer-Martin improved medium (TM improved) (contains vancomycin, colistin, anisomycin, and trimethoprim [Remel, Inc.])
 - d. TM improved without vancomycin, sold as a biplate with TM improved (Remel, Inc.)
 - e. Martin-Lewis medium with lincomycin (Hardy Diagnostics)
 - f. GC-Lect (contains lincomycin, vancomycin at 200 µg/100 ml, colistin, trimethoprim, and amphotericin B) (BD Diagnostic Systems)
 - g. New York City medium with the same antimicrobial agents as GC-Lect (BD Diagnostic Systems)

h. The following media provide both CO₂ generation and the media in a self-contained system that promotes survival of *N. gonorrhoeae* during transportation.

- (1) JEMBEC (BD Diagnostic Systems; Remel, Inc.)
- (2) Gono-Pak (BD Diagnostic Systems; Remel, Inc.)
- (3) InTray GC (BioMed Diagnostics)

3. Biplate formulations containing both CHOC and selective agar are available from several manufacturers.

B. Gram stain reagents (procedure 3.2.1)

C. Direct amplification tests

Refer to reference 19.

D. Identification tests

1. Oxidase reagent (see procedure 3.17.39)

2. 30% Hydrogen peroxide (Sigma, St. Louis, Mo.) for superoxol test (2, 23)

3. Nutritionally basic media, such as nutrient agar, TSA, or Mueller-Hinton (MH) agar

4. *Neisseria* identification systems

- a. Carbohydrate utilization assay: cysteine Trypticase agar (CTA) base with 1% carbohydrates (glucose, maltose, lactose, and sucrose) or carbohydrate degradation media with 20% carbohydrates (Remel, Inc.; see procedure 3.17.9)

b. Coagglutination

- (1) Gonogen (New Horizons Diagnostics; BBL Microbiology, BD Diagnostic Systems)

- (2) Phadebact GC monoclonal antibody test (Boule Diagnostics AB, Huddinge, Sweden)

- c. Monoclonal antibody assay: GonoGen II (New Horizons Diagnostics; BBL Microbiology, BD Diagnostic Systems)

d. Substrate utilization

- (1) RapID NH, BactiCard Neisseria, NET (Remel, Inc.)

- (2) Gonocheck II (EY Laboratories, Inc., San Mateo, Calif.)

- (3) Neisseria Screen (Key Scientific Products, Round Rock, Tex.)

III. MATERIALS (continued)

- (4) BBL CRYSTAL (BD Diagnostic Systems)
- (5) *Neisseria/Haemophilus* ID kit, API NH (bioMérieux)
- e. Fluorescent antibody: Syva MicroTrak *N. gonorrhoeae* cul-
- ture confirmation test (Trinity Biotech plc., Co. Wicklow, Ireland)
- f. Probe hybridization: AccuProbe *N. gonorrhoeae* culture confirmation test (Gen-Probe, Inc.)

ANALYTICAL CONSIDERATIONS**IV. QUALITY CONTROL**

- A. Verify that media meet expiration date and QC parameters per current Clinical and Laboratory Standards Institute (CLSI; formerly NCCLS) M22 document. See procedures 14.2 and 3.3.1 for further procedures, especially for in-house-prepared media.
- B. Test each lot of identification kits and reagents, according to package insert or procedure 14.2. Generally the following QC organisms are used for sugar or kit tests. See Table 3.9.3-1 for expected reactions.
 - 1. *N. gonorrhoeae* ATCC 43069
 - 2. *Neisseria meningitidis* ATCC 13077
 - 3. *Neisseria lactamica* ATCC 23970
 - 4. *Moraxella catarrhalis* ATCC 25240
- C. Perform QC of CHOC and selective media by lot; incubate for 24 to 48 h in 5 to 7% CO₂ at 35°C.

CHOC

Test organism	Result
<i>Neisseria gonorrhoeae</i> ATCC 43069 or ATCC 43070	Growth
<i>Haemophilus influenzae</i> ATCC 10211	Growth

Selective media for pathogenic *Neisseria* spp.

Test organism	Result
<i>Neisseria gonorrhoeae</i> ATCC 43069 or ATCC 43070	Growth
<i>Proteus mirabilis</i> ATCC 43071	Partial inhibition—test only if medium contains trimethoprim
<i>Staphylococcus epidermidis</i> ATCC 12228	Partial inhibition

V. PROCEDURES**A. Direct test**

- 1. Gram stain
 - a. Perform smears only on male urethra specimens.
 - b. Roll swab across slide after culture has been inoculated.
 - c. Fix smear with methanol. (See procedure 3.2.1 for staining and reading smear.)
- 2. Direct probe assays (19)

B. Culture methods

- 1. Inoculation
 - a. Process specimen as soon as received.
 - b. Inoculate medium by rolling the swab over one quadrant of the plate and streaking for isolation. If medium is received that has already been inoculated (either in a “Z” streak or down the center of the plate), it is best to cross-streak for isolation to ensure separation of mixed colony types.



Observe standard precautions.

Table 3.9.3-1 Biochemical reactions of *Neisseria* and related oxidase-positive diplococci and rods that may grow on Thayer-Martin or similar selective agar^c

Organism(s)	Superoxol, 30% H ₂ O ₂	Growth on basic agar media at 35°C ^c	Colistin (10 µg) disk	Glucose	Maltose	Lactose, ONPG, or BGAL	Sucrose	PRO	GLUT	Butyrate ^d
<i>Neisseria gonorrhoeae</i>	4+	—	R	+ —	—	—	—	+	—	—
<i>Neisseria meningitidis</i>	2–4+	V	R	+ —	—	—	—	V	+	—
<i>Neisseria lactamica</i>	2+	+	R	+ —	+	—	—	+	—	—
<i>Neisseria cinerea</i> ^b	2+	+	V	V	—	—	—	+	—	—
<i>Neisseria flavescens</i> ^b	2+	+	S	—	—	—	—	+	—	—
<i>Neisseria elongata</i> ^b	2+	+	S	—	—	—	—	V	—	—
Other nonpathogenic <i>Neisseria</i> spp. ^b	2+	+	V	+ —	—	—	V	V	V	—
<i>Moraxella catarrhalis</i>	2–4+	+	V	—	—	—	—	V	—	+
<i>Kingella</i> species	—	+	R	+ —	V	—	—	V	—	—

^a Abbreviations: PRO, prolyl-aminopeptidase; BGAL, β -galactosidase; GLUT, δ -glutamyl-aminopeptidase; V, variable reactions; + —, reaction is generally positive, but rare negative results occur, resulting in critical misidentifications if other tests are not also performed; R, resistant; S, susceptible. Polymyxin B can be substituted for colistin, or susceptibility can usually be determined by growth or lack of growth on Thayer-Martin or other selective agar with colistin or polymyxin B. Reactions are from package inserts, from <http://www.CDC.gov/ncidod/dastir/gcdif/neident/index.html>, and from references 18 and 27.

^b This organism(s) does not usually grow on selective media for *N. gonorrhoeae*. *N. subflava* and *N. flavescens* colonies are yellow; *N. subflava* is the only species other than *N. meningitidis* to be GLUT positive.

^c Nutrient agar, MH agar, or TSA without blood at 35°C.

^d See procedure 3.17.7. Do not read after time period in package insert, as this delay may result in false-positive reactions. Many *Moraxella* spp. and *Actinobacter* spp. are butyrate positive. Isolate must be a diplococcus for identification of *M. catarrhalis* to be accurate.

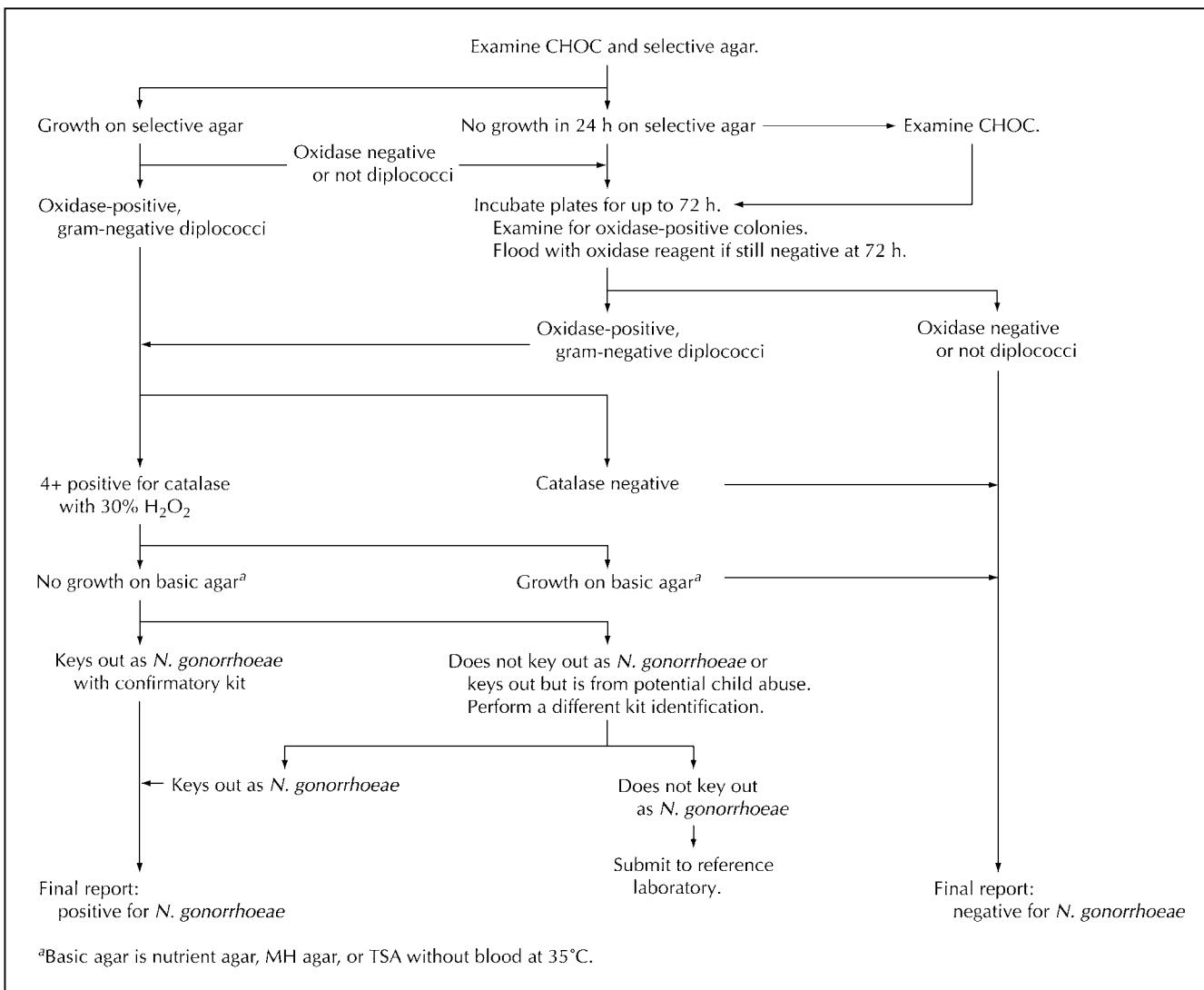


Figure 3.9.3–1 Laboratory diagnosis of *N. gonorrhoeae* by culture.

V. PROCEDURES (continued)

2. Incubation

- a. Incubate plates at 35 to 37°C in 5% CO₂ contained system.
- b. Alternatively, use a CO₂-generating contained system to provide the proper atmosphere if a CO₂ incubator is unavailable.
- c. If no other method is available, place in a jar and light a candle. Seal the jar quickly. When the candle is extinguished, the atmosphere contains approximately 3 to 5% CO₂.

3. Save specimen in case confirmation of identification is needed.

C. Culture examination

- 1. Examine all plated media for macroscopic evidence of growth at 24 h.
- 2. If no visible growth is observed on the culture media
 - a. Return cultures to incubator quickly to minimize loss of viability in the absence of CO₂.
 - b. Read aerobic plates daily for a total of 72 h before reporting as negative.

V. PROCEDURES (continued)

- c. Prior to discarding plates, flood plates with oxidase reagent. If a purple color colony is observed, immediately subculture to CHOC, since oxidase reagent is toxic to bacteria.
3. Cultures with growth (see Fig. 3.9.3–1)
 - a. Examine for typical colonies that are small, translucent, raised, gray, and mucoid with entire margins. When picked from the agar surface, they tend to come off as whole colonies.
 - b. Perform a Gram stain and oxidase test on suspicious colonies. If the Gram stain shows gram-negative diplococci with flattened adjacent sides or gram-negative cocci in clusters and the oxidase is positive, the isolate is presumed to be either a *Neisseria* sp. or *M. catarrhalis*.
 - c. If the specimen is sent for diagnosis of carriage of *N. meningitidis*, examine CHOC carefully and identify any oxidase-positive, gram-negative diplococci. These strains may not grow on selective agar.
 - d. Confirm identification to the species level. If the isolate is from an extra-genital site or from a child, perform two confirmatory assays that utilize distinct identification mechanisms (e.g., monoclonal antibody and carbohydrate utilization).
 - (1) Superoxol test (references 2 and 23 and procedure 3.17.10)
 - (a) Drop 30% hydrogen peroxide reagent onto colony.
 - (b) *N. gonorrhoeae* will give immediate, explosive bubbling.
 - (c) Other species, except some *N. meningitidis* and *M. catarrhalis* strains, show weak reactions. A positive result should only be considered presumptive for the identification of *N. gonorrhoeae*.
 - (2) Subculture typical colonies to a nutritionally basic medium, such as nutrient agar, TSA, or MH agar, and to CHOC for proof of viability. Failure of viable organisms to grow on one of these basic media after overnight incubation at 35°C with growth on CHOC indicates a presumptive pathogenic *Neisseria* sp.



It is imperative that these cultures be handled in a biosafety hood.

D. Culture confirmation

Choose one method as the primary method for use in the laboratory.

1. Growth-dependent carbohydrate degradation (procedure 3.17.9)

- a. CTA supplemented with 1% carbohydrates
 - (1) Inoculate tubes containing glucose, maltose, sucrose, and lactose (*o*-nitrophenyl-β-D-galactopyranoside [ONPG] may be substituted for lactose) and a control tube containing no additional carbohydrate source.
 - (2) Incubate at least overnight, depending on inoculum density. *N. gonorrhoeae* produces acid only in glucose.
- b. Alternatively, use carbohydrate degradation media with 20% carbohydrates.
 - (1) Use a heavy inoculum and incubate for 1 to 4 h, since the test depends on the presence of preformed enzyme activity and uses a buffered low-peptone base with 2% glucose, lactose, and sucrose and 0.3% maltose (28).
 - (2) Use the same interpretation as for growth carbohydrate degradation tests.

2. BBL GonoGen

- **NOTE:** BBL GonoGen is a monoclonal antibody-based coagglutination test for the confirmatory identification of *N. gonorrhoeae* from culture. The assay is based on the recognition of a major outer membrane protein (protein I) of the organism.

V. PROCEDURES (continued)

- a. Suspend several colonies of suspected *N. gonorrhoeae* from culture medium in a tube containing 0.5 ml of distilled water to the equivalent of a McFarland no. 3 standard (Appendix 3.16–1).
- b. Heat the suspension at 100°C for 10 min, cool to room temperature, and vortex to remove aggregates.
- c. Place 1 drop of reagent G on a separate circle of a glass slide for each test isolate and for positive and negative controls.
- d. Add 1 drop of the heat-treated suspension of organism to the drop of reagent G and mix. Add 1 drop each of the positive and negative control suspension to separate drops of reagent G and mix.
 - (1) A positive reaction produces clumping of the reagents with partial or total clearing of the organism suspension in 2 min or less.
 - (2) Test positive reactions of test isolates against the staphylococcus control reagent (reagent S) to verify specificity.
3. Phadebact GC monoclonal test

NOTE: The Phadebact GC monoclonal test is a coagglutination assay in which two pools of monoclonal antibodies directed against a gonococcus-specific membrane protein (protein I) are coupled to the protein A of non-viable staphylococci. When the monoclonal antibody reagent is mixed with a sample containing gonococci, a matrix is formed causing a visible agglutination reaction.

 - a. Prepare a slightly turbid suspension of cells in 0.5 ml of 0.9% saline from colonies presumptively identified as *N. gonorrhoeae*.
 - b. Heat the suspension in a boiling water bath for at least 5 min.
 - c. Cool the suspension to room temperature.
 - d. Place 1 drop of the gonococcal monoclonal reagent on a disposable slide.
 - e. Add 1 drop of the boiled colony suspension to the gonococcal reagent.
 - f. Mix the drops thoroughly but gently with a disposable loop.
 - g. Rock the slide and read the result within 1 min.
 - h. A positive and negative control should be included in each run.
 - i. A positive reaction is denoted by the appearance of visible agglutination.
4. GonoGen II

NOTE: GonoGen II is a monoclonal antibody-based colorimetric test developed for the confirmatory identification of *N. gonorrhoeae* from culture. The assay is based on the recognition of a major outer membrane protein (protein I) of the organism.

 - a. Label a 12- by 75-mm test tube for each isolate to be tested and for a positive and negative control.
 - b. Add 500 µl of GonoGen II solubilizing buffer to each tube and prepare a suspension of the test isolates equivalent to a McFarland no. 1 standard (Appendix 3.16–1).
 - c. Add 1 drop of the GonoGen II murine monoclonal antibody solution (antibody against protein I surface antigens of *N. gonorrhoeae*) to each tube, mix well, and incubate at room temperature for at least 5 min.
 - d. Transfer 2 drops of each test and control suspension into a separate test reaction well and allow the solution to absorb into the reaction well matrix.
 - e. A pink to red residual dot in the test well or a color reaction more intense than the negative control should be interpreted as a positive result.
5. BactiCard Neisseria

NOTE: BactiCard Neisseria relies on four chromogenic substrates impregnated in a test reaction card to detect preformed enzymes produced by pathogenic *Neisseria* species. Enzyme activity will presumptively differentiate *N. gonorrhoeae*, *N. meningitidis*, *N. lactamica*, and *M. catarrhalis* (26).

V. PROCEDURES (continued)

- a. Rehydrate each substrate test circle with 1 drop of BactiCard rehydrating fluid.
- b. From a pure culture 18 to 48 h old, growing on selective medium (not CHOC), smear several colonies of the isolate onto each substrate test circle with a wooden applicator stick.
- c. Incubate at room temperature for 2 min.
- d. Observe each test circle for the production of a blue-green color that indicates a positive test for enzyme activity or a pink to red color after addition of the color developer.
 - (1) *N. gonorrhoeae* is positive only for prolyl-iminopeptidase and negative for β -galactosidase, butyrate esterase, and δ -glutamyl-aminopeptidase (Table 3.9.3–1).
 - (2) Certain strains of *Kingella*, *Neisseria cinerea*, and *Neisseria subflava* could provide reactions consistent with *N. gonorrhoeae*. Care must be taken to only test isolates recovered from selective media that do not grow on nutrient agar (see Table 3.9.3–1).
6. Gonocheck II, NET and Neisseria Screen
 - **NOTE:** Gonocheck II or NET or Neisseria Screen relies on three chromogenic substrates in a test tube to detect preformed enzymes produced by pathogenic *Neisseria* species. Enzyme activity will presumptively differentiate *N. gonorrhoeae*, *N. meningitidis*, and *N. lactamica* (27).
 - a. Dispense 4 drops of reagent buffer into reagent tube.
 - b. Remove 5 to 10 colonies and emulsify into tube.
 - c. Incubate at 37°C for 30 min.
 - d. Yellow color is indicative of *N. meningitidis* (positive for δ -glutamyl-aminopeptidase), and blue color (positive for indolyl- β -D-galactosidase) is indicative of *N. lactamica* (Table 3.9.3–1). If the yellow color is faint, incubate for 60 min to confirm identification.
 - e. If there is no color change, remove white cap and recap with red cap (Gonocheck II). Invert to allow reagent in cap to mix with solution. (Alternatively, add reagent to the tube for Neisseria Screen and NET.) A red to pink color (positive for prolyl-iminopeptidase) when returned to the upright position indicates positivity for *N. gonorrhoeae*.
 - f. Certain strains of *Kingella*, *Neisseria cinerea*, and *Neisseria subflava* could provide reactions consistent with *N. gonorrhoeae*. Care must be taken to only test isolates recovered from selective media or from CHOC that do not grow on nutrient agar (see Table 3.9.3–1).
7. MicroTrak *N. gonorrhoeae* culture confirmation test
 - **NOTE:** The MicroTrak test uses a fluorescein-labeled monoclonal antibody in a fluorescent microscopy assay to positively identify presumptive isolates of *N. gonorrhoeae*.
 - a. Perform the assay on 18- to 24-h colonies. Subculture colonies older than 24 h before testing.
 - b. Place 5 μ l of distilled or deionized water in a 6-mm slide well.
 - c. Touch five colonies of the isolate to be tested with a loop and gently emulsify the cells into the drop of water to form an even suspension on the slide well. Prepare wells of known positive and negative controls in a similar fashion.
 - d. Allow the slide to air dry, and gently heat fix the smear.
 - e. Stain the slide with MicroTrak *N. gonorrhoeae* culture confirmation reagent (30 μ l per well) for 15 min at 37°C in a humidified chamber.
 - f. Remove excess reagent and rinse the slide for 5 to 10 s in a gentle stream of water. Shake off the excess water, and air dry the smear.
 - g. Add a drop of mounting fluid and place a coverslip on the well.

V. PROCEDURES (continued)

- h.** Read the slide using a fluorescent microscope with a $100\times$ oil objective. A positive test will demonstrate apple-green fluorescent diplococci along with appropriate reactions for the positive and negative control wells.

8. AccuProbe *N. gonorrhoeae* culture identification test

■ **NOTE:** The AccuProbe system uses a single-stranded DNA probe with a chemiluminescent label that recognizes an rRNA sequence of *N. gonorrhoeae*. Following the release of the target RNA, the DNA probe forms a stable DNA:RNA hybrid. A selection reagent inactivates unhybridized probe and the labeled DNA:RNA hybrids are detected in a luminometer.

- a.** Label a sufficient number of probe reagent tubes to test the number of culture isolates and a positive (*N. gonorrhoeae*) and negative (*N. meningitidis*) control.
- b.** Pipette 50 μl of lysis reagent into all tubes.
- c.** Transfer a 1- μl loopful of cells or several small colonies (<48 h old) to the tubes and twirl the loop to remove the cells.
- d.** Pipette 50 μl of hybridization buffer into all tubes, and mix by vortexing.
- e.** Incubate tubes for 15 min at 60°C in a water bath or dry-heat block.
- f.** Remove and cool the tubes. Pipette 300 μl of selection reagent to each tube and mix by vortexing.
- g.** Incubate tubes for 5 min at 60°C in a water bath or dry-heat block.
- h.** Cool the tubes to room temperature and read results in the luminometer within 30 min. Cutoff values for positive and negative signals are established by the manufacturer for the type of luminometer used.

■ **NOTE:** The performance of each confirmatory assay discussed above in terms of the definitive identification of *N. gonorrhoeae* varies considerably in the literature. For example, the reported sensitivity of the fluorescent MicroTrak *N. gonorrhoeae* culture confirmation test ranges from 76 to 100%, while the sensitivity of the AccuProbe *N. gonorrhoeae* culture identification test has consistently been found to be 100% (3, 5, 10, 17, 18, 26, 29).

Based on these findings, a *single negative result produced by any of the confirmatory tests does not rule out an identification of N. gonorrhoeae*. Repeat testing should be performed on a freshly subcultured isolate in conjunction with lack of growth on nutrient agar and/or carbohydrate utilization tests (see Table 3.9.3–1). Oxidase-positive, gram-negative diplococci that do not grow on nutrient agar but are repeatedly negative by any one assay or are uniformly negative for carbohydrate utilization should be referred to a reference laboratory for additional testing or evaluated by another identification method.

■ **NOTE:** Biochemical and/or enzymatic bacterial identification systems that contain *N. gonorrhoeae* in their database but have not been specifically designed for culture confirmation of *N. gonorrhoeae* (e.g., API NH [bioMérieux], IDS RapID NH panel [Remel, Inc.], BBL CRYSTAL *Neisseria/Haemophilus* ID kit [BD Diagnostic Systems], Vitek NHI card [bioMérieux], and Microscan HNID [Dade Behring]) are reviewed elsewhere (12) but can be useful adjuncts for this purpose.

POSTANALYTICAL CONSIDERATIONS

VI. REPORTING RESULTS

- A.** Report the Gram stain results as soon as possible, usually within 1 h of receipt (procedure 3.2.1).

- 1.** Interpret Gram stain for gram-negative intracellular diplococci.
- 2.** Notify physician if smear is positive.

■ **NOTE:** In contrast to almost all male urethral discharges with gram-negative diplococci, such morphotypes in vaginal, cervical, or other specimens are not diagnostic for *N. gonorrhoeae*.

VI. REPORTING RESULTS (continued)

- B. Refer to procedure 3.3.2 for general reporting guidelines.
- C. Report negative cultures as "No *Neisseria gonorrhoeae* organisms isolated." Additionally, a comment can be added as follows.
 1. "Specimen overgrown by normal microbiota. Please submit another specimen."
 2. "Specimen contaminated with yeast cells, which are inhibitory to *Neisseria gonorrhoeae*."
 3. "Specimen received 24 h after collection. Rates of recovery of *Neisseria gonorrhoeae* from specimens with delayed transport can be significantly reduced."
- D. Report positive cultures with probable genus and species as soon as preliminary tests are completed.
 1. Document notification to physician of positive findings.
 2. Generally report *N. gonorrhoeae* to the local health department.
 3. In cases involving children or the possibility of legal intervention, isolates of *N. gonorrhoeae* should be frozen for long-term retrieval.
- E. Antimicrobial susceptibility testing (AST)
 1. Do not perform routine AST of *N. gonorrhoeae*. Perform AST only in cases of treatment failure (8) and for antimicrobial agents for which universal susceptibility has not been established. Refer to CLSI guidelines (9) for special media and conditions of AST.
 2. Do not perform beta-lactamase test, because currently recommended therapies circumvent beta-lactamase production by *N. gonorrhoeae* (8).

VII. INTERPRETATION

- A. A positive culture indicates infection with the organism.
- B. Because of the labile nature of the organism, a negative culture does not rule out infection.

VIII. LIMITATIONS

- A. False-positive reports can result from misidentification of the organism.
- B. False-negative results can be caused by delayed or inappropriate transport.
- C. A single negative result produced by any of the confirmatory tests does not rule out an identification of *N. gonorrhoeae*. Further confirmatory testing should be performed.
- D. When carbohydrate degradation tests are used, a few strains of *N. gonorrhoeae* will be glucose negative and a few strains of *N. meningitidis* will be maltose negative. A second non-carbohydrate-based method should be used to identify these discrepant strains.
- E. Use reagent-grade carbohydrates if you make your own media to avoid glucose contamination in other carbohydrate reagents.
- F. The growth from a positive CTA glucose utilization test should be examined by Gram stain to ensure that the reaction is due only to *N. gonorrhoeae* and not a contaminant.
- G. More than one confirmatory method for identification of *N. gonorrhoeae* is essential for potential cases of child abuse, since nearly every method has some errors.
- H. Avoid use of candle jars, since superior products to generate CO₂ are available. If using candle jars, use only white wax candles. Colored candles yield toxic substances.
- I. *N. cinerea*, usually a saprophyte found in the pharynx, can be an occasional pathogen. It has been reported in cases of conjunctivitis, bacteremia, and peritonitis. The microorganism gives a positive glucose reaction in some rapid sys-

VIII. LIMITATIONS (continued)

tems and is also positive for hydroxyprolylaminopeptidase. Hence, this organism can be mistaken for *N. gonorrhoeae* unless additional testing is performed (6, 11). Further, differentiation of *N. cinerea* from glucose-negative *N. gonorrhoeae* can be difficult when using substrate-based identification kits (7). *N. cinerea* is, however, susceptible to colistin and thus will likely not be recovered on media containing this agent. In addition, most strains will grow well on nutritionally basic agar media such as TSA, MH agar, or nutrient agar.

- J.** Monoclonal antibodies might not react with all strains of *N. gonorrhoeae*, and false-positive monoclonal antibody reactions have been reported with *N. meningitidis* (20).

REFERENCES

- Arbique, J. C., K. R. Forward, and J. LeBlanc. 2000. Evaluation of four commercial transport media for the survival of *Neisseria gonorrhoeae*. *Diagn. Microbiol. Infect. Dis.* **36**:163–168.
- Arko, R. J., and T. Odugbemi. 1984. Superoxol and amylase inhibition tests for distinguishing gonococcal and nongonococcal cultures growing on selective media. *J. Clin. Microbiol.* **20**:1–4.
- Beebe, J. L., M. P. Rau, S. Flageolle, B. Calhoon, and J. S. Knapp. 1993. Incidence of *Neisseria gonorrhoeae* isolates negative by Syva direct fluorescent-antibody test but positive by Gen-Probe Accuprobe test in a sexually transmitted disease clinic population. *J. Clin. Microbiol.* **31**:2535–2537.
- Beverly, A., J. R. Bailey-Griffin, and J. R. Schwebke. 2000. InTray GC medium versus modified Thayer-Martin agar plates for diagnosis of gonorrhea from endocervical specimens. *J. Clin. Microbiol.* **38**:3825–3826.
- Boehm, D. M., M. Bernhardt, T. A. Kurzynski, D. R. Pennell, and R. F. Schell. 1990. Evaluation of two commercial procedures for rapid identification of *Neisseria gonorrhoeae* using a reference panel of antigenically diverse gonococci. *J. Clin. Microbiol.* **28**:2099–2100.
- Bourbeau, P., V. Holla, and S. Piemontese. 1990. Ophthalmia neonatorum caused by *Neisseria cinerea*. *J. Clin. Microbiol.* **28**:1640–1641.
- Boyce, J. M., and E. B. Mitchell, Jr. 1985. Difficulties in differentiating *Neisseria cinerea* from *Neisseria gonorrhoeae* in rapid systems used for identifying pathogenic *Neisseria* species. *J. Clin. Microbiol.* **22**:731–734.
- Centers for Disease Control and Prevention. 1998. Guidelines for treatment of sexually transmitted diseases. *Morb. Mortal. Wkly. Rep.* **47**(RR-1):70–73.
- Clinical and Laboratory Standards Institute. 2006. *Performance Standards for Antimicrobial Susceptibility Testing*. Sixteenth informational supplement. Approved standard M100-S16. Clinical and Laboratory Standards Institute, Wayne, Pa.
- Dillon, J. R., M. Carballo, and M. Pauze. 1988. Evaluation of eight methods for identification of pathogenic *Neisseria* species: Neisseria-Kwik, RIM-N, Gonobio-Test, Minitek, Gonochek II, GonoGen, Phadebact Monoclonal GC OMNI Test, and Syva MicroTrak Test. *J. Clin. Microbiol.* **26**:493–497.
- Dossett, J. H., P. C. Appelbaum, J. S. Knapp, and P. A. Totten. 1985. Proctitis associated with *Neisseria cinerea* misidentified as *Neisseria gonorrhoeae* in a child. *J. Clin. Microbiol.* **21**:575–577.
- Evangelista, A. T., A. L. Truant, and P. Bourbeau. 2002. Rapid systems and instruments for the identification of bacteria, p. 22–49. In A. L. Truant (ed.), *Manual of Commercial Methods in Microbiology*. ASM Press, Washington, D.C.
- Evans, G. L., D. L. Kopyta, and K. Crouse. 1989. New selective medium for the isolation of *Neisseria gonorrhoeae*. *J. Clin. Microbiol.* **27**:2471–2474.
- Farhat, S. E., M. Thibault, and R. Devlin. 2001. Efficacy of a swab transport system in maintaining viability of *Neisseria gonorrhoeae* and *Streptococcus pneumoniae*. *J. Clin. Microbiol.* **39**:2958–2960.
- Granato, P. A., J. L. Paepke, and L. B. Werner. 1980. Comparison of modified New York City medium with Martin-Lewis medium for recovery of *Neisseria gonorrhoeae* from clinical specimens. *J. Clin. Microbiol.* **12**:748–752.
- Greenwood, J. R., J. Voss, R. F. Smith, H. Wallace, C. Peter, M. Nachtigal, T. Maier, J. Wilber, and A. Butsumyo. 1986. Comparative evaluation of New York City and modified Thayer-Martin media for isolation of *Neisseria gonorrhoeae*. *J. Clin. Microbiol.* **24**:1111–1112.
- Janda, W. M., L. M. Wilcoski, K. L. Mandel, P. Ruther, and J. M. Stevens. 1993. Comparison of monoclonal antibody methods and a ribosomal ribonucleic acid probe test for *Neisseria gonorrhoeae* culture. *Eur. J. Clin. Microbiol. Infect. Dis.* **12**:177–184.
- Janda, W. M., and J. S. Knapp. 2003. *Neisseria* and *Moraxella catarrhalis*, p. 585–608. In P. R. Murray, E. J. Baron, J. H. Jorgensen, M. A. Pfaller, and R. H. Yolken (ed.), *Manual of Clinical Microbiology*, 8th ed. ASM Press, Washington, D.C.
- Jerris, R. C., and C. M. Black. 2002. *Chlamydia trachomatis*, p. 128–200. In A. L. Truant (ed.), *Manual of Commercial Methods in Microbiology*. ASM Press, Washington, D.C.

REFERENCES (continued)

20. Kellogg, J. A., and L. K. Orwig. 1995. Comparison of GonoGen, GonoGen II, and MicroTrak direct fluorescent antibody test with carbohydrate fermentation for confirmation of culture isolates of *Neisseria gonorrhoeae*. *J. Clin. Microbiol.* **33**:474–476.
21. Olsen, C. C., J. R. Schwebke, W. H. Benjamin, Jr., A. Beverly, and K. B. Waites. 1999. Comparison of direct inoculation and Copan transport systems for isolation of *Neisseria gonorrhoeae* from endocervical specimens. *J. Clin. Microbiol.* **37**:3583–3585.
22. Rechard, C. A., L. M. Rupkey, W. E. Brady, and E. W. Hook III. 1989. Comparison of GC-Lect and modified Thayer-Martin media for isolation of *Neisseria gonorrhoeae*. *J. Clin. Microbiol.* **27**:808–811.
23. Saginur, R., B. Clecner, J. Portnoy, and J. Mendelson. 1982. Superoxol (catalase) test for identification of *Neisseria gonorrhoeae*. *J. Clin. Microbiol.* **15**:475–477.
24. Sng, E. H., V. S. Rajan, K. L. Yeo, and A. J. Goh. 1982. The recovery of *Neisseria gonorrhoeae* from clinical specimens: effects of different temperatures, transport times, and media. *Sex. Transm. Dis.* **9**:74–78.
25. Thayer, J. D., and J. E. Martin. 1966. Improved medium selective for cultivation of *N. gonorrhoeae* and *N. meningitidis*. *Public Health Rep.* **81**:559–562.
26. Turner, A., K. R. Gough, and A. E. Jephcott. 1995. Comparison of three methods for culture confirmation of *Neisseria gonorrhoeae* strains currently circulating in the UK. *J. Clin. Pathol.* **48**:919–923.
27. Yajko, D. M., A. Chu, and W. K. Hadley. 1984. Rapid confirmatory identification of *Neisseria gonorrhoeae* with lectins and chromogenic substrates. *J. Clin. Microbiol.* **19**:380–382.
28. Yong, D. C. T., and A. Prytula. 1978. Rapid micro-carbohydrate test for confirmation of *Neisseria gonorrhoeae*. *J. Clin. Microbiol.* **8**:643–647.
29. Young, H., and A. Moyes. 1993. Comparative evaluation of AccuProbe culture identification test for *Neisseria gonorrhoeae* and other rapid methods. *J. Clin. Microbiol.* **31**:1996–1999.

SUPPLEMENTAL READING

- Centers for Disease Control and Prevention.** 2000. Gonorrhea—United States, 1998. *Morb. Mortal. Wkly. Rep.* **49**:538–542.
- Chapel, T. A., M. Smelter, and R. Dassel.** 1976. The effect of delaying incubation in a CO₂-enriched environment on gonococci. *Health Lab. Sci.* **13**:45–48.
- Evangelista, A., and H. Beilstein.** 1992. *Cumitech 4A, Laboratory Diagnosis of Gonorrhea*. Coordinating ed., C. Abramson. American Society for Microbiology, Washington, D.C.
- Evans, K. D., E. M. Peterson, J. I. Curry, J. R. Greenwood, and L. M. de la Maza.** 1986. Effect of holding temperature on isolation of *Neisseria gonorrhoeae*. *J. Clin. Microbiol.* **24**:1109–1110.
- Ingram, D. L., V. D. Everett, L. A. Flick, T. A. Russell, and S. T. White-Sims.** 1997. Vaginal gonococcal cultures in sexual abuse evaluations: evaluation of selective criteria for preteenaged girls. *Pediatrics* **99**:E8.
- Iwen, P. C., R. A. Walker, K. L. Warren, D. M. Kelly, J. Linder, and S. H. Hinrichs.** 1996. Effect of off-site transportation on detection of *Neisseria gonorrhoeae* in endocervical specimens. *Arch. Pathol. Lab. Med.* **120**:1019–1022.
- Koumans, E. H., R. E. Johnson, J. S. Knapp, and M. E. St. Louis.** 1998. Laboratory testing for *Neisseria gonorrhoeae* by recently introduced nonculture tests: a performance review with clinical and public health considerations. *Clin. Infect. Dis.* **27**:1171–1180.
- Palmer, H. M., H. Mallinson, R. L. Wood, and A. J. Herring.** 2003. Evaluation of the specificities of five DNA amplification methods for the detection of *Neisseria gonorrhoeae*. *J. Clin. Microbiol.* **41**:835–837.
- Ratner, J. B., H. Tinsley, R. E. Keller, and C. W. Stratton.** 1985. Comparison of the effect of refrigerated versus room temperature media on the isolation of *Neisseria gonorrhoeae* from genital specimens. *J. Clin. Microbiol.* **21**:127–128.
- Rosey, C. E., and E. M. Britt.** 1984. Urine as a holding medium for *Neisseria gonorrhoeae*. *Sex. Transm. Dis.* **11**:301–303.
- Shapiro, R. A., C. J. Schubert, and R. M. Siegel.** 1999. *Neisseria gonorrhoea* [sic] infections in girls younger than 12 years of age evaluated for vaginitis. *Pediatrics* **104**:e72.

3.9.4

Haemophilus ducreyi Cultures

[Updated March 2007]

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Haemophilus ducreyi is the etiologic agent of a sexually transmitted genital ulcer disease known as chancroid. The disease is common in Africa, Asia, and Latin America, but there are over 1,000 reported cases each year in the United States, mostly from the South and the southeastern region (12). Chancroid presents as an acute, localized infection with necrotizing ulcers. The soft chancre, which contains short streptococcal gram-negative rods, can

resemble the ulcers of herpes simplex or syphilis. Two lesions often appear together which are soft on palpation, having a deep ulcerative center (1). In most cases, the disease is diagnosed clinically, but reports of sensitivities using that method alone range from 40 to 80% (12). Ruling out syphilis and other genital infections can increase the sensitivity. Recently, it has been noted that human immunodeficiency virus infection prolongs the disease

and can modify its presentation. Gram stains from the leading edge of the ulcer can be helpful early in the infection before the ulcer is contaminated with other microbiota, but culture is the definitive method of diagnosis. Unfortunately the organism is labile and difficult to grow without specialized media. West et al. (13) and others (12) have used PCR methods to detect the organism.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING

NOTE: Refer to procedure 3.3.1 for additional details. The following can be copied for preparation of a specimen collection instruction manual for physicians and caregivers. It is the responsibility of the laboratory director or designee to educate physicians and caregivers on proper specimen collection.

A. Specimen collection (1)

Refer to procedure 3.9.1 for further details on specimen collection.

1. Prior to collection, obtain two different selective agar plates (*see item III.A*) from the laboratory, if they are available.
2. Clean the surface of the lesion with 0.85% NaCl.
 - a. If there is a crust on the lesion, remove it.
 - b. Moisten swab with saline and collect specimen by vigorously rubbing the base of the lesion.
 - c. Alternatively, scrape the base of the ulcer with a sterile scalpel blade.
 - (1) Irrigate with sterile saline.
 - (2) Then rub the base vigorously with a sterile swab, or aspirate fluid with a flamed smoothed Pasteur pipette or needle and syringe.
3. Abscess
NOTE: Intact bubo aspirates are rarely positive for the organism unless they have ruptured.
 - a. Disinfect skin with alcohol and iodine.
 - b. Aspirate fluid with a needle and syringe.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING (continued)

B. Specimen transport

1. Plates
 - a. Inoculate selective agar plates (see item III.A) with specimen.
 - b. Submit to laboratory immediately for incubation.
2. Transport medium
 - a. Inoculate Amies transport medium or a specially formulated THIO-hemin-based medium containing L-glutamine and albumin (3).
 - b. Place immediately on ice or in the refrigerator until and during transport.
3. Label specimens with the patient's demographic information and the site of collection. Attach requisition listing the patient's name, address, medical record number, sex, age, collection location, site of collection, specific test request, and physician of record.

C. Rejection criteria

1. *H. ducreyi* is quite labile. Culture should not be attempted unless special medium is available or specimens can be transported in suitable transport medium on ice to a laboratory with specialized media.
2. Prior antimicrobial treatment reduces the ability to isolate the organism (4).

III. MATERIALS

A. Media

Inoculate any two selective media plus CHOC.

1. Selective media

NOTE: Plates that are commercially available are from Remel, Inc., as CHOC with vancomycin and CHOC with fetal bovine serum and vancomycin. Other media listed below must be prepared in-house.

a. GC agar base with 1% Iso-VitaleX (BD Diagnostic Systems), 5% fetal bovine serum, 1% hemoglobin, and 3 µg of vancomycin per ml (3, 4)

b. GC agar base with 5% Fildes enrichment, 5% horse blood, and 3 µg of vancomycin per ml

c. 5% Fresh rabbit blood agar with 3 µg of vancomycin per ml (11)

d. Mueller-Hinton agar with 5% chocolatized horse blood, 1% IsoVitaleX, and 3 µg of vancomycin per ml (3, 4)

NOTE: Some isolates will grow on one medium but not others (12); some lots of fetal bovine serum are inhibitory. Use of two media and repeating cultures at 48 h increased the isolation rate to 92% for men with ulcers who had no

prior antimicrobial use or evidence of syphilis (5). Oberhofer and Black (8) demonstrated that Mueller-Hinton base was clearly superior to TSA base, although there was little difference between 5% sheep blood and supplemented CHOC. Vancomycin is used to inhibit gram-positive microbiota but may inhibit some strains of *H. ducreyi*.

2. Nonselective media: CHOC

B. Identification methods

1. Gram stain (procedure 3.2.1)
2. Catalase test (procedure 3.17.10)
3. Oxidase test (procedure 3.17.39). Do not use *N,N-dimethyl-p-phenylenediamine dihydrochloride*.
4. Indole test (procedure 3.17.23)
5. Aminolevulinic acid (ALA) test for porphyrin synthesis (procedure 3.17.3)
6. Sodium polyanethol sulfonate (SPS) disk (procedure 3.17.45) (Remel, Inc.)
7. Optional: one of the following
 - a. RapID NH (Remel, Inc.) (6)
 - b. RapID ANA (Remel, Inc.) (10)

C. Other supplies

1. Incubator at 33°C
2. CO₂-generating bag for culture incubation

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

- A. Verify that plate media meet expiration date and QC parameters per current Clinical and Laboratory Standards Institute (formerly NCCLS) document M22. See procedures 14 and 3.3.1 for further procedures.
- B. Perform QC with each lot of selective agar. Incubate aerobically overnight at 33°C in an airtight container with a CO₂-generating system.

Test organism	Result
<i>Haemophilus ducreyi</i> ATCC 33940	Growth in 48 h
<i>Staphylococcus epidermidis</i> ATCC 12228	Inhibition of growth if vancomycin in agar

V. PROCEDURE



Observe standard precautions.

A. Inoculation

- NOTE:** Use of biosafety cabinet will avoid contamination of the culture or specimen as well as protect laboratory processing personnel. If the specimen is not received on plates, inoculate plates from the swab in transport medium. Use CHOC without vancomycin and two selective media with vancomycin.

B. Direct smear

1. Prepare a Gram stain from the swab after plate inoculation.
 2. Stain slide (procedure 3.2.1) and observe for gram-negative coccobacilli arranged in short chains, clumps, or whorls with occasional “rail track” or “school of fish” arrangements (7).
- NOTE:** Direct exam has a sensitivity of about 60% (4).

C. Incubation

1. Incubate plates at 33 to 34°C in 5% CO₂, using a CO₂-generating system to provide the proper atmosphere and moisture. Keep plates moist using sterile-water-moistened filter paper.
- NOTE:** Recovery is substantially increased if the incubation is at 33°C rather than 35°C (9).
2. Observe for growth after at least 48 h of incubation. Hold negative plates for 5 days.

D. Culture examination

1. Observe plates for variable-size, smooth, dome-shaped colonies that are buff-yellow to gray. Whole colonies move across the agar when touched with a loop (1, 7). Colonies are often pinpoint at 48 h and increase to 2 mm in diameter.
 2. Perform Gram stain; colonies will clump in saline. Gram-negative coccobacilli should be identified further.
 3. Rapidly perform the following tests from a young culture and determine if the reactions agree with those listed below (7).
 - a. No growth on MAC
 - b. Catalase negative
 - c. Oxidase positive—reaction may be slow and require 15 to 20 s
 - d. Indole negative
 - e. ALA negative (no fluorescence or porphyrin production)
 4. If the above reactions are consistent with the identification, confirm the identification as follows.
 - a. Test with SPS on CHOC.
- NOTE:** *H. ducreyi* is susceptible, with a zone ≥12 mm; no other *Haemophilus* species are susceptible. The addition of 0.002% Tween 80 may aid in dispersion of the cells (10). *Capnocytophaga* is another genus of

V. PROCEDURE (continued)

- gram-negative coccobacillus that is reported to be SPS susceptible, but *Capnocytophaga* is ALA positive (10).
- b.** Optional: perform either the RapID NH (6) or the RapID ANA (10) for further confirmation using enzyme reactions. *H. ducreyi* is asaccharolytic.
 - c.** Submit to a reference laboratory if further confirmation is desired, especially until proficiency in the recognition of the colony morphology is determined.
 - E.** Hold positive culture plates for at least 7 days should antimicrobial susceptibility testing be needed. Since strains are usually beta-lactamase positive, there is no need to test for this enzyme (12).

POSTANALYTICAL CONSIDERATIONS**VI. REPORTING RESULTS**

- A.** If the culture is negative, report "No *Haemophilus ducreyi* isolated."
- B. Positive reporting**
 - 1.** *H. ducreyi* organisms are catalase-negative, oxidase-positive, gram-negative coccobacilli that are SPS sensitive and ALA negative.
 - 2.** Report the presence of *H. ducreyi* as soon as preliminary tests are completed.
 - 3.** Do not report any enumeration.
 - 4.** Generally report positive results to the physician and to the local health department.
 - 5.** Document notification of reporting to the physician and health department.

VII. INTERPRETATION

- A.** A positive culture indicates infection in a patient with an ulcerative lesion.
- B.** The CDC (2) recommends treating *H. ducreyi* with azithromycin, 1 g orally in a single dose; ceftriaxone, 250 mg intramuscularly in a single dose; or erythromycin base, 500 mg per os four times a day for 7 days.
- C.** Mixed infections with other agents known to cause ulcerative sexually transmitted diseases are not uncommon. The presence of *H. ducreyi* does not rule out these other infections, which should be considered in the evaluation of the patient.

VIII. LIMITATIONS

- A.** Methods that employ PCR for the detection of *H. ducreyi* are more rapid than culture techniques and may have an increased sensitivity (12).
- B.** False-negative cultures can result from prior antimicrobial therapy, strain growth variability, and sampling and transport techniques. Culture using one medium can have a sensitivity from 65 to 75% (12); using two media will increase the sensitivity to more than 84%.
- C.** False-negative reports can result from misreading the oxidase test as negative.
- D.** False-positive results can be caused by misinterpretation of the confirmatory tests.

REFERENCES

1. Baron, E. J., G. H. Cassell, D. A. Eschenbach, J. R. Greenwood, S. M. Harvey, N. E. Madinger, E. M. Paterson, and K. B. Waites. 1993. *Cumitech 17A, Laboratory Diagnosis of Female Genital Tract Infections*. Coordinating ed., E. J. Baron. American Society for Microbiology, Washington, D.C.
2. Centers for Disease Control and Prevention. 1998. 1998 guidelines for treatment of sexually transmitted diseases. *Morb. Mortal. Wkly. Rep.* **47**(RR-1):1–118.
3. Dangor, Y., F. Radebe, and R. C. Ballard. 1993. Transport media for *Haemophilus ducreyi*. *Sex. Transm. Dis.* **20**:5–9.
4. Dieng Sarr, A., N. C. Toure Kane, N. D. Samb, C. S. Boye, I. K. Diaw, G. Diouf, I. N'Doye, and S. M'Boup. 1994. Importance of culture media choice in the isolation of *Haemophilus ducreyi*. Experience in Senegal. *Bull. Soc. Pathol. Exot.* **87**:22–27. (In French.)

REFERENCES (continued)

5. Dylewski, J., H. Nsanze, G. Maitha, and A. Ronald. 1986. Laboratory diagnosis of *Haemophilus ducreyi*: sensitivity of culture media. *Diagn. Microbiol. Infect. Dis.* **4**:241–245.
6. Hannah, P., and J. R. Greenwood. 1982. Isolation and rapid identification of *Haemophilus ducreyi*. *J. Clin. Microbiol.* **16**:861–864.
7. Lubwama, S. W., F. A. Plummer, J. Ndinya-Achola, H. Nsanze, W. Namaara, L. J. D'Costa, and A. R. Ronald. 1986. Isolation and identification of *Haemophilus ducreyi* in a clinical laboratory. *J. Med. Microbiol.* **22**:175–178.
8. Oberhofer, T. R., and A. E. Black. 1982. Isolation and cultivation of *Haemophilus ducreyi*. *J. Clin. Microbiol.* **15**:625–629.
9. Schmid, G. P., Y. C. Faur, J. A. Valu, S. A. Sikandar, and M. M. McLaughlin. 1995. Enhanced recovery of *Haemophilus ducreyi* from clinical specimens by incubation at 33 versus 35°C. *J. Clin. Microbiol.* **33**:3257–3259.
10. Shawar, R., J. Sepulveda, and J. E. Claridge. 1990. Use of the RapID-ANA system and sodium polyanetholesulfonate disk susceptibility testing in identifying *Haemophilus ducreyi*. *J. Clin. Microbiol.* **28**:108–111.
11. Sottnek, F. O., J. W. Biddle, S. J. Kraus, R. E. Weaver, and J. A. Stewart. 1980. Isolation and identification of *Haemophilus ducreyi* in a clinical study. *J. Clin. Microbiol.* **12**:170–174.
12. Trees, D. L., and S. A. Morse. 1995. Chancroid and *Haemophilus ducreyi*: an update. *Clin. Microbiol. Rev.* **8**:357–375.
13. West, B., S. M. Wilson, J. Changalucha, S. Patel, P. Mayaud, R. C. Ballard, and D. Mabey. 1995. Simplified PCR for detection of *Haemophilus ducreyi* and diagnosis of chancroid. *J. Clin. Microbiol.* **33**:787–790.

SUPPLEMENTAL READING

- Albritton, W. L.** 1989. Biology of *Haemophilus ducreyi*. *Microbiol. Rev.* **53**:377–389.
- Dangor, Y., R. C. Ballard, S. D. Miller, and H. J. Koornhof.** 1990. Antimicrobial susceptibility of *Haemophilus ducreyi*. *Antimicrob. Agents Chemother.* **34**:1303–1307.
- Hammond, G. W.** 1996. A history of the detection of *Haemophilus ducreyi*, 1889–1979. *Sex. Transm. Dis.* **23**:93–96.
- Johnson, S. R., D. H. Martin, C. Cammarata, and S. A. Morse.** 1995. Alterations in sample preparation increase sensitivity of PCR assay for diagnosis of chancroid. *J. Clin. Microbiol.* **33**:1036–1038.
- Kilian, M.** 2003. *Haemophilus*, p. 623–635. In P. R. Murray, E. J. Baron, J. H. Jorgensen, M. A. Pfaffer, and R. H. Yolken (ed.), *Manual of Clinical Microbiology*, 8th ed. ASM Press, Washington, D.C.
- Lagergard, T., A. Frisk, and B. Trollfors.** 1996. Comparison of the Etest with agar dilution for antimicrobial susceptibility testing of *Haemophilus ducreyi*. *J. Antimicrob. Chemother.* **38**:849–852.
- Morse, S. A.** 1989. Chancroid and *Haemophilus ducreyi*. *Clin. Microbiol. Rev.* **2**:137–157.

3.10

Ocular Cultures

[Updated March 2007]

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Inflammatory eye conditions may be due to a variety of diseases, and microorganisms play a major role in both acute and chronic diseases (Table 3.10–1). The detection of infectious agents depends on knowledge of the site of infection and the severity of the process, because a variety of organisms cause infections of the eye.

Unlike the procedures with other specimen types, it may be important for the physician to inoculate culture media at the bedside rather than transport the specimen to the laboratory for processing. This procedure describes the clinical syndromes associated with bacterial infections of the eye, the organisms associated with these

syndromes, and the procedure for isolation of these infectious agents. In addition to aerobic bacterial culture, Table 3.10–2 indicates the media to inoculate for anaerobic, fungal, and mycobacterial cultures. Refer to the respective sections of the handbook for workup of the microorganisms that are not covered in this procedure.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING



Observe standard precautions.

■ **NOTE:** Most eye specimens are collected by an ophthalmologist. These specimens are inoculated onto culture media at the bedside, in the clinic or the physician's office. A variety of techniques are used to collect material from different parts of the eye. The conjunctiva is constantly contaminated by various bacteria from the environment and ocular adnexa. Therefore, specimens from the conjunctiva serve as a control when compared with specimens collected by more aggressive or invasive techniques. See section 2 for general principles of collection.

■ **NOTE:** Refer to procedure 3.3.1 for additional details on specimen collection. The following can be copied for preparation of a specimen collection instruction manual for physicians and other caregivers. It is the responsibility of the laboratory director or designee to educate physicians and caregivers on proper specimen collection.

- A. Provide fresh media to the clinical areas routinely collecting ocular cultures, and instruct physicians to immediately transport inoculated media and slides to the laboratory.
- B. Obtain viral and chlamydial samples before topical anesthetics are instilled.
 1. Obtain samples for chlamydial cultures with calcium alginate swabs.
 2. For viral cultures, use Dacron or cotton swabs with nonwood shafts.
- C. Collection by anatomic site

■ **NOTE:** *Specimen collection must be performed by a qualified physician.*

 1. Conjunctiva (bacterial conjunctivitis) and lid margin (if staphylococcal blepharoconjunctivitis is suspected)
 - a. Obtain the specimen with a sterile, premoistened cotton or calcium alginate swab.
 - b. Roll the calcium alginate or cotton swab over the conjunctiva before topical medications are applied.

Table 3.10–1 Clinical description of common ocular infections

Clinical syndrome	Location of infection	Predisposing factors	Clinical symptoms/signs	Specimen(s) collected
Conjunctivitis	Acute and chronic inflammation of the conjunctiva, the mucous membrane covering the sclera	Direct inoculation of organisms from fomites, hands, and the environment. Hematogenous spread may also occur but is rare.	Reddening of the conjunctival surface, increased tearing, and purulent discharge of one or both eyes	Swab of conjunctiva surface/pus
Dacryoadenitis/ dacryocystitis	Dacryoadenitis is infection of the lacrimal glands. Dacryocystitis is an infection of the lacrimal sac.	Obstruction of the nasolacrimal duct	Redness of the inner eyelid and corner of the eye with induration, pain, and increased tearing. Pus may be expressed from the lacrimal duct and a fistula may form.	Superficial wound cultures of the external lacrimal duct or pus
Canaliculitis	Inflammation of the canaliculus, the passage that connects the punctum to the lacrimal sac	Extension of dacryocystitis and dacryoadenitis	Swelling, pain, and tenderness at the corner of the eye. Patients may also have conjunctivitis and redness of the eyelid.	Superficial wound cultures of the external lacrimal duct or pus
Bacterial keratitis	Acute and chronic inflammation of the cornea. May be superficial infection of the corneal epithelium or deep stromal ulceration that may perforate and result in anterior chamber of deeper infection of the eye.	Prior ocular disease Contact lens wear Use of topical corticosteroids Burn patients Alcoholic patients Immunocompromised patients	Redness of the eye, inflammation of the conjunctiva, increased pain, decreased vision, and photophobia. Patients may feel like there is “sand” in the eye that results in increased tearing and formation of exudates. Bacterial keratitis requires prompt investigation and therapy to avoid permanent loss of vision.	Corneal scrapings
Bacterial endophthalmitis	Inflammation of the ocular cavities and intraocular tissue (uvea and retina)	Trauma or injury Surgery Corneal perforation due to keratitis Bacteremia with seeding of the eye	Postoperative infection usually manifest within 72 h after surgery. Endogenous infection on the eye occurs secondary to bacteremia. Endophthalmitis presents with pain, decreased vision, lid edema, conjunctival redness, severe iridocyclitis (uveitis), and hypopyon (pus in the eye). Endophthalmitis is a medical emergency that requires immediate surgical debridement, installation of intraocular antibiotics, and prompt treatment with systemic antibiotic therapy to minimize permanent loss of vision.	Aqueous and vitreous fluid is collected by aspiration.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING (continued)

- c. Culture both eyes with separate swabs.
- d. Immediately inoculate the material at the bedside onto BAP and CHOC as listed in Table 3.10–2.
- e. Inoculate the swab from the right conjunctiva in horizontal streaks, and inoculate the swab from the left conjunctiva in vertical streaks, each on one half of the same agar plate.
- f. Inoculate specimens from the right and left lid margins, if collected, by making an R and an L to represent the respective sites on another agar plate.

Table 3.10–1 (continued)

Clinical syndrome	Location of infection	Predisposing factors	Clinical symptoms/signs	Specimen(s) collected
Preseptal cellulitis	Inflammation of the periorbital soft tissues	Trauma or injury Extension of facial cellulitis	Periorbital cellulitis must be clinically differentiated from orbital cellulitis. Periorbital cellulitis presents with redness, induration, and pain in the eyelids and soft tissues surrounding one or both eyes. Vision is usually preserved, except that the patient may not be able to fully open the eye due to swelling. Conjunctival edema and redness may be present, but there is no chemosis, proptosis, or extraocular muscle palsy as occurs in orbital cellulitis.	Superficial wound culture Blood cultures
Orbital cellulitis	Infection of the deeper orbital tissues that may extend to the retro-orbital space and brain, resulting in cavernous sinus thrombosis and/or other intracranial infection	Trauma and injury Surgery Extension of sinus or paranasal infection Extension of endophthalmitis	Patients are systemically unwell, with fever and leukocytosis, and have gross periorbital redness, induration, and severe pain in the eye. Eyelid edema and chemosis of the conjunctiva are extensive, and patients may not be able to open the eye. Limited ocular motility occurs due to swelling, and extension to the retro-orbital space results in proptosis. Palsy of one or more extraocular muscle movements suggests extension to the brain. Orbital cellulitis is a medical emergency that requires immediate investigation for underlying cause and extent of infection, and prompt treatment with systemic antibiotic therapy to minimize permanent loss of vision.	Superficial wound culture Blood cultures Sinus aspirates

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING (continued)

- g. Obtain conjunctival scrapings for a smear preparation as follows.
 - (1) Instill 1 or 2 drops of proparacaine hydrochloride.
 - (2) Using a Kimura spatula, gently scrape across the lower right tarsal conjunctiva.
 - (3) Smear the material in a circular area 1 cm in diameter on a clean glass slide.
 - (4) Prepare at least two slides.
 - (5) Immerse the slides in 95% methyl alcohol or 100% methanol for 5 min.
 - (6) Repeat steps for the left conjunctiva.

Table 3.10–2 Handling of specimens for diagnosis of ocular infections

Clinical condition	Primary isolation media ^a	Primary pathogen(s)	Comments
Conjunctivitis	BAP CHOC MAC (if hospitalized patient) Fungal media ^c AFB media ^d Viral culture	<i>Haemophilus influenzae</i> <i>Staphylococcus aureus</i> <i>Streptococcus pneumoniae</i> <i>Streptococcus pyogenes</i> <i>Moraxella</i> spp. <i>Neisseria gonorrhoeae</i>	<i>Enterobacteriaceae</i> and <i>P. aeruginosa</i> may be important in hospitalized and/or immunocompromised patients. DFA ^b should also be done for <i>Chlamydia trachomatis</i> in neonates.
Keratitis	BAP CHOC MAC Fungal media ^c AFB media ^d Viral culture	Corneal trauma/ulcer <i>Pseudomonas aeruginosa</i> <i>S. aureus</i> <i>S. pneumoniae</i> Viridans group streptococci <i>Moraxella</i> spp. AFB—rapid growers <i>Nocardia</i> spp. Herpes simplex and herpes zoster viruses Contact lens associated: Gram-negative bacteria, including <i>P. aeruginosa</i> , <i>Serratia</i> spp. <i>Bacillus</i> spp. <i>Acanthamoeba</i> spp.	Keratitis is caused by a variety of organisms, depending upon the mechanism of corneal injury. Fungi, AFB, and <i>Nocardia</i> spp. should be ruled out in chronic infection. Corneal ulcers should have viral cultures, particularly for patients with trigeminal herpes zoster infection.
Endophthalmitis	BAP CHOC MAC Anaerobic BAP Fungal media ^c AFB media ^d Viral culture	Postsurgical or traumatic <i>S. aureus</i> Coagulase-negative staphylococci Viridans group streptococci <i>Bacillus</i> spp. <i>P. aeruginosa</i> <i>P. acnes</i> Anaerobes, including <i>Clostridium</i> spp. Bacteremia <i>S. aureus</i> <i>H. influenzae</i> <i>S. pneumoniae</i> <i>N. gonorrhoeae</i> <i>Neisseria meningitidis</i> Gram-negative bacteria, including <i>P. aeruginosa</i> Any other bacteria or yeasts/fungi causing bacteremia or fungemia	Endophthalmitis is caused by a variety of organisms, depending upon the mechanism of infection. Fungi, AFB, and <i>Nocardia</i> spp. should be ruled out in chronic postsurgical and traumatic infections. Viral cultures should be done, particularly for patients with trigeminal herpes zoster infection. The inner eye chambers may be seeded by any bacterial or fungal organism causing bacteremia or fungemia, so blood cultures should be obtained.
Periorbital cellulitis	BAP CHOC MAC (if secondary to trauma) Anaerobic BAP (if secondary to trauma)	<i>S. aureus</i> <i>S. pyogenes</i> <i>H. influenzae</i>	Localized soft tissue infection around the eye. <i>H. influenzae</i> may be recovered in infants and young children.
Orbital cellulitis	BAP CHOC MAC Anaerobic BAP Fungal media ^c	<i>S. aureus</i> <i>S. pneumoniae</i> Gram-negative bacilli, including <i>P. aeruginosa</i> <i>H. influenzae</i> <i>S. pyogenes</i> Anaerobes	Mixed aerobic and anaerobic infection occurs secondary to trauma or sinusitis. Blood cultures should be obtained. Fungi, including <i>Aspergillus</i> spp. and <i>Mucor</i> spp., may be important in diabetic or other immunocompromised patients.

^a Bacterial culture includes a Gram stain, fungal culture includes a calcofluor white stain, and AFB culture includes an auramine-rhodamine or Ziehl-Neelsen stain.

^b DFA, direct fluorescent antibody testing.

^c Fungal media may include IMA, BHI, or potato flake agar.

^d AFB media may include Lowenstein-Jensen or Middlebrook agar and broth culture.

**II. SPECIMEN COLLECTION,
TRANSPORT, AND HANDLING**
(continued)

2. Bacterial keratitis
 - a. Instill 1 or 2 drops of proparacaine hydrochloride.
 - b. Obtain conjunctival samples as described above, and then obtain corneal scrapings from the advancing edge of the ulcer by scraping multiple areas of ulceration and suppuration with a sterile Kimura spatula, using short, firm strokes in one direction. (Keep the eyelid open, and be careful not to touch the eyelashes.)
 - c. Obtain approximately three to five scrapings per cornea.
 - d. Inoculate each set of scrapings onto BAP and CHOC, using a C formation for each scraping.
 - e. Prepare smears by applying the scrapings in a gentle circular motion over a clean glass slide or by compressing material between two clean glass slides and pulling the slides apart (*see* Fig. 3.2.1–1).
3. Bacterial endophthalmitis
 - a. Collect an aspirate of the vitreous fluid or perform a paracentesis of the anterior chamber using a needle aspiration technique to collect intraocular fluid.
 - b. Collect specimens for conjunctival cultures along with the fluid to determine the significance of indigenous microbiota.
 - c. If a small volume of fluid is collected, inoculate cultures at the bedside by inoculating 1 or 2 drops of fluid onto culture media (Table 3.10–2).
 - d. Alternatively, submit in anaerobic transport tube or capped syringe after replacing the needle with a Luer-Lok.
4. Preseptal cellulitis
 - a. Cleanse the skin with alcohol and tincture of iodine or iodophor.
 - b. In the absence of an open wound, the physician makes a stab incision in either the upper or lower lid with a no. 11 Baird-Parker blade.
 - c. If there is an open wound, collect the purulent material with a syringe and needle.
 - d. Inoculate media (Table 3.10–2), and prepare slides as described above for conjunctivitis.
 - e. Inject some material into an anaerobic transport vial, and process specimens for anaerobes as described in section 4 of this handbook.
5. Orbital cellulitis
 - a. Obtain aspirate or biopsy sample of the wound, and process as described above for preseptal cellulitis. Additionally, inoculate fungal media or submit to the laboratory for inoculation (Table 3.10–2).
 - b. Collect blood cultures.
6. Dacryoadenitis
 - a. Collect a specimen of the purulent discharge by using a swab, as described above for conjunctivitis. Inoculate media (Table 3.10–2).
 - b. Do not perform a needle aspiration of the lacrimal gland.
7. Dacryocystitis
 - a. Obtain conjunctival cultures.
 - b. Press the lacrimal sac to remove exudate material for culture and smear, or collect exudate in a needle and syringe.
 - c. Place aspirated material in a transport vial, and transport to the laboratory.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING

(continued)

8. Canaliculitis
 - a. Compress the inner aspect of the eyelid to express pus.
 - b. Follow procedure outlined above for conjunctivitis.
 - c. Inoculate media (Table 3.10–2), and prepare slides as described above for conjunctivitis.
 - d. Additionally, inoculate fungal media or submit to the laboratory for inoculation (Table 3.10–2).
 - e. Inject some material into an anaerobic transport vial, and process specimens for anaerobes as described in section 4 of this handbook.
- D. Rejection criteria
 1. Request that a swabbed specimen of the conjunctiva accompany any specimen collected by invasive technique.
 2. Even in cases of suspected unilateral conjunctivitis, indicate that bilateral bacterial cultures are mandatory.
 3. When inoculated plates are delayed in transit, notify the physician that the culture may be compromised or contaminated.

III. MATERIALS

- | | |
|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| A. Media <ol style="list-style-type: none"> 1. BAP 2. CHOC 3. MAC 4. Anaerobic BAP (<i>see</i> section 4 for options) 5. THIO or other anaerobic broth for anaerobic culture 6. Media for fungal and mycobacterial cultures as listed in Table 3.10–2 (<i>see</i> sections 7 and 8) | B. Gram stain reagents and slides
C. Specimen collection devices <ol style="list-style-type: none"> 1. Kimura platinum spatula 2. Cotton, polyester, or calcium alginate swab 3. Needle and syringe 4. Aerobic swab transport system 5. Anaerobic transport system 6. Topical anesthetic—proparacaine hydrochloride (0.5%) |
|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

- A. Verify that media meet expiration date and QC parameters per current Clinical and Laboratory Standards Institute (formerly NCCLS) M22 document. See procedures 14 and 3.3.1 for further procedures, especially for in-house-prepared media.
- B. Swabbed specimens from the conjunctiva are used as a control along with other specimens collected by more aggressive or invasive techniques.
- C. Conjunctival cultures are used to determine the presence of indigenous microbiota.

V. PROCEDURE



Observe standard precautions.

A. Direct smears

1. Gram stain
 - a. Prepare two or three smears from the clinical material if glass slides do not accompany the specimen. If cheesy concentrations are present, crush them onto a slide for Gram stain.
 - b. Place slides in 95 or 100% methanol for 5 to 10 min to fix material.
 - c. Perform a Gram stain (*see* procedure 3.2.1).

V. PROCEDURE (continued)

- d. Examine the stained smear for the presence of somatic cells and extra- and intracellular organisms.
 - (1) The presence of PMNs suggests a bacterial infection.
 - (2) The presence of mononuclear cells may indicate viral conjunctivitis. Refer to section 10 of this handbook for detailed viral procedures.
 - (3) Refer to the Gram stain procedure (3.2.1) for details on interpretation of various morphologies observed on the smear.
 - (4) Refer to section 9 for examination for microsporidia.
- NOTE:** Pigment granules that resemble gram-positive cocci may be present on the Gram-stained smear. They can be differentiated from cocci because they are large, oval, and brown.

2. Giemsa stain

- a. For suspected chlamydial infections, prepare smears and examine them for intracytoplasmic inclusions.
- b. Alternatively, perform direct fluorescent-antibody test or culture for *Chlamydia* (procedures 10.6 and 10.7) or PACE 2 nucleic acid probe (Gen-Probe, Inc.) (procedure 12.2.2, part 1).

3. Calcofluor white stain

- a. Prepare a calcofluor white stain if fungi or *Acanthamoeba* cysts are suspected.
- b. For details, refer to procedure 9.3.8.

B. Culture inoculation, examination, and interpretation

1. Inoculate culture media according to Table 3.10-2, if plates are not received. Streak in quadrants for isolation of colonies.
 - a. If a scant specimen of intraocular fluid is submitted in a syringe, use broth to wash out the syringe by drawing up a small amount of broth.
 - b. Use the broth to inoculate plate media with 2 drops per plate.
 - c. Place the balance of the broth and specimen in broth culture tube. Avoid creating an aerosol.
 - d. For culture for *Acanthamoeba*, refer to procedure 9.9.2.
2. Incubate cultures at 35°C in 5 to 7% CO₂ for 72 h.
3. Refer to procedure 3.3.2 for instructions on incubation and examination of broth cultures. Hold broth cultures from invasively collected ocular specimens for 10 days (1, 3) to detect infections with *Propionibacterium acnes*.
4. Examine daily for the presence of microorganisms.
5. Estimate and report the number of each organism on each plate. The presence of moderate numbers of colonies or many colonies on one or more culture plates should indicate the bacterial etiology of the infection.
6. For quantitation of C streaks
 - a. 1+ : less than half of the C streaks are positive per plate
 - b. 2+ : more than half of the streaks, but not all, are positive
 - c. 3+ : all streaks are positive for bacteria
7. Identify the organisms by using the tests recommended in procedures 3.3.2, 3.18.1, and 3.18.2.

V. PROCEDURE (continued)

8. Consult the physician prior to identifying rare colonies of indigenous microflora from noninvasively collected specimens, including the following.
 - a. Coagulase-negative staphylococci
 - b. Diphtheroids
 - c. Viridans group streptococci
 - d. *Moraxella catarrhalis*
 - e. *P. acnes*
 - f. *Peptostreptococcus* spp.
9. Correlate culture with the Gram stain of the direct smear.

POSTANALYTICAL CONSIDERATIONS**VI. REPORTING RESULTS**

- A. Telephone positive reports from noninvasively collected specimens to the physician as soon as possible.
- B. Report the relative number and morphology of all microorganisms seen, the presence and numbers of somatic cells (especially PMNs), and whether the organisms were observed intracellularly as well as extracellularly.
- C. Report the quantity and organism identity for each morphological type observed on culture media.
- D. If indigenous microorganisms are present and it has been determined that they may be contaminants, include a comment such as "Possible contamination" and/or "Presence of indigenous conjunctival microbiota."

VII. INTERPRETATION

- A. All organisms grown in any amount from critical eye specimens (i.e., aqueous and vitreous fluid) should be identified and have AST results reported.
- B. All organisms present in the direct smear that grow on primary culture plates are considered clinically significant and should be worked up.
- C. The following criteria may assist the laboratory to determine the clinical significance of bacterial isolates from critical eye specimens that may otherwise be considered indigenous microbiota.
 1. Isolates of coagulase-negative staphylococci, diphtheroids, *P. acnes*, or viridans group streptococci that grow on more than one medium are generally considered significant.
 2. Indigenous microbiota isolates may also be clinically significant when growth occurs only on one medium or in broth. In these cases a comment may be added to the final report indicating that the clinical significance of organisms that are part of the skin indigenous microbiota must be determined by the clinical picture.

VIII. LIMITATIONS

- A. False-positive cultures can result from contamination of the specimen or the inoculated plates with skin microbiota.
- B. Conversely, false-negative reports can result from considering corynebacteria as contaminating microbiota when they can be pathogens. For example, *Corynebacterium macginleyi* has been implicated in conjunctivitis and corneal ulcers (2).
- C. False-negative results can occur if antimicrobial agents are given prior to collection of the specimens.
- D. Even with the best techniques, culture often fails to yield the infecting organism. Currently, use of DNA probes is being investigated as a more sensitive alternative to culture.

REFERENCES

1. Chern, K. C., D. M. Meisler, G. S. Hall, S. M. Myers, R. E. Foster, Z. N. Zakov, and C. Y. Lowder. 1996. Bacterial contamination of anaerobic vitreous cultures: using techniques employed for endophthalmitis. *Curr. Eye Res.* **15**:697–699.
2. Funke, G., M. Pagano-Niederer, and W. Bernauer. 1998. *Corynebacterium macginleyi* has to date been isolated exclusively from conjunctival swabs. *J. Clin. Microbiol.* **36**:3670–3673.
3. Hall, G. S., K. Pratt-Rippin, D. M. Meisler, J. A. Washington, T. J. Roussel, and D. Miller. 1994. Growth curve for *Propionibacterium acnes*. *Curr. Eye Res.* **13**:465–466.

SUPPLEMENTAL READING

- Armstrong, R. A.** 2000. The microbiology of the eye. *Ophthalmic Physiol. Opt.* **20**:429–441.
- Baum, J.** 1995. Infections of the eye. *Clin. Infect. Dis.* **21**:479–486.
- Brady, S. E., E. J. Cohen, and D. H. Fischer.** 1988. Diagnosis and treatment of chronic post-operative bacterial endophthalmitis. *Ophthalmic Surg.* **19**:590–594.
- Brook, I.** 1980. Anaerobic and aerobic flora of acute conjunctivitis in children. *Arch. Ophthalmol.* **98**:833–835.
- Brook, I.** 1988. Presence of anaerobic bacteria in conjunctivitis associated with wearing contact lenses. *Ann. Ophthalmol.* **20**:397–399.
- Donzis, P. B., B. J. Mondino, and B. A. Weissman.** 1988. Bacillus keratitis associated with contaminated contact lens care systems. *Am. J. Ophthalmol.* **105**:195–197.
- Friedlaender, M. H.** 1995. A review of the causes and treatment of bacterial and allergic conjunctivitis. *Clin. Ther.* **17**:800–810.
- Israele, V., and J. D. Nelson.** 1987. Periorbital and orbital cellulitis. *Pediatr. Infect. Dis.* **6**:404–410.
- Joondeph, B. C., H. W. Flynn, D. Miller, and H. C. Joondeph.** 1989. A new culture method for infectious endophthalmitis. *Arch. Ophthalmol.* **107**:1334–1337.
- Kinnear, F. B., and C. M. Kirkness.** 1995. Advances in rapid laboratory diagnosis of infectious endophthalmitis. *J. Hosp. Infect.* **30**(Suppl):253–261.
- Klotz, S. A., C. C. Penn, G. J. Negesky, and S. I. Butrus.** 2000. Fungal and parasitic infections of the eye. *Clin. Microbiol. Rev.* **13**:662–685.
- Kresloff, M. S., A. A. Castellarin, and M. A. Zarbin.** 1998. Endophthalmitis. *Surv. Ophthalmol.* **43**:193–224.
- Mandell, G. L., R. G. Douglas, and J. E. Bennett** (ed.). 1990. *Principles and Practice of Infectious Diseases*, 3rd ed. Churchill Livingstone, New York, N.Y.
- McNatt, J., S. D. Allen, L. A. Wilson, and V. R. Dowell.** 1978. Anaerobic flora of the normal human conjunctival sac. *Arch. Ophthalmol.* **96**:1448–1450.
- Okhravi, N., P. Adamson, and S. Lightman.** 2000. Use of PCR in endophthalmitis. *Ocul. Immunol. Inflamm.* **8**:189–200.
- Perkins, R. E., R. B. Knudsen, M. V. Pratt, I. Abrahamsen, and H. B. Leibowitz.** 1975. Bacteriology of normal and infected conjunctiva. *J. Clin. Microbiol.* **1**:147–149.
- Smith, R. E., and J. R. Nobe.** 1989. Eye infections, p. 213–232. In S. M. Finegold and W. L. George (ed.), *Anaerobic Infections in Humans*. Academic Press, Inc., New York, N.Y.
- Smolin, G., K. Tabbara, and J. Witcher.** 1984. *Infectious Diseases of the Eye*. The Williams & Wilkins Co., Baltimore, Md.
- Weissgold, D. J., and D. J. D'Amico.** 1996. Rare causes of endophthalmitis. *Int. Ophthalmol. Clin.* **36**:163–177.
- Wilhelmus, K. R., T. J. Liesegang, M. S. Osato, and D. B. Jones.** 1994. *Cumitech 13A, Laboratory Diagnosis of Ocular Infections*. Coordinating ed., S. C. Specter. American Society for Microbiology, Washington, D.C.

3.11

RESPIRATORY TRACT CULTURES

3.11.1

Guidelines for Performance of Respiratory Tract Cultures

[Updated March 2007]

Specimens from the upper respiratory tract (throat specimens, nasopharyngeal swabs, nasal discharges) can be easily obtained but are contaminated with resident microbiota. In addition, many microorganisms present in the nares and throat are found in both the disease and the carrier states (2, 3, 4). It is estimated that 60% of children sporadically carry *Streptococcus pneumoniae* in their nasal passages by the age of 2 years (2). Because of this contamination, these specimens often do not provide accurate, clinically useful information for diagnosis of bacterial respiratory infection caused by organisms such as *S. pneumoniae*, *Haemophilus influenzae*, and *Moraxella catarrhalis*. On the other hand, these specimens are useful for the diagnosis of specific pathogens, whose presence in symptomatic patients most often indicates disease (i.e., *Streptococcus pyogenes*, *Bordetella pertussis*, *Corynebacterium diphtheriae*, and respiratory viruses). Nasal cultures are also performed as a part of the infection control of hospitalized patients to detect carriage of oxacillin-resistant *Staphylococcus aureus* or as part of a staphylococcal outbreak. In the latter case, nasal carriage by hospital employees may also be important (procedure 13.17). However, culture of nasopharyn-

geal specimens to detect carriage of potential pathogens such as *Neisseria meningitidis*, *S. pneumoniae*, and *H. influenzae* should be discouraged. Since these pathogens are all part of the normal oropharyngeal flora, the clinical relevance of culturing them from this site cannot be determined. In addition, reporting of normal oropharyngeal flora from this site may result in the patient receiving an unnecessary course of antibiotic therapy, thus fostering the development of resistance. Antibiotic prophylaxis of individuals in close contact with a patient with meningocephemia should be directed by the CDC guidelines, and should not be withheld based on the nasopharyngeal culture result (1). Likewise, nasopharyngeal specimen cultures for yeast or mold colonization in otherwise healthy asymptomatic individuals should be discouraged for similar reasons. Nasopharyngeal cultures for the detection of either *Aspergillus* spp., *Mucor* spp., or other fungi may be warranted for immunocompromised patients who are suspected of having an invasive infection.

Thus, a request for “routine” bacterial culture of noninvasively collected nasal or throat specimens, usually submitted to the laboratory on swabs or as a wash, aspirate,

or discharge, should *not* be processed by the laboratory (refer to cystic fibrosis procedure [3.11.3] for exception for deep pharyngeal cultures from these patients). It is important that these cultures be done only when detection of a specific pathogen is sought and not be performed routinely to detect any organism that is present. When requests are received by the laboratory, the caregiver should be contacted to determine if the specimen was sent for detection of one of the specific pathogens (Table 3.11.1–1). If the specimen is being sent to diagnose a bacterial cause of lower respiratory disease, otitis media, or sinusitis, inform the caregiver that a more invasive specimen, such as endotracheal aspirate, maxillary sinus puncture, or tympanocentesis fluid, respectively, is needed to make the diagnosis (see Table 3.11.1–1 for specific specimens and procedural references). Procedures that follow in this portion present collection, processing, and interpretation to detect individual respiratory pathogens and procedures for bacterial etiology of upper and lower respiratory disease from specific anatomic sites. A procedure is also listed specifically for processing specimens from cystic fibrosis patients. Refer to Table 3.11.1–1 for the appropriate specimens and procedures.

REFERENCES

1. Bilukha, O. O., and N. Rosenstein. 2005. Prevention and control of meningococcal disease. Recommendations of the Advisory Committee on Immunization Practices (ACIP). *Morb. Mortal. Wkly. Rep.* **54**(RR-7):1–21.
2. Dagan, R., R. Melamed, M. Mualem, L. Piglansky, and P. Yagupsky. 1996. Nasopharyngeal colonization in southern Israel with antibiotic-resistant pneumococci during the first 2 years of life: relation to serotypes likely to be included in pneumococcal conjugate vaccines. *J. Infect. Dis.* **174**:1352–1355.
3. Faden, H., M. Heimerl, G. Goodman, P. Winkelstein, and C. Varma. 2002. New technique (the NOW test) for rapid detection of *Streptococcus pneumoniae* in the nasopharynx. *J. Clin. Microbiol.* **40**:4748–4749.
4. Robinson, D. A., K. M. Edwards, K. B. Waites, D. E. Briles, M. J. Crain, and S. K. Hollingshead. 2001. Clones of *Streptococcus pneumoniae* isolated from nasopharyngeal carriage and invasive disease in young children in central Tennessee. *J. Infect. Dis.* **183**:1501–1507.

3.11.1.1

Table 3.11.1-1 Appropriate specimens for diagnosis of bacterial and yeast upper and lower respiratory diseases

Clinical condition	Primary pathogen(s)	Specimens	Procedure reference(s)
Candidiasis (oral thrush)	<i>Candida albicans</i>	Swab of buccal mucosa, tongue, or oro-pharynx	8.3 (KOH, calcofluor white stain)
Cystic fibrosis	<i>Pseudomonas aeruginosa</i> <i>S. aureus</i> <i>Burkholderia cepacia</i> <i>Aspergillus</i> spp.	Deep throat (young children) Lower respiratory ^a	3.11.3
Diphtheria	<i>C. diphtheriae</i>	Nasopharyngeal swab ^b	3.11.7
Epiglottitis	<i>H. influenzae</i>	Blood culture	3.4.1
Esophagitis ^c	<i>C. albicans</i> Herpes simplex virus Cytomegalovirus	Esophageal biopsy samples should be sent for yeast and viral culture as well as histopathology	8.3, 8.4
Bacterial pharyngitis	Group A streptococcus (<i>S. pyogenes</i>) <i>N. gonorrhoeae</i>	Throat swab (use charcoal if for <i>Neisseria gonorrhoeae</i> culture)	3.9.3
Laryngitis, bronchitis	<i>Mycoplasma pneumoniae</i> Respiratory viruses	Lower respiratory ^a	3.15
Lemierre's disease	<i>Fusobacterium necrophorum</i>	Nasopharyngeal swab for viruses	
Otitis externa	<i>S. aureus</i> <i>S. pyogenes</i> <i>P. aeruginosa</i>	Blood culture Swab of inner ear canal	3.4.1 3.11.5
Otitis media	<i>S. pneumoniae</i> <i>H. influenzae</i> <i>M. catarrhalis</i> <i>S. pyogenes</i> <i>Alloiococcus otitis</i>	Swab of myringotomy tube drainage Tympanocentesis fluid	3.11.5
Pertussis	<i>B. pertussis</i>	Nasopharyngeal swab or aspirate ^b	3.11.6
Chronic bronchitis	<i>S. pneumoniae</i> <i>S. aureus</i> , including MRSA ^d <i>H. influenzae</i> Gram-negative bacilli Respiratory viruses	Lower respiratory ^a	3.11.2
Pneumonia	Community-acquired pneumonia <i>S. pneumoniae</i> <i>S. aureus</i> , including MRSA <i>H. influenzae</i> <i>Klebsiella pneumoniae</i> Anaerobes (if aspiration) <i>Pasteurella</i> spp. <i>Legionella</i> spp. <i>M. pneumoniae</i> <i>Chlamydia pneumoniae</i> <i>Chlamydia psittaci</i> Respiratory viruses <i>Pneumocystis carinii</i> (PCP ^e) (HIV ^f risk factors or seropositive) Agents of bioterrorism, including <i>Bacillus anthracis</i> , <i>Brucella</i> spp., <i>Francisella tularensis</i> , <i>Yersinia pestis</i> , and <i>Burkholderia pseudomallei</i> <i>Mycobacterium tuberculosis</i>	Lower respiratory ^a	3.11.2, 3.15, 3.11.4

(continued)

Table 3.11.1–1 Appropriate specimens for diagnosis of bacterial and yeast upper and lower respiratory diseases (*continued*)

Clinical condition	Primary pathogen(s)	Specimens	Procedure reference(s)
Pneumonia (<i>cont.</i>)	Hospital-acquired pneumonia, including VAP ^g <i>S. aureus</i> Gram-negative bacilli, including <i>P. aeruginosa</i> and other nonfermenters Anaerobes (if aspiration) Immunocompromised patient Primary pathogen(s) listed above Unusual bacteria such as <i>Rhodococcus equi</i> <i>P. carinii</i> (PCP) Fungi AFB ^h Viruses	Lower respiratory ^a	3.11.2, 3.15, 3.11.4
Empyema	<i>S. aureus</i> <i>S. pyogenes</i> <i>S. pneumoniae</i> Anaerobes <i>M. tuberculosis</i>	Pleural fluid	3.5
Lung abscess	<i>S. aureus</i> <i>S. pyogenes</i> <i>K. pneumoniae</i> <i>P. aeruginosa</i> Anaerobes	Lung aspirate or biopsy sample	3.5, 3.11.2, 3.13.1
Sinusitis	Acute <i>S. aureus</i> <i>S. pneumoniae</i> <i>H. influenzae</i> <i>M. catarrhalis</i> Chronic Agents of acute sinusitis Gram-negative bacilli, including <i>P. aeruginosa</i> Anaerobes	Sinus aspirate	3.11.9
Vincent's angina	<i>Borrelia vincentii</i> (spirochetes) Anaerobes (fusiform rods)	Swab of gingiva	3.2.1 (Gram stain) or 7.2 (Ziehl-Neelsen stain but dilute 1:10 with water)
Staphylococcal carriage	<i>S. aureus</i> , including MRSA	Swab of nares	13.11

^a Lower respiratory includes sputa, endotracheal tube aspirates, auger suction samples, lung aspirates, bronchial washings, brochoalveolar lavage specimens, protected brush specimens, and lung biopsy specimens. Procedures for detection of respiratory viruses (section 10), *M. tuberculosis* (section 7), aerobic *Actinomyces* (section 6), and fungi, such as *Histoplasma capsulatum* and *Coccidioides immitis* (section 8), should be included, depending on the patient's exposure history and clinical situation and condition.

^b Specimen is also used to diagnose infection caused by respiratory viruses (section 10).

^c Herpes simplex virus and cytomegalovirus are important causes, and biopsy samples should be sent for histopathology and virology testing.

^d MRSA, methicillin-resistant *S. aureus*.

^e PCP, *P. carinii* pneumonia.

^f HIV, human immunodeficiency virus.

^g VAP, ventilator-associated pneumonia.

^h AFB, acid-fast bacilli.

3.11.2

Lower Respiratory Tract Cultures

[Updated March 2007]

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Infections of the lower respiratory tract are the sixth leading cause of mortality in the United States, with 2,000,000 to 3,000,000 cases per year, resulting in 500,000 hospitalizations (1). For community-acquired pneumonia (CAP) in adults and the elderly, *Streptococcus pneumoniae* is the cause in 6 to 10% of all cases and 60% of the bacterial cases (4, 7, 8), hence the utility of the pneumococcal vaccine to prevent this disease.

Culture of lower respiratory secretions can be helpful, but in reality it is limited, with no agent isolated in 40 to 60% of cases (1). This lack of culture sensitivity may be due to the low sensitivity (50%) of sputum culture for *S. pneumoniae*, especially if specimens are not immediately processed. In addition, many agents of pneumonia are difficult to grow (e.g., *Legionella*, *Chlamydia pneumoniae*, and *Mycoplasma pneumoniae*). It is estimated that *C. pneumoniae* is the second most common cause of pneumonia and that *M. pneumoniae* accounts for most cases of ambulatory CAP identified by serologic methods (7, 15). *Haemophilus influenzae* and *Legionella* are the third and fourth most common bacterial causes of CAP requiring hospitalization. In addition to the difficulty in culturing the pathogens, specimens are easily contaminated with upper respiratory secretions, which may obscure the presence of lower respiratory pathogens.

Because of the difficulty in detection of the pathogen and the delay that is inherent in culture, early guidelines for diagnosis and treatment of CAP suggested that culture was of very limited value. More recent guidelines of the Infectious Disease

Society of America (IDSA) (1), the American Thoracic Society (ATS) (13), and the Canadian Infectious Disease Society (7) now recommend culture in limited situations, but each society has different criteria for culture. Many guidelines include a blood culture to detect pneumococcal pneumonia; 8 to 10% of patients with CAP will have positive blood cultures, with 60% of these containing *S. pneumoniae* (7). Generally respiratory culture and Gram stain can be helpful when the patient is ill enough to be admitted to the hospital, according to the IDSA. The ATS limits cultures to those where drug resistance is suspected or an unusual pathogen is being considered (e.g., areas where dimorphic fungi, mycobacteria, or *Legionella* is endemic). If pleural fluid is present, thoracentesis should also be performed. Appropriate antimicrobial therapy is necessary to decrease mortality; however, the overuse of antimicrobial agents is a cause for concern for the development of antimicrobial resistance (3). Each of the guidelines recommends specific empiric therapy based on the severity of illness and disease presentation.

The sputum Gram stain has variability in sensitivity and specificity, depending on the specimen and the skill of the reader (7, 12, 13, 16). A great variability and a lack of reproducibility on repeat smear preparations were shown by Nagendra et al. (12); however, there was less variability for culture of the specimens. Generally smears are only of value if there are numerous inflammatory cells and a preponderance of gram-positive diplococci (57% sensitivity) or gram-negative rods suggestive of *Haemophilus* (82% sensitivity)

(16). The specificity of the diagnosis by Gram stain can be increased if the Quellung test is performed on the direct specimen (7). The proper evaluation of the specimen by Gram stain is critical and is used to ensure that only appropriate specimens are processed. Refer to Appendix 3.2.1–2 of the Gram stain procedure for acceptability criteria.

Nosocomial pneumonia can be caused by a large number of pathogens, including anaerobes, as in aspiration pneumonia (8). The CDC has issued guidelines for the prevention of nosocomial pneumonia (2). *Staphylococcus aureus* and gram-negative rods are common causes of ventilator-associated pneumonia (VAP), particularly in patients that have had a prolonged hospital course. Patients with severe CAP, immunocompromised patients with progressive pneumonia, or those with other types of nosocomial pneumonia (e.g., postoperative or mediastinal hypostatic) may also develop respiratory failure and require ventilator support. Diagnosis of the microbial pathogens causing VAP requires pulmonologists or intensivists to perform a bronchoalveolar lavage (BAL) for collection of samples from the alveoli of the lung, including protected brush specimens (PBS) for anaerobe culture. Quantitative bacterial culture of BAL samples has a sensitivity of 82 to 91% provided that a threshold of $\geq 10^4$ CFU/ml for primary pathogen identification is used (9). BAL samples from immunocompromised patients may also be tested for *Pneumocystis jiroveci* (formerly *P. carinii*) pneumonia (PCP; now PJB), fungal culture, acid-fast bacillus (AFB) culture, *Legionella* culture, and respiratory viruses. Culture of endotra-

cheal tube (e.g., bronchial secretions and sputa) samples from ventilated patients may be clinically misleading due to colonization of the bronchus by organisms that

have not been inoculated into the deeper lung. Primary bacterial pathogens of nosocomial pneumonia are usually resistant to one or more commonly used antibiotic

agents, so microbiology culture results are essential for directing appropriate therapy for this serious infection.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING



Observe standard precautions.

■ **NOTE:** Refer to procedure 3.3.1 for additional details. The following can be copied for preparation of a specimen collection instruction manual for physicians and caregivers. It is the responsibility of the laboratory director or designee to educate physicians and caregivers on proper specimen collection.

A. Collection of sputa, ET tube aspirates, and auger suction specimens

1. Expectorated sputum

- a. Do *not* have the patient rinse mouth and gargle with nonsterile water prior to sputum collection, since this can introduce contaminating microbiota.

■ **NOTE:** Commensal mycobacteria from tap water can contaminate mycobacterial cultures but are rarely an issue for routine bacteriology culturing. For specialized cultures (e.g., mycobacteria and legionellae), supply sterile saline or water to gargle prior to collection.

- b. Instruct the patient not to expectorate saliva or postnasal discharge into the container.
- c. Collect expectorated sputum specimens into a sterile container with a screw cap that is tightly secured prior to transport.
- d. The specimen should have a label that provides the sample type (i.e., ET tube aspirate, auger suction versus expectorated or induced sputa), date and time of collection, and the same patient demographic information as that provided on the requisition.

2. Induced sputum

■ **NOTE:** Induced sputa should only be tested for the presence of *Mycobacterium tuberculosis* and other *Mycobacterium* spp. (1). Although PJP (formerly PCP) testing may also be performed by request of the physician, this test should only be done on induced sputa from human immunodeficiency virus (HIV)-infected/AIDS patients. PJP sputum testing is not diagnostic for other types of immunocompromised patients in whom there is a much lower burden of cysts in the alveoli.

- a. Using a wet toothbrush and *sterile* water or saline, brush the buccal mucosa, tongue, and gums for 5 to 10 min prior to the procedure. Do not use toothpaste.
- b. Rinse the patient's mouth thoroughly with *sterile* water or saline.
- c. Using an ultrasonic nebulizer, have the patient inhale approximately 20 to 30 ml of 3% NaCl.
- d. Collect induced sputum specimens into a sterile container with a screw cap that is tightly secured prior to transport.
- e. The specimen should have a label that provides the sample type (i.e., ET tube aspirate, auger suction versus expectorated or induced sputa), date and time of collection, and the same patient demographic information as that provided on the requisition.

3. ET tube aspirates and auger suction specimen

- a. Aspirate the specimen into a sterile sputum trap (i.e., Luken trap) and aseptically transfer the sputum sample to a sterile screw-cap container with the cap tightly secured before transport. Luken traps frequently leak during transport, which contaminates the sample.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING

(continued)

- b. Neonates, infants, and small children cannot provide an expectorated sputum sample, so a sample is collected from the upper airway using an auger. Frequently a very small amount of secretions or sputum is obtained by this method, so the sample may be submitted in the catheter in a sterile container.
 - c. The specimen should have a label that provides the sample type (i.e., ET tube aspirate, auger suction versus expectorated or induced sputa), date and time of collection, and the same patient demographic information as that provided on the requisition.
 - B. Collection of lower lung samples by bronchoscopy—performed by pulmonologists, intensivists, or other trained physicians. Major clinical contraindications to bronchoscopy include severe hypoxemia, severe hypocapnia, and thrombocytopenia or other coagulopathy due to the risk of bleeding into the lung.
 - 1. Bronchoscopy specimens include BAL samples, bronchial washings, PBS, and transbronchial biopsy specimens.
 - 2. BAL specimens and PBS may be cultured semiquantitatively or quantitatively for bacterial pathogens. BAL samples and PBS should be quantitatively cultured if they are from an intensive care unit (ICU) patient suspected of having severe pneumonia, particularly VAP (*see Appendix 3.11.2–1 for quantitative methods*).
 - 3. Precautions
 - a. To avoid excess blood in the recovered fluid, obtain bronchial wash and BAL specimens before brushing or biopsy specimens. Blood may alter the concentration of cellular and noncellular components.
 - b. Avoid suctioning through the working channel before retrieval of specimens to avoid contamination of the specimens.
 - c. Avoid the injection of topical anesthetic agents as much as possible, as the injection method may lead to contamination of the specimen. Aerosol application of anesthetic agents is preferred.
 - 4. Bronchoscopy procedures
 - **NOTE:** There may be variability in the way individual physicians or groups of physicians perform a bronchoscopy and collect samples from various locations in the lung. A standard procedure should be developed by physicians collecting bronchoscopy samples in consultation with the laboratory so that an adequate amount of sample is collected for the tests ordered, and the fractions are clearly labeled according to their lung locations and order of collection.
 - a. Bronchial wash samples
 - **NOTE:** Bronchial wash samples are collected from the major bronchi, including the main bronchus, at the bifurcation and the right and left bronchi depending on the extent of disease. This procedure is most commonly done to diagnose cancer in patients who have bronchial lesions or masses. Diagnostic tests for infection may be done concomitantly to rule out infection, particularly if there is bronchial obstruction and pneumonia secondary to this complication.
 - (1) Pass the bronchoscope transnasally or transorally in nonintubated patients or via the ET tube in intubated patients.
 - (2) Inject sterile nonbacteriostatic 0.85% NaCl (generally 20- to 30-ml aliquots in adults and 5-ml aliquots in children) from a syringe through a biopsy channel of the bronchoscope.
 - (3) Gently suction the recovered bronchial wash specimen into a sterile container (e.g., sterile suction trap on the bronchoscope). Due to the smaller volumes of fluid injected into the bronchus during collection of a bronchial wash sample, a smaller sample is received by the laboratory than during a BAL collection.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING *(continued)*

- (4) Bronchial wash samples are frequently split between the cytopathology and microbiology laboratories.
- (5) Bronchial wash samples from different lung locations (e.g., right and left bronchi) should not be pooled.

b. Transbronchial biopsy samples

Lung biopsy samples may be obtained during bronchoscopy by inserting a forcep on the biopsy channel of the bronchoscope. Individual samples are immediately placed into a sterile container with nonbacteriostatic saline for microbiological testing. Additional samples may be placed into a sterile container with formalin for histopathological testing, or into a sterile container with RPMI 1640 for flow cytometry analysis.

c. BAL

■ NOTE: The laboratory will not be able to distinguish a BAL from a bronchial wash based on the appearance of the container or the fluid in the container. BAL samples are collected from the distal bronchioles and alveoli after the bronchoscope has been wedged into the distal airway lumen in one or more lung segments (e.g., RUL, RML, RLL, LUL, and LLL) depending on the extent of the infection.

- (1) Pass the bronchoscope transnasally or transorally in nonintubated patients or via the ET tube in intubated patients.
- (2) Collect BAL samples by carefully wedging the tip of the bronchoscope into an airway lumen and instilling as large a volume of 0.85% nonbacteriostatic saline as possible per aliquot (i.e., 25 to 30 ml for adults and 5 to 10 ml for children). Several aliquots may be used in the same lung location to wash the alveoli in order to collect enough fluid to perform all of the required tests. Each BAL sample returned from separate lavage of the area contains secretions distal to the bronchioles and alveoli.
- (3) Gently suction the recovered specimen into a sterile container before administering the next aliquot. (In general, 50 to 75% of the saline instilled is recovered in the lavage effluent.) Keep aliquots separate during collection.
- (4) Discard the initial fluid as contaminated and submit the rest for culture and staining.
■ NOTE: In the laboratory, aliquots from the same site may be combined for microbiology cultures and smears, but aliquots from separate sites (for example, right upper lobe and right lower lobe) should be combined only after consultation with the physician of record.

d. PBS

- (1) Instill a brush to collect cellular material from the airway wall. This is the best specimen for viral culture and cytology studies.
- (2) Only a PBS is acceptable for anaerobic bacterial culture. Obtain by inserting a telescoping double catheter plugged with polyethylene glycol at the distal end (to prevent contamination of the bronchial brush) through the biopsy channel of the bronchoscope.
- (3) Place the PBS in a sterile vial containing 1 ml of nonbacteriostatic saline.

5. Lung aspirates

Lung aspiration is performed by a pulmonologist or interventional radiologist. Pulmonary lesions or abscess cavities that are accessible to aspiration are located in the periphery of the lung. This procedure must be performed with caution since a pneumothorax and/or bronchocutaneous fistula may be created. The presence of thrombocytopenia or a coagulopathy are contraindications to the procedure.

**II. SPECIMEN COLLECTION,
TRANSPORT, AND HANDLING**
(continued)

- a. A computed tomography scan is used to direct the aspiration needle through the chest wall directly into peripheral lung lesion or abscess cavity.

- b. Material is aspirated from the lesion or cavity.

- c. Multiple samples may be collected if the lesion is large or there are multiple lesions or cavities that are peripherally accessible.

- d. Fluid or pus obtained by aspiration should be immediately transferred to a sterile container and promptly transported to the laboratory. If anaerobic culture is required, a portion of the sample should also be placed into a sterile tube containing prerduced anaerobically sterilized media.

■ NOTE: Syringes with the needle attached are not acceptable for culture due to the sharp and biohazard risk to laboratory staff. Syringes with a capped Luer-Lok are also not acceptable because the sample may be contaminated if the sample leaks during transport or during handling of the sample by the laboratory (e.g., the sample may congeal in the syringe during transport and be very difficult or impossible to retrieve without contamination).

6. OLB samples

Open lung biopsy (OLB) samples are collected by thoracic surgeons during an operative procedure. Patients undergoing this procedure must be clinically able to withstand an operative procedure with a minimal risk of bleeding.

- a. One or more pieces of tissue are collected from one or more lung segments depending on the extent of the infection. OLB pieces of 0.5 to 1.0 cm square are harvested. Larger biopsy samples increase the risk of pulmonary hemorrhage.

- b. Multiple OLB pieces may be collected from a large area of disease in a particular lung segment, or individual OLB pieces may be harvested from multiple lesions or diseased lung areas.

- c. OLB pieces should be immediately placed into a sterile container with nonbacteriostatic saline for microbiological testing. Individual OLB pieces should be placed into a sterile container with formalin for histopathological analysis.

7. Pleural fluid—see procedure 3.5**C. Transport of sputa, endotracheal aspirates, and auger suction specimens**

1. Since the samples are not placed into buffered transport media, it is important that they be received and handled by the laboratory as soon after collection as possible.

2. Store sputum specimens at 2 to 8°C until samples can be transported or processed by the laboratory.

■ NOTE: A delay in processing of more than 2 h may result in a decreased ability to recover fastidious pathogens, such as *S. pneumoniae* and *H. influenzae*, and/or overgrowth of upper respiratory microbiota (7).

3. Sputum samples should be placed into biohazard bags prior to transport to minimize contamination of the external environment if leakage occurs during transport.

D. Transport of bronchoscopy specimens

1. Bronchial wash specimens, BAL specimens, PBS, lung aspirates, and OLB specimens should be immediately transported and handled by the laboratory as soon as possible after collection, since saline may be toxic to the recovery of some primary pathogens.

2. Store specimens at 2 to 8°C until samples can be transported or processed by the laboratory.

■ NOTE: A delay in processing of more than 1 to 2 h may result in a decreased ability to recover fastidious pathogens, such as *S. pneumoniae* and *H. influenzae*, and/or overgrowth of upper respiratory microbiota (7).

**II. SPECIMEN COLLECTION,
TRANSPORT, AND HANDLING**
(continued)

3. Bronchial wash specimens, BAL specimens, lung aspirates, and OLB specimens should be placed into biohazard bags prior to transport to minimize contamination of the external environment if leakage occurs during transport.
4. For bronchial wash, BAL, and OLB specimens, the microbiology laboratory should assess whether enough sample has been collected to perform all of the requested tests. Also ensure that samples have been sent to cytopathology and histopathology if ordered.
5. Bronchial wash, BAL, and OLB specimens are acceptable for testing for other pneumonia pathogens, including *Legionella*, viruses, fungi, and mycobacteria.
6. BAL and OLB specimens are also acceptable for testing for PJP and mycoplasmas.
7. If insufficient sample has been collected to perform all of the microbiology tests ordered on a bronchial wash, BAL, or OLB specimen, then the laboratory must contact the physician to establish the testing priority. Tests should then be done according to the priority list established with the physician (i.e., start with the highest-priority test and distribute the sample in descending order of clinical priority so that as many tests as possible for the amount of submitted sample are performed).

E. Rejection criteria for sputum and endotracheal aspirate specimens

1. Reject duplicate specimens received on the same day unless the initial sample was inadequate for culture according to microscopic evaluation.
2. Do not accept repeat cultures at intervals of less than every 48 h.
3. Reject the following specimens for diagnosis of lower respiratory tract disease.
 - a. 24-h sputum collection
 - b. Contaminated sputum and endotracheal specimens per Gram stain rejection criteria (Appendix 3.2.1–2)
 - c. Specimens that are visually saliva only
 - d. Specimens that are visibly contaminated by toothpaste or other substances
 - e. Nasal washes and aspirates or swabs of nares (see Table 3.11.1–1 and procedure 3.11.9 for appropriate specimens to diagnose sinusitis)
 - f. Throat specimens, since they are not diagnostic of lower respiratory tract infection (6)
 - g. Anaerobic culture should not be done of sputum samples because they are highly contaminated with normal anaerobic flora of the upper respiratory tract.

F. Rejection criteria for bronchial wash specimens, BAL specimens, lung aspirates, and OLB specimens

1. Bronchial wash specimens, BAL specimens, lung aspirates, and OLB specimens should never be rejected by the laboratory, since the patient has undergone an invasive procedure in order to have them collected.
2. For specimens delayed in transit more than 2 h without refrigeration, indicate on the report that the delay in transit may compromise the culture results.
3. Bronchial wash samples should not be cultured quantitatively; only a semi-quantitative bacterial culture result is required.
4. BAL samples should be cultured quantitatively for bacterial pathogens from patients with suspected VAP.
5. PBS is the preferred sample for anaerobic culture, since both bronchial wash and BAL samples and transbronchial biopsy samples are contaminated by respiratory anaerobic microbiota.
6. Anaerobic culture should be performed on lung aspirates, pleural fluid, and OLB specimens by request or when the original specimen Gram stain demonstrates morphotypes suggestive of anaerobic infection.

III. MATERIALS

- A. Direct tests**
1. Gram stain (procedure 3.2.1)
 2. *S. pneumoniae* antisera (stored at 4°C). See Quellung procedure (3.17.42).
 - a. Methylene blue—0.3% in water
 - b. Normal rabbit serum
 3. NOW urinary antigen assay (Binax, Inc., Portland, Maine) (10, 11)
- B. Primary media**
1. BAP
 2. CHOC or horse blood agar (HBA), or HBA with 20,000 IU of bacitracin per liter (HBAB)
 3. MAC or EMB
- C. Identification methods**
- Refer to procedure 3.3.2 for supplies for identification of the common agents of pneumonia.
- D. Other supplies/equipment**
1. Incubator at 35°C with 5% CO₂ or other CO₂-generating system
 2. Inoculating sticks and loops
 3. Petri dishes and filter paper
 4. Bacitracin (10-IU disk) and optochin disks (optional)

ANALYTICAL CONSIDERATIONS**IV. QUALITY CONTROL**

- A.** Verify that media meet expiration date and QC parameters per current Clinical and Laboratory Standards Institute (CLSI; formerly NCCLS) M22 document. See procedures 14.2 and 3.3.1 for further procedures, especially for in-house-prepared media.
- B.** Test each lot of CHOC according to procedure 3.3.1.
- C.** QC HBA with bacitracin as indicated below; without bacitracin, test only with *H. influenzae*. Incubate at 35°C in 5% CO₂.

Test organism	Result
<i>Haemophilus influenzae</i> ATCC 10211	Growth
<i>Staphylococcus epidermidis</i> ATCC 12228	Partial to complete inhibition

- D.** If used on direct specimen plates, perform QC on each lot or shipment of bacitracin (10-IU disk) and optochin disks. Use CHOC for bacitracin and BAP for optochin. See procedure 3.17.38 for optochin QC. For bacitracin QC, incubate CHOC at 35°C in CO₂.
- NOTE:** This is *not* the bacitracin disk historically used to detect group A beta-hemolytic streptococci.

Test organism	Bacitracin (10 IU) zone size
<i>Haemophilus influenzae</i> ATCC 10211	No zone
<i>Staphylococcus epidermidis</i> ATCC 12228	>12 mm

V. PROCEDURE

It is imperative that these cultures be handled in a biosafety hood.

- A. Processing and culture examination of sputa, auger suction, and ET aspirate specimens**
- 1. Inoculation and incubation**
 - a. Process all specimens as soon after receipt as possible in order to maintain the viability of primary pathogen(s).
 - b. Select the most purulent or blood-tinged portion of the specimen to microscopically examine.
 - c. Perform a Gram stain of the specimen as soon after receipt as possible to microscopically assess cellular and bacterial components.
 - d. For sputum samples collected from adults and older children, if the specimen is inadequate for culture based on microscopic criteria (*see* procedure 3.2.1, including Appendix 3.2.1–2 for details about the preparation

V. PROCEDURE (continued)

and reading of smears), reject the sample and immediately phone the physician or nursing unit to collect another sample.

- e. Perform a Gram stain on auger suction and endotracheal aspirates. Although these types of specimens are analyzed like sputa, microscopic criteria for specimen adequacy have not been established.
 - f. If there is a predominance of gram-positive diplococci, perform either a direct bile test (procedure 3.17.6) or a direct Quellung test (procedure 3.17.42).
 - g. Process microscopically adequate sputum, auger suction, and endotracheal aspirate specimens in a BSL II cabinet, since aerosols can result in laboratory-acquired infections.
 - h. Using a sterile swab, stick, or pipette, inoculate specimen to BAP, CHOC (or HBA or HBAB), and MAC (or EMB). The following biochemical tests may also be performed at inoculation.
 - (1) Add a 10-U bacitracin disk (not Taxo A) to CHOC and HBA to inhibit upper respiratory microbiota and improve detection of *H. influenzae*.
 - (2) Add staphylococcal spots using *S. aureus* ATCC 25923 to BAP to demonstrate satelliting of *Haemophilus* on direct plates.
 - (3) Add an optochin disk to the second quadrant of BAP to demonstrate inhibition by *S. pneumoniae* for direct detection on primary plates.
 - **NOTE:** Adding an optochin disk is not recommended routinely but may be helpful for laboratories with a large number of specimens from patients with CAP.
 - i. Incubate plates at 35 to 37°C in 5% CO₂ for a minimum of 48 h; 72 h is preferred.
2. Culture examination (see procedure 3.3.2 for identification tests and procedure 3.17 for biochemical procedures; additional identification charts are in procedures 3.18.1 and 3.18.2)
 - a. Observe plates at 24 h.
 - b. Incubate plates for an additional 24 to 48 h, which is useful to detect slow-growing, fastidious gram-negative rods, such as *Bordetella* spp. or molds such as *Aspergillus* spp.
 - c. Even if there was growth at 24 h, plates should be examined again at 48 h in order to detect additional isolates not found at 24 h.
 - d. The original specimen Gram stain should be used as a guide to interpret the culture.
 - (1) Primary pathogen(s) grown in culture should be present in the original specimen Gram smear.
 - (2) If one or more isolates grow on culture that were not reported from the original Gram stain, then the smear should be read a second time by a different person than the one who initially read the slide.
 - (3) Follow Table 3.11.2-1 for reporting bacterial morphotypes from the Gram stain that may be primary pathogens.
 - e. Identify the organisms that grow in significant amounts, defined as bacterial morphotypes that are potential primary pathogens that may or not be part of the normal respiratory microbiota. Table 3.11.2-1 provides guidelines for the reporting of primary pathogens from these types of specimens. Significant growth is defined as follows.
 - (1) Moderate to heavy growth of an isolate in the second or greater quadrant of the plate
 - (2) Small amounts of a primary pathogen in the culture that are consistent with the predominant morphotype seen in the original specimen Gram stain associated with PMNs

Table 3.11.2-1 Guidelines for reporting of primary pathogens for lower respiratory cultures

Organism	Amount (semiquantitative) ^a	Amount (CFU/ml) (quantitative PBS/BAL) ^b	Action	Comments
May be part of normal microbiota				
Group A streptococcus, <i>S. pyogenes</i>	Any	Any	Identification/no AST	AST should be performed for the penicillin-allergic patient due to the increasing resistance to erythromycin and clindamycin; D-test should be done to detect inducible resistance.
<i>S. pneumoniae</i>	≤Normal microbiota ^c	<10 ³	No identification/report normal microbiota	AST should be done to detect resistance to penicillin and erythromycin. Levaquin-resistant strains have also been reported.
	Moderate to heavy and >normal microbiota	≥10 ³ –10 ⁴	Identification/AST	
<i>S. aureus</i>	≤Normal microbiota	<10 ³	Rule out MRSA ^d /report normal microbiota if no MRSA	MRSA should be ruled out. Increasing disease due to invasive community-acquired MRSA.
	Moderate to heavy and >normal microbiota	≥10 ³ –10 ⁴	Identification/AST	
<i>M. catarrhalis</i>	≤Normal microbiota	<10 ³	No identification/report normal microbiota	AST should be performed if strain is beta-lactamase negative.
	Moderate to heavy and >normal microbiota	≥10 ³ –10 ⁴	Identification/AST	
<i>H. influenzae</i>	≤Normal microbiota	<10 ³	No identification/report normal microbiota	Type in neonates, infants, and small children.
	Moderate to heavy and >normal microbiota	≥10 ³ –10 ⁴	Identification/AST/beta-lactamase	
<i>N. meningitidis</i>	≤Normal microbiota	<10 ³	No identification/report normal microbiota	Type on request.
	Moderate to heavy and >normal microbiota	≥10 ³ –10 ⁴	Identification/AST/beta-lactamase	
Group B, C, and G streptococci	≤Normal microbiota	<10 ³	No identification/report normal microbiota	AST should be performed for the penicillin-allergic patient due to the increasing resistance to erythromycin and clindamycin; D-test should be done to detect inducible resistance.
	Moderate to heavy and >normal microbiota	≥10 ³ –10 ⁴	Identification/no AST	
Other normal microbiota ^c	NA ^e	<10 ³ ≥10 ³ –10 ⁴	No identification Identification/AST of organism present in pure growth	Reporting of normal microbiota from lower respiratory cultures may lead to inappropriate antimicrobial treatment. Coagulase-negative staphylococci, enterococci, and other normal microbiota should only be reported from BAL or PBS cultures if there is a heavy amount or pure growth.
Anaerobes	NA	PBS only <10 ³ ≥10 ³ –10 ⁴	Limited identification to list anaerobes present Identification/AST	Only PBS are acceptable for anaerobe culture. Anaerobes are an important cause of aspiration or obstructive pneumonia.

(continued)

Table 3.11.2–1 Guidelines for reporting of primary pathogens for lower respiratory cultures (*continued*)

Organism	Amount (semiquantitative) ^a	Amount (CFU/ml) (quantitative PBS/BAL) ^b	Action	Comments
Bioterrorism agents				
<i>Brucella</i> spp.	Any	Any	Limited identification according to CDC algorithm. Cultures should be handled in a BSL II cabinet.	Immediate referral for further analysis (<i>see</i> section 16)
<i>F. tularensis</i>	Any	Any	Limited identification according to CDC algorithm. Cultures should be handled in a BSL II cabinet.	Immediate referral for further analysis (<i>see</i> section 16)
<i>Y. pestis</i>	Any	Any	Limited identification according to CDC algorithm. Cultures should be handled in a BSL II cabinet.	Immediate referral for further analysis (<i>see</i> section 16)
<i>Bacillus anthracis</i>	Any	Any	Limited identification according to CDC algorithm. Cultures should be handled in a BSL II cabinet.	Immediate referral for further analysis (<i>see</i> section 16)
Gram-negative bacilli				
<i>Pasteurella</i> spp.	Moderate to heavy and >normal microbiota	<10 ³ ≥10 ³ –10 ⁴	Identification/AST	Patients usually have contact with animals, particularly horses
<i>Enterobacteriaceae</i>	One or two types ≤Normal microbiota Moderate to heavy and >normal microbiota	<10 ³ ≥10 ³ –10 ⁴	No identification/AST unless ICU and/or immunocompromised patient Identification/AST	<i>Klebsiella</i> pneumonia more common in alcoholics; colonization of the upper airway with enterics more common in patients in hospitals. AST should be done to rule out ESBL-producing or <i>ampC</i> -carrying strains.
<i>P. aeruginosa</i>	Any	Any	Identification/AST	<i>P. aeruginosa</i> pneumonia has a high mortality rate. AST should be done to rule out antimicrobial resistance, including screening for metallo-beta-lactamase-producing strains in hospitalized patients.
Other nonfermenters (<i>Bordetella bronchiseptica</i> , <i>Stenotrophomonas maltophilia</i> , <i>Acinetobacter</i> spp., <i>B. cepacia</i> , etc.)	≤Normal microbiota Moderate to heavy and >normal microbiota	<10 ³ ≥10 ³ –10 ⁴	Identification/AST	<i>B. bronchiseptica</i> is an unusual cause of CAP. Other nonfermenters are important causes of nosocomial pneumonia. These organisms may also be transmitted in the hospital, and infection control should be notified about positive cultures. <i>B. cepacia</i> is mainly found in CF ^e patients (<i>see</i> procedure 3.11.3).

(continued)

Table 3.11.2-1 (continued)

Organism	Amount (semiquantitative) ^a	Amount (CFU/ml) (quantitative PBS/BAL) ^b	Action	Comments
Mixed gram-negative bacilli, >2 morphotypes	≤Normal microbiota Moderate to heavy and >normal microbiota	<10 ³ ≥10 ³ –10 ⁴	No identification/AST Limited identification unless patient is in ICU or immunocompromised	List all types of gram-negative bacilli present (e.g., “Growth of mixed gram-negative bacilli, including 2 lactose fermenters and a non-lactose fermenter that is not <i>P. aeruginosa</i> ”). Include a comment that the plates will be held if further analysis is clinically required.
Gram-positive bacilli				
<i>Corynebacterium</i> spp.	≤Normal microbiota Moderate to heavy and >normal microbiota pure growth	<10 ³ ≥10 ³ –10 ⁴	No identification/report normal microbiota Identification/AST	Urea-positive strains such as <i>C. pseudodiphtheriticum</i> may cause pneumonia.
<i>R. equi</i>	Any	Any	Identification/AST	Important cause of CAP in immunocompromised patients, particularly those with HIV/AIDS. Patients may have contact with horses.
Aerobic actinomycetes (<i>Nocardia</i> spp., <i>Streptomyces</i>)	Any	Any	Identification/AST	Important cause of pneumonia in immunocompromised patients
Yeasts				
<i>Cryptococcus neoformans</i>	Any	Any	Identification	May cause pneumonia in otherwise healthy and immunocompromised patients. Isolates should be subtyped to rule out <i>C. neoformans</i> subtype <i>gattii</i> .
Fungi				
Molds not considered saprophytic contaminants (e.g., <i>Aspergillus</i> spp., <i>Mucor</i> spp., agents of systemic mycoses)	Any	Any	Identification	Important causes of pneumonia in immunocompromised patients. CF patients may be colonized with <i>Aspergillus</i> spp. (see procedure 3.11.3).

^a The original specimen Gram stain should be correlated with the bacterial morphotypes grown on culture. Significant growth of a primary pathogen is defined as moderate to heavy growth into the second or greater quadrant of the plate that is more than the background normal microbiota. Smaller amounts of a primary pathogen may be clinically important if it is an almost pure growth (i.e., little or no growth of normal microbiota) and the bacteria are consistent with the morphology seen in the Gram stain associated with PMNs.

^b Counts of ≥10³ CFU/ml are considered significant for quantitative PBS cultures, and counts of ≥10⁴ CFU/ml are considered significant for BAL specimens.

^c Normal respiratory tract microbiota is defined as viridans group streptococci, commensal *Neisseria* spp., *N. meningitidis*, diphtheroids, coagulase-negative staphylococci, *Rothia*, group F streptococci, anaerobes, *Haemophilus* spp. (not *H. influenzae*), enterococci, *Candida* spp., *Eikenella*, *Actinobacillus*, *Capnocytophaga*, and *Moraxella*.

^d MRSA, methicillin-resistant *S. aureus*.

^e NA, not applicable.

^f ESBL, extended-spectrum beta-lactamase.

^g CF, cystic fibrosis.

V. PROCEDURE (continued)

(3) Colonies in the first quadrant of the plate provided there is little or no other normal respiratory microbiota on the plate (i.e., pure growth of a primary pathogen) and the Gram smear shows purulence (i.e., PMNs).

f. Subculture to BAP and/or CHOC to obtain isolated colonies for accurate identification from mixed cultures.

B. Processing and culture examination of bronchial wash and BAL specimens**1. Inoculation and incubation**

a. Process all bronchial wash and BAL specimens immediately after receipt in order to maintain the viability of primary pathogen(s).

b. Process bronchial wash and BAL specimens in a BSL II cabinet, since aerosols can result in laboratory-acquired infections.

c. Perform a Gram stain of the specimen as soon after receipt as possible to microscopically assess cellular and bacterial components. See procedure 3.2.1, including Appendix 3.2.1–2, for details on preparation and reading of smears. Use a cytocentrifuge to prepare BAL specimens for Gram stain.

d. Bronchial wash specimens should be inoculated and incubated using the same methods as for sputum specimens (see items V.A.1.a to V.A.1.g). Semiquantitative bacterial culture results may be reported for primary pathogens.

e. There are two methods for performing quantitative cultures on BAL samples: the serial dilution method and the calibrated loop method (1, 2). After incubation, the colonies are counted and multiplied by the appropriate dilution factor to determine the number of bacteria present per milliliter of fluid (see Appendix 3.11.2–1).

f. Identify organisms present in significant amounts in quantitative BAL culture (defined as $\geq 10^4$ CFU/ml) and in quantitative PBS (defined as $\geq 10^3$ CFU/ml) of a primary pathogen. See Table 3.11.2–1 for guidelines for reporting primary pathogens from these types of specimens.

g. The original Gram stain result should be used to guide further analysis of BAL and PBS cultures. A primary pathogen isolated in smaller amounts as pure growth on the primary plates should also be identified if a consistent bacterial morphotype was found in the Gram stain.

C. Processing and culture examination of other invasive specimens from the lower respiratory tract

1. See procedure 3.13 for processing and bacterial culture of lung aspirates.

2. See procedure 3.13 for processing and bacterial culture of lung tissue (e.g., transbronchial biopsy and OLB specimens).

3. See procedure 3.5 for processing and bacterial culture of pleural fluid.

D. Identification of primary pathogens of lower respiratory tract infection

The following species are important respiratory pathogens. See Table 3.3.2–5 for rapid identification methods.

1. *Streptococcus* species

a. Examine for beta-hemolytic colonies and identify catalase-negative cocci in chains and pairs.

(1) Use pyrrolidonyl- β -naphthylamide (PYR) test to identify *S. pyogenes*, which is reported in any amount.

(2) Examine for colonies with a small zone of hemolysis and identify group B streptococcus in pediatric patients, if present, in any amount.

(3) Identify other beta-hemolytic streptococci in significant amounts only if they are predominant. Do not report small colony types of beta-hemolytic streptococci or group F streptococci, as they are part of the upper respiratory microbiota.

V. PROCEDURE (continued)

- b.** Examine alpha-hemolytic colonies for morphology consistent with *S. pneumoniae*. These will be inhibited by optochin.
- (1) Add a drop of 10% bile to colonies that resemble pneumococci.
 - (2) If colony dissolves, report *S. pneumoniae*.
 - (3) Quickly pick similar colonies. Perform antimicrobial susceptibility testing (AST) (see section 5). Confirm purity with optochin disk to detect contamination of AST results.
 - (4) If colonies are not dissolved by bile, but still resemble pneumococci, confirm identification with optochin susceptibility.
- NOTE: Some pneumococci are bile resistant and others are resistant to optochin (14). No one test is 100% accurate, and the combination of these two tests will prevent erroneous reporting. The DNA probe (Gen-Probe) is another accurate method to confirm identification when the optochin zone is less than 14 mm.
- 2.** Fastidious gram-negative rods (these microorganisms grow slowly or not at all on MAC). Perform spot tests on significant numbers to differentiate them from normal respiratory microbiota.
- a.** *H. influenzae* organisms are coccobacilli that grow on CHOC but not on BAP, except with staphylococci or other microorganisms to demonstrate satellitizing. Perform ALA (aminolevulinic acid) test (procedure 3.17.3) to confirm identification and beta-lactamase test for penicillin susceptibility for *H. influenzae* isolates.
 - b.** *Francisella tularensis* organisms are coccobacilli that can grow on CHOC but do not grow on BAP, even with staphylococci for satellitizing. They are weakly catalase positive and oxidase and urease negative. They are beta-lactamase positive.
 - c.** *Legionella* organisms are gram-negative rods that may grow on CHOC but will not grow on BAP, even with staphylococci for satellitizing. They are motile and have a characteristic colony and Gram stain morphology (see procedure 3.11.4)
 - d.** The major significant *Bordetella* organisms which grow on BAP are catalase and urease positive. They may be visible only after 48 h. See Table 3.11.6–1.
 - e.** *Pasteurella* organisms are indole and oxidase positive and represent normal mouth microbiota of animals.
 - f.** Identify *Yersinia pestis* in any amount. It presents as a non-lactose-fermenting rod on MAC or EMB, but it may appear as pinpoint colonies on BAP at 24 h of incubation.
 - g.** Do not identify most other fastidious gram-negative rods, such as *Eikenella*, unless they are predominant and present in large amounts, since they are part of the normal upper respiratory microbiota and rarely cause respiratory disease.
- 3.** Gram-negative diplococci
- a.** Examine colonies present in significant amounts that move when pushed. Confirm as *Moraxella catarrhalis*. Since more than 90% of *M. catarrhalis* organisms are beta-lactamase positive, testing is not helpful to treatment.
 - b.** Examine CHOC for any oxidase-positive colonies that do not grow or grow poorly on BAP. Confirm identification of *Neisseria gonorrhoeae* and *N. meningitidis* (see Table 3.9.3–1). Do not perform AST.
- 4.** Gram-negative rods that grow well on either MAC or EMB
- a.** Identify and perform AST on enteric gram-negative rods, particularly *Klebsiella pneumoniae*, if there is only one morphology in significant amounts with no other pathogens in greater amounts.

V. PROCEDURE (continued)

- b. For inpatients, regardless of the presence of other pathogens, examine for significant numbers of *Pseudomonas aeruginosa*, *Acinetobacter*, *Burkholderia*, and *Stenotrophomonas* organisms because they are typically resistant to multiple antimicrobials and can be implicated in nosocomial epidemics.
- c. If more than one type of other gram-negative rods are present in equal numbers, perform minimal testing to be able to describe the organisms (e.g., indole, oxidase, odor and morphology on MAC, colony pigment, and reaction on Kligler's iron agar [KIA] or triple sugar iron agar [TSI]).
- d. See procedure 3.11.3 for special requirements for cystic fibrosis patients.
- 5. Staphylococci
 - a. Identify *S. aureus* if present in significant amounts.
 - (1) Perform AST if the Gram stain shows predominant cocci in clusters associated with WBCs and no other pathogen in significant amounts.
 - (2) If the patient is an inpatient, based on infection control policy, check for resistance to oxacillin, even if organism is present in low numbers.
 - b. Identify coagulase-negative staphylococci, without species identification or AST, only if they are in 90% pure culture. Otherwise include in respiratory microbiota.
- 6. Do not report *Enterococcus* unless the culture is 90% pure and the identification is confirmed with extensive biochemical tests. See procedure 3.18.1. Many gram-positive cocci in the normal respiratory tract are PYR positive and even bile-esculin and LAP (leucine aminopeptidase) positive.
- 7. Gram-positive rods
 - a. Rule out *Nocardia* in any amount and *Rhodococcus equi* (mucoid and urease positive) from immunocompromised patients (see procedures 6.1 and 6.2 and Table 3.18.1–6).
 - b. Examine for large, spore-forming, gram-positive rods. If present, identify *Bacillus anthracis* and *Bacillus cereus*.
 - c. Limit identification of *Corynebacterium*, using a commercial kit for gram-positive rods, to those present in numerous and predominant amounts and when either of the following is true.
 - (1) The organism is rapid urea positive. (*Corynebacterium pseudodiphtheriticum* is urea positive.)
 - (2) The specimen is from an intubated patient from an intensive care unit.
 - d. Generally, do not pursue other gram-positive rods, as they are unlikely to cause pneumonia.
- 8. Identify molds (section 8), unless the organism is consistent with a laboratory or environmental contaminant (e.g., *Penicillium*).
 - NOTE:** Biphasic fungi (*Histoplasma capsulatum* and *Coccidioides immitis*) can be isolated from plates held for >48 h, even as the yeast-phase colony morphology. Use caution in examination of older cultures.
- 9. Rule out *Cryptococcus* (section 8) and do not identify other yeasts further.
 - NOTE:** *Candida* organisms are not a cause of pneumonia, except possibly in oncology (e.g., leukemia) or lung transplant patients or in neonates. Even in those cases, growth of *Candida* in lower respiratory specimens, regardless of species, does not correlate with disease (5). Yeasts are normal inhabitants of the mouth.
- 10. If bacteria were seen on smear but did not grow on culture, extend the incubation and inoculate other media (e.g., for *Legionella*, *Bordetella pertussis*, and *Mycobacterium*).



It is imperative that these cultures be handled in a biosafety hood.

POSTANALYTICAL CONSIDERATIONS**VI. REPORTING RESULTS**

- A. Report the presence of cells and bacteria from Gram stains of lower respiratory specimens as outlined in procedure 3.2.1. If significant bacterial morphotypes are found in the Gram stain, which do not grow in culture, a comment about this discrepancy should be provided on the report.
- B. Report "No growth of pathogens or normal upper respiratory tract microorganisms" if there is no growth on any plates.
 - NOTE:** This may be indicative of antimicrobial inhibition of normal microbiota.
- C. Positive reporting
 - 1. Report preliminary and final results as "Isolates consistent with microorganisms encountered in the upper respiratory tract" if no pathogens are isolated.
 - 2. Report all pathogens and susceptibility tests performed using Table 3.11.2–1 as a guide.
 - 3. Provide preliminary reports as indicated in procedure 3.3.2.
 - 4. Report AST in accordance with CLSI guidelines (4) and section 5.

VII. INTERPRETATION

- A. A positive culture with *S. pneumoniae* or *H. influenzae* generally indicates infection with that organism, although carriage of these organisms may lead to false-positive results.
- B. A positive culture with a predominant gram-negative rod or *S. aureus* generally indicates infection with that agent if the smear suggests an infectious process involving the corresponding morphology.
- C. A negative bacterial culture does not rule out lower respiratory infection. The primary pathogen is frequently not recovered from patients with pneumonia, either because they have already been started on antimicrobial therapy when samples are taken or because they have infection with another type of organism (e.g., virus, parasite, fungus, mycoplasma, or mycobacterium) that will not be recovered by bacterial culture.
- D. Although published guidelines by several expert groups are controversial about the clinical utility of sputum bacterial cultures for the diagnosis of CAP (1, 7, 13), lower respiratory tract specimens should be collected and analyzed to determine the microbial cause of infection in patients with nosocomial pneumonia or VAP.
- E. CAP guidelines recommend treatment of ambulatory patients with either amoxicillin or amoxicillin-clavulanate or a macrolide. A fluoroquinolone with enhanced activity against penicillin-resistant strains of *S. pneumoniae* (i.e., Levaquin) should be used in patients with CAP, particularly those with severe infection who require admission to a hospital (1, 3, 7, 13).
- F. Bacterial isolates from patients with nosocomial pneumonia and VAP are more likely to have resistance to one of the more commonly used agents. Primary bacterial pathogens should have AST reported to direct appropriate therapy.
- G. Immunocompromised patients with progressive pneumonia are more likely to have infection due to *Legionella* or a nonbacterial cause of infection. Lower respiratory tract specimens (i.e., BAL and/or OLB specimens) should be collected early in the course of the infection in order to optimize the recovery of unusual pneumonia pathogens, including *Legionella*, *P. jiroveci* (formerly *P. carinii*), viruses, fungi, mycoplasmas, and mycobacteria. Laboratories should refer to the specific sections of this handbook that provide detailed procedures for the analysis of clinical specimens to detect these pathogens.

VIII. LIMITATIONS

- A. Some primary pathogens of pneumonia do not grow in routine bacterial culture. Laboratories should refer to the specific sections of this handbook that provide detailed procedures for the analysis of clinical specimens to detect these pathogens (e.g., *Legionella*, viruses, fungi, mycoplasmas, and mycobacteria).
- B. False-negative bacterial cultures usually result from improper collection and/or delayed transport of lower respiratory specimens to the laboratory. Routine bacterial culture may also be negative if the specimens were collected after the patient was started on antimicrobial therapy.
- C. False-positive results are usually caused by contamination of the specimen by normal respiratory flora and its subsequent growth on culture and overinterpretation by the laboratory. Contamination of a lower respiratory sample may also occur by leakage during transport.

REFERENCES

1. Bartlett, J. G., S. F. Dowell, L. A. Mandell, T. M. File, Jr., D. M. Musher, and M. J. Fine. 2000. Guidelines from the Infectious Diseases Society of America—practice guidelines for the management of community-acquired pneumonia in adults. *Clin. Infect. Dis.* **31**:347–382.
2. Centers for Disease Control and Prevention. 1997. Guidelines for prevention of nosocomial pneumonia. *Morb. Mortal. Wkly. Rep.* **46**(RR-1):1–79.
3. Chow, A. W., C. B. Hall, J. Klein, R. B. Kammer, R. D. Meyer, and J. S. Remington. 1992. General guidelines for the evaluation of new anti-infective drugs for the treatment of respiratory tract infections. *Clin. Infect. Dis.* **15**(Suppl. 1):S62–S88.
4. Clinical and Laboratory Standards Institute. 2006. *Performance Standards for Antimicrobial Susceptibility Testing*. Sixteenth informational supplement. Approved standard M100-S16. Clinical and Laboratory Standards Institute, Wayne, Pa.
5. Kontoyiannis, D. P., B. T. Reddy, H. A. Torres, M. Luna, R. E. Lewis, J. Tarrand, G. P. Bodey, and I. I. Raad. 2002. Pulmonary candidiasis in patients with cancer: an autopsy study. *Clin. Infect. Dis.* **34**:400–403.
6. Korppi, M., M. L. Katila, R. Kallikoski, and M. Leinonen. 1992. Pneumococcal finding in a sample from upper airways does not indicate pneumococcal infection of lower airways. *Scand. J. Infect. Dis.* **24**:445–451.
7. Mandell, L. A., T. J. Marrie, R. F. Grossman, A. W. Chow, R. H. Hyland, and The Canadian Community-Acquired Pneumonia Working Group. 2000. Canadian guidelines for the initial management of community-acquired pneumonia: an evidence-based update by the Canadian Infectious Diseases Society and the Canadian Thoracic Society. *Clin. Infect. Dis.* **31**:383–421.
8. Marrie, T. J. 2000. Community-acquired pneumonia in the elderly. *Clin. Infect. Dis.* **31**:1066–1078.
9. Mayhall, C. G. 2001. Ventilator-associated pneumonia or not? Contemporary diagnosis. *Emerg. Infect. Dis.* **7**:200–204.
10. Michelow, I. C., J. Lozano, K. Olsen, C. Goto, N. K. Rollins, F. Ghaffar, V. Rodriguez-Cerrato, M. Leinonen, and G. H. McCracken, Jr. 2002. Diagnosis of *Streptococcus pneumoniae* lower respiratory infection in hospitalized children by culture, polymerase chain reaction, serological testing, and urinary antigen detection. *Clin. Infect. Dis.* **34**:E1–E11.
11. Murdoch, D. R., R. T. Laing, G. D. Mills, N. C. Karalus, G. I. Town, S. Mirrett, and L. B. Reller. 2001. Evaluation of a rapid immunochromatographic test for detection of *Streptococcus pneumoniae* antigen in urine samples from adults with community-acquired pneumonia. *J. Clin. Microbiol.* **39**:3495–3498.
12. Nagendra, S., P. Bourbeau, S. Brecher, M. Dunne, M. LaRocco, and G. Doern. 2001. Sampling variability in the microbiological evaluation of expectorated sputa and endotracheal aspirates. *J. Clin. Microbiol.* **39**:2344–2347.
13. Niederman, M. S., L. A. Mandell, A. Anzueto, J. B. Bass, W. A. Broughton, G. D. Campbell, N. Dean, T. File, M. J. Fine, P. A. Gross, F. Martinez, T. J. Marrie, J. F. Plouffe, J. Ramirez, G. A. Sarosi, A. Torres, R. Wilson, and V. L. Yu. 2001. Guidelines for the management of adults with community-acquired pneumonia. Diagnosis, assessment of severity, antimicrobial therapy, and prevention. *Am. J. Respir. Crit. Care Med.* **163**:1730–1754.
14. Pikis, A., J. M. Campos, W. J. Rodriguez, and J. M. Keith. 2001. Optochin resistance in *Streptococcus pneumoniae*: mechanism, significance, and clinical implications. *J. Infect. Dis.* **184**:582–590.
15. Principi, N., S. Esposito, F. Blasi, L. Allegra, and the Mowgli Study Group. 2001. Role of *Mycoplasma pneumoniae* and *Chlamydia pneumoniae* in children with community-acquired lower respiratory tract infections. *Clin. Infect. Dis.* **32**:1281–1289.
16. Rosón, B., J. Carratalà, R. Verdaguer, J. Dorca, F. Manresa, and F. Gudiol. 2000. Prospective study of the usefulness of sputum Gram stain in the initial approach to community-acquired pneumonia requiring hospitalization. *Clin. Infect. Dis.* **31**:869–874.

SUPPLEMENTAL READING

- Carroll, K. C.** 2002. Laboratory diagnosis of lower respiratory tract infections: controversy and conundrums. *J. Clin. Microbiol.* **40**:3115–3120.
- Centers for Disease Control and Prevention.** 2000. Preventing pneumococcal disease among infants and young children: recommendations of the Advisory Committee on Immunization Practices (ACIP). *Morb. Mortal. Wkly. Rep.* **49**(RR-9):1–35.
- Ryan, K. J., T. F. Smith, and W. R. Wilson.** 1987. *Cumitech 7A, Laboratory Diagnosis of Lower Respiratory Tract Infections*. Coordinating ed., J. A. Washington II. American Society for Microbiology, Washington, D.C.

APPENDIX 3.11.2–1**Quantitative Culture of Protected Specimen Brush and Bronchoalveolar Lavage Fluid Specimens****PREANALYTICAL CONSIDERATIONS****I. PRINCIPLE**

Quantitative cultures increase the specificity of the diagnosis, but the sensitivity is dependent on the threshold chosen for a positive result. Many authors report a colony count of $\geq 10^4$ CFU/ml to be consistent with bacterial pneumonia, whereas counts below 10^4 CFU/ml likely indicate contamination with oronasal microbiota (1, 2, 5, 6, 7). This theory has been validated in numerous clinical studies, and quantitative culture of BAL fluid specimens has become the reference method for the diagnosis of pneumonia by BAL (3). For PBS, a colony count of $\geq 10^3$ CFU/ml is consistent with bacterial pneumonia. Taking the dilution factor of both specimen effluents into consideration, this represents a count of bacteria in the secretions of the lung of 10^5 to 10^6 CFU/ml (2).

There are two methods of performing quantitative cultures: the serial dilution method and the calibrated-loop method (1, 2). After incubation the colonies are counted and multiplied by the appropriate dilution factor to determine the number of bacteria present in 1 ml of fluid.

II. MATERIALS

- Primary media
 - BAP or
 - CHOC
- Other supplies/equipment
 - Pipette method
 - One 100- μ l pipettor with sterile tips
 - Two 16- by 125-mm snap-cap tubes containing 5 ml of sterile phosphate-buffered saline or suitable broth
 - Loop method
 - 0.001-ml (1- μ l)
 - 0.01-ml (10- μ l) loop
 - Disposable loops are color coded according to volume delivery.
 - Sterile bent glass or plastic disposable sterile rods (hockey sticks) to spread inoculum (Excel Scientific, Wrightwood, Calif., [760] 249-6371).
 - Petri dish turntable to rotate plates (optional)
 - Incubator at 35°C with 5% CO₂ or other CO₂-generating system

ANALYTICAL CONSIDERATIONS**III. QUALITY CONTROL**

- For calibrated-pipette QC, refer to Appendix 3.12–4. If calibrated loops are used, verify that they are accurate by one of the methods in Appendix 3.12–3.
- NOTE:** Jacobs et al. (4) have indicated that actual BAL fluid should be used to validate the accuracy of the loops. Some, but not all, loops provide accurate results.

B. Refer to procedure 3.11.2 for medium QC testing.**IV. PROCEDURES**

- Inoculation (see Fig. 3.11.2–A1).
 - Vortex specimen vial vigorously for 30 to 60 s.
 - Label a BAP or CHOC for each dilution. Do not use selective medium. If CHOC contains bacitracin, use BAP. Otherwise use CHOC for quantitative culture. Inoculate other media according to procedure 3.11.2 for other lower respiratory tract pathogens.



Observe standard precautions.



It is imperative that these cultures be handled in a biosafety hood.

APPENDIX 3.11.2–1 (continued)**3. Pipette method**

- Transfer 100 μ l of PBS to each plate marked “ $\times 10$.” Each colony from this plate = 10 CFU/ml.
- Transfer 10 μ l of PBS or BAL specimen to each plate marked “ $\times 100$.” Each colony from this plate = 100 CFU/ml.
- For BAL (and PBS, optional), do the following.
 - Label one tube of 5 ml of phosphate-buffered saline “1:100.”
 - Transfer 50 μ l of “undiluted” fluid from the vial to the tube labeled “1:100.” Vortex.
 - Transfer 100 μ l of “1:100” dilution to each plate marked “ $\times 10^3$.” Each colony from this plate = 10^3 CFU/ml.
- For BAL fluid only, do the following.
 - Label one tube of 5 ml of phosphate-buffered saline “1:10,000.”
 - Vortex.
 - Transfer 50 μ l of “1:100” dilution to the tube labeled “1:10,000.”
 - Transfer 100 μ l of the “1:10,000” dilution to each plate marked “ $\times 10^5$.” Each colony from this plate = 10^5 CFU/ml.
- Spread the inoculum over the surface of each plate evenly by using a sterile glass or disposable plastic rod.*
 - Place plate on spinning turntable for even greater distribution of colonies.
 - Begin with the most dilute inoculum and proceed to the least dilute using the same sterile rod for each plate.

4. Loop method

- PBS
 - Vortex for 30 to 60 s.
 - Place 100 μ l (2 drops) of specimen onto each plate marked “ $\times 10$.” Each colony from this plate = 10 CFU/ml.
 - Using a 1:100 loop, place 10 μ l on a plate labeled “ $\times 100$.” Each colony from this plate = 100 CFU/ml.
- BAL fluid
 - Vortex for 30 to 60 s.
 - Using a 1:100 loop, place 10 μ l on a plate labeled “ $\times 100$.” Each colony from this plate = 100 CFU/ml.
 - Using a 1:1,000 loop, place 1 μ l on a plate labeled “ $\times 1,000$.” Each colony from this plate = 1,000 CFU/ml.
- Spread the inoculum over the surface of each plate evenly by using a sterile glass or disposable plastic rod.*

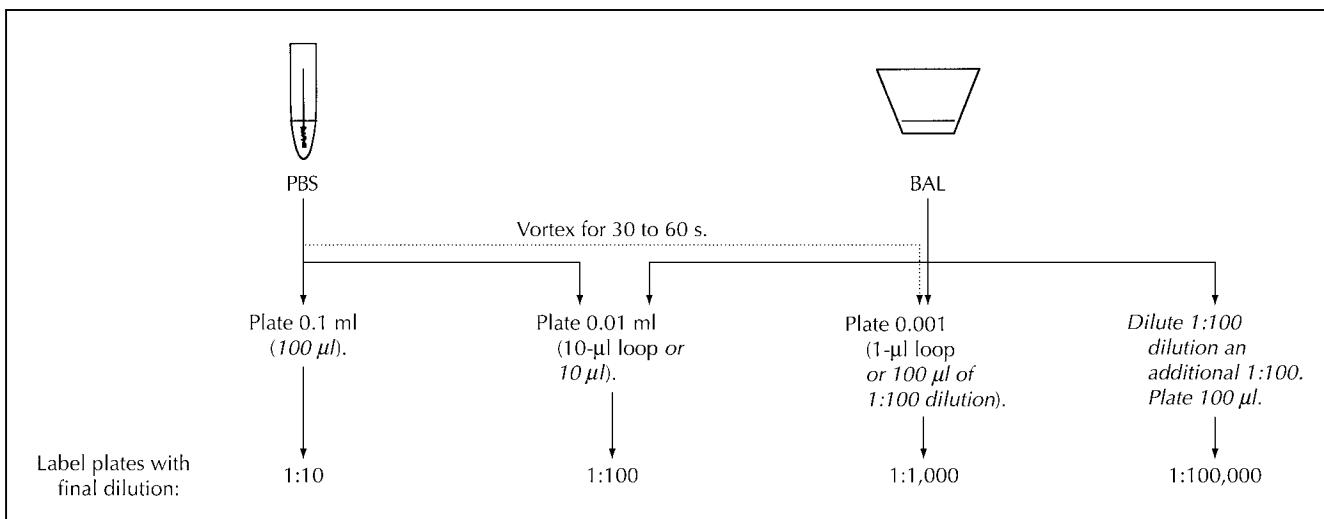


Figure 3.11.2–A1 Quantitative culture methods. Serial dilution method is in italics. Dashed line is optional. Adapted from reference 2.

APPENDIX 3.11.2-1 (continued)

- (1) Place plate on spinning turntable for even greater distribution of colonies.
- (2) Begin with the most dilute inoculum and proceed to the least dilute using the same sterile rod for each plate.
- (3) Alternatively, spread the inoculum in only half the plate and use the second half to streak in two quadrants for isolation of large numbers of microorganisms.

- B. Incubate the aerobic plates in a 5 to 10% CO₂, 35°C incubator.
- C. Inoculate each additional medium with 100 µl of specimen (2 drops) and streak for isolated colonies, according to procedure 3.11.2 or procedure 3.11.3.
- D. Prepare a Gram stain from the BAL sample, using the cytocentrifuge.
- E. Centrifuge the rest of the BAL specimen for culture for viruses, mycobacteria, *Legionella*, and fungi and for smears for viruses, *Pneumocystis*, AFB, and fungi, on request. Alternatively, use the unprotected brush as the best specimen for smears, if it is available.

POSTANALYTICAL CONSIDERATIONS**V. INTERPRETATION**

- A. Count the colonies from the dilution with the greatest number of colonies without confluence. Multiply by the dilution factor listed for each dilution (e.g., 40 colonies on the “× 1,000” plate is 40×10^3 CFU or 4×10^4 CFU).
- B. Count each morphotype individually.
- C. If growth is too numerous to count on the highest dilution, report the count as greater than that dilution.
- D. Counts for BAL fluid of less than 10^4 are considered to indicate contamination.
- E. Counts for PBS of less than 10^3 are considered to indicate contamination.

VI. REPORTING RESULTS

- A. Gram stain—report smear as indicated in procedure 3.2.1. Any organism seen in a cytocentrifuged smear of BAL fluid is considered indicative of bacterial pneumonia (6).
- B. Quantitative culture: report the amount of each primary pathogen found as CFU per milliliter of specimen. If the culture count is lower than the threshold, identification and AST are not routinely performed unless there is a pure growth of a primary pathogen.
- C. *Candida* species are most often contaminants of the procedure and generally should not be identified to the species level.

VII. LIMITATIONS

- A. Counts near the thresholds may be difficult to interpret accurately.
- B. Not all pathogens will grow on the media used and may result in a false negative culture.
- C. Anaerobes are implicated in disease, especially in the elderly or those obtunded with aspiration pneumonia. Cultures are generally not cost-effective or helpful because they vary in sensitivity from 20 to 60%, possibly due to aeration during collection and dilution of the specimen.
- D. Loop cultures are not as accurate as pipette cultures, but both are quite sensitive provided that a rod is used to spread the colonies on the plate. Spreading with the loop is *not* as sensitive a method and will underestimate the quantity of organisms present.
- E. Even a single dose of antimicrobial therapy prior to specimen collection can negate the ability to isolate the causative agent of disease from the specimen.

APPENDIX 3.11.2-1 (continued)

References

1. Baselski, V. S., M. El-Torky, J. J. Coalson, and J. P. Griffin. 1992. The standardization of criteria for processing and interpreting laboratory specimens in patients with suspected ventilator-associated pneumonia. *Chest* **102**:571S–579S.
2. Baselski, V. S., and R. G. Wunderink. 1994. Bronchoscopic diagnosis of pneumonia. *Clin. Microbiol. Rev.* **7**:533–558.
3. Ewig, S. 1996. Diagnosis of ventilator-associated pneumonia: nonroutine tools for routine practice. *Eur. Respir. J.* **9**:1339–1341.
4. Jacobs, J. A., E. I. G. B. De Brauwer, E. I. M. Cornelissen, and M. Drent. 2000. Accuracy and precision of quantitative calibrated loops in transfer of bronchoalveolar lavage fluid. *J. Clin. Microbiol.* **38**:2117–2121.
5. Kahn, F. W., and J. M. Jones. 1988. Analysis of bronchoalveolar lavage specimens from immunocompromised patients with a protocol applicable in the microbiology laboratory. *J. Clin. Microbiol.* **26**:1150–1155.
6. Thorpe, J. E., R. P. Baughman, P. T. Frame, T. A. Wesseler, and J. L. Staneck. 1987. Bronchoalveolar lavage for diagnosing acute bacterial pneumonia. *J. Infect. Dis.* **155**:855–861.
7. Torres, A., J. Puig de la Bellacasa, A. Xaubet, J. Gonzalez, R. Rodriguez-Roisin, M. T. Jimenez de Anta, and A. Agusti Vidal. 1989. Diagnostic value of quantitative cultures of bronchoalveolar lavage and telescoping plugged catheters in mechanically ventilated patients with bacterial pneumonia. *Am. Rev. Respir. Dis.* **140**:306–310.

3.11.3

Respiratory Cultures from Cystic Fibrosis Patients

[Updated March 2007]

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Chronic lung infection is responsible for 75 to 85% of deaths in patients with cystic fibrosis (CF) (5, 7). Life expectancy has increased steadily over the past 50 years in large part because of improvements in management of lung disease in this patient population. The purpose of the CF culture is to provide a method for culturing respiratory secretions from patients with CF in order to isolate organisms associated with pulmonary disease in these patients. The number of microbial species associated with CF lung disease is relatively limited. Emphasis is placed on the recovery of those organisms, including mucoid and nonmucoid *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Burkholderia cepacia* complex (which includes *B. cepacia* genomovars I, III, and VI; *Burkholderia*

multivorans; *Burkholderia stabilis*; *Burkholderia vietnamiensis*; *Burkholderia ambifaria*; *Burkholderia anthina*; and *Burkholderia pyrrocinia*), *Stenotrophomonas maltophilia* and other non-glucose-fermenting, gram-negative rods, and *Haemophilus influenzae* and *Streptococcus pneumoniae* (5, 7, 9, 12, 16). The vast majority of *B. cepacia* complex isolates that will be recovered from CF patients will be *B. cepacia* genomovar III, *B. multivorans*, and *B. vietnamiensis* (Table 3.11.3-1) (10). Nontuberculous mycobacteria and *Aspergillus* spp. may also play a role in CF lung disease and should be actively sought at the caregivers' request in these patients (7). Common respiratory viruses such as respiratory syncytial virus in children and influenza virus in all patients may

also cause respiratory symptoms and should be considered during periods of disease activity in the community (7). In selected centers, lung transplantation is performed in CF patients. Special culture needs of this patient population should be applied to specimens obtained from lung recipients with CF disease since they continue to harbor the same types of organisms as they did pretransplantation (17). Patients with CF may be colonized or chronically infected with microbiota which may change little over a long period of time; therefore, an approach is adopted which is designed to maximize service to the patient while minimizing duplication in the laboratory and assisting in cost containment.

Table 3.11.3-1 Characteristics of *B. cepacia* complex and related organisms^a

Test	<i>B. cepacia</i> genomovars I and III	<i>B. multivorans</i>	<i>B. stabilis</i>	<i>B. vietnamiensis</i>	<i>B. gladioli</i>	<i>Pandoraea</i>	<i>R. pickettii</i>
Oxidase	+ ^b	+	+	+	-	- (+) ^d	+
Oxidation of:							
Glucose	+	+	+	+	+	- (+)	+
Maltose	(+) ^c	+	+	+	-	-	+
Lactose	+	+	+	+	-	-	+
Xylose	+	+	+	+	+	-	+
Sucrose	+	-	(+)	+	-	-	-
Adonitol	+	+	+	-	+	-	-
Lysine decarboxylase	+	(+)	+	+	-	-	-
Ornithine decarboxylase	(+)	-	+	-	-	-	-
ONPG	+	+	-	+	+	-	-

^a Data are from reference 12; all isolates are colistin or polymyxin B resistant.

^b +, >75% of isolates gave a positive result.

^c (+), 20 to 75% of isolates gave a positive result.

^d - (+), <20% of isolates gave a positive result.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING

A. Specimen collection

1. Collect the following specimens for culture of respiratory secretions from CF patients.
 - a. Deep pharyngeal (also referred to as “cough or gagged” throat specimens)
 - (1) Place a plastic shaft, Dacron- or rayon-tipped swab in the back of the throat and induce coughing.
 - (2) Remove the swab when coughed secretions have been collected.
 - **NOTE:** This technique is used in children, usually <10 years of age, who are unable to produce sputum. *Do not use* this technique in children who are able to produce sputum.
 - b. Sputum
 - c. Endotracheal aspirates (on ventilated patients)
 - d. Bronchoscopically obtained specimens, including bronchoalveolar lavage (BAL) specimens, protected specimen brushings, and transbronchial biopsy specimens. See quantitative culture method (Appendix 3.11.2–1).

2. Use the same transport devices for these specimens obtained from CF patients that would be used for non-CF patients.
3. If the specimen cannot be transported and processed within 4 h, it may be held at 4°C for up to 24 h without affecting the recovery of the major pathogens of interest in CF patients.

B. In addition to specimen labeling which meets good laboratory practices, identify the specimen as being obtained from a “cystic fibrosis patient” to allow appropriate specimen processing and workup.

C. Rejection criteria

1. Since bronchoscopic specimens are obtained at significant expense and some degree of risk to the patient, all attempts should be made to process these specimens, even if they are compromised. However, on the final reports, note that specimen quality may have been compromised.
2. Process no more than one specimen per month from nontransplant CF patients who are outpatients without physician or other caregiver consultation.
3. Process no more than two specimens per admission for nontransplant CF patients who are inpatients without physician or caregiver consultation.
4. If a swab is received labeled as a sputum, contact caregiver prior to processing, to determine source of specimen. It is likely a deep pharyngeal specimen.
■ **NOTE:** The microbiota responsible for chronic lung disease in CF patients is very stable, with patients being infected with organisms such as *S. aureus* or *P. aeruginosa* for months to years.
5. No guidelines currently exist for frequency of culture from CF lung transplant recipients. Process specimens by request.
6. Do not use culture rejection criteria for sputum or endotracheal aspirates based on Gram stain quality (Appendix 3.2.1–2), since they are of little value in CF patients. Specimens from CF patients will grow potential pathogens >90% of the time, although approximately 40% would be rejected based on the Gram stain evaluation (14).

III. MATERIALS**A. Media for culture**

1. CHOC or horse blood agar with or without 20,000 U of bacitracin per liter (5) for recovery of *H. influenzae* (Remel, Inc.; BD Diagnostic Systems)

NOTE: The addition of bacitracin will inhibit most strains of streptococci, staphylococci, and *Neisseria* and *Micrococcus* species, but *Escherichia coli*, some *Neisseria* species, and strains of *Candida*, *Klebsiella*, *Proteus*, and *Pseudomonas* spp. as well as other bacteria may grow on the medium.

2. Mannitol salt agar (MSA) for recovery of *S. aureus*

3. MAC

4. Columbia colistin-nalidixic acid agar (CNA)

5. *B. cepacia* selective agar (BCSA) (Remel, Inc.; Hardy Diagnostics) (11)

NOTE: This medium contains lactose and sucrose with phenol red and crystal violet to differentiate colonies that oxidize these carbohydrates from those that do not, as well as polymyxin B, gentamicin, and vancomycin to inhibit most other bacteria.

B. Biochemical tests

1. See procedures 3.3.2 and 3.18.2 for standard laboratory tests and flowcharts helpful in identification of organisms recovered from CF patients.

2. For nonfermenting, gram-negative rods, use a commercial kit designed to identify them (e.g., Vitek GNI, GNI Plus, or API 20NE [bio-Mérieux, Inc.]; RapID NF Plus or N/F System [Remel, Inc.]; Microscan GNP [Dade-Behring Microscan]; Crysta E/NF [BD Diagnostic Systems]). Refer to procedure 3.1 for vendor contact information.
3. In addition, the following tests are helpful.
 - a. Lysine and ornithine decarboxylase (procedure 3.17.15)
 - b. *o*-Nitrophenyl-β-D-galactopyranoside (ONPG) (procedure 3.17.37)
 - c. OF sugars (glucose, lactose, maltose, mannitol, adonitol, sucrose, and xylose [procedure 3.17.9])
 - d. Colistin or polymyxin B disks (procedure 3.17.4)
 - e. DNase (procedure 3.17.16)
4. Etests (AB Biodisk, Solna, Sweden) for trimethoprim-sulfamethoxazole, ticarcillin-clavulanic acid, and ceftazidime for susceptibility testing of *S. maltophilia*
5. Antimicrobial disks for susceptibility testing (procedure 5.1) or microdilution MIC method with overnight incubation (procedure 5.2)

ANALYTICAL CONSIDERATIONS**IV. QUALITY CONTROL**

- A. Verify that media meet expiration date and QC parameters per current Clinical and Laboratory Standards Institute (CLSI; formerly NCCLS) M22 document. See procedures 14.2 and 3.3.1 for further procedures, especially for in-house-prepared media.

B. QC the following media by lot when received.

1. *Haemophilus* media with bacitracin (see procedure 3.11.2 for details)
2. CHOC (see procedure 3.2.1 for details)
3. BCSA

Test organism	Incubation			Result
	Time (h)	Temp (°C)	Atmosphere	
<i>B. cepacia</i> ATCC 25416	24–72	35	Aerobic	Growth
<i>P. aeruginosa</i> ATCC 27853	24	35	Aerobic	Partial to complete inhibition
<i>S. aureus</i> ATCC 25923	24	35	Aerobic	Partial to complete inhibition

IV. QUALITY CONTROL (continued)

- C. Perform standard QC for reagents, commercial identification systems, and antimicrobial susceptibility testing according to package inserts and CLSI documents (see sections 5 and 14).

V. PROCEDURE



Observe standard precautions.

A. Microscopic examination

1. Perform Gram stain on bronchoscopically obtained specimens and report results according to procedure 3.2.1.
2. Perform Gram stains on deep pharyngeal, sputum, and endotracheal aspirates by request only.

NOTE: Screening for specimen quality is inappropriate since limited organisms are associated with CF lung disease and their presence on culture is considered clinically significant regardless of Gram stain findings. See Table 3.11.3–2 for further details.
3. Perform acid-fast bacillus stains on request for sputum, endotracheal aspirates, and bronchoscopically obtained specimens from CF patients.
4. Perform fungal stains on request from sputum, endotracheal aspirates, and bronchoscopically obtained specimens from CF patients.

Table 3.11.3–2 Processing of organisms

Organism	Oropharyngeal microbiota component	Identify	Special processing
Mold	NA ^a	Any amount	Identify <i>Aspergillus</i> spp. and other fungi to the species level on an annual basis.
<i>Enterobacteriaceae</i>	Not predominant or pharyngeal site	Predominant	Perform susceptibility testing.
<i>S. aureus</i>	NA	Any amount	Perform complete susceptibility testing yearly; perform oxacillin screening on each culture.
<i>H. influenzae</i>	Not predominant or pharyngeal site	Predominant	Perform beta-lactamase test. If colony is mucoid or wet, consult the lab director regarding possibly sending out for serotyping.
<i>S. pneumoniae</i>	Not predominant or pharyngeal site	Predominant	Screen for penicillin resistance with oxacillin disk. Perform penicillin Etest upon request.
<i>P. aeruginosa</i> , <i>B. cepacia</i> , other non-glucose-fermenting, gram-negative rods	NA	Any amount	Perform susceptibility testing.
Rapidly growing mycobacterium	NA	Any amount	Identify to species level.

^a NA, not applicable.

V. PROCEDURE (continued)**B. Culture methods**

1. Inoculate each of the five media listed above and incubate CHOC or horse blood agar and CNA in 5% CO₂ at 35°C.
 - a. Incubate MAC, BCSA, and MSA without CO₂ at 35°C.
 - b. Inoculate the CHOC or horse blood agar quantitatively, if from bronchoscopy, according to Appendix 3.11.2–1.
 - c. For deep throat specimens, omit the CNA and CHOC or horse blood agar, since the specimen will only be examined for *P. aeruginosa*, *B. cepacia*, and *S. aureus*.
2. Because of the high rate of *P. aeruginosa* contamination, decontaminate all CF specimens in which the detection of acid-fast bacilli is requested using *N*-acetyl-L-cysteine–sodium hydroxide decontamination followed by 5% oxalic acid decontamination before inoculating standard mycobacterium isolation medium (18). See section 7 for general procedures.
3. Examine all plates at 24, 48, and 72 h of incubation. Examine BCSA plates also at 96 h for growth.
 - a. BCSA medium—*B. cepacia* complex will appear as small to large, pink, yellow, or metallic colonies. The medium surrounding the colonies will change from red to yellow. Proceed with identification from growth as indicated in Fig. 3.11.3–1.
 - b. MSA—for isolation of *S. aureus*, which will ferment the mannitol and produce a yellow zone around the colony
 - (1) Multiple strains of *S. aureus* may be present in one specimen; be alert to subtle differences in colonial morphology.
 - (2) In particular, thymidine-dependent strains of *S. aureus* may arise in patients treated with long-term trimethoprim-sulfamethoxazole; these strains frequently present with colonies which are somewhat smaller, flatter, and grayer than the parent strain (8).
 - (3) Pay close attention to colonial morphology of staphylococci on the CNA and horse blood agar or CHOC as well as the MSA. Strains of *S. aureus* which fail to ferment mannitol, or which do so only after many days of incubation, have been recovered from CF patients.
 - c. MAC—identify lactose-positive organisms according to the criteria in Table 3.11.3–2 and the methods in procedure 3.18.2. Subculture various morphotypes of non-lactose-fermenting, gram-negative rods from their primary plate to BAP. This will enable evaluation of their probable species morphology based on the following general guidelines.
 - (1) *P. aeruginosa*: beta-hemolytic, metallic, grape odor or mucoid colony morphology, oxidase positive, and indole negative. Both mucoid and nonmucoid phenotypes of *P. aeruginosa* should be identified using odor and colistin susceptibility. Perform further identification using commercial kits or biochemical tests, if odor is lacking. Refer to procedures 3.3.2 and 3.18.2.
 - (2) *S. maltophilia*: non-hemolytic, slight yellow pigment, and oxidase negative, at least initially.
■ NOTE: *S. maltophilia* isolates have been misidentified as *B. cepacia*, especially strains which are colistin resistant. *S. maltophilia* isolates growing on BCSA are colistin resistant. Key biochemical characteristics of *S. maltophilia* are an inability to oxidize mannitol, strong oxidation of maltose, and a positive reaction on DNase agar after a full 72 h of incubation. Unknown isolates should be considered DNase negative only after 72 h of incubation (6, 9).
 - (3) *Alcaligenes* and *Achromobacter* spp.: nonhemolytic, small colony type, oxidase positive, and not grape-like or metallic smelling; may be sweet smelling.

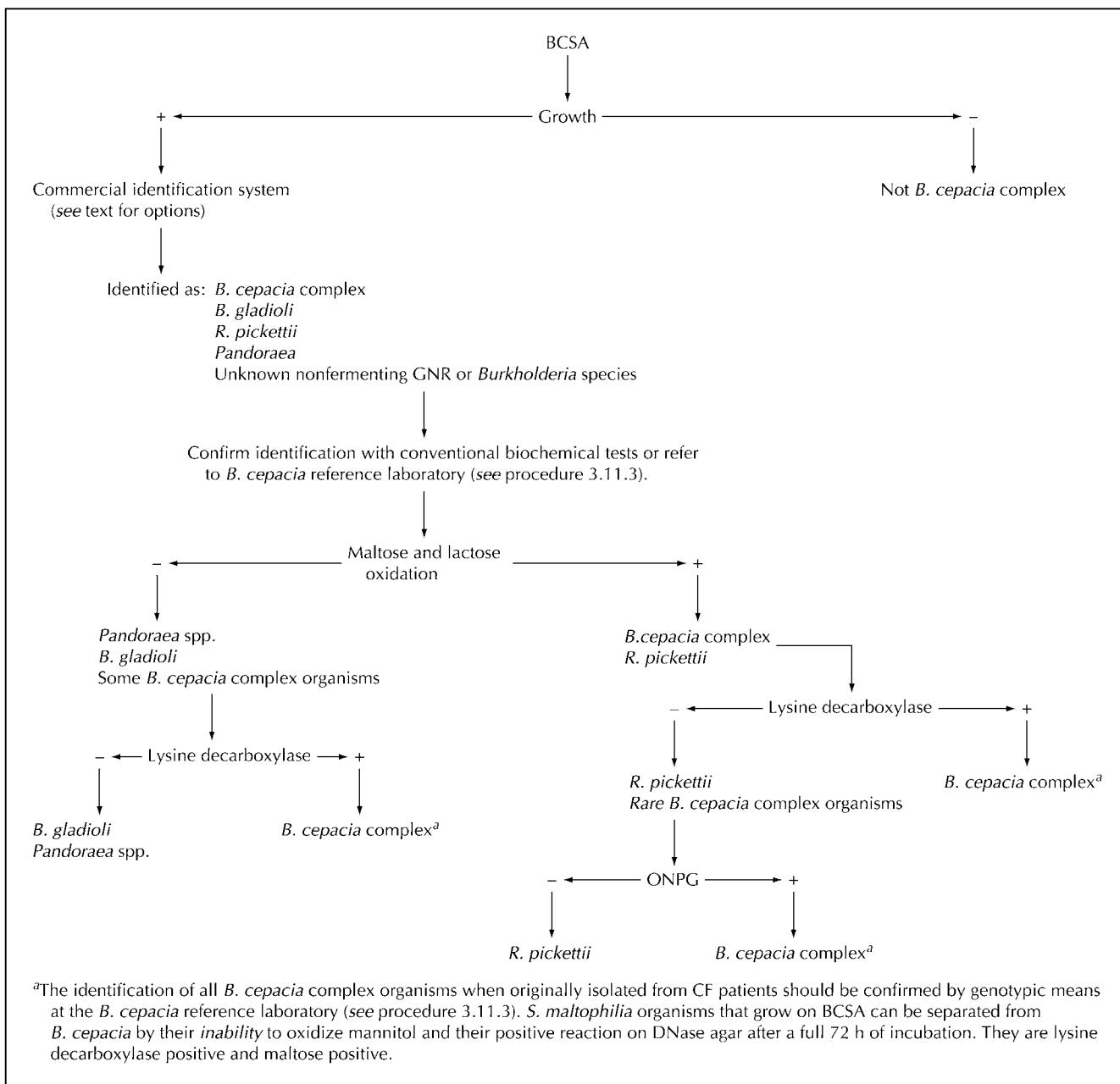


Figure 3.11.3–1 Flowchart for the identification of *B. cepacia* complex. GNR, gram-negative rod.

V. PROCEDURE (continued)

(4) *B. cepacia* complex and related organisms: nonhemolytic, foul odor, oxidase variable. For details on identification of *B. cepacia* complex organisms to the species level by conventional methods, refer to Table 3.11.3–1.

■ **NOTE:** Confirm the identity of any organism from a CF patient identified by a commercial system as *B. cepacia*, as a *B. cepacia*-like organism, including a *Burkholderia* sp., *B. gladioli*, a *Pandoraea* sp., and *Ralstonia pickettii*, or as an unidentified gram-negative rod by genotypic means (1, 4, 12, 13). The United States Cystic Fibrosis

V. PROCEDURE (continued)

Foundation has established a reference laboratory at the University of Michigan for this purpose. Call (734) 936-9769 or e-mail Jli-puma@umich.edu for details on submission of isolates.

- d. Horse blood agar or CHOC—identify *Haemophilus*-like colonies from this medium by following criteria in Table 3.11.3–2 and methods in procedure 3.3.2.
- e. CNA medium—identify *S. pneumoniae* isolates from this medium by following criteria in Table 3.11.3–2 and methods in procedure 3.3.2.
- f. Molds may grow on BCSA, CNA medium, CHOC, or horse blood agar. Identify these organism by following criteria in Table 3.11.3–2 on an annual basis.
- g. Rapidly growing mycobacteria may grow on CHOC/horse blood agar or CNA medium; identify if typical colony morphology is observed.
4. Further workup of positive cultures should follow guidelines in Table 3.11.3–2.
 - a. For quantitative cultures, identify all organisms and do susceptibility testing for all organisms listed in Table 3.11.3–2 which are found at concentrations of $>10^3$ CFU/ml in protected specimen brushings and $>10^4$ CFU/ml in BAL samples. If multiple potential pathogens (≥ 3) are found above the quantitative cutoff, consult with the physician or caregiver concerning further workup of the specimen.
 - b. Susceptibility testing guidelines
 - (1) *S. aureus*: complete susceptibility testing of *S. aureus* isolates should be done once a year at a minimum and on a more frequent basis as requested by the physician. Individual isolates should be screened for oxacillin resistance (procedure 13.17). Isolates should also be confirmed as either oxacillin resistant or susceptible (procedure 5.4).
 - (2) *H. influenzae*: perform beta-lactamase screening.
 - (3) *P. aeruginosa*: CF patients are colonized and may develop infection from multiple morphotypes of this organism. Each mucoid and non-mucoid morphotype should be separately identified and tested either by disk diffusion, Etest, or microdilution MIC determination (2). Patient isolates should be subjected to AST no more than once a month for outpatients, and weekly during acute respiratory exacerbations requiring hospital admission. Automated susceptibility test systems with an incubation time of less than 24 h should not be used for testing *P. aeruginosa* isolates from CF patients.
■ NOTE: The laboratory should keep track of the different morphotypes of *P. aeruginosa* isolated from individual CF patients in order to recognize when new morphotypes that may be more resistant to antimicrobial agents emerge.
 - (4) *S. maltophilia*: test for MIC by either broth microdilution or Etest
(5) The Etest panel can be limited to trimethoprim-sulfamethoxazole, ticarcillin-clavulanic acid, and ceftazidime.
■ NOTE: Reliable disk diffusion breakpoints to trimethoprim-sulfamethoxazole, minocycline, and levofloxacin are available in CLSI document M100-S16 (3).
 - (5) *B. cepacia* complex, *Alcaligenes*, and related organisms: test either by disk diffusion or by microdilution MIC testing. Report disk diffusion results using *P. aeruginosa* breakpoints with the disclaimer “nonstandardized susceptibility results” (5).
■ NOTE: Reliable disk diffusion breakpoints for *B. cepacia* to ceftazidime, minocycline, and meropenem are available in CLSI document M100-S16 (3).

V. PROCEDURE (continued)

5. Hold positive culture plates for 7 days at room temperature after laboratory results have been finalized.
6. Hold specimen for 7 days at 4°C to resolve any problem with the specimen.

POSTANALYTICAL CONSIDERATIONS**VI. REPORTING RESULTS**

- A. If no growth is observed, report "No growth."
- B. If only normal microbiota is observed, report as such.
- C. Report all potential pathogens according to criteria listed in Table 3.11.3–2.
 1. For deep pharyngeal cultures, report the presence in any amount of *S. aureus*, *P. aeruginosa*, and *B. cepacia* only.
 2. For quantitative cultures, report all potential pathogens (Table 3.11.3–2) that are in concentrations of $>10^3$ CFU/ml for protected specimen brushings and $>10^4$ CFU/ml for BAL samples.
 3. For colistin-resistant gram-negative rods that are being referred to the CF reference laboratory, report as "probable XX," where XX is the most likely identification from the flowchart. Add a note: "Sent to *B. cepacia* reference laboratory for definitive identification." If the identification is truly in doubt, report the organism as "Unidentified gram-negative rod" and refer to the CF reference laboratory.
- D. Document all results either as hard copy or by computerized work card.

VII. INTERPRETATION

- A. The finding of *S. aureus*, *P. aeruginosa*, and *B. cepacia* complex regardless of quantity is always considered clinically significant (5, 7).
 1. Sputum cultures from CF patients have been shown to correlate well with the presence of these organisms in the lower airways (5, 7, 15).
 2. The presence of any of these three organisms in deep pharyngeal cultures should be reported. Other organisms in Table 3.11.3–2 should not be reported in pharyngeal cultures since they can be considered part of the oropharyngeal microbiota.
- B. The finding of *B. cepacia* complex organisms in particular is viewed as important in the CF community, since patients with these organisms are often segregated from *B. cepacia*-negative CF patients both socially and medically. In addition, infection with these organisms may be considered a contraindication for lung transplantation, an important life-saving option in the terminal phase of this disease. Therefore, accurate identification of this complex of bacteria is critical for patient care, especially in light of the fact that there is not convincing evidence that the organisms with which it most frequently confused, *B. gladioli*, *R. pickettii*, and *Pandoraea* spp., play a significant role in CF lung disease (1, 4, 13).
- C. *Aspergillus* spp., especially *Aspergillus fumigatus*, are associated with allergic bronchopulmonary aspergillosis (ABPA). ABPA is typically a clinical diagnosis with the finding of *Aspergillus* on culture supportive of the diagnosis of ABPA (7).
- D. Other agents listed in Table 3.11.3–2 have been associated with pulmonary exacerbation of CF and should be reported according to criteria in Table 3.11.3–2.

VIII. LIMITATIONS

- A. Patients with CF who are experiencing pulmonary exacerbation of their lung disease often respond to combinations of antimicrobials to which their organisms are resistant both individually and in combination by in vitro testing (5, 7).
- B. The interpretation of deep pharyngeal culture results is problematic. The absence of CF pathogens in these specimens is a good predictor of their absence in the lower airways. However, the presence of *S. aureus*, *P. aeruginosa*, and *B. cepacia* is not so strong a predictor of their presence in the lower airway. Nevertheless, caregivers of CF patients will treat the finding of these organisms in this specimen type as clinically significant (15). Other pathogens listed in Table 3.11.3–2 should not be reported from this specimen type.

REFERENCES

1. Blecker-Shelly, D., T. Spikler, E. J. Gracely, T. Coenye, P. Vandamme, and J. J. LiPuma. 2000. Utility of commercial systems for the identification of *Burkholderia cepacia* complex from cystic fibrosis sputum culture. *J. Clin. Microbiol.* **38**:3112–3115.
2. Burns, J. L., L. Saiman, S. Whittier, D. Larone, J. Krzewinski, A. Liu, S. A. Marshall, and R. N. Jones. 2000. Comparison of agar diffusion methodologies for antimicrobial susceptibility testing of *Pseudomonas aeruginosa* isolates from cystic fibrosis patients. *J. Clin. Microbiol.* **38**:1818–1822.
3. Clinical and Laboratory Standards Institute. 2006. *Performance Standards for Antimicrobial Susceptibility Testing*. Sixteenth informational supplement. Approved standard M100-S16. Clinical and Laboratory Standards Institute, Wayne, Pa.
4. Coenye, T., P. Vandamme, J. R. W. Govan, and J. J. LiPuma. 2001. Taxonomy and identification of the *Burkholderia cepacia* complex. *J. Clin. Microbiol.* **39**:3427–3436.
5. Cystic Fibrosis Foundation. 1994. Microbiology and infectious disease in cystic fibrosis, p. 1–26. In Cystic Fibrosis Foundation, *Concepts of Care*, vol. V, section 1. Cystic Fibrosis Foundation, Bethesda, Md.
6. Denton, M., and K. G. Kerr. 1998. Microbiological and clinical aspects of infections associated with *Stenotrophomonas maltophilia*. *Clin. Microbiol. Rev.* **11**:57–80.
7. Gilligan, P. H. 1991. Microbiology of airway disease in patients with cystic fibrosis. *Clin. Microbiol. Rev.* **4**:35–51.
8. Gilligan, P. H., P. A. Gage, D. F. Welch, M. J. Muszynski, and K. R. Wait. 1987. Prevalence of thymidine-dependent *Staphylococcus aureus* in patients with cystic fibrosis. *J. Clin. Microbiol.* **25**:1258–1261.
9. Gilligan, P. H., G. Lum, P. A. R. Vandamme, and S. Whittier. 2003. *Burkholderia*, *Stenotrophomonas*, *Ralstonia*, *Brevundimonas*, *Comamonas*, *Delftia*, *Pandoraea*, and *Acidovorax*, p. 729–748. In P. R. Murray, E. J. Baron, J. H. Jorgensen, M. A. Pfaffer, and R. H. Yolken (ed.), *Manual of Clinical Microbiology*, 8th ed. ASM Press, Washington, D.C.
10. Heath, D. G., K. Hohmekler, C. Carriker, K. Smith, J. Routh, J. J. LiPuma, R. M. Aris, D. Weber, and P. H. Gilligan. 2002. Six-year analysis of *Burkholderia cepacia* complex isolates among cystic fibrosis patients at a referral center for lung transplantation. *J. Clin. Microbiol.* **40**:1188–1193.
11. Henry, D. A., M. Campbell, C. McGimpsey, A. Clarke, L. Louden, J. L. Burns, M. H. Roe, P. Vandamme, and D. Speert. 1999. Comparison of isolation media for the recovery of *Burkholderia cepacia* complex from respiratory secretions of patients with cystic fibrosis. *J. Clin. Microbiol.* **37**:1004–1007.
12. Henry, D. A., E. Mahenthiralingam, P. Vandamme, T. Coenye, and D. P. Speert. 2001. Phenotypic methods for determining genovar status of the *Burkholderia cepacia* complex. *J. Clin. Microbiol.* **39**:1073–1078.
13. Kiska, D. L., A. Kerr, M. C. Jones, J. A. Caracciolo, B. Eskridge, M. Jordan, S. Miller, D. Hughes, N. King, and P. H. Gilligan. 1996. Accuracy of four commercial systems for identification of *Burkholderia cepacia* and other gram-negative nonfermenting bacilli recovered from patients with cystic fibrosis. *J. Clin. Microbiol.* **34**:886–891.
14. Nair, B., J. Stapp, L. Stapp, L. Bugni, J. Van Dalsen, and J. L. Burns. 2002. Utility of Gram staining for evaluation of the quality of cystic fibrosis sputum samples. *J. Clin. Microbiol.* **40**:2791–2794.
15. Ramsey, B. W. 1996. What is the role of upper airway bacterial cultures in patients with cystic fibrosis? *Pediatr. Pulm.* **21**:265–266.
16. Shreve, M. R., S. Butler, H. J. Kaplowitz, H. R. Rabin, D. Stokes, M. Light, and W. E. Regelmann for North America Scientific Advisory Group and Investigators of the Epidemiologic Study of Cystic Fibrosis. 1999. Impact of microbiology practice on cumulative prevalence of respiratory tract bacteria in patients with cystic fibrosis. *J. Clin. Microbiol.* **37**:753–757.
17. Steinbach, S., L. Sun, R.-Z Jiang, P. Flume, P. Gilligan, T. M. Egan, and R. Goldstein. 1994. Persistent, clonal *Pseudomonas cepacia* infection in cystic fibrosis lung transplant recipients and clinical patients. *N. Engl. J. Med.* **331**:981–987.
18. Whittier, S., R. L. Hopfer, M. R. Knowles, and P. H. Gilligan. 1993. Improved recovery of mycobacteria from respiratory secretions of patients with cystic fibrosis. *J. Clin. Microbiol.* **31**:861–864.

3.11.4

Legionella Cultures

[Updated March 2007]

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Legionellosis is the collective name used to refer to (primarily pulmonary) infections caused by members of the genus *Legionella*. There are currently 45 named species in the genus, and several species contain more than one serotype. Many, but not all, species have caused human infection. Legionellae thrive in warm moist environments, both natural and artificial, and their multiplication is enhanced by the presence of free-living amoebae, in which the organisms multiply in these environments. Whether the few species which have not been isolated from humans are of lower virulence or simply live in environments with which humans do not come into contact is unknown. Transmission to humans typically occurs as aerosols. Legionellosis is not contagious.

Culture of respiratory secretions is among the most sensitive methods for the diagnosis of *Legionella* infection. The legionellae are intracellular pathogens, residing primarily in macrophages within

the alveoli. Like other intracellular pathogens, they may not be present in every sputum sample and, when present, their numbers may be low. Since many patients with legionellosis do not produce sputum, collection of multiple respiratory specimens may be beneficial.

Urinary antigen testing has supplanted direct immunofluorescence antibody (DFA) testing of sputum for rapid diagnosis of legionellosis (see Appendix 3.11.4-1). Although urine antigen testing is designed to detect infections due to *Legionella pneumophila* serogroup 1, patients with infections due to other species and serogroups may sometimes have a positive test (1, 4). While *L. pneumophila* serogroup 1 represents the most commonly recognized cause of legionellosis, it may account for as little as 50% of cases (8, 9); thus, a negative urinary antigen test is not sufficient to presume that a patient does not have legionellosis.

Testing for antibodies to *Legionella* is not useful in the care of critically ill patients and is not recommended for routine use. Most patients with culture-confirmed legionellosis do not develop detectable antibodies until >3 weeks after infection, and some highly immunosuppressed individuals never produce detectable antibodies (3). Likewise, high titers in acute-phase serum samples are not, in themselves, diagnostic. Many persons have preexisting antibody from prior (subclinical) *Legionella* infection. Antibody tests using paired serum samples may be useful, however, as a tool in tracking nosocomial and community outbreaks.

The procedures presented here include the detection of legionellae by culture of clinical specimens. Refer to procedure 13.5 for culture of environmental samples for legionellae.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING

A. Specimen collection (see procedure 3.11.2 for details on collection of lower respiratory specimens)

■ NOTE: *L. pneumophila* survives in up to 3% salt solutions at temperatures below 30°C; in fact, small amounts of salt (0.1 to 0.5%) enhance survival (5). Saline is not toxic to the organism, as previously thought.

1. Respiratory secretions (sputum, bronchial and tracheal aspirates, bronchial washings)
 - a. Place expectorated specimen in a sterile screw-cap cup.
 - b. Specimens collected by bronchoscopy or aspiration may remain in the Luken trap for transport so long as the free ends of the tubing are securely joined together to prevent leakage of the specimen during transport.
 - c. Collect at least 3 ml of specimen for *Legionella* culture.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING (continued)

2. Bronchoalveolar lavage (BAL) fluid
Submit a minimum of 50 ml of fluid in a sterile container.
 3. Sterile body fluids (pleural, pericardial, peritoneal)
 - a. Submit at least 5 ml in a sterile tube or in the syringe used for collection.
 - b. Remove the needle and cap the syringe with a Luer-Lok before transport.
 4. Bronchial brushings
 - a. Cut the brush off at a point about 30 to 40 mm from the end.
 - b. Place the brush into a small tube containing no more than 0.5 ml of sterile saline or TSB.
 5. Lung tissue
 - a. Place a piece of tissue approximating the size of a dime onto a gauze square moistened with sterile nonbacteriostatic saline.
 - b. Place the gauze square and the tissue into a sterile specimen cup with a screw cap.
 6. Other tissues and wound specimens (including prosthetic heart valves)
 - a. Perform culture for legionellae on such specimens, especially if routine bacterial cultures prove to be negative.
NOTE: Legionellae survive in the specimen when stored in the refrigerator for long periods of time. However, they may also be present in a backup broth used for culture but will not grow or be detected in the broth. If the routine culture is negative, and the specimen is no longer available, subculture the broth to selective medium for legionellae. Post-surgical wound infections due to use of contaminated water for wound care have been reported.
 - b. Collect tissues as for lung tissues.
 - c. Collect external wound specimens on swabs after cleansing the site with sterile saline.
 7. Blood
There are no reliable methods for recovering legionellae from blood. "Blind subculture" of "negative" standard blood culture bottles sometimes results in recovery of the organism. The sensitivity of this method is not sufficient for routine use.
- B. Timing and transport**
1. Submit samples in the acute phase of infection, preferably before the beginning of antimicrobial therapy.
 2. Transport to laboratory quickly. While legionellae may survive extended transport, their isolation may be compromised by overgrowth of commensal bacteria in the specimens.
 3. If specimens are being transported to a remote laboratory, place samples on wet ice for transport.
 4. For extended transport times (>1 day), freeze samples (-70°C) and transport on dry ice.
- C. Rejection criteria**
1. Respiratory secretions (sputum, etc.) submitted for routine bacterial culture are screened for adequacy by Gram stain; *do not* apply these criteria for specimens submitted for *Legionella* culture (6).
NOTE: Patients with Legionnaires' disease typically produce sputum which is thin and watery and may contain few WBCs. Additionally, only normal oral bacterial morphotypes may be seen in Gram stains because legionellae do not stain with Gram stain reagents in clinical specimens.
 2. BAL specimens should never be rejected, since they are collected by an invasive procedure. Since *Legionella* organisms are present in very low numbers, as large a volume of sample as possible (i.e., ideally up to 25 to 30 ml) should be concentrated and centrifuged before culture to optimize the recovery of *Legionella*.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING

(continued)

3. Do not perform “quantitative BAL cultures” for legionellae; their concentration in such specimens is always low.
4. Pleural fluid specimens of less than 5 ml should be cultured only after alerting the physician that such specimens are unreliable for the recovery of *Legionella*.
5. Reject “test-of-cure” cultures, since they should not be used to monitor a patient’s response to therapy.

III. MATERIALS



Include QC information on reagent container and in QC records.

A. Media

1. Inoculate two media for each culture.
 - a. Buffered charcoal-yeast extract agar (BCYE- α)

NOTE: This nonselective medium contains ferric pyrophosphate, cysteine, α -ketoglutarate, and charcoal.
 - b. BCYE- α with polymyxin, anisomycin, cefamandole, and α -ketoglutarate (BMPA- α)

NOTE: While useful for specimens containing other microorganisms, this medium may be inhibitory to *Legionella* species (such as *Legionella micdadei*) which are susceptible to cefamandole. It should never be used alone.
2. Store at 4°C in sealed bags to keep it moist. Do not store for any period out of the bags.

B. Other supplies

1. Inoculating loop
2. Pasteur pipettes
3. Tissue homogenization apparatus (stomacher or other mechanical homogenizer) and TSB
4. Centrifuge to allow for 1,500 \times g and centrifuge tubes for BAL samples (50 ml) and body fluids (10 to 15 ml)

C. Reagents

1. KCl acid-wash solution, pH 2.2
 - a. Hydrochloric acid (0.2 M): add 2 ml of 1 M HCl to 8 ml of distilled water.
 - b. Potassium chloride (0.2 M): dissolve 1.5 g of KCl in 100 ml of distilled water.
 - c. Add 3.9 ml of 0.2 M HCl to 25 ml of 0.2 M KCl. Adjust pH to 2.2 \pm 0.1 at 25°C with HCl or KCl; filter sterilize.
 - d. Aseptically dispense 1-ml volumes into sterile screw-cap tubes containing a few glass beads. Store at room temperature. Shelf life is 1 year.
- NOTE:** KCl solution (pH 2.2, 0.2 M) may be purchased from Remel, Inc. (20 tubes/pack, catalog no. 062621; 100 tubes/case, catalog no. 062620)

Formula is HCl (0.2 M) at 135 ml plus potassium chloride (0.2 M) at 865 ml.
2. BCYE without cysteine (Remel, Inc.)
3. 5% Solution of powdered skim milk in sterile distilled water
4. Beta-lactamase test (procedure 5.3)
5. Hippurate test (procedure 3.17.21)
6. DFA reagents (see Appendix 3.11.4-2)

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

- A. Verify that media are within expiration period. Do not use if media appear to have dried, even if they have not passed the expiration date.

NOTE: In particular, the effectiveness of BMPA- α declines with age as the antimicrobial agents degrade. Media which are stored for extended periods may dry out, causing the concentrations of ingredients to rise and making the media inhibitory.

B. Source of QC organisms

NOTE: True QC of *Legionella* media is difficult, since the organisms rapidly become adapted to growth on artificial media within one or two passages. Media which pass internal QC procedures may not be presumed always to be of adequate quality.

IV. QUALITY CONTROL (continued)

1. Aliquot respiratory or tissue samples from a patient with Legionnaires' disease, for the best source of QC. Store at -70°C with approximately a 1-year expiration date. Lacking a clinical specimen, obtain a low-passage isolate of *L. pneumophila* from a reference laboratory.
2. Suspend the isolate in a 5% solution of sterile skim milk and store in small aliquots at -70°C .
- C. Preparation of QC cultures. Use the methods outlined in Clinical and Laboratory Standards Institute (CLSI; formerly NCCLS) standard M22-A3 (2) and procedure 4.2.
 1. Prepare a culture of the stored *Legionella* isolate on BCYE- α .
 2. Suspend colonies from this first stock culture in sterile TSB to match the turbidity of a no. 1.0 McFarland standard.
 3. Mix this adjusted culture with an equal volume of 10% (wt/vol) skim milk solution. Divide this final suspension into small aliquots and freeze at -70°C .
- D. QC testing
 1. Remove one of the frozen aliquots from the freezer, thaw, and dilute 1/100 in TSB.
 2. Inoculate the volume of a 10- μl inoculating loop onto the surface of the media to be tested.
 3. Incubate for 48 h at 35°C and count the colonies. Depending on the exact concentration of the original suspension, there should be between 50 and 200 colonies on each inoculated plate.
 4. Keep detailed records of the colony counts for each lot of medium. Over time, the viability of the stored inoculum may decline, and a decrease in the number of colonies on the tested plates could be the result of decline in viability of the inoculum rather than a decrease in the quality of the media. In order to assess this possibility, test the lot in question again with a fresh inoculum which has not been frozen.
 5. Verify that BMPA- α is able to inhibit growth of similar suspensions containing *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Candida albicans*.

V. PROCEDURES



It is imperative that these cultures be handled in a biosafety hood.

- A. Commercial multiplex PCR assays have recently become available for the detection of *L. pneumophila*. The Pneumoplex (Prodesse Inc.) PCR assay simultaneously detects *Mycoplasma pneumoniae* and *Chlamydia pneumoniae* as well as *L. pneumophila*, *L. micdadei*, and *Bordetella pertussis* (7); however, this assay is for research use only.
- B. Culture inoculation
 1. For all specimens, inoculate BCYE- α and BMPA- α plates.
 - a. Label plates with patient name and identifying number.
 - b. Record the date and time of inoculation.
 - c. Streak plates in several sectors to produce isolated colonies after incubation.
 - d. After initial processing, save a portion of specimen at 4°C for 7 days in case the specimen is contaminated and acid treatment is indicated.
 2. Respiratory secretions
 - a. Touch a swab to the most purulent portions of the specimen containing blood or mucus, if present.
 - b. Use separate swabs to inoculate each plate.

V. PROCEDURES (continued)

- c. If the specimen is from a patient with cystic fibrosis or a patient known to have colonization with gram-negative rods, acid treat the specimen and inoculate an additional BAP and BCYE- α plate.
 - (1) Place 0.1 ml of sputum sample into 0.9 ml of KCl solution, and vortex.
 - (2) Allow sample to incubate for 5 min at room temperature.
 - (3) Inoculate 0.1-ml portions of the treated specimen onto BCYE- α and BMPA- α .
3. BAL specimen
 - a. Centrifuge 30 to 50 ml or more of specimen at $1,500 \times g$ for 20 min.
 - b. Decant all but 0.3 to 0.5 ml of the supernatant. Vortex to resuspend sediment into the remaining supernatant.
 - c. Use a sterile Pasteur pipette to dispense 2 drops of the specimen onto each plate.
4. Sterile body fluids
 - a. Concentrate at least 10-fold, when possible, by centrifuging at least 5 ml at $1,500 \times g$ for 20 min.
 - b. Decant all but 0.5 ml of the supernatant. Resuspend any sediment in the remaining supernatant by vigorous shaking or vortexing.
 - c. Use a sterile Pasteur pipette to dispense 2 drops of the specimen onto each plate.
5. Bronchial brushings
 - a. Vigorously agitate the brush in the TSB to remove the cellular material adherent to it.
 - b. Use a sterile Pasteur pipette to dispense 2 drops of the specimen onto each plate.
6. Tissue
 - a. Place a piece of tissue no larger than a dime into 5 ml of TSB.
 - b. Homogenize the tissue using a mechanical tissue grinder or a stomaching device.
 - c. Use a sterile Pasteur pipette to dispense 2 drops of the specimen onto each plate.

C. Examination of cultures

1. Incubate cultures on buffered media in ambient air (*i.e.*, without increased CO_2) at 35 to 37°C for 7 days with adequate humidity (50 to 70% relative humidity) to prevent drying of media during incubation.
NOTE: Dried media are inhibitory to growth of *Legionella*. If low incubator humidity is a problem, place sterile gauze moistened with sterile water in bottom of jar and place plates on gauze in the jar.
2. Examine culture plates *daily* with the lids removed.
 - a. Examine plates early each morning so that suspicious colonies can be tested with FA reagents that day for early diagnosis of disease.
 - b. It is imperative to examine the plates using a dissecting microscope with a magnification of $\times 20$ to $\times 50$. Even colonies not yet visible to the naked eye will have the colony morphology typical of *Legionella*.
 - c. Use a focused light source aimed at a high oblique angle to illuminate the surface of the plate.
 - d. Avoid exposure to contaminating airborne molds during examination. Work in a biological safety hood, if possible.
3. At 24 h, if there is heavy growth of non-*Legionella* bacteria on the BCYE- α and BMPA- α plates, reprocess the original specimen using acid treatment, as described above for respiratory secretions containing gram-negative rods.
NOTE: Reprocessing of the culture may not be required if the “routine” culture of the sample yields organisms consistent with the patient’s diagnosis.

V. PROCEDURES (continued)

Consultation with the patient's physician regarding the likelihood of legionellosis may be appropriate prior to undertaking acid treatment of the specimen.

4. When viewed as described above, *Legionella* organisms appear as circular colonies with an entire edge, having a distinctive "ground-glass" appearance (see Fig. 3.11.4-1) and an iridescent blue, green, or pink hue. Only a few other bacteria of medical importance have this appearance. Older colonies are opaque, with a white center and opalescent perimeter.

■ **NOTE:** Colonies resembling *Legionella* may not be present on both media. *L. micdadei* and a few other species may be inhibited by the cefamandole in BMPA- α . On BCYE- α , growth of large amounts of other gram-negative bacteria and yeasts may inhibit the growth of *Legionella*.

Caution: Exercise caution when examining non-Legionella gram-negative bacteria present on BCYE- α . Some highly infectious organisms, such as *Francisella tularensis*, may grow preferentially on BCYE- α . Additionally, fungi such as *Coccidioides immitis* and *Histoplasma capsulatum* may also grow on BCYE- α .

D. Preliminary identification of isolates

1. Gram stain suspicious colonies. Legionellae will appear as small gram-negative rods, which may stain faintly. Typically they are about the size of *Haemophilus* spp.; however, it is not unusual for there to be long filamentous forms present as well.
2. Examine plates with presumptive legionella morphology immediately using a long-wave UV lamp (Wood's lamp) in a darkened room, since some legionellae exhibit autofluorescence.
 - a. Bright blue-white fluorescence
 - (1) *Legionella bozemanii*
 - (2) *Legionella dumoffii*
 - (3) *Legionella gormanii*
 - (4) *Legionella anisa*
 - (5) *Legionella tucsonensis*
 - (6) *Legionella cherrii*
 - (7) *Legionella parisiensis*
 - (8) *Legionella steigerwaltii*

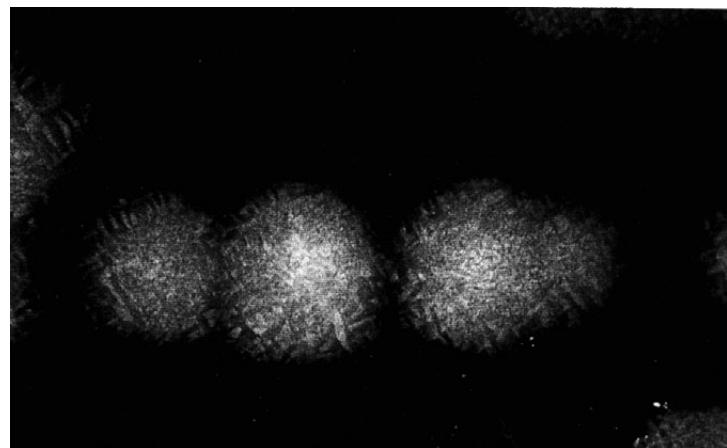


Figure 3.11.4-1 Colonies of *L. pneumophila* on BCYE- α showing ground-glass appearance.

V. PROCEDURES (continued)

- b.** Yellow-green fluorescence
 - (1) *Legionella wadsworthii*
 - (2) *Legionella birminghamensis*
- c.** Red fluorescence
 - (1) *Legionella erythra*
 - (2) *Legionella rubrilucens*
- 3.** Subculture suspicious colonies to BCYE- α and either a BAP or a plate of BCYE- α from which cysteine has been omitted. Do not use CHOC, since some *Legionella* isolates will grow on this medium.
 - a.** Incubate plates at 35 to 37°C for 24 to 48 h.
 - b.** Isolates which grow on BCYE- α but not on BAP or on the unsupplemented BCYE plate should be considered as presumptive *Legionella* spp. However, since rapid diagnosis is important to patient care, do not wait for these subculture results to perform confirmatory FA testing on typical colonies.
- 4.** Perform rapid hippurate test and beta-lactamase test on suspicious colonies.
 - NOTE:** There are few biochemical tests which can be used to identify legionellae. Testing for the ability to hydrolyze hippurate may be of routine value, especially for laboratories that do not perform DFA testing. All serotypes of *L. pneumophila* hydrolyze hippurate, but a few less common species (e.g., *Legionella spiritensis*, *Legionella waltersii*) may also. Nevertheless, since *L. pneumophila* is the predominant species isolated from human infections, the finding that an isolate hydrolyzes hippurate is reasonably powerful suggestive evidence of its identity.
- 5.** Use the beta-lactamase test to separate *L. pneumophila* (positive) from other species, such as *L. micdadei* (negative), as an aid in choosing which antisera to test for identification.

E. Definitive identification of isolates

- 1.** For definitive identification, refer isolates to the local or state health department laboratory or a reference laboratory.
 - NOTE:** Definitive identification of *Legionella* to the species level requires testing not generally available in routine clinical microbiology laboratories, including the determination of the major branched-chain fatty acids contained in the cells and/or genetic analysis.
- 2.** Identify the most prevalent species, *L. pneumophila* and *L. micdadei*, by testing the isolated colonies with DFA or indirect FA reagents, which are available commercially.
 - a.** Perform FA tests as soon as suspicious colonies are isolated and preliminary rapid tests are performed.
 - b.** Do not wait for subculture to confirm that the isolate does not grow on BAP, as delays may affect patient care.
 - c.** See Appendix 3.11.4–2 for procedure.

POSTANALYTICAL CONSIDERATIONS**VI. REPORTING RESULTS****A. Negative cultures**

1. Preliminary report: "No *Legionella* isolated to date."
2. Final report: "No *Legionella* isolated after 7 days."

B. Positive cultures

1. Report "Positive for *Legionella* spp." if isolate has only been identified with polyvalent DFA reagents.
2. If serogroup or species has been determined, then report using the full species and/or serogroup designation, e.g., "Positive for *Legionella micdadei*."
3. Notification
 - a. For hospitalized patients, notify the patient's physician immediately. Notify infection control immediately also.
 - b. For outpatients or recently discharged patients, notify the patient's physician immediately.
 - c. Report positive cultures and urinary antigen tests to local or state health departments using whatever means is customary, following local disease reporting guidelines.
4. Storage of isolates
Molecular methods for typing *Legionella* isolates are available from some reference laboratories. Since an individual patient may be a part of a developing outbreak, it is prudent to save clinical *Legionella* isolates in 5% sterile skim milk at -70°C for possible future typing studies.

C. Contaminated cultures

If culture is overgrown with bacteria, yeast, or mold, report "Culture terminated due to growth of contaminating microbiota."

VII. LIMITATIONS

- A. Legionellae are reliably susceptible to macrolides (azithromycin, erythromycin), quinolones (e.g., levofloxacin), and rifampin. Acquisition of resistance during therapy does not occur. In vitro susceptibility testing is not useful for selecting the "best" antimicrobial agent or for any other therapeutic purpose and should not be performed.
- B. Legionellae are intracellular pathogens and may persist in sputum for several weeks after the institution of antimicrobial therapy. Test-of-cure cultures should not be used to monitor a patient's response to therapy, since patients who are recovering from Legionnaires' disease may continue to shed organisms. The patient's clinical response is the best indicator of a response to therapy.
- C. Culture may be the most sensitive indicator of disease, but a negative culture does not rule out infection. Inhibitors of growth, such as the presence of yeasts, sampling error in collection, and prior treatment with antimicrobial agents, can affect the sensitivity of culture.
- D. Legionellae have been isolated on CHOC in rare instances.
- E. *F. tularensis*, *Bordetella pertussis*, and *Afipia* will grow on BCYE- α and not on BAP. The former two species are not motile and the latter does not have the colony morphology of *Legionella* spp.
- F. Some species of *Legionella* will not react with current antisera. Suspicious colonies should be submitted to a reference laboratory for further identification.

REFERENCES

1. **Benson, R. F., P. W. Tang, and B. S. Fields.** 2000. Evaluation of the Binax and Biostest urinary antigen kits for detection of Legionnaires' disease due to multiple serogroups and species of *Legionella*. *J. Clin. Microbiol.* **38**:2763–2765.
2. **Clinical and Laboratory Standards Institute.** 2004. *Quality Assurance for Commercially Prepared Microbiological Culture Media*, 3rd ed. Approved standard M22-A3. Clinical and Laboratory Standards Institute, Wayne, Pa.
3. **Edelstein, P. H., R. D. Meyer, and S. M. Finegold.** 1980. Laboratory diagnosis of Legionnaires' disease. *Am. Rev. Respir. Dis.* **121**:317–327.
4. **Helbig, J. H., S. A. Uldum, P. C. Luck, and T. G. Harrison.** 2001. Detection of *Legionella pneumophila* antigen in urine samples by the BinaxNOW immunochromatographic assay and comparison with both Binax *Legionella* Urinary Enzyme Immunoassay (EIA) and Biostest *Legionella* Urin Antigen EIA. *J. Med. Microbiol.* **50**:509–516.
5. **Heller, R., C. Holler, R. Sussmuth, and K. O. Gundermann.** 1998. Effect of salt concentration and temperature on survival of *Legionella pneumophila*. *Lett. Appl. Microbiol.* **26**:64–68.
6. **Ingram, J. G., and J. F. Plouffe.** 1994. Danger of sputum purulence screens in culture of *Legionella* species. *J. Clin. Microbiol.* **32**:209–210.
7. **Khanna, M., J. Fan, K. Pehler-Harrington, C. Waters, P. Douglass, J. Stallock, S. Kehl, and K. J. Henrickson.** 2005. The Pneumoplex assays, a multiplex PCR-enzyme hybridization assay that allows simultaneous detection of five organisms, *Mycoplasma pneumoniae*, *Chlamydia pneumoniae*, *Legionella pneumophila*, *Legionella micdadei*, and *Bordetella pertussis*, and its real-time counterpart. *J. Clin. Microbiol.* **43**:565–571.
8. **Marston, B. J., H. B. Lipman, and R. F. Breiman.** 1994. Surveillance for Legionnaires' disease. Risk factors for morbidity and mortality. *Arch. Intern. Med.* **154**:2417–2422.
9. **Reingold, A. L., B. M. Thomason, B. J. Brake, L. Thacker, H. W. Wilkinson, and J. N. Kuritsky.** 1984. *Legionella* pneumonia in the United States: the distribution of serogroups and species causing human illness. *J. Infect. Dis.* **149**:819.

SUPPLEMENTAL READING

- Benin, A. L., R. F. Benson, and R. E. Besser.** 2002. Trends in Legionnaires disease, 1980–1998: declining mortality and new patterns of diagnosis. *Clin. Infect. Dis.* **35**:1039–1046.
- Edelstein, P. H.** 1998. Antimicrobial chemotherapy for Legionnaires disease: time for a change. *Ann. Intern. Med.* **129**:328–330.
- Edelstein, P. H.** 1998. Legionnaires' disease. *N. Engl. J. Med.* **338**:200–201.
- Grant, W. W., V. S. Baselski, and R. G. Wunderink.** 2001. *Legionella* and community-acquired pneumonia: a review of current diagnostic tests from a clinician's viewpoint. *Am. J. Med.* **110**:41–48.
- Koneman, E. W., S. D. Allen, W. M. Janda, P.C. Schreckenberger, and W. C. Winn, Jr.** 1997. *Color Atlas and Textbook of Diagnostic Microbiology*, 5th ed, p. 473–489. J. B. Lippincott, Philadelphia, Pa.
- Stout, J. E., and V. L. Yu.** 1997. Legionellosis. *N. Engl. J. Med.* **337**:682–687.
- Stout, J. E., J. D. Rihs, and V. L. Yu.** 2003. *Legionella*, p. 809–823. In: P. R. Murray, E. J. Baron, J. H. Jorgensen, M. A. Pfaffer, and R. H. Yolken (ed.), *Manual of Clinical Microbiology*, 8th ed. ASM Press, Washington, D.C.

APPENDIX 3.11.4-1

Legionella Urinary Antigen Test**I. PRINCIPLE**

Urinary antigen testing has supplanted direct immunofluorescence testing of sputum for rapid diagnosis of legionellosis. While urinary antigen tests are generally of excellent specificity, the sensitivity (70 to 90%) is less than that of culture. These tests detect infections due to *Legionella pneumophila* serogroup 1 and perhaps some other serogroups as well (1, 2). When possible, antigen tests should be performed in conjunction with culture or respiratory secretions to enable the recovery of species and serotypes not detected by the urinary antigen test and also to provide organisms for molecular typing in the event that the patient is part of an outbreak of Legionnaires' disease. (Also see procedure 11.4.)

II. SPECIMEN

- A. Collect at least 5 ml of urine by clean catch or from a catheter.
- B. Submit in a sterile screw-cap container. Storage and transport in urine preservative tubes containing boric and formic acids are also acceptable.
- C. Store samples at 4°C until tested.
- D. Allow patient samples to come to room temperature before testing.

III. MATERIALS

- A. NOW *Legionella* urinary antigen test kit (Binax, Inc., Portland, Maine)
- B. For larger-volume laboratories, use similar tests available in EIA or ELISA format.
 1. *Legionella* urinary antigen EIA kit (Binax, Inc.)
 2. Bartels *Legionella* urinary antigen ELISA (Intracel Corp., Rockville, Md.)
 3. Urine *Legionella* antigen ELISA (Wampole Laboratories, Cranbury, N.J.)

IV. QUALITY CONTROL

- A. Remove control swabs (positive and negative) from pouch.
- B. Place each swab into a test card as described for patient specimens.
- C. Unlike for test specimens, add 6 drops of reagent A to the control specimens.
- D. Seal the test cards, as described for the assay, and incubate for 15 min.

V. ASSAY PROCEDURE

- A. Open test card pouch just before use. Label the test card with the sample accession number and other appropriate information.
- B. Dip a test swab (supplied as part of the test kit) into the urine sample, completely covering the head of the swab. Remove the swab from the urine.
- C. If the swab drips, touch the swab to the side of the container to remove the excess sample.
- D. Insert the swab through the bottom hole on the test card and push it forward until the top of the swab is visible in the top hole of the card.
- E. Hold the vial of reagent A vertically, 1 to 1.5 in. above the test card. Add 2 drops of reagent A to the bottom hole of the card.
- F. Peel off the adhesive tape from the right side of the card. Close and seal, pressing down on the edge of the test card. Incubate at room temperature.
- G. Start timer and read result after 15 min of incubation.

APPENDIX 3.11.4–1 (continued)**VI. INTERPRETATION**

- A. Determine results after 15 min of incubation. Reading results beyond this point may produce inaccurate results.
- B. If no pink lines are seen in the test window, the assay is invalid and should be repeated.
- C. A negative sample will produce a pink upper control line. The lower test line in the window will not be visible.
- D. A positive sample will display two pink lines. Both the upper control line and the lower test line will be visible.

VII. REPORTING RESULTS

- A. Positive urinary antigen

Report “Positive for urinary antigen of *Legionella pneumophila* serogroup 1.”

- B. Negative urinary antigen

1. Report “Negative for urinary antigen of *Legionella pneumophila* serogroup 1.”
2. Also add the following comment: “Other serogroups and species of *Legionella* which may cause human disease are not detected by this test. Culture of respiratory secretions is recommended if infection with *Legionella* is still suspected.”

VIII. LIMITATIONS

- A. This test detects primarily *L. pneumophila* serogroup 1 and should not be relied on solely for the diagnosis of legionellosis. A negative test does not rule out the possibility of legionellosis caused by other serogroups and species.
- B. *Legionella* urinary antigen may persist in the urine of successfully treated patients for weeks to months following recovery. Sequential urinary antigen tests should not be performed to monitor response to therapy.
- C. In one study comparing two EIAs and the NOW test, the sensitivities of the assays were 88 to 100% for severe cases of pneumonia but dropped to 40 to 53% for milder cases of the disease (3).

References

1. Benson, R. F., P. W. Tang., and B. S. Fields. 2000. Evaluation of the Binax and Biotest urinary antigen kits for detection of Legionnaires' disease due to multiple serogroups and species of *Legionella*. *J. Clin. Microbiol.* **38**:2763–2765.
2. Helbig, J. H., S. A. Uldum, P. C. Luck, and T. G. Harrison. 2001. Detection of *Legionella pneumophila* antigen in urine samples by the Binax NOW immunochromatographic assay and comparison with both Binax *Legionella* Urinary Enzyme Immunoassay (EIA) and Biostest *Legionella* Urin Antigen EIA. *J. Med. Microbiol.* **50**:509–516.
3. Yzerman, E. P. F., J. W. den Boer, K. D. Lettinga, J. Schellekens, J. Dankert, and M. Peeters. 2002. Sensitivity of three urinary antigen tests associated with clinical severity in a large outbreak of Legionnaires' disease in The Netherlands. *J. Clin. Microbiol.* **40**:3232–3236.

APPENDIX 3.11.4–2**Detection of *Legionella* by Fluorescent Antibody****I. PRINCIPLE**

Rabbit antibodies prepared against *Legionella* species and serogroups are conjugated with fluorescein isothiocyanate (FITC). The antibodies will bind to the homologous *Legionella* species, making them visible when viewed under UV light. Direct immunofluorescence is most commonly used for the identification of *Legionella* isolates from cultures.

Direct immunofluorescence may also be used to detect legionellae in clinical specimens; however, the sensitivity of this procedure may be low, depending on the skill and experience of the operator (1). It should be used cautiously by inexperienced laboratorians for critical specimens such as lung tissue. Some laboratories use it as an adjunct to examination of highly contaminated sputum specimens in conjunction with acid decontamination and culture (J. Stout, personal communication). (Also see procedure 11.3.)

APPENDIX 3.11.4–2 (continued)**II. SPECIMENS****A. Tissue, fresh**

1. Use a sterile scalpel to cut a fresh face on the surface of the tissue. Homogenize the tissue in sterile water to make an approximately 10% suspension.
2. Alternatively, use a sterile scalpel to cut a fresh face on the surface of the tissue. Grasping the tissue with forceps, press the newly exposed face of the tissue to a clean slide.
3. Make one slide for each antibody conjugate employed and one slide for a negative control conjugate.
4. Air dry and heat fix.
5. Fix smears in 1% formalin for 10 min. Drain and rinse off formalin with a gentle stream of filtered water.

B. Tissue, formalin fixed

1. Use a scalpel to cut a fresh face on the tissue. Place the tissue in a petri dish and use the scalpel to scrape a fine puree of debris and tissue fluids from the newly cut surface of the tissue.
2. Use the scalpel blade to transfer portions of the puree to clean microscope slides.
3. Prepare one slide for each antibody conjugate employed and one slide for the negative control conjugate.
4. Air dry and heat fix.

C. Respiratory secretions

1. If present, select purulent-appearing portions of the sample. Use a swab to prepare thin smears of the sample on clean microscope slides. Prepare one slide for each antibody conjugate employed and one slide for the negative control conjugate.
2. Air dry and heat fix.
3. Fix in 1% formalin for 10 min. Drain and rinse with a gentle stream of filtered water.

D. Any bacterial colony with morphology resembling *Legionella* spp.

1. Emulsify colonies into 1% formalin to match the turbidity of a no. 0.5 McFarland standard in saline or phosphate-buffered saline (PBS [Appendix 3.11.4–3]). Dilute the suspension 1/100 in PBS. *Note:* Making these suspensions too dense may quench the fluorescence.
2. Use a separate slide for each antibody conjugate to be tested.
3. Fix in 1% formalin for 10 min. Drain and rinse with a gentle stream of filtered water.

III. MATERIALS**A. Reagents**

1. *Legionella* antisera, conjugated with FITC. Minimally, use conjugates reactive with *L. pneumophila* serogroups 1 to 6 and *L. micdadei*, except where local experience indicates an increased prevalence of other *Legionella* species. In these latter instances, antibody conjugates reactive with a broader range of species or serogroups are available.

a. SciMedx, Inc., Danville, N.J.

(1) Polyvalent *L. pneumophila* serogroup 1 to 6 reagent and monovalent *L. micdadei* reagent or

(2) Polyvalent *L. pneumophila* serogroups 1 to 6, *L. bozemani*, *L. dumoffii*, *L. gormanii*, *Legionella longbeachae* serogroups 1 and 2, *L. micdadei*, *Legionella jordanis*

b. Zeuss Scientific, Raritan, N.J.

Polyvalent *L. pneumophila* serogroup 1 to 6 reagent

2. FITC-conjugated negative control serum (normal rabbit globulins)

3. PBS, pH 7.5, filtered (*see* Appendix 3.11.4–3)

4. Buffered glycerol mounting fluid, pH 9.0

5. Formalin (1%) in 0.85% saline (10% formalin diluted 1/10 with saline)

6. Distilled water, filtered (*see* below)

7. Low-fluorescence immersion oil

NOTE: Filter all aqueous reagents made with locally produced deionized water through a 0.22-μm-pore-size filter before use. Legionellae may colonize water-deionizing systems and produce false-positive tests.

APPENDIX 3.11.4–2 (continued)**B. Alternative methods—indirect FA staining**

■ NOTE: Test kits employing indirect FA methods for staining of *Legionella* are also available and have performance similar to that of the DFA staining method described above. Contact the vendors for additional information, other supplies needed, and specific instructions for testing.

1. Remel, Inc. (Lenexa, Kans.)—*Legionella* Poly-ID test kit

L. pneumophila serogroups 1 to 6, *L. dumoffii*, *L. longbeachae* serogroups 1 and 2, *L. bozemanii*, *L. gormanii*, *L. micdadei*, *L. wadsworthii* serogroup 1, *Legionella oakridgensis*, *Legionella feeleii* serogroup 1, *Legionella sainthelensi* serogroup 1, *L. jordanis* serogroup 1, *Legionella anisa* serogroup 1, *L. spiritensis* serogroup 1, *Legionella hackeliae*, *Legionella maceachernii*, *Legionella james-townensis*, *L. cherrii* serogroup 1, *L. steigerwaltii* serogroup 1, *L. parisiensis*, *L. rubrilucens* serogroup 1, *L. erythra* serogroup 1.

2. Bio-Rad Laboratories (Hercules, Calif.)—MONOFLUO monoclonal immunofluorescence test kit contains monoclonal antibody reactive with all known serogroups of *L. pneumophila*.
3. Zeuss Scientific—*Legionella* IFA test system
Legionella pneumophila serogroups 1 to 4

C. Supplies

1. Clean plastic wash bottles
2. Microscope slides suitable for FA testing. FA slides with delineated circles are recommended (Precision Laboratory Products, Middleton, Wis., <http://www.precisionslides.com>).
3. Tuberculin syringes or 50-μl pipettor to dispense antiserum
4. Wooden applicator sticks
5. Humidified staining chamber: a large covered petri dish lined with filter paper or covered glass baking dish lined with a paper towel. Chamber should contain a rack (e.g., glass rods) so that the slides do not sit directly on the moistened paper.

IV. QUALITY CONTROL**A. Prepare bacterial control slides containing *Legionella* for use with each test.**

1. *L. pneumophila* serogroup 1 may be used as a positive control for conjugates containing antibody against *L. pneumophila* and as a negative control for *L. micdadei* antibody conjugates.
2. *L. micdadei* may be used as a positive control for conjugates containing *L. micdadei* antibody and as a negative control for conjugates containing *L. pneumophila* antibody.
3. For polyvalent conjugates containing *L. micdadei* antibody conjugates, use another species of *Legionella* or *Escherichia coli* (e.g., ATCC 25922) as a negative control.
4. Prepare suspensions of the control organisms as described above for cultures, or purchase formalin- or heat-killed suspensions of control organisms from vendor that supplies antibody reagents.

B. Place a drop of the diluted suspension of each control organism on one slide for each conjugate used and on one slide for the negative antibody conjugate. Thus, each control slide should contain both *L. pneumophila* and *L. micdadei* or another species if other species are being tested.

C. Prepare an extra slide for each specimen being tested, to use as a negative specimen control.

D. Do not report patient results if positive control slides do not produce a 3+ or greater fluorescence or the negative control slides produce more than 1+ fluorescence. See item VI for definitions of degree of fluorescence.

V. PROCEDURE**A. Place the slides in a humidified chamber on racks.**

- B. Add 25 to 50 μl of the *L. pneumophila* polyvalent conjugate to each circle on one of the specimen slides and one of the bacterial control slides. Only one conjugate should be applied to any one slide, although one slide may contain several specimens.

APPENDIX 3.11.4–2 (continued)

- C. Use a wooden applicator stick to spread the conjugate drop over the entire area delineated by the circles on all of the slides. Use caution to avoid carrying over conjugate or bacteria between the circles. *Use a separate applicator stick for each circle or break off the end of the applicator before spreading the conjugate in the next circle.*
- D. Similarly, add the *L. micdadei* conjugate to the specimen slides and a bacterial control slide.
- E. Add 25 to 50 µl of the negative antibody conjugate to each circle on the control slide prepared from each of the specimens being tested and to a bacterial control slide. Spread within each circle, as described above.
- F. Place the slides (test slides and control slides) into the humidified chamber, making certain that they do not touch each other. Cover the chamber to exclude light and allow the slides to incubate at room temperature for 20 to 30 min.
- G. Rinse the antibody off of the slides with a gentle stream of PBS from a squirt bottle. While rinsing, hold the slide horizontally and allow the PBS to run across one circle at a time to minimize the chance that stained bacilli will be transferred between the circles.
- H. Place the slides back onto the rack in the chamber or on a staining rack in a sink. Fill each circle with PBS and allow the slides to sit for 10 min. Do not allow the PBS to run between the circles, since stained bacilli from one sample may be carried to the next circle. Rinse with deionized water.
- I. Wash and rinse the slides one more time with PBS and deionized water as described above. Allow slides to air dry.
- J. Place a drop of buffered glycerol mounting fluid and a no. 1 coverslip on each slide. Examine immediately.
- K. Examine slides using transmitted or incident UV light. Check with your microscope supplier for the proper combination of barrier and exciter filters needed for use with FITC.

VI. INTERPRETATION

- A. Scan the slides with a 40× objective. Legionellae from cultures may appear as rods or as long filaments which stain apple-green on their periphery. Examine the bacterial control slides, where the bacteria will be easily visualized, first.
- B. Examine positive areas of the slides with a 100× oil immersion objective to quantitate the fluorescence.
 1. 4+: cell wall appears as a brilliant yellow-green ring around a less bright central area of the bacilli.
 2. 3+: cell wall stains bright yellow-green.
 3. 2+: cell wall is dull yellow-green, barely discernible from central area of cell.
 4. 1+: diffuse faint staining of cells; cell center is indistinguishable from cell wall.
- C. Fluorescence in the “test” conjugate of ≥3+ and no fluorescence in the control conjugate is a positive test as long as there is ≥3+ fluorescence of the appropriate bacterial control suspension.
- D. Similarly examine the negative control slides containing the negative conjugate. They should display no more than 1+ fluorescence.
- E. Examine slides made from clinical specimens (especially respiratory secretions) for at least 5 min, since they may contain few bacilli. Bacteria in clinical specimens may appear either intra- or extracellular.

VII. REPORTING RESULTS

- A. Report positive or negative results on direct tests as “Positive (or negative) for *Legionella* (add the antiserum name that produced the positive result) by direct fluorescent-antibody test.”
- B. For cultures, use the positive results of the species-specific antiserum to definitively identify the species present.

VIII. LIMITATIONS

- A. Dim fluorescence (≤2+) of the test conjugate and dim fluorescence of the control conjugate indicate probable cross-reaction with a non-*Legionella* bacterium.
- B. Dim fluorescence of the test conjugate and no fluorescence in the control conjugate indicate possible cross-reaction with a species or serotype of *Legionella* other than the ones against which the test conjugate was produced. This result may also be

APPENDIX 3.11.4–2 (continued)

produced by some cross-reacting non-*Legionella* organisms, such as *P. aeruginosa*, *Pseudomonas fluorescens*, *B. pertussis*, and *Bacteroides* spp. These cross-reacting bacteria can be recognized because the size and shape of the fluorescent cells will be different from those of the fluorescent cells of *Legionella* spp. Compare the morphologies of bacteria in these slides to those of the positive bacterial control slides.

- C. The sensitivity of direct immunofluorescence testing on clinical samples may range from <50% to about 75%, depending on the skill and experience of the operator.

Reference

1. Edelstein, P. H., R. D. Meyer, and S. M. Finegold. 1980. Laboratory diagnosis of Legionnaire's disease. *Am. Rev. Respir. Dis.* **121**:317–332.

APPENDIX 3.11.4–3**Phosphate-Buffered Saline, pH 7.5 ± 0.1**

- A. Stock buffer (pH will not be 7.5)

Na ₂ HPO ₄ (anhydrous reagent grade)	13.8 g
NaH ₂ PO ₄ ·H ₂ O (reagent grade)	1.8 g
NaCl (reagent grade)	85.0 g
Distilled water to make final volume of	1,000.0 ml

- B. Working solution (pH 7.5 ± 0.1; 0.01 M buffer; 0.85% NaCl)

concentrated stock solution	100.0 ml
distilled water to make final volume of	1,000.0 ml

- C. When commercially available reagent packets are used

1. Add one envelope of the powdered mixture to a 1-liter volumetric flask, and fill the flask to 1 liter with freshly deionized water.
 2. Mix until all powder is dissolved. Buffer should be clear and colorless.
 3. Measure the pH. Adjust to pH 7.5 ± 0.1 with HCl or NaOH.
 4. Assign a 1-month expiration date. Store at 2 to 8°C.
- D. Filter buffer if made with locally produced deionized water through a 0.22-μm-pore-size filter before use.

3.11.5

Otitis Cultures

[Updated March 2007]

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Otitis media is an infection of the middle ear and accounts for more than 30 million office visits per year for a cost of over \$2 billion in the United States (1). More than 80% of children have one or more episodes of otitis media by age 6 years, with the highest number of cases between 2 and 6 years of age (17). Hearing loss and deficits in learning are a few of the complications (14). The most common agents of otitis media are *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Moraxella catarrhalis*, and *Alloiococcus otitis* (2, 3, 9, 14), although *Streptococcus pyogenes* is found on a seasonal basis (12, 14). (Note: Although the name *Alloiococcus otitis* is in common usage [6], that name has not been officially adopted; *Alloiococcus otitis* is the official name.) Amoxicillin and amoxicillin-clavulanate are the drugs of choice for initial treatment (15). Although *S. pneumoniae* organisms are becoming increasingly more resistant, the concentrations of amoxicillin that can be achieved in the middle ear fluid are sufficient to eliminate all but the most resistant strains (12). Treatment generally resolves the infection, but treatment failures occur and

surgical intervention can be necessary (4, 11, 12, 15). Tympanocentesis and culture of the middle ear fluid constitute a valuable tool for definitive diagnosis, to guide therapy, to evaluate treatment failures, and for research studies to determine the efficacy of antimicrobials against the most common agents. However, the diagnosis is usually made on clinical grounds, because of the invasive nature of tympanocentesis. Culture is usually reserved for persistent infections. Recently, multiplex PCR methods for the detection of common agents have been used to improve sensitivity in the diagnosis (8, 16). Historically pediatricians cultured the nasopharynx to predict the pathogens in the middle ear fluid. This practice is no longer recommended, since the presence of pathogens in the nasopharynx does not predict the pathogens present in the middle ear (7). However, lack of isolation of any pathogen has a 96% negative predictive value for lack of a pathogen in the middle ear fluid (7).

Otitis externa is an infection of the external auditory canal. Unique problems occur with this infection because of the narrow and tortuous nature of the canal

and its tendency to trap foreign objects, wax, and water. Infections are classified as acute and chronic. Acute infections are often referred to as “swimmer’s ear.” *Pseudomonas aeruginosa* is a frequent cause of freshwater otitis, and *Vibrio alginolyticus* is a cause in oceanic swimmers, although other aerobic bacteria can be involved. Localized infections with *Staphylococcus aureus* or *S. pyogenes* can also occur. Contaminating skin bacterial microbiota (corynebacteria and staphylococci) can be present, which are not significant. More invasive infections are caused by extension of bacteria into the adjacent soft tissues and bone, with the formation of a cholesteatoma. Chronic otitis is usually caused by bacterial infection, and although *P. aeruginosa* may be predominant, a variety of anaerobes may also be present. Tissue and bone samples from the inner ear should also be cultured for fungi, *Nocardia*, and mycobacteria, which may also be etiologic agents of chronic infection (12). Underlying diseases such as tertiary syphilis may also cause chronic otitis, but this presentation is rare in the developed world (12).

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING



Observe standard precautions.

NOTE: Refer to procedure 3.3.1 for additional details. The following can be copied for preparation of a specimen collection instruction manual for physicians and other caregivers. It is the responsibility of the laboratory director or designee to educate physicians and caregivers on proper specimen collection.

A. Specimen collection

1. External ear

- a. Insert sterile swab into ear canal until resistance is met.
- b. Rotate swab and allow fluid to collect on swab.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING (continued)

2. Tympanocentesis fluid
 - **NOTE:** Because of the invasive nature of the collection process, these specimens are usually submitted primarily to diagnose middle ear infections only if previous therapy has failed.
 - a. Clean the external canal with mild detergent.
 - b. Using a syringe aspiration technique, the physician will obtain the fluid from the ear drum.
 - c. Send the specimen in a sterile container or in the syringe capped with a Luer-Lok and with the needle removed.
 - d. If the eardrum is ruptured, collect exudate by inserting a sterile swab through an auditory speculum.
- B. Specimen transport
 1. Submit to laboratory
 - a. Submit swabs in tube of transport medium or in BD Culturette EZ.
 - b. Submit aspirates in a sterile container or in the original syringe capped with a Luer-Lok to prevent leakage.
 2. Label specimens with demographic information, date and time of collection, and site of collection.
 3. List the diagnosis of otitis media, chronic otitis, or otitis externa.

III. MATERIALS

A. Primary media

1. BAP
2. CHOC
3. MAC or EMB
4. BHI agar prepared with 5% rabbit blood for detection of *A. otitis* (The following companies supply defibrinated rabbit blood and dehydrated BHI agar for preparation of media: Remel, Inc.; BD Diagnostic Systems; and Hardy, Inc.)
 - **NOTE:** *A. otitis* occasionally grows on BAP with 5% sheep blood in 5 days but does not grow on CHOC, Mueller-Hinton agar (MH) with lysed horse blood, buffered charcoal-yeast extract agar, brucella agar, or Columbia colistin-

nalidixic acid agar (3). Growth generally takes 72 h on rabbit blood agar (3). Routine testing for this organism is beyond the capabilities of most laboratories, but awareness of the species may be important to communicate to the physician.

B. Identification methods

1. Gram stain (procedure 3.2.1)
2. Refer to procedure 3.3.2 for identification of the common agents of otitis.

C. Other supplies

1. Incubator at 35°C with 5% CO₂ or a CO₂-generating system
2. Inoculating sticks and loops
3. Petri dishes and filter paper

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

- A. Verify that plate media meet expiration date and QC parameters per current Clinical and Laboratory Standards Institute (CLSI; formerly NCCLS) document M22. See procedures 14.2 and 3.3.1 for further procedures.
- B. Test each lot of CHOC per procedure 3.3.1.

V. PROCEDURE



Observe standard precautions.

A. Inoculation

1. Inoculate specimen to BAP, CHOC, and MAC.
2. Firmly roll swab over one-sixth (no more) of the agar surface, or aspirate 3 or 4 drops of fluid onto agar. Streak carefully for isolation in four quadrants to minimize overgrowth by other microorganisms.

B. Incubation

1. Incubate plate at 35 to 37°C in 5% CO₂ for a minimum of 48 h.
2. For cultures of invasively collected ear fluid samples, incubation may be extended to 4 days, if results are negative. Anaerobic cultures may be indicated (see section 4).

V. PROCEDURE (continued)**C. Gram stain**

1. Perform a Gram stain from the swab or fluid (procedure 3.2.1).
2. Note the presence of WBCs and bacteria.

D. Culture examination

1. External otitis: observe plates at 24 and 48 h for growth of enteric gram-negative rods, pseudomonads, vibrios, streptococci, coryneforms, and *S. aureus*.
 - a. Since only one pathogen is generally responsible for otitis externa, mixed cultures of gram-negative rods should be minimally identified.
 - b. For gram-negative rods, perform spot oxidase and indole tests to avoid misidentifications.
 - (1) *V. alginolyticus* is an oxidase- and indole-positive, gram-negative rod that grows well on MAC. It will grow on MH with 4% salt (10). Place a colistin or polymyxin B disk on MH. It is further identified by most kit systems, although the reactions are improved if the inoculum is made in saline rather than water (see Table 3.18.2–8).
 - (2) *P. aeruginosa* is oxidase positive and indole negative and often has a characteristic odor or blue-green or brown pigment, for definitive identification. If the characteristic odor or these pigments are lacking, it can be definitively identified by positive growth at 42°C and production of a fluorescent pigment on agar media (procedure 3.17.17).
 - (3) Other gram-negative rods are less common and are usually identified by commercial kits. Exception: swarming *Proteus* spp. are identified by indole, ampicillin susceptibility, and ornithine (see Table 3.3.2–5).
 - (4) Perform antimicrobial susceptibility testing (AST) on the predominant microorganism. Evaluate swarming *Proteus* organisms on MAC or EMB, where they generally do not swarm, to be sure they are the predominant microorganism before performing AST; they can be an important pathogen from a patient with diabetes mellitus.
 - c. Examine cultures for predominant gram-positive cocci. Generally *S. aureus* and *S. pyogenes* are the most common gram-positive cocci involved in otitis externa.
 - (1) Identify *S. pyogenes* and other beta-hemolytic streptococci (Table 3.3.2–5).
 - (2) Identify and perform AST on *S. aureus* (Table 3.3.2–5 and section 5, respectively)
 - d. Identify yeasts and molds, if present. *Aspergillus* and *Candida albicans* have been implicated in chronic infections.
 - e. Since resident cutaneous microbiota (coagulase-negative staphylococci and coryneforms) are normal in the external ear canal, they should not be further evaluated.
2. Otitis media: observe plates at 24 h and up to 4 days for cultures of middle ear fluid. Pursue all organisms present, since the specimen is collected by an invasive procedure and any microorganism can be considered the agent of disease.
 - a. Fastidious gram-negative rods and diplococci
 - (1) Identify *H. influenzae* and *M. catarrhalis* according to Table 3.3.2–5 and perform beta-lactamase test on *H. influenzae*. Since more than 90% of *M. catarrhalis* organisms are beta-lactamase positive, testing is not helpful to treatment.
 - (2) *Bordetella trematum* is an oxidase-negative, catalase-positive, gram-negative rod that has been implicated in ear infections (18). It is motile, frequently reduces nitrate, and may or may not grow on MAC (see Table 3.11.6–1).

V. PROCEDURE (continued)

- b.** Observe for growth of gram-positive cocci. Refer to Table 3.3.2–5 for rapid identification tests to identify the following.
 - (1) *S. pyogenes* and other beta-hemolytic streptococci
 - (2) *S. pneumoniae*
 - (a) Confirm negative bile solubility test with optochin disk.
 - (b) Perform AST, using standardized methods (reference 5 and section 5), per laboratory protocol and physician policy.
- c.** Normal skin microbiota (coagulase-negative staphylococci and corynebacteria) are not generally identified to the species level unless they are the only predominant species in the culture and are present in large numbers.
 - (1) Identify *Turicella otitidis*, a long coryneform rod implicated in otitis media (6). It is catalase positive, asaccharolytic, and CAMP test positive.
 - (2) Examine for *Nocardia* in chronic infections (see procedure 6.1).
- 3.** Examine, on request, for *A. otitis* from middle ear fluid. Prepare BHI agar with 5% rabbit blood and inoculate with specimen. Incubate plates for 5 days; increased CO₂ is not required.
 - ☒ A few strains have been reported to grow on sheep blood agar, but it is not optimal (3).
 - a.** *A. otitis* is slow growing and produces pinpoint colonies that are moist and slightly yellow (3). No hemolysis is observed. Eventually the colonies adhere to the agar.
 - b.** No growth is seen on CHOC (3).
 - c.** On Gram stain, *A. otitis* organisms are gram positive cocci in clusters and tetrads without chains and cannot be distinguished from staphylococci.
 - d.** Perform the following tests to confirm the identification. Note the expected reactions (13).
 - (1) Catalase negative or weak
 - (2) Pyrrolidonyl-β-naphthylamide (PYR) positive
 - (3) Leucine aminopeptidase (LAP) positive
 - (4) Vancomycin susceptible

POSTANALYTICAL CONSIDERATIONS

VI. REPORTING RESULTS

- A.** Gram stain—report smear as indicated in procedure 3.2.1.
- B.** Negative results
 - 1. Report preliminary and final results as “No growth.”
 - 2. Indicate the number of days the culture was incubated.
 - 3. If bacteria were seen on smear but did not grow on culture, extend the incubation and make a notation on the report to indicate the discrepancy.
- C.** Positive reporting
 - 1. Indicate the presence of skin microbiota, without identification.
 - 2. If the culture is mixed but with no predominating pathogen, indicate the genera and do not report further: e.g., “Mixed microbiota present, consisting of three morphologies of gram-negative rods, molds, and skin microorganisms; contact laboratory if further testing is clinically indicated.”
 - 3. Report all pathogens and susceptibility tests performed, using preliminary reports as indicated in procedure 3.3.2 and CLSI guidelines (5). Also refer to section 5.
 - 4. For *Vibrio*, report that the organism is resistant to colistin or polymyxin B if there was no zone around the disk, since ear drops often contain this antimicrobial.

VII. INTERPRETATION

- A. A positive external ear culture with a predominant gram-negative rod, beta-hemolytic streptococci, or *S. aureus* generally indicates infection with that agent.
- B. A positive middle ear culture with *S. pneumoniae*, *H. influenzae*, *M. catarrhalis*, and *A. otitis* generally indicates infection with that organism.
- C. A negative culture cannot rule out otitis media. In fact, in chronic infections, a pathogen is often not isolated.
- D. Controversy regarding the need for treatment of otitis media has been found in recent literature, but most physicians agree on its benefits (15). *A. otitis* is resistant to sulfamethoxazole-trimethoprim and often to erythromycin (3), but it does not have a beta-lactamase.
- E. Otitis externa is usually treated with ear drops containing a variety of agents, including 2% acetic acid, hydrocortisone, and antimicrobial agents, such as quinolones, neomycin, and either gentamicin or polymyxin B.

VIII. LIMITATIONS

- A. Methods that employ PCR for the detection of pathogens responsible for otitis media are more sensitive than culture techniques and increase the rate of detection of a pathogen to 75% (16).
- B. False-negative cultures can result from overgrowth of the culture with normal cutaneous microbiota.
- C. False-positive results can be caused by overinterpretation of the culture results.
- D. *T. otitidis*, a coryneform rod, has been infrequently isolated from ear fluid and may be a cause of otitis media (6).
- E. *A. otitis* is difficult to culture and may not be detected.

REFERENCES

1. Bluestone, C. D. 1989. Modern management of otitis media. *Pediatr. Clin. N. Am.* **36**:1371–1377.
2. Bluestone, C. D., J. S. Stephenson, and L. M. Martin. 1992. Ten-year review of otitis media pathogens. *Pediatr. Infect. Dis. J.* **11**:S7–S11.
3. Bosley, G. S., A. M. Whitney, J. M. Pruckler, C. W. Moss, M. Daneshvar, T. Sih, and D. F. Talkington. 1995. Characterization of ear fluid isolates of *Alloiococcus otitis* from patients with recurrent otitis media. *J. Clin. Microbiol.* **33**:2876–2880.
4. Chow, A. W., C. B. Hall, J. Klein, R. B. Kammer, R. D. Meyer, and J. S. Remington. 1992. General guidelines for the evaluation of new anti-infective drugs for the treatment of respiratory tract infections. *Clin. Infect. Dis.* **15**(Suppl. 1):S62–S88.
5. Clinical and Laboratory Standards Institute. 2006. *Performance Standards for Antimicrobial Susceptibility Testing*. Sixteenth informational supplement. Approved standard M100-S16. Clinical and Laboratory Standards Institute, Wayne, Pa.
6. Funke, G., S. Stubbs, M. Altweig, A. Carlotto, and M. D. Collins. 1994. *Turicella otitidis* gen. nov., sp. nov., a coryneform bacterium isolated from patients with otitis media. *Int. J. Syst. Bacteriol.* **44**:270–273.
7. Gehanno, P., G. Lenoir, B. Barry, J. Bons, I. Boucot, and P. Berche. 1996. Evaluation of nasopharyngeal cultures for bacteriologic assessment of acute otitis media in children. *Pediatr. Infect. Dis. J.* **15**:329–332.
8. Hendolin, P. H., A. Markkanen, J. Ylikoski, and J. J. Wahlforss. 1997. Use of multiplex PCR for simultaneous detection of four bacterial species in middle ear effusions. *J. Clin. Microbiol.* **35**:2854–2858.
9. Hendolin, P. H., U. Kärkkäinen, A. Markkanen, T. Himi, and J. Ylikoski. 1999. High incidence of *Alloiococcus otitis* in otitis media with effusion. *Pediatr. Infect. Dis. J.* **18**:860–865.
10. Janda, J. M., C. Powers, R. G. Bryant, and S. L. Abbott. 1988. Current perspectives on the epidemiology and pathogenesis of clinically significant *Vibrio* spp. *Clin. Microbiol. Rev.* **1**:245–267.
11. Klein, J. O. 1993. Microbiologic efficacy of antibacterial drugs for acute otitis media. *Pediatr. Infect. Dis. J.* **12**:973–975.
12. Klein, J. O. 2000. Otitis externa, otitis media, mastoiditis, p. 669–675. In G. L. Mandell, J. E. Bennett, and R. Dolin (ed.), *Principles and Practice of Infectious Diseases*, 5th ed. Churchill Livingstone, New York, N.Y.
13. LaClaire, L. L., and R. R. Facklam. 2000. Comparison of three commercial rapid identification systems for the unusual gram-positive cocci *Dolosigranulum pigrum*, *Ignavigranum ruoffiae*, and *Facklamia* species. *J. Clin. Microbiol.* **38**:2037–2042.
14. McCarthy, J. M. 1995. Bacterial susceptibility and tympanocentesis in acute otitis media. *Pediatr. Infect. Dis. J.* **14**:S45–S50.
15. Pichichero, M. E. 2000. Recurrent and persistent otitis media. *Pediatr. Infect. Dis. J.* **19**:911–916.

REFERENCES (continued)

16. Post, J. C., R. A. Preston, J. J. Aul, M. Larkins-Pettigrew, J. Rydquist-White, K. W. Anderson, R. M. Wadowsky, D. R. Reagan, E. S. Walker, L. A. Kingsley, A. E. Magit, and G. D. Ehrlich. 1995. Molecular analysis of bacterial pathogens in otitis media with effusion. *JAMA* **273**:1598–1604.
17. Teele, D. W., J. O. Klein, B. Roener, and The Greater Boston Otitis Media Study Group. 1989. Epidemiology of otitis media during the first seven years of life in children in greater Boston: a prospective cohort study. *J. Infect. Dis.* **160**:83–94.
18. Vandamme, P., M. Heyndrickx, M. Vancaenehye, B. Hoste, P. De Vos, E. Falsen, K. Kersters, and K. H. Hinz. 1996. *Bordetella trematum* sp. nov., isolated from wounds and ear infections in humans, and reassessment of *Alcaligenes denitrificans* Ruger and Tan 1983. *Int. J. Syst. Bacteriol.* **46**:849–858.

SUPPLEMENTAL READING

- Bannatyne, R. M., C. Clausen, and L. R. McCarthy.** 1979. *Cumitech 10, Laboratory Diagnosis of Upper Respiratory Tract Infections*. Coordinating ed., I. B. R. Duncan. American Society for Microbiology, Washington, D.C.
- Giebink, G. S.** 1989. The microbiology of otitis media. *Pediatr. Infect. Dis. J.* **8**(Suppl. 1):18–20.
- Hendolin, P. H., L. Paulin, and J. Ylikoski.** 2000. Clinically applicable multiplex PCR for four middle ear pathogens. *J. Clin. Microbiol.* **38**:125–132.

3.11.6

Bordetella Cultures

[Updated March 2007]

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

A resurgence of cases of pertussis (whooping cough) and outbreaks in North America in the last decade created a renewed interest in establishing a laboratory diagnosis of the disease. Compared to the period from 1990 to 1993, the period from 1994 to 1996 had average incidences of pertussis among age groups 5 to 9, 10 to 19, and ≥ 20 years that increased 40, 106, and 93%, respectively, in the United States (6). Clinical diagnosis may be elusive, since the typical cough with whoop occurs late in the illness (weeks 3 to 6) and symptoms are often nonspecific and virus-like, especially in older children and adults. In young children, the disease may be severe, with choking, apnea, or cyanosis, and with significant morbidity and mortality. Adults can serve as a reservoir for transmission of the disease to unvaccinated infants or may suffer from the disease themselves.

Treatment in the early stages of infection reduces the severity of illness and further spread of the disease. Thus, an accurate early laboratory diagnosis remains a key tool in control and prevention. Pertus-

sis is caused primarily by *Bordetella pertussis* and occasionally by *B. parapertussis*. Other species of *Bordetella*, such as *B. holmesii*, *B. hinpii*, and *B. bronchiseptica*, can cause respiratory infections in humans but not specifically “whooping cough.”

Bordetella spp. are small, faintly staining, gram-negative coccobacilli. They are nonmotile obligate aerobes that do not utilize carbohydrates, and they grow slowly in vitro; some can be recovered from the respiratory tracts of animals. *B. pertussis*, in particular, is very fastidious and must be cultured initially on special media. *B. parapertussis* is less fastidious. *B. parapertussis*, *B. bronchiseptica*, *B. holmesii*, and *B. hinpii* grow on blood agar within 2 to 3 days.

Laboratory confirmation of pertussis is subject to many constraints, and the primary method used, culture, is considered no more than 50% sensitive. Unfortunately, prior to 1996, culture was the only acceptable laboratory criterion for confirming a case of pertussis. Diagnosis by culture is insensitive because of issues re-

lated to specimen collection and transport, the fastidious nature of the organism, and interference from other bacterial microbiota. In June 1996, the Council for State and Territorial Epidemiologists revised the laboratory criteria to include positive PCR assays for *B. pertussis* as official laboratory confirmation of pertussis (1). Therefore, PCR has become the test of choice for the diagnosis of pertussis (3, 13, 20). For PCR testing protocol, see procedure 12.2.3, part 11. Reagents (polyclonal and monoclonal) are available for direct fluorescent-antibody (DFA) testing, but a positive DFA result without culture or PCR confirmation is not considered suitable confirmation of pertussis in any state in the United States. Culture still has a role in isolation of *B. pertussis* and related species, especially for antimicrobial susceptibility testing (AST) in cases of treatment failure (8). This procedure describes the processing and culturing of specimens for *Bordetella* spp. Refer to Appendix 3.11.6-1 for the DFA procedure and procedure 12.2.3.11, part 11, for PCR.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING

NOTE: Refer to procedure 3.3.1 for additional details. The following can be copied for preparation of a specimen collection instruction manual for physicians and caregivers. It is the responsibility of the laboratory director or designee to educate physicians and caregivers on proper specimen collection.

A. Specimen collection

NOTE: Cough plates and nasal swabs, previously thought to be ideal specimens, are no longer recommended for culture of *Bordetella*.

1. Timing

- a. Collect as soon as possible after symptoms develop.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING

(continued)

- b. Collect specimens up to 4 weeks after onset, provided that antimicrobial therapy has not been started.
- **NOTE:** The organism is rarely found by culture after the fourth week of illness, and the percentage of positive culture results decreases with time. Positive results have been reported using PCR with specimens collected as late as 60 days after onset of symptoms.
2. Collection of nasopharyngeal specimen
- **NOTE:** *B. pertussis* exhibits a tropism and binds specifically to ciliated respiratory epithelial cells. Since the nasopharynx is lined with these cells, it is a far superior site for detection of the bacterium.
- a. Nasopharyngeal swabs (refer to Fig. 3.11.6-1A)
- **NOTE:** Nasopharyngeal swabs *cannot* be used for PCR since both the alginate component and the aluminum shaft inhibit PCR-based assays (19).
- (1) Use a *calcium alginate or Dacron fiber tip swab* on a fine flexible wire. Bend the wire so that it mimics the curve of the nasal airway and gently pass the swab through the nostril to the posterior nasopharynx. Do not force the swab; resistance will be felt when the posterior nasopharynx is reached.
 - (2) Rotate the swab and leave it in place for up to 30 s or until the patient coughs. Withdraw as quickly as possible.
 - (3) *Repeat procedure through the second nostril.*
 - (4) Submit both swabs for culture and DFA testing.
- b. Nasal wash: syringe method (refer to Fig. 3.11.6-1B)
- (1) Use a 3- to 5-ml syringe with a 2-in. 18-gauge tubing attachment. Fill the syringe with saline.
 - (2) Instruct the patient not to swallow during the procedure.

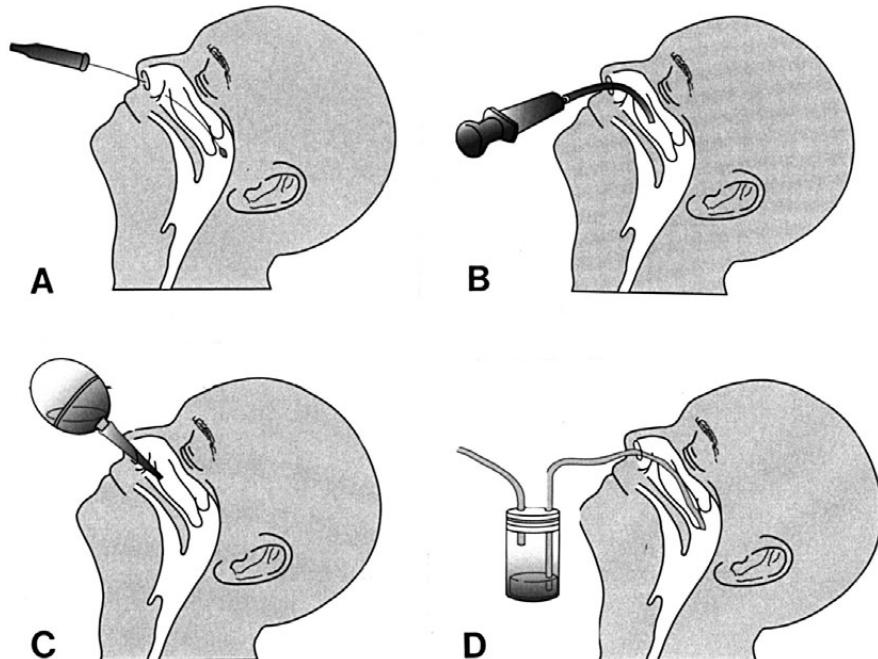


Figure 3.11.6-1 Collection of nasal pharyngeal swab(s) (A), nasal wash specimen(s) by syringe method (B), nasal wash specimen(s) by bulb method (C), and nasal aspirate specimen(s), assisted by vacuum (D). Diagrams courtesy of BD Diagnostic Systems, Sparks, Md., with permission.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING

(continued)

- (3) With the patient's head hyperextended (approximately 70° angle), quickly instill approximately 5 ml of sterile 0.85% NaCl into one nostril.
- (4) Immediately aspirate the saline solution back into the syringe, *or*
- (5) Tilt the head forward and allow the fluid to run out of the nares into a sterile container, *or*
- (6) Aspirate the fluid by inserting a rubber bulb syringe into each nostril.
- (7) Place the specimen in a sterile container.
- c. Nasal wash: bulb method (refer to Fig. 3.11.6–1C)
 - (1) Suction 3 to 5 ml of sterile 0.85% NaCl into a 1- to 2-oz tapered rubber bulb.
 - (2) Instruct the patient not to swallow during the procedure.
 - (3) With the patient's head hyperextended (approximately 70° angle), insert the bulb into one nostril until the nostril is occluded.
 - (4) Quickly instill the sterile saline into the nostril with one squeeze of the bulb.
 - (5) Immediately release the bulb to collect the nasal wash specimen.
 - (6) Empty the bulb contents into a sterile container and transport.
- d. Nasal aspirate: vacuum assisted (refer to Fig. 3.11.6–1D)
 - (1) Connect a mucus trap (i.e., Luken's tube) to a suction pump and catheter, turn on suction, and adjust to suggested suction pressure (*see chart below*).
 - (2) Insert the end of the catheter though the external nares to the posterior pharynx.
 - (3) Apply suction while slowly withdrawing the catheter, allowing the catheter to remain in the nasopharynx no longer than 10 s.
 - (4) After aspiration, flush material out of the catheter with a small volume (1 to 1.5 ml) of sterile saline.

Patient age	Catheter size (French) ^a	Suction pressure (mm Hg)
Premature infant	6	80–100
Infant	8	80–100
Toddler	10	100–120
School age	12	100–120
Adolescent/adult	14	120–150

^a To determine length of catheter tubing, measure distance from tip of nose to external opening of ear.

■ **NOTE:** Nasopharyngeal aspiration or wash yields sufficient material for numerous diagnostic procedures and gives a 11% higher yield on culture than nasopharyngeal swabs (7). Aspirate specimens are easily divided and saved, are suitable for all testing methodologies, and can be frozen for long periods (2 years at –70°C).

■ **NOTE:** Nasopharyngeal specimen collection directions are taken in part with permission from BD Diagnostic Systems, Sparks, Md.

B. Specimen transport

1. Inoculate plates at bedside.

■ **NOTE:** The best culture results are achieved when specimens are plated directly onto culture media at the bedside; unfortunately, for many this is not possible or practical to establish as a routine. Bedside plating results in a higher positive culture rate than that obtained with nasopharyngeal aspirates taken at the same time and cultured the same day in the laboratory.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING (continued)

2. Alternatively, submit in transport tube; choose a transport medium based on the length of time the specimen will be in transit.
 - a. Transport tubes
 - (1) 0.5 to 1% casein hydrolysate (Casamino Acid; Remel, Inc.)
 - (2) Amies transport medium
 - (3) Half-strength charcoal blood agar stab, also called Regan-Lowe transport medium (15) (prepared media available from Hardy Diagnostics)
 - (a) Weigh 25.5 g of CM119 charcoal agar (Oxoid, Inc., Ogdensburg, N.Y.) into 900 ml of water, autoclave, and cool.
 - (b) Add 100 ml of sterile defibrinated horse blood (available from most medium vendors) and 40 mg of cephalexin.
 - (c) Dispense into screw-cap vials to fill half full, seal, and store for up to 2 months at 4°C. Label containers with the expiration date.
 - b. If delay is brief (≤ 2 h), use 0.5 to 1% casein hydrolysate (Casamino Acid) solution (Remel, Inc.) as a transport medium. With nasopharyngeal aspirates, add the solution to the specimen container and mix well.
 - c. If specimens will be plated within 24 h of collection, use Amies medium with charcoal.
 - d. If delay is prolonged (>24 h but ≤ 3 days), use Regan-Lowe transport medium.
 - (1) Incubate at 35°C for 2 days.
 - (2) Then hold at room temperature during transport.
 - (3) For this choice, transit time should not exceed 3 days.
 - e. If transit time will exceed 3 days, use Regan-Lowe transport medium.
 - (1) Incubate at 35°C for 2 days.
 - (2) Then hold at 4°C during transport.
 - (3) Organisms can remain viable for as long as 8 days with this choice.

C. Rejection criteria

1. Do not perform culture if transport conditions are not followed.
2. Throat specimens, nares swabs, and sputum are unacceptable specimens.
3. Do not perform cultures from specimens collected on rayon or cotton swabs, as they contain fatty acids that inhibit growth.

III. MATERIALS

A. Primary tests

See Appendix 3.11.6-1 for DFA reagents.

B. Media

1. Regan-Lowe agar media containing Oxoid CM119 charcoal agar (51 g/liter), 10% defibrinated horse blood, and 0.04 g of cephalexin per liter (BD Diagnostic Systems; Hardy Diagnostics; Remel, Inc.). Some formulas use cyclodextrin in place of charcoal.

- a. Store commercially prepared plates at 4°C for 4 to 8 weeks in sealed container.
- b. Prepare in-house from Regan-Lowe base dehydrated medium (CM119).
 - (1) Dehydrated base has a 4-year shelf life.
 - (2) Dehydrated media can be purchased with or without 40 mg of cephalexin per liter.

- (3) Add 10% horse blood to autoclaved and cooled medium prior to use.
- (4) Plates are good for 4 to 8 weeks from date of preparation (10).

NOTE: Bordet-Gengou agar has been supplanted by Regan-Lowe agar, which has a longer shelf life and greater ability to isolate the organism and is easier to prepare (10). Some *Bordetella* spp. are inhibited by cephalexin, and some investigators recommend using media with and without antimicrobial agents (9). Unfortunately, medium without antimicrobial agents becomes overgrown by contaminants very quickly, and this practice does not appear to increase the positivity rate. As a result, most laboratories do not follow this recommendation.

III. MATERIALS (continued)

- 2. BAP
- 3. MAC—used to verify growth requirements
- C. Identification methods**
 1. Gram stain (procedure 3.2.1)
 2. Catalase test (procedure 3.17.10)
 3. Oxidase test (procedure 3.17.39)
 4. Rapid urea test (procedure 3.17.48)
 5. Nitrate medium (procedure 3.17.35)
 6. Mueller-Hinton agar for pigment
- 7. Serologic reagents
 - a. *B. pertussis* agglutination anti-serum (BD Diagnostic Systems no. 210590, formerly Difco 2310-50-0) (*B. parapertussis* agglutination reagent is not available.)
 - b. DFA antisera (see Appendix 3.11.6-1).
- D. Other supplies**
 1. Incubator at 35°C
 2. Fluorescent microscope

ANALYTICAL CONSIDERATIONS**IV. QUALITY CONTROL**

- A. See Appendix 3.11.6-1 for QC of DFA reagents.
- B. Test each lot of serologic agglutination reagents prior to use and, additionally, at least every 6 months with a positively (*B. pertussis* ATCC 9797) and negatively (*Escherichia coli* ATCC 25922) reacting control.
- C. For biochemical tests, see each biochemical procedure for QC requirements.
- D. Perform QC on each lot of Regan-Lowe agar. Incubate aerobically overnight at 35°C.
 1. Verify that media meet expiration date and QC parameters per current Clinical and Laboratory Standards Institute (formerly NCCLS) M22 document. See procedures 14.2 and 3.3.1 for further details.
 2. For controls, it is better to freeze aliquots of clinical specimens that have not been cultivated through several passages.
 - a. Freeze in sheep blood, skim milk, or 15% glycerol at -70°C (see item V in procedure 14.2).
 - b. Use these isolates or ATCC strains for control of media, by following inoculum preparation described in procedure 14.2.

Test organism	Result
<i>Bordetella pertussis</i> ATCC 9797 or in-house strain	Growth
<i>Bordetella parapertussis</i> ATCC 15311	Growth
<i>Staphylococcus aureus</i> ATCC 25923	Partial to complete inhibition

V. PROCEDURE

Observe standard precautions.

- A. See Appendix 3.11.6-1 for DFA tests.
 - ▣ **NOTE:** Always perform culture with DFA because of the insensitivity of DFA and the subjectivity of interpretation of DFA tests.
- B. Commercial multiplex PCR assays have recently become available for the detection of *B. pertussis*. The ProPertussis (Prodesse Inc.) assay detects *B. pertussis*. The Pneumoplex (Prodesse Inc.) PCR assay simultaneously detects *Mycoplasma pneumoniae* and *Chlamydia pneumoniae* as well as *Legionella pneumophila*, *Legionella micdadei*, and *B. pertussis* (12). PCR detection of *B. pertussis* has been shown to be able to distinguish this organism from other clinically important *Bordetella* spp. and has a much higher sensitivity than other detection methods (16).
- C. Culture inoculation
 1. Firmly roll swab over one-third of the Regan-Lowe agar surface, and streak carefully for isolation in four quadrants to minimize overcrowding by breakthrough microorganisms that may release inhibitory substances to suppress growth of *Bordetella*.
 2. Inoculate a BAP and incubate with the Regan-Lowe agar for comparison of growth.

V. PROCEDURE (continued)

D. Incubation

1. Place plates in a plastic bag or moist chamber with a sterile moistened filter paper to avoid drying. *B. pertussis* is very susceptible to drying.
2. Place at 35°C in an aerobic atmosphere (*without* 5% CO₂).
■ NOTE: An incubation temperature of 37°C will not support the growth of many strains of *B. pertussis*; maintaining 35°C is critical.
3. Incubate plates for 5 to 12 days.
■ NOTE: While most colonies of *B. pertussis* and *B. parapertussis* are detected in 5 to 7 days, maximum recovery can take as long as 12 days. Katzko et al. (11) showed that 16% of *B. pertussis* organisms and 50% of *B. parapertussis* organisms were recovered only after extended incubation.

E. Culture examination

1. Observe BAP at 24 h and daily thereafter for a total of 72 h.
 - a. *B. pertussis* does not grow on BAP, but other species do (Table 3.11.6–1).
 - b. *B. parapertussis* colonies are smooth and opaque and usually hemolytic.
 - c. *B. bronchiseptica* colonies are gray-white, somewhat flat, and dull and may have hazy hemolysis.
2. Observe Regan-Lowe plates at 48 h and daily thereafter with the use of a magnifying lens.
 - a. *B. pertussis* colonies appear at a minimum of 72 h as small, convex, gray, smooth, and very shiny, like drops of mercury. They may vary in size and run together.
 - b. *B. parapertussis* colonies appear similar to those of *B. pertussis* but are grayer and less domed, and they grow more rapidly.
 - c. *Legionella* may grow on Regan-Lowe plates and will appear as ground-glass colonies.
3. Gram stain colonies suggestive of *Bordetella*, especially those growing on Regan-Lowe plates and not on BAP.
 - a. Apply the counterstain (carbol fuchsin or basic fuchsin preferred) for 1 to 2 min to enhance staining intensity.
 - b. Observe for gram-negative coccobacilli or short rods consistent with *Bordetella* spp.
4. Perform catalase and oxidase tests. *B. pertussis* is positive for both. *B. parapertussis* is catalase positive and oxidase negative.

Table 3.11.6–1 Biochemical differentiation of *Bordetella* species of importance in respiratory cultures^a

Test	<i>B. pertussis</i>	<i>B. parapertussis</i>	<i>B. holmesii</i> (CDC NO-2)	<i>B. trematum</i>	<i>B. bronchiseptica</i> ^b	<i>B. avium</i> ^c	<i>B. hinzii</i> ^c
Motility	—	—	—	+	+	+	+
Urease	—	+	—	—	+	—	—
Oxidase	+	—	—	—	+	+	+
Catalase	+	+	V (W)	+	+	+	+
Growth on MAC	—	+(D)	+ (D)	+	+	+	+
Soluble pigment (on peptone agar, e.g., MH)	NA	Brown	Brown	—	—	W, amber	—
Nitrate/with gas ^d	NA	—/—	—/—	V	+/-	—/—	—/—
Growth on BAP (hemolysis)	— (NA)	+ (beta)	+ (V green)	+ (—)	+ (rare beta)	+ (V beta)	+ (—)

^a Table extrapolated from references 4, 17, 18, and 21. NA, not applicable; W, weak; V, variable; D, delayed.

^b *B. bronchiseptica* grows on salmonella-shigella agar and is tartrate negative, unlike other closely related organisms (*Oligella ureolytica* and *Ralstonia paucula* [IVc-2]). Also see Table 3.18.2–12 for other tests to separate these genera. *Brucella* spp. are similar to *B. bronchiseptica*, but the former are not motile and generally do not grow on MAC.

^c *B. avium* is malonate negative, and *B. hinzii* is malonate positive; *B. avium* is not a human pathogen (4).

^d The nitrate reaction is first; if the gas reaction is known, it is listed second, preceded by a slash.

V. PROCEDURE (continued)

5. Immediately confirm suspicious colonies.
 - a. Perform DFA for *B. pertussis* and *B. parapertussis* (Appendix 3.11.6–1).
 - b. For *B. pertussis*, perform slide agglutination test.
 - (1) Add a drop of a turbid saline suspension (no. 3 McFarland standard) from a colony suspension to a drop of the working dilution (1:10) of the *B. pertussis* antiserum on a slide.
 - (2) Also add a drop of suspension without antiserum for comparison.
 - (3) Observe for agglutination under $\times 30$ magnification.
 - (4) A 4+ rapid and complete reaction (large clumps and clear background) is considered a positive result.
 - (5) No agglutination should be visible in the saline control. If agglutination in the control does occur, the culture is rough and not suitable for serologic techniques.
 - c. Additional testing is not necessary unless results are equivocal.
6. If DFA or agglutination is negative, confirm species with biochemical tests.
 - a. Perform rapid urea test.
 - (1) Both *B. parapertussis* and *B. bronchiseptica* are urea positive.
 - (2) *B. pertussis* is negative.
 - *Brucella spp.* are oxidase, catalase, and urea positive. Work in a biological safety cabinet until this genus has been eliminated from consideration. *Brucella spp.* grow on blood agar, generally not on MAC, and are not motile.
 - b. If isolate grows on BAP, inoculate Mueller-Hinton agar for pigment production.
 - c. See additional tests for identification of *Bordetella* spp. in Table 3.11.6–1 or submit to reference laboratory for confirmation.
7. Do not perform AST routinely. Seal positive culture plates and hold for at least 7 days at 4°C, should AST be needed for determination of erythromycin resistance.

POSTANALYTICAL CONSIDERATIONS**VI. REPORTING RESULTS****A. Negative results**

1. If the culture is negative, report “No *Bordetella pertussis* or *Bordetella parapertussis* isolated.”
2. Report the number of days the culture was incubated.

B. Positive results

1. If the culture is positive, report the appropriate species identified, such as “*Bordetella pertussis* isolated.”
2. Report the presence of *B. pertussis* or *B. parapertussis* as soon as preliminary tests are completed.
 - **NOTE:** The report of *Bordetella* is of critical value because patients with pertussis are placed in isolation rooms.
Call positive results to the attending physician(s) or caregiver and to infection control for inpatients. Document notification.
3. Pertussis is a reportable disease in most states; however, a positive DFA result without culture or PCR confirmation (procedure 12.2.3, part 11) is *not* considered suitable confirmation of pertussis in any state in the United States.

VII. INTERPRETATION

- A. The isolation of *B. pertussis* is always significant. Patients who are symptomatic by definition have pertussis; those who do not have symptoms are carriers and represent a public health risk. Both should be treated.
- B. Virtually all *B. pertussis* organisms are susceptible to erythromycin (the drug of choice to treat colonization), negating the need for AST (5). However, erythromycin resistance has been recently reported, and AST may be indicated in

VII. INTERPRETATION (continued)

cases of treatment failure. Testing is nonstandard, since it has to be performed on Regan-Lowe media. Both disk diffusion and the Etest method have been used (8).

- C. *B. bronchiseptica* has been isolated from lower respiratory tract specimens, usually after 48 h of incubation. Rule out this pathogen, using Table 3.11.6–1, when examining such specimens.

VIII. LIMITATIONS

- A. Methods that employ PCR are more rapid and sensitive than culture or DFA techniques.
- B. *Legionella* spp. cross-react with *B. pertussis* antiserum (14).
- C. Some *B. pertussis* organisms can grow on buffered charcoal-yeast extract medium with α -ketoglutarate, used for recovery of *Legionella*.
- D. False-positive results can be caused by misinterpretation of the confirmatory tests.
- E. The polyclonal antibody reagents used for DFA are capable of cross-reacting with several non-*B. pertussis* bacteria, resulting in false-positive results (2).
- F. The monoclonal DFA reagent may produce a positive result with some strains of *B. bronchiseptica* (package insert, Altachem Pharma Inc.)
- G. Treatment with antimicrobial agents can affect both DFA and culture, resulting in false negatives.

REFERENCES

1. Centers for Disease Control and Prevention. 1997. Case definitions for infectious conditions under public health surveillance. *Morb. Mortal. Wkly. Rep.* **46**(RR-10):1–57.
2. Ewanowich, C. A., L. W. L. Chui, M. G. Paranchych, M. S. Peppler, R. G. Marusyk, and W. L. Albritton. 1993. Major outbreak of pertussis in northern Alberta, Canada: analysis of discrepant direct fluorescent-antibody and culture results by using polymerase chain reaction methodology. *J. Clin. Microbiol.* **31**:1715–1725.
3. Farrell, D. J., G. Daggard, and T. K. S. Mukkur. 1999. Nested duplex PCR to detect *Bordetella pertussis* and *Bordetella parapertussis* and its application in diagnosis of pertussis in nonmetropolitan southeast Queensland, Australia. *J. Clin. Microbiol.* **37**:606–610.
4. Funke, G., T. Hess, A. von Graevenitz, and P. Vandamme. 1996. Characteristics of *Bordetella hinzzii* strains isolated from a cystic fibrosis patient over a 3-year period. *J. Clin. Microbiol.* **34**:966–969.
5. Gordon, K. A., J. Fusco, D. J. Biedenbach, M. A. Pfaller, and R. N. Jones. 2001. Antimicrobial susceptibility testing of clinical isolates of *Bordetella pertussis* from northern California: report from the SENTRY antimicrobial surveillance program. *Antimicrob. Agents Chemother.* **45**:3599–3600.
6. Guris, D., P. M. Strebel, B. Bardenheier, M. Brennan, R. Tachdjian, E. Finch, M. Wharton, and J. R. Livengood. 1999. Changing epidemiology of pertussis in the United States: increasing reported incidence among adolescents and adults, 1990–1996. *Clin. Infect. Dis.* **28**:1230–1237.
7. Hallander, H. O., E. Reizenstein, B. Renemark, G. Rasmussen, L. Mardin, and P. Olin. 1993. Comparison of nasopharyngeal aspirates with swabs for culture of *Bordetella pertussis*. *J. Clin. Microbiol.* **31**:50–52.
8. Hill, B. C., C. N. Baker, and F. C. Tenover. 2000. A simplified method for testing *Bordetella pertussis* for resistance to erythromycin and other antimicrobial agents. *J. Clin. Microbiol.* **38**:1151–1155.
9. Hoppe, J. E., and M. Schlagenhauf. 1989. Comparison of three kinds of blood and two incubation atmospheres for cultivation of *Bordetella pertussis*. *J. Clin. Microbiol.* **27**:2115–2117.
10. Hoppe, J. E., and R. Vogl. 1986. Comparison of three media for culture of *Bordetella pertussis*. *Eur. J. Clin. Microbiol. Infect. Dis.* **5**:361–363.
11. Katzko, G., M. Hofmeister, and D. Church. 1996. Extended incubation of culture plates improves recovery of *Bordetella* spp. *J. Clin. Microbiol.* **34**:1563–1564.
12. Khanna M., J. Fan, K. Pehler-Harrington, C. Waters, P. Douglass, J. Stallock, S. Kehl, and K. J. Henrickson. 2005. The Pneumoplex assays, a multiplex PCR-enzyme hybridization assay that allows simultaneous detection of five organisms, *Mycoplasma pneumoniae*, *Chlamydia pneumoniae*, *Legionella pneumophila*, *Legionella micdadei*, and *Bordetella pertussis*, and its real-time counterpart. *J. Clin. Microbiol.* **43**:565–571.
13. Meade, B. D., and A. Bollen. 1994. Recommendations for the use of the polymerase chain reaction in the diagnosis of *Bordetella pertussis* infections. *J. Med. Microbiol.* **41**:51–55.
14. Ng, V. L., L. Weir, M. K. York, and W. K. Hadley. 1992. *Bordetella pertussis* versus non-*L. pneumophila* *Legionella* spp.: a continuing diagnostic challenge. *J. Clin. Microbiol.* **30**:3300–3301.

REFERENCES (continued)

15. Regan, J., and F. Lowe. 1977. Enrichment medium for isolation of *Bordetella*. *J. Clin. Microbiol.* **6**:303–309.
16. Templeton, K. E., S. A. Scheltinga, A. van der Zee, B. M. Diederken, A. M. van Kruisjissen, H. Goossens, E. Kuijper, and E. C. Claas. 2003. Evaluation of real-time PCR for detection of and discrimination between *Bordetella pertussis*, *Bordetella parapertussis*, and *Bordetella holmesii* for clinical diagnosis. *J. Clin. Microbiol.* **41**:4121–4126.
17. Vandamme, P., M. Heyndrickx, M. Vancanneyt, B. Hoste, P. De Vos, E. Falsen, K. Kersters, and K. H. Hinz. 1996. *Bordetella trematum* sp. nov., isolated from wounds and ear infections in humans, and reassessment of *Alcaligenes denitrificans* Ruger and Tan 1983. *Int. J. Syst. Bacteriol.* **46**:849–858.
18. Vandamme, P., J. Hommez, M. Vancanneyt, M. Monsieurs, B. Hoste, B. Cookson, C. H. Wirsing von Konig, K. Kersters, and P. J. Blackall. 1995. *Bordetella hinpii* sp. nov., isolated from poultry and humans. *Int. J. Syst. Bacteriol.* **45**:37–45.
19. Wadowsky, R. M., S. Laus, T. Libert, S. States, and G. D. Ehrlich. 1994. Inhibition of PCR-based assay for *Bordetella pertussis* by using calcium alginate fiber and aluminum shaft components of a nasopharyngeal swab. *J. Clin. Microbiol.* **32**:1054–1057.
20. Wadowsky, R. M., R. H. Michaels, T. Libert, L. A. Kingsley, and G. D. Ehrlich. 1996. Multiplex PCR-based assay for detection of *Bordetella pertussis* in nasopharyngeal swab specimens. *J. Clin. Microbiol.* **34**:2645–2649.
21. Weyant, R. S., C. W. Moss, R. E. Weaver, D. G. Hollis, J. G. Jordan, E. C. Cook, and M. I. Daneshvar. 1995. *Identification of Unusual Pathogenic Gram-Negative Aerobic and Facultatively Anaerobic Bacteria*, 2nd ed. Williams & Wilkins, Baltimore, Md.

SUPPLEMENTAL READING

- American Academy of Pediatrics.** 2003. Pertussis, p. 472–486. In L. K. Pickering (ed.), *2000 Red Book: Report of the Committee on Infectious Diseases*, 26th ed. American Academy of Pediatrics, Elk Grove Village, IL.
- Friedman, R. L.** 1988. Pertussis: the disease and new diagnostic methods. *Clin. Microbiol. Rev.* **1**:365–376.
- Jackson, L. A., J. D. Cherry, S. P. Wang, and J. T. Grayston.** 2000. Frequency of serological evidence of *Bordetella* infections and mixed infections with other respiratory pathogens in university students with cough illnesses. *Clin. Infect. Dis.* **31**:3–6.
- Müller, F. C., J. E. Hoppe, and C. H. Wirsing von König.** 1997. Laboratory diagnosis of pertussis: state of the art in 1997. *J. Clin. Microbiol.* **35**:2435–2443.

APPENDIX 3.11.6–1

Detection of *Bordetella pertussis* by Direct Fluorescent Antibody

I. PRINCIPLE

The DFA test performed on nasopharyngeal specimens is an important adjunct to culture for early presumptive diagnosis of pertussis. DFA tests are rapid but are too insensitive and subjective to be done in lieu of culture. Success in interpreting DFA examinations of clinical material is directly proportional to the experience of the reader. A positive DFA result without culture or PCR confirmation is *not* considered suitable confirmation of pertussis in any state in the United States.

Reagents (polyclonal and monoclonal) are available for testing, but the polyclonal reagent has variable sensitivity and low specificity. The monoclonal reagent contains fluorescein-conjugated anti-*B. pertussis* monoclonal antibody and rhodamine-conjugated anti-*B. parapertussis* monoclonal antibody. Limited information is available at this time concerning the sensitivity and specificity of the monoclonal reagent (2).

DFA is performed directly on the patient specimen but can also be used to confirm an isolate grown in culture as *B. pertussis* or *B. parapertussis*.

II. SPECIMENS

- A. Nasopharyngeal specimen (*see* procedure 3.11.6 for collection)
- B. Smears prepared from presumptive *B. pertussis* or *B. parapertussis* cultures, to confirm identification

III. MATERIALS

A. DFA reagents

1. Polyclonal reagents: BD Diagnostic Systems (formerly Difco reagents)
 - a. Bacto chicken anti-*B. pertussis* fluorescein isothiocyanate (FITC) conjugate (formerly Difco no. 2359-56; BD Diagnostic Systems catalog no. 223591).
 - b. Bacto chicken anti-*B. parapertussis* FITC conjugate (formerly Difco no. 2378-56; BD Diagnostic Systems catalog no. 223781).
 - c. Reagent preparation
 - (1) Bring lyophilized reagents to room temperature.

APPENDIX 3.11.6–1 (continued)

- (2) To rehydrate, add 5 ml of sterile distilled or deionized water to each vial and rotate gently to completely dissolve contents.
 - (3) Determine the working dilution of each FITC conjugate immediately after rehydration, since it varies with each lot of reagent, the fluorescent microscope and filters used, and the age of the bulb.
 - (a) Prepare twofold dilutions of each FITC conjugate ranging from 1:5 to 1:80 in phosphate-buffered saline (PBS) buffer and test using stock strains.
 - (b) Choose the working dilution as one dilution less than the highest dilution yielding a 4+ fluorescence. For example, if the last dilution yielding a 4+ fluorescence is 1:40, then 1:20 (one less dilution) is the working dilution for that reagent.
 - (4) Aliquot the working dilution of each conjugate and store at –20°C until the expiration date of original vial.
 - (5) Label with reagent name, lot number, dilution, and preparation and expiration date.
2. Monoclonal reagents
- a. Accu-Mab Plus *B. pertussis/parapertussis* DFA (Altachem Pharma Inc., Edmonton, Alberta, Canada)
 - (1) Catalogue no. BP002-01 (one-vial, 40-test kit)
 - (2) Catalog no. BP002-04 (four-vial, 160-test kit).
 - b. Supplied ready for use in PBS, pH 7.2, in an amber glass serum vial
 - c. Store at 2 to 8°C.
- B. Supplies
1. Wash buffer: PBS, 0.01 M phosphate, 0.15 M sodium chloride (pH 7.2 to 7.4)
 - a. Stock solution
 - (1) Dissolve 2.56 g of NaH₂PO₄ · H₂O (or 2.89 g of NaH₂PO₄ · 2H₂O) and 11.94 g of Na₂HPO₄ in 500 ml of distilled H₂O.
 - (2) Add 97.66 g of NaCl, and bring to a total volume of 1,000 ml.
 - (3) Sterilize by autoclaving or filtration and adjust pH to 7.2.
 - (4) Store at 4°C for up to 6 months.
 - b. Working solution
 - (1) Dilute stock 1:10 in sterile distilled H₂O.
 - (2) Store at 4°C for up to 2 weeks.
 2. McFarland standard no. 3 (Appendix 3.16–1)
 3. 0.5 to 1% Casamino Acids (catalog no. 060462; Remel, Inc.)
 4. 95% Ethanol
 5. Staining tray and moisture chamber
 6. FA mounting fluid, pH 7.2; (9 parts reagent-grade glycerin to 1 part PBS)
 7. 25-µl pipette for monoclonal reagents
 8. Miscellaneous: microscope slides, immersion oil, wooden applicator sticks, staining jar, coverslips
- C. Fluorescent microscope with excitation and emission filters for FITC conjugate (and rhodamine conjugate for monoclonal reagent). Check with your microscope supplier for the proper combination of barrier and excitation filters needed for use with conjugates.
- IV. QUALITY CONTROL**
- A. Keep both the reagent and stained slides in reduced light to minimize photobleaching of the fluorescent dyes.
 - B. Always use aseptic technique when opening reagents for use.
 1. Perform a visual inspection of the vial for turbidity and/or mold growth prior to each use.
 2. Do not use reagents if any signs of contamination (turbidity or growth) are present.
 - C. Prior to use of new lots and each time a fluorescent antigen test is performed, test a positively and negatively reacting control with *each antiserum*.
 1. Positive controls must fluoresce brilliant apple-green with the fluorescein filters and bright orange-red with the rhodamine filters, as indicated in the chart below.
 2. For positive controls, coccobacilli must be brightly fluorescent at the edges and have dark centers.

APPENDIX 3.11.6–1 (continued)

3. A positive control should show a 4+ reaction and the negative control should not exceed a 1+ reaction according to the chart below. See grading system in item VI below.
4. If a positive control is less than 3+, or if a negative control exceeds 1+, the test should be repeated and patient specimen slides should not be read and/or reported.

Test organism	Polyclonal reagent (apple-green)		Monoclonal reagent	
	<i>B. pertussis</i> conjugate	<i>B. parapertussis</i> conjugate	Fluorescein filter (apple-green)	Rhodamine filter (orange-red)
<i>B. pertussis</i> ATCC 9797	Positive, 3–4+	Negative or 1+	Positive, 3–4+	Negative or 1+
<i>B. parapertussis</i> ATCC 15311	Negative or 1+	Positive, 3–4+	Negative or 1+	Positive, 3–4+
<i>Bordetella</i> (not <i>B. pertussis</i> , <i>B. parapertussis</i> , or <i>B. bronchiseptica</i>)—optional			Negative or 1+	Negative or 1+

D. Preparation of positive and negative control slides

1. Store stock strains of QC organisms frozen at –70°C indefinitely (see item V in procedure 14.2) or on sealed plates at 4°C for 1 month.
2. Pick appropriate colonies from Regan-Lowe agar and emulsify in 2 ml of sterile distilled or deionized water (use PBS wash buffer for monoclonal reagents) until a cell density equal to a no. 3 McFarland standard is achieved. (An ideal smear will contain 10 to 100 organisms/oil immersion field.)
3. From the cell suspension smear a drop or large loopful of specimen on two areas of each clean glass microscope slide. Each area should be 10 to 15 mm in diameter. Do this for each control organism used. Only one area per slide is needed for monoclonal reagent.
4. Allow the smears to air dry and then fix the slide by a 1-min immersion in 95% ethanol. Remove slides, and allow them to air dry (or for monoclonal reagents, flood with 95% ethanol and allow the ethanol to evaporate at room temperature).
5. Place in a slide storage box, and keep at –20°C for up to 1 year. However, condensation and frost buildup can cause problems over time. Fixed control slides may also be kept in a desiccator in the dark at room temperature for up to 1 year.
6. Each time the test is performed, remove one *B. pertussis* and one *B. parapertussis* control slide from the freezer and allow them to come to room temperature.
7. Test each control slide in the same manner as each patient or culture specimen is tested.

■ NOTE: *B. pertussis* ATCC 12742 and *B. parapertussis* ATCC 15237 are both mutant strains lacking lipopolysaccharide structures that are required for the monoclonal reagent to attach. They must *not* be used as positive controls in this procedure.

V. PROCEDURE

A. Preparation of slides

1. Specimens

- a. If swab is received, place in a tube containing 0.5 ml of sterile 0.1% casein hydrolysate, and vortex for 20 s.

■ NOTE: This step is very important, as smears made directly from the specimen swabs often have so much background fluorescence from nasal secretions that interpretation becomes difficult if not impossible.

- b. For aspirate specimens, vortex the aspirate for 20 s and then use the aspirate directly.

APPENDIX 3.11.6–1 (continued)

2. Cultures

- a. Pick appropriate colonies from Regan-Lowe agar and emulsify in 2 ml of sterile distilled or deionized water (*use PBS wash buffer for monoclonal reagents*) until a cell density equal to a no. 3 McFarland standard is achieved.
- b. An ideal smear will contain 10 to 100 organisms/microscopic field.

B. DFA procedure (polyclonal reagents)

1. Process patient specimen, culture, and control slides in the same manner.
2. Smear a drop or large loopfull of emulsified specimen or culture in two separate areas on a glass slide. Each area should be 10 to 15 mm in diameter.
3. Draw a line with a wax pencil to separate sides or, preferably, use Teflon-coated slides with wells (Precision Laboratory Products, Middleton, Wis., <http://www.precisionslides.com>).
4. Fix slides.
 - a. Heat fix gently using either a flame or hot plate until warm to the touch *or*
 - b. Immerse for 1 min in 95% ethanol.
5. Add several drops (1 drop = 35 µl) of the appropriate FA working conjugate to the fixed smears.
 - a. Use FITC-conjugated *B. pertussis* antiserum on one half of each slide and FITC-conjugated *B. parapertussis* antiserum on the other half.
 - b. Keep the same antiserum location on each slide (i.e., *B. pertussis* antiserum on left side of each slide).
6. Spread the conjugate over the entire area using a wooden applicator stick.
7. Place the slide(s) in a staining tray or moisture chamber.
8. Incubate at room temperature for 30 min.
9. Remove excess conjugate.
10. Place slide(s) in a staining jar containing PBS wash buffer for 10 min; change buffer two times during wash step.
11. Rinse for 2 min in distilled water.
12. Remove the slide(s), allow to drain, and air dry or blot with bibulous paper.
13. Add a small drop of mounting fluid (pH 7.2) to the center of each stained area, and mount each with a coverslip.
14. Examine slides using a fluorescent microscope with filters for fluorescein conjugate.
15. Examine control slides with 100× objective before proceeding to specimen slides.
16. Screen specimen slides with 40× objective and confirm with 100× objective.

C. DFA procedure (monoclonal reagents)

1. Process patient specimen, culture, and control slides in the same manner.
2. Smear a drop or large loopful of emulsified specimen or culture on a glass slide approximately 10 to 15 mm in diameter.
3. Fix slides immediately.
 - a. Flood the smear with 95% ethanol and allow the ethanol to evaporate at room temperature.
 - b. In addition, if specimen was transported or mixed in casein hydrolysate, dip the slide several times in PBS wash buffer to remove casein and allow slide(s) to dry.
4. Add 25 µl of the DFA reagent to each fixed smear.
5. Spread the reagent over the entire area using a wooden applicator stick.
6. Place the slide(s) in a moisture chamber for 30 min at room temperature. Do not allow the reagent to dry on the slide(s) or results will not be accurate.
7. Place slide(s) in a staining jar containing PBS wash buffer for 5 min.
8. Place positive control slides in a separate staining jar.
9. Repeat wash with fresh wash buffer for an additional 5 min. (If background fluorescence is found to be too high, increase wash steps to 10 min each.)
10. Dip slide(s) for 1 min in distilled water.
11. Blot excess water from slide(s) with lint-free tissue or bibulous paper and allow to air dry.
12. Add a small drop of mounting fluid (pH 7.2) to the center of each stained area, and mount each with a coverslip.

APPENDIX 3.11.6–1 (continued)

13. Examine slides with fluorescent microscope with filters for both fluorescein and rhodamine.
 - a. Since fluorescein fades more rapidly than rhodamine after light exposure, examine each slide with fluorescein filters before proceeding to rhodamine filters.
 - b. Examine control slides with 100 \times objective before proceeding to specimen slides.
 - c. Screen specimen slides with 40 \times objective and confirm with 100 \times objective.

VI. INTERPRETATION

- A. Score fluorescence from 1+ to 4+.

1. Score immediately when each field is first viewed since the dyes, especially fluorescein, may fade rapidly after exposure to the excitation beam of the microscope.
2. Use the following grading for controls and specimens with fluorescein conjugate and filters.
 - a. 4+: brilliant apple-green fluorescence with clear-cut cell outline and sharply defined center
 - b. 3+: bright apple-green peripheral fluorescence with clear-cut cell outline and sharply defined center.
 - c. 2+: definite but dull apple-green peripheral fluorescence with cell outline less well defined
 - d. 1+: very dim, barely visible apple-green fluorescence with cell outline indistinguishable from center
3. Use the following grading for controls and specimens with rhodamine conjugate and filters.
 - a. 4+: brilliant orange-red fluorescence with clear-cut cell outline and sharply defined center
 - b. 3+: bright orange-red peripheral fluorescence with clear-cut cell outline and sharply defined center
 - c. 2+: definite but dull orange-red peripheral fluorescence with cell outline less well defined
 - d. 1+: very dim, barely visible orange-red fluorescence with cell outline indistinguishable from center

- B. A positive cell must appear as a small oval-shaped or coccobacillary cell with an intense (3+ to 4+) fluorescence surrounding a *dark cell center*.
 1. Ignore occasional nonspecifically staining diplococci, or diphtheroid-like rods.
 2. Consider typical morphology as well as fluorescence in the interpretation.

- C. For a positive result

1. Polyclonal reagents

- a. Slide positive for *B. pertussis* must show *four or more cells* with the characteristic morphology and 3 to 4+ apple-green fluorescence on the area stained with *B. pertussis* antiserum, with no or 1+ fluorescence on the area stained with *B. parapertussis* antiserum.
- b. Slide positive for *B. parapertussis* must show *four or more cells* with the characteristic morphology and 3 to 4+ apple-green fluorescence on the area stained with *B. parapertussis* antiserum, with no or 1+ fluorescence on the area stained with *B. pertussis* antiserum.

2. Monoclonal reagents

- a. Slide positive for *B. pertussis* must show one cell with the characteristic morphology and 3 to 4+ yellow-green fluorescence with fluorescein filters and no or 1+ fluorescence with rhodamine filters.
- b. Slide positive for *B. parapertussis* must show one cell with the characteristic morphology and 3 to 4+ orange-red fluorescence with rhodamine filters and no or 1+ fluorescence with fluorescein filters.
- c. Depending on your microscope filters, positive *B. parapertussis* cells may give a faint orange fluorescence (maximum, 1+) when viewed using the fluorescein filter. This faint orange fluorescence should not be confused with the expected bright green fluorescence of positive *B. pertussis* cells.

APPENDIX 3.11.6–1 (continued)

- d. Depending on your microscope filters, positive *B. pertussis* cells may give a pale yellow fluorescence (maximum, 1+) when viewed using the rhodamine filter. This pale yellow fluorescence should not be confused with the expected bright orange-red fluorescence of positive *B. parapertussis* cells.

■ NOTE: The manufacturer claims that the monoclonal reagents are so specific that a slide should be considered positive for *B. pertussis* or *B. parapertussis* if a single cell with the characteristic morphology and appropriate fluorescence is observed. The single evaluation of the monoclonal reagent published to date used the stringent criterion of a minimum of four fluorescing organisms with typical morphology to qualify as a positive, but it was felt that the manufacturer's recommendation of a single cell was valid (2).

VII. REPORTING RESULTS**A. Negative results**

1. Report the DFA test result as "Negative for *Bordetella pertussis* and *Bordetella parapertussis* by direct fluorescent antibody testing."
2. If an isolate from a culture does not fluoresce with reagents, report "No *Bordetella pertussis* or *Bordetella parapertussis* isolated."

B. Positive results

1. For positive DFA test, report "Positive for *Bordetella pertussis* or *Bordetella parapertussis* by DFA."
2. For positive DFA test from culture, report "Positive for *Bordetella pertussis* or *Bordetella parapertussis*" depending on which conjugate gave a positive result.

VIII. LIMITATIONS

- A. Methods that employ PCR are more rapid and sensitive than culture or DFA techniques.
- B. Polyclonal reagent for DFA has variable sensitivity and low specificity.
- C. False-positive results can be caused by misinterpretation of the confirmatory tests.
- D. The polyclonal antibody reagents used for DFA are capable of cross-reacting with several non-*B. pertussis* bacteria, resulting in false-positive results (1).
- E. The monoclonal DFA reagent may produce a positive result with some strains of *B. bronchiseptica* (package insert, Altachem Pharma Inc.).
- F. *Legionella* spp. cross-react with *B. pertussis* antiserum (3).
- G. Treatment with antimicrobial agents can affect both DFA and culture, resulting in false negatives.

References

1. Ewanowich, C. A., L. W. L. Chui, M. G. Paranchych, M. S. Peppler, R. G. Marusyk, and W. L. Albritton. 1993. Major outbreak of pertussis in northern Alberta, Canada: analysis of discrepant direct fluorescent-antibody and culture results by using polymerase chain reaction methodology. *J. Clin. Microbiol.* **31**:1715–1725.
2. McNichol, P., S. M. Giercke, M. Gray, D. Martin, B. Brodeur, M. S. Peppler, T. Williams, and G. Hammond. 1995. Evaluation and validation of a monoclonal immunofluorescent reagent for direct detection of *Bordetella pertussis*. *J. Clin. Microbiol.* **33**:2868–2871.
3. Ng, V., L. Weir, M. K. York, and W. K. Hadley. 1992. *Bordetella pertussis* versus non-*L. pneumophila* *Legionella* spp.: a continuing diagnostic challenge. *J. Clin. Microbiol.* **30**:3300–3301.

Supplemental Reading

- Gilchrist, M. J. R.** 1990. Laboratory diagnosis of pertussis. *Clin. Microbiol. Newslet.* **12**:49–53.
- Halperin, S. A., R. Bortolussi, and A. J. Wort.** 1989. Evaluation of culture, immunofluorescence, and serology for the diagnosis of pertussis. *J. Clin. Microbiol.* **27**:752–757.
- Müller, F. C., J. E. Hoppe, and C. H. Wirsing von König.** 1997. Laboratory diagnosis of pertussis: state of the art in 1997. *J. Clin. Microbiol.* **35**:2435–2443.
- Streubel, P. M., S. L. Cochi, K. M. Farizo, B. J. Payne, S. D. Hanauer, and A. L. Baughman.** 1993. Pertussis in Missouri: evaluation of nasopharyngeal culture, direct fluorescent antibody testing, and clinical case definitions in the diagnosis of pertussis. *Clin. Infect. Dis.* **16**:276–285.
- Young, S. A., G. L. Anderson, and P. D. Mitchell.** 1987. Laboratory observations during an outbreak of pertussis. *Clin. Microbiol. Newslet.* **9**:176–179.

3.11.7

Corynebacterium diphtheriae Cultures

[Updated March 2007]

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Diphtheria is an acute, toxin-mediated, communicable infectious disease primarily of the upper respiratory tract (throat, larynx) and occasionally of the skin. It is caused by toxigenic lysogenized strains of *Corynebacterium diphtheriae*, of which there are four biotypes: mitis, intermedius, belfani, and gravis. Diphtheria exacts a high mortality rate, approximating 10%. A less severe diphtheria-like disease (pharyngitis and atypical symptoms, such as endocarditis, septic arthritis, and other forms of systemic disease) caused by nontoxigenic strains has been reported. The classic pseudomembrane of the pharynx may be lacking in mild cases, which usually mimic streptococcal pharyngitis.

Although the number of cases reported in the United States has significantly declined, studies of diphtheria immunity levels among adults in the United States have shown that from 20 to 90% do not possess adequate immunity against the disease (10). In 1996, isolation of toxigenic *C. diphtheriae* from the blood of an American Indian woman living in the Northern Plains region of the United States prompted public health officials to conduct diphtheria surveillance in the patient's community, resulting in the recovery of 10 *C. diphtheriae* isolates (2, 9, 10). There is currently no geographic concentration of cases in the United States. An-

timicrobial agents have little or no effect on the clinical outcome, and laboratory confirmation of diphtheria often requires several days, yet diagnosis and antimicrobial treatment are essential in limiting further spread (4, 11).

This procedure presents proper specimen collection and transport and culture for *C. diphtheriae*. Some laboratories may not be able to store the culture medium because of few requests and may elect to submit specimens to a reference laboratory for culture. It is a CAP requirement that the laboratory have available a mechanism to provide culture for *C. diphtheriae*, when requested.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING



Observe standard precautions.

NOTE: Refer to procedure 3.3.1 for additional details on specimen collection. The following can be copied for preparation of a specimen collection instruction manual for physicians and other caregivers. It is the responsibility of the laboratory director or designee to educate physicians and caregivers on proper specimen collection.

A. Specimen collection

1. Contact the clinical microbiology laboratory prior to specimen collection for diphtheria, because special techniques and/or media are required for the isolation of these agents.
2. In case of respiratory diphtheria, obtain material for culture on a swab (either a cotton- or a polyester-tipped swab) from the inflamed areas in the nasopharynx (4).
 - a. If membranes are present and can be removed, swab from beneath the membrane.
 - b. Collect from several areas to increase sensitivity.
 - c. Collect nasopharyngeal swabs from suspected carriers.
 - d. For details, see Specimen Collection, Transport, and Handling for culture of beta-hemolytic streptococci, procedure 3.11.8.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING

(continued)

3. If cutaneous diphtheria is suspected, collect skin (aspirate/swab), throat (swab), and nasopharynx (swab) specimens (1, 11).

B. Specimen transport

1. Use any routine swab collection systems, such as Amies or BD Culturette EZ, because the organism is not fastidious.
 NOTE: BD Culturette EZ is preferred because the polyurethane adsorbs and releases >95% of the organisms, unlike other swabs that absorb the organisms, making release inefficient.
2. If inoculation of media is anticipated to exceed 24 h, use silica gel transport packages or sterile, desiccated silica gel crystals in tubes (4). However, it is best to avoid delays in culture inoculation to provide speed and accuracy in the diagnosis.
3. Follow the guidelines for the packaging and shipping of infectious substances as outlined in the respective Department of Transportation (DOT) or International Air Transport Association (IATA) regulations for shipping of specimens and/or cultures suspected of containing *C. diphtheriae* (see procedure 15.5).
4. Include patient demographics on the culture request form (age, sex, and name), including vaccination and travel history. Record collection time and source of specimen.

C. Rejection criteria

1. Specimens not representative of the anatomic site from which *C. diphtheriae* is commonly found
2. Specimens not accompanied by appropriate information (patient name, age, and vaccination history; source of specimen)
3. Specimens submitted in unapproved packaging and shipping containers
4. Specimen transport time exceeds 24 h and specimen is not contained in silica gel transport medium.

III. MATERIALS

A. Media

1. BAP inoculated for the detection of hemolytic streptococci and *C. diphtheriae* present in large numbers. Addition of a 50-µg disk of fosfomycin (BD Diagnostic Systems) to the initial inoculum will inhibit most contaminating oral microbiota and allow coryneforms to grow (4).
2. Selective media (see Appendix 3.11.7-1 for details)
 - a. Cystine tellurite blood agar (CTBA) (Remel, Inc.) or serum tellurite agar with lamb serum (BD Diagnostic Systems)
 NOTE: This medium inhibits oronasal microbiota and turns colonies of corynebacteria, staphylococci, and yeasts black.
 - b. Tinsdale (TIN) agar base medium (Remel, Inc.)
 NOTE: This medium contains cysteine and forms a halo around colonies that produce cystinase.
3. Slants (optional)
 - a. Loeffler agar slant (LAS) (Remel, Inc.) or Loeffler blood serum with beef serum (BD Diagnostic Systems) *or*
 - b. Pai agar slant (formula in Appendix 3.11.7-1)
4. Todd-Hewitt broth with 3% sterile rabbit blood (optional). Defibrinated rabbit blood is available from most medium vendors and from Hemostat (Los Angeles, Calif., [800] 572-6888) or Quad-5 (Ryegate, Mont., [406] 568-2911).

B. Reagents

1. Gram stain reagents (see procedure 3.2.1)
2. Alkaline Loeffler methylene blue stain (LMBS) (optional)
 - a. Dissolve 0.3 g of methylene blue in 30 ml of 95% ethanol.
 - b. Then add 100 ml of 0.01% potassium hydroxide.
3. 3% Hydrogen peroxide for catalase (see procedure 3.17.10)

III. MATERIALS (continued)

4. Conventional biochemical media: see procedures 3.17 and 3.18.1.
 - a. Sugar fermentation media (procedure 3.17.9)
 - (1) Preferably cysteine Trypticase agar for determining oxidation (found at surface of the tube) and fermentation (acid or alkali production in entire tube) for acid production from glucose, maltose, sucrose, mannitol, and xylose, *or*
 - (2) Rapid sugar fermentation tubes (Remel, Inc.)
 - b. Nitrate broth (procedure 3.17.35)
 - c. Motility media (procedure 3.17.31)
 - d. Urea hydrolysis (procedure 3.17.48)
 - e. Esculin hydrolysis (procedure 3.17.5)
 - f. A beta-hemolysin-producing strain (e.g., *Staphylococcus aureus* ATCC 25923) for CAMP reaction (procedure 3.17.8)
5. Commercial identification systems: RapID CB Plus system (Remel, Inc.), API (RAPID) Coryne system (bioMérieux), Biolog GP plate (Biolog, Hayward, Calif.), BBL CRYSTAL GP system (BD Diagnostic Systems)

■ NOTE: Publications related to the performance of these systems are limited to the first three systems (5, 6, 7, 8). It is recommended that the use of any of the above-listed commercial identification systems be supplemented with macroscopic morphology and Gram stain characteristics. Any isolate that is unidentifiable and deemed to be clinically significant should be sent to a creditable reference laboratory with expertise in identification of corynebacteria, especially for identification of toxin-producing *C. diphtheriae*.

C. Other supplies

1. Incubator at 35°C
2. Microscope

ANALYTICAL CONSIDERATIONS**IV. QUALITY CONTROL**

- A. Verify that media meet expiration date and QC parameters per current Clinical and Laboratory Standards Institute (CLSI; formerly NCCLS) M22 document. See procedures 14.2 and 3.3.1 for further procedures.
- B. QC checks need not be performed in-house on commercially purchased BAP. However, each new lot number and each new shipment of CTBA, LAS, Pai, and TIN must be tested with stock strains of *C. diphtheriae* (preferably biotype intermedius) for characteristic growth (11). Incubate aerobically overnight at 35°C.

Test organism	Result
<i>Corynebacterium diphtheriae</i> bv. intermedius ATCC 9675 or ATCC 51696	CTBA: growth, gray to black colonies, some with dark centers LAS: growth Pai: growth TIN: black colony with halo
<i>Streptococcus pyogenes</i> ATCC 19615	TIN: black, pinpoint without halos
<i>Staphylococcus aureus</i> ATCC 25923	CTBA: inhibition (partial to complete)

- C. Test each lot of reagents and kits used for identification with organisms known to produce reliable positive and negative reactions.
- D. Test methylene blue stain with a characteristic *Corynebacterium* and another gram-positive microorganism with each use of the test.

V. PROCEDURE



Observe standard precautions.

■ NOTE: Refer to Fig. 3.11.7-1 for a flowchart of steps in the isolation, detection, and identification of *C. diphtheriae*.

A. Inoculation

■ NOTE: Use of a biosafety cabinet will avoid contamination of the culture or specimen as well as protect laboratory processing personnel.

1. If the swab appears desiccated, was collected several days prior to receipt, or is received in silica gel, place it into Todd-Hewitt broth supplemented with 3% sterile rabbit blood, incubate it overnight, and then inoculate isolation media.
2. Inoculate the specimen to CTBA and BAP; streak for isolation. Incubate media at 35°C aerobically.

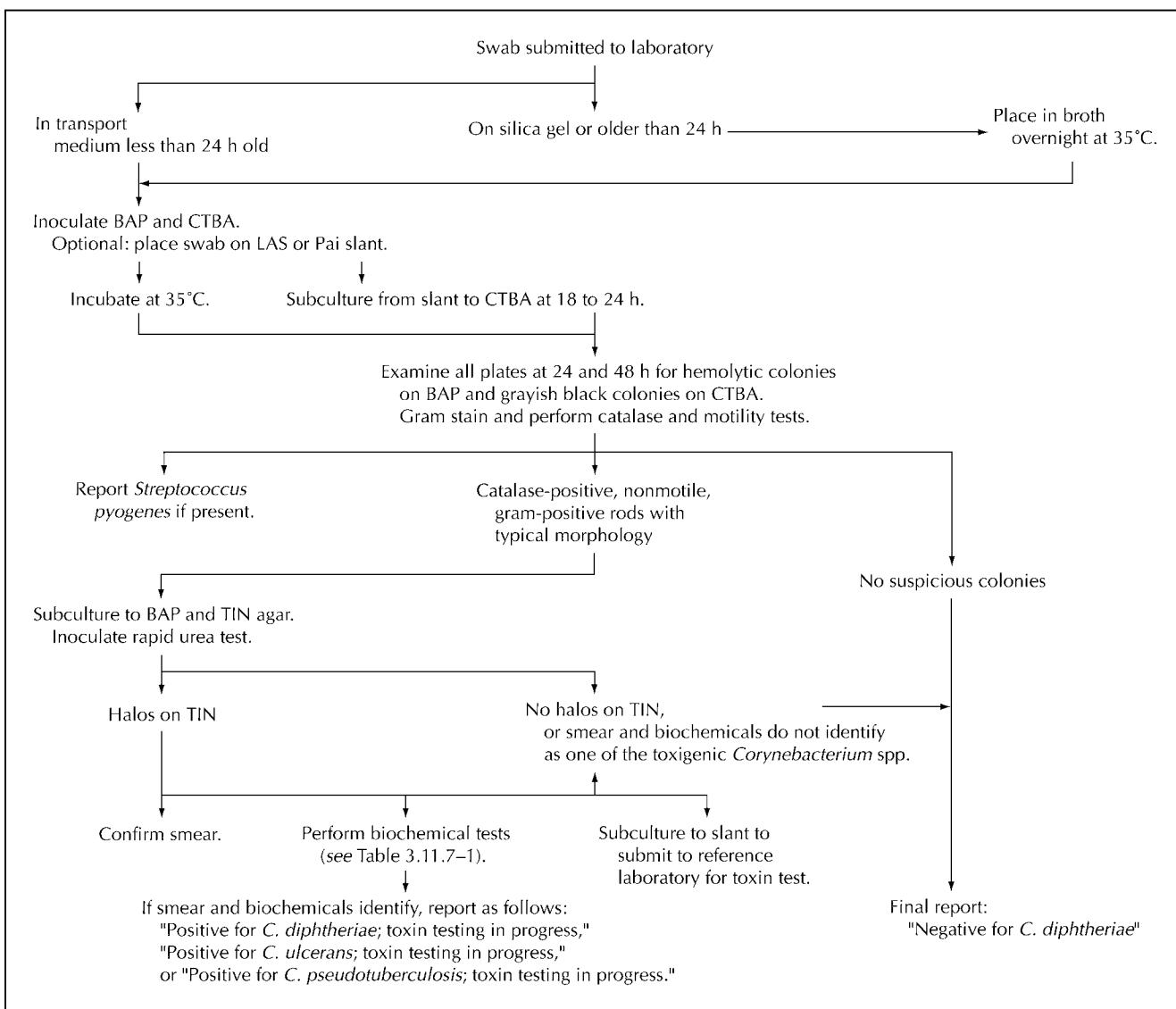


Figure 3.11.7-1 Flowchart for evaluation of culture for *C. diphtheriae*.

V. PROCEDURE (continued)

3. Optionally, inoculate an LAS or a Pai agar slant, and leave the swab on the slant during incubation.
 - a. Incubate media at 35°C aerobically.
 - b. After 18 to 24 h of incubation, subculture the agar slant to a second plate of CTBA.

B. Culture evaluation

1. Examine all agar plates at 24 and 48 h for colonies typical of *C. diphtheriae* (see descriptions below). Pursue any beta-hemolytic colonies on BAP and dark colonies on CTBA.
2. Subculture colonies that are catalase positive and exhibit typical morphology on Gram stain (Fig. 3.11.7-2) to the following.
 - a. BAP to provide growth for identification procedures
 - b. Modified TIN plate. Stab the agar in several places for halos.
3. Incubate plates aerobically at 35°C and examine at 24 and 48 h. Do not incubate in CO₂.

C. Morphology of *C. diphtheriae*

1. Cellular morphology (Fig. 3.11.7-2)
 - a. Gram stain: pleomorphic gram-positive rods that occur in angular arrangements (commonly referred to as palisades or Chinese letters); possibly coccobacillary forms, most notably in older cultures

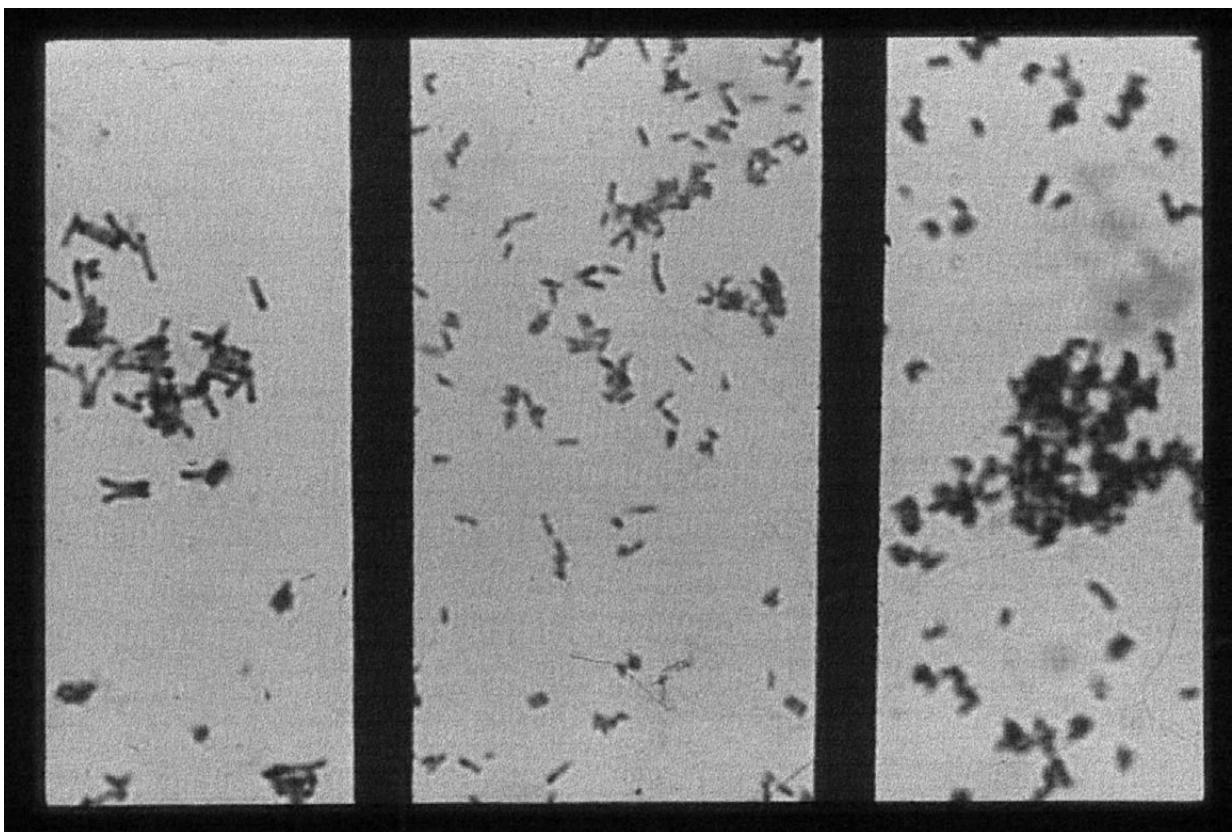


Figure 3.11.7-2 Microscopic morphology of three types of *C. diphtheriae* grown on Loeffler media and stained with Loeffler methylene blue: from right to left they are biotypes gravis, mitis, and intermedius. Photos reproduced from Clinical Microbiology, ASM Committee on Educational Materials, 1985.

V. PROCEDURE (continued)

- b.** LMBS: pleomorphic beaded rods whose ends may be swollen, producing a club shape; angular arrangements; reddish purple metachromatic granules or bars apparent
- 2.** Colonial morphology
 - a.** CTBA: grayish black (gunmetal gray) and 1 to 3 mm in size, with a garlic-like odor
 - (1) Biotype intermedius: smallest, flat
 - (2) Biotypes mitis and gravis: larger, convex, smooth or wrinkled
 - b.** BAP (Fig. 3.11.7-3)
 - (1) Biotype intermedius: smaller, flat, creamy, transparent, nonhemolytic
 - (2) Biotypes mitis and gravis: larger, convex, with weak beta-hemolysis
 - (3) *Streptococcus pyogenes*: gram-positive cocci in chains; production of beta-hemolysis
 - c.** TIN: brownish black zone (halo) around the colony darkens with age.
- D.** Identification procedures
 - 1.** Examine TIN at 24 and 48 h for brownish black halos.
 - 2.** Make a suspension of suspected colony on any of the agars in Todd-Hewitt broth. Use this suspension to do the following.
 - a.** Prepare stains
 - (1) Examine LMBS or Gram stain black colonies to confirm characteristic cellular morphology. *Staphylococcus* spp. may produce a characteristic halo on TIN.
 - (2) Perform wet mount motility to eliminate *Bacillus* spp.

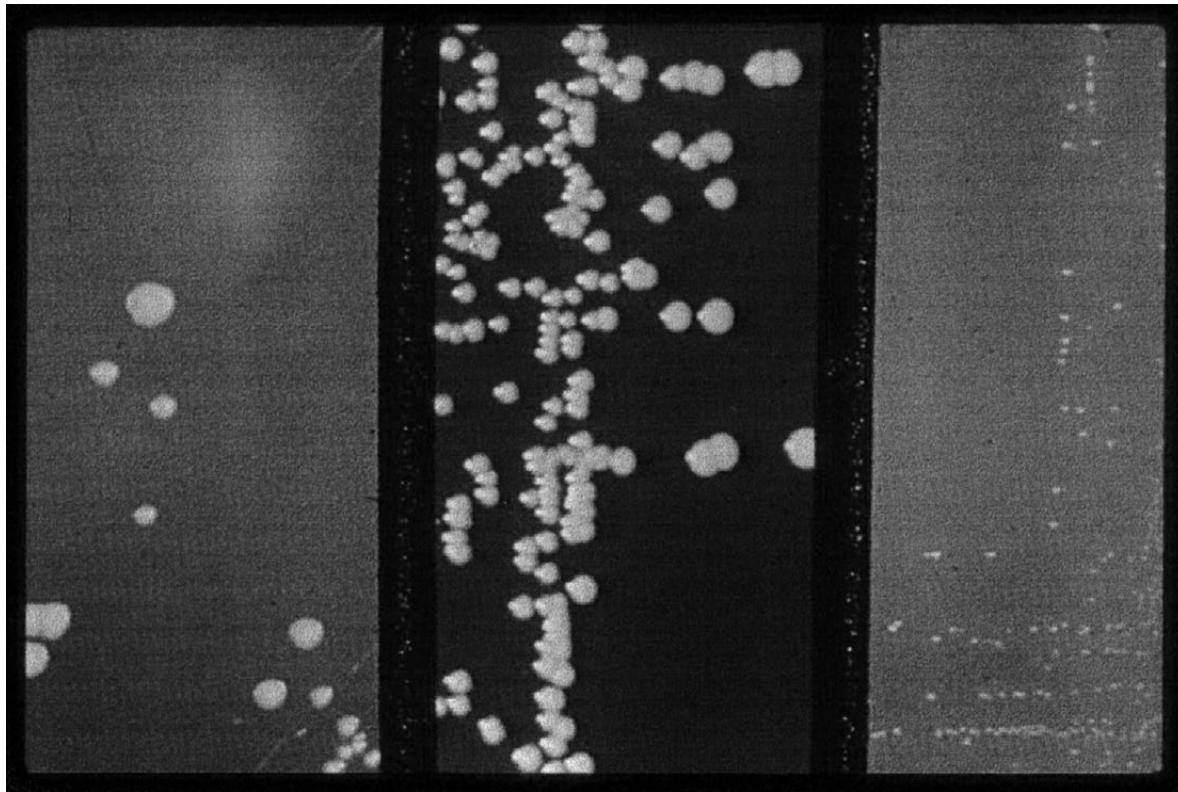


Figure 3.11.7-3 Colonial morphology of *C. diphtheriae* grown on BAP for 48 h; from right to left they are biotypes gravis, mitis, and intermedius. Photos reproduced from Clinical Microbiology, ASM Committee on Educational Materials, 1985.

V. PROCEDURE (continued)

- b.** Inoculate CAMP test.
- c.** Inoculate biochemical tests.
 - (1) Conventional
 - (a) Urea
 - i. For agar slant, overlay urea slant with sterile mineral oil.
 - ii. Or use rapid disk test (preferred) (procedure 3.17.48)
 - a. *Corynebacterium ulcerans* and *Corynebacterium pseudotuberculosis* produce a characteristic halo on TIN and are urea positive (Table 3.11.7-1).
 - **NOTE:** *C. ulcerans* has been known to cause diphtheria-like disease and can elaborate diphtheria toxin. *C. pseudotuberculosis* can also produce the toxin.
 - b. *C. diphtheriae* is urea negative.
 - (b) Nitrate
 - (c) Sugar fermentation
 - i. Conventional media may be supplemented with a drop or two of sterile rabbit serum to enhance growth (see procedure 3.17.9). Incubate aerobically at 35°C and examine daily for 3 days, or
 - ii. Inoculate 1 drop of each rapid sugar identification medium heavily with a loopful of colony, making an even suspension. Incubate for 1 h in a 37°C water bath. Interpret as for conventional tests.
 - (2) As an alternative to the above-mentioned biochemical tests, inoculate a commercial identification test kit and read according to the manufacturer's instructions.

E. If identification procedures indicate *C. diphtheriae*, *C. ulcerans*, or *C. pseudotuberculosis*, ship (per DOT or IATA packaging and shipping guidelines) LAS or Pai or other slant to a public health laboratory for toxigenicity testing. The isolate must be toxigenic to confirm a clinical diagnosis of diphtheria (11). Contact the local public health laboratory to determine where it wishes the isolate to be sent. The CDC Diphtheria Laboratory performs toxin testing 7 days a week.

F. Antimicrobial susceptibility testing
In the absence of published CLSI guidelines for susceptibility testing of corynebacteria (3), it is recommended that if testing is requested, tests be performed on Mueller-Hinton agar with sheep blood using the Etest method (procedure 5.8). Report the MIC without an interpretive breakpoint, unless it is obviously resistant.

Table 3.11.7-1 Key biochemical reactions to identify toxic *Corynebacterium* species^a

Species	Halos on TIN	Motility	Nitrate	Urea	Hemolysis	Can have diphtheria toxin	Reverse CAMP
<i>C. diphtheriae</i>	+	—	+ ^b	—	V	+	—
<i>C. ulcerans</i>	+	—	—	+	+	+	+
<i>C. pseudotuberculosis</i>	+	—	V	+	+	+	+
<i>C. pseudodiphtheriticum</i>	Unknown	—	+	+	—	—	—

^a Table extrapolated from text and tables in reference 4. +, positive; —, negative; V, variable. *C. ulcerans* is trehalose and glycogen positive; *C. pseudotuberculosis* is not. Many commercial kits do an excellent job of identification of these species. *C. diphtheriae*, *C. ulcerans*, and *C. pseudotuberculosis* are pyrazinamidase negative; *C. pseudodiphtheriticum* and many common corynebacteria are positive. For laboratories that must screen numerous isolates for *C. diphtheriae*, tablets to rapidly test for pyrazinamidase activity are available from Key Scientific.

^b *C. diphtheriae* biotype belfani is nitrate reductase negative.

POSTANALYTICAL CONSIDERATIONS

VI. REPORTING RESULTS

- A. If the culture is negative, report “No *Corynebacterium diphtheriae* isolated after 3 days.”
- B. Positive culture results
 - 1. If biochemical results are consistent with *C. diphtheriae*, report “Presumptive for *Corynebacterium diphtheriae*; sent to public health laboratory for confirmation and toxigenicity testing.” Do not report any enumeration.
 - 2. Notify physician and infection control of positive results. Diphtheria is a reportable disease in most states.
 - 3. Document notification.

VII. INTERPRETATION

- A. Pleomorphic gram-positive rods that usually grow well on BAP and are catalase positive, nonmotile, CAMP negative, and urea negative and form halos on TIN are presumptively *C. diphtheriae*.
- B. Confirm such results with further biochemical kit or sugar tests.
- C. Phenotypic identification of *C. diphtheriae* does not mean that the organism is a toxin producer. Toxin genes are carried on plasmids which can be lost. Toxin production must be demonstrated.

VIII. LIMITATIONS

- A. Smears made directly from clinical specimens are not reliable for diagnostic purposes, since metachromatic granules are found in other bacteria as well (11). Methylene blue stain (Loeffler) smears from media incubated for a short time are no longer recommended, for the same reason.
- B. *C. diphtheriae* must be a toxigenic strain to be diagnostic of diphtheria.
- C. TIN is not suitable as a primary plating medium, since it may not support the growth of some strains of *C. diphtheriae*.
- D. Do not read TIN too soon, since several organisms may give some slight browning on this medium in 18 h.
- E. Incubation at 5 to 10% CO₂ retards the development of halos on TIN.
- F. Black colonies on CTBA can be due to other organisms capable of reducing tellurite to tellurium. Other *Corynebacterium* spp., staphylococci, and some streptococci possess this ability.
- G. *C. diphtheriae* rapidly loses viability in saline. Use broth for suspensions of the organism or to moisten specimen swabs.
- H. Sterile rabbit serum will enhance growth of *C. diphtheriae*, in particular biotype intermedius, and is especially advised in biochemical media.
- I. Toxigenicity testing (modified Elek test) requires skill and experience in the preparation of reagents and interpretation of results and is therefore best left to public health laboratories or other reference laboratories that perform these procedures with some regularity.

REFERENCES

1. Bannatyne, R. M., C. Clausen, and L. R. McCarthy. 1979. Cumitech 10, *Laboratory Diagnosis of Upper Respiratory Tract Infections*. Coordinating ed., I. B. R. Duncan. American Society for Microbiology, Washington, D.C.
2. Centers for Disease Control and Prevention. 1997. Toxigenic *Corynebacterium diphtheriae*—Northern Plains Indian community, August–October 1996. *Morb. Mortal. Wkly. Rep.* **46**:506–510.
3. Clinical and Laboratory Standards Institute. 2006. *Performance Standards for Antimicrobial Susceptibility Testing*. Sixteenth informational supplement. Approved standard M100-S16. Clinical and Laboratory Standards Institute, Wayne, Pa.
4. Funke, G., and K. A. Bernard. 2003. Coryneform gram-positive rods, p. 472–501. In P. R. Murray, E. J. Baron, J. H. Jorgensen, M. A. Pfaffer, and R. H. Yolken (ed.), *Manual*

REFERENCES (continued)

- of Clinical Microbiology*, 8th ed. ASM Press, Washington, D.C.
5. **Funke, G., K. Peters, and M. Aravena-Roman.** 1998. Evaluation of the RapID CB Plus system for identification of coryneform bacteria and *Listeria* spp. *J. Clin. Microbiol.* **36**:2439–2442.
 6. **Funke, G., F. N. R. Renaud, J. Freney, and P. Riegel.** 1997. Multicenter evaluation of the updated and extended API (RAPID) Coryne database 2.0. *J. Clin. Microbiol.* **35**:3122–3126.
 7. **Hudspeth, M. K., S. H. Gerardo, D. M. Citron, and E. J. C. Goldstein.** 1998. Evaluation of the RapID CB Plus system for identification of coryneform species and other gram-positive rods. *J. Clin. Microbiol.* **36**:543–547.
 8. **Lindenmann, K., A. von Graevenitz, and G. Funke.** 1995. Evaluation of the Biolog system for the identification of asporogenous, aerobic gram-positive rods. *Med. Microbiol. Lett.* **4**:287–296.
 9. **Marston, C. K., F. Jamieson, F. Cahoon, G. Lesiak, A. Golaz, M. Reeves, and T. Popovic.** 2001. Persistence of a distinct *Corynebacterium diphtheriae* clonal group within two communities in the United States and Canada where diphtheria is endemic. *J. Clin. Microbiol.* **39**:1586–1590.
 10. **Popovic, T., C. Kim, J. Reiss, M. Reeves, H. Nakao, and A. Golaz.** 1999. Use of molecular subtyping to document long-term persistence of *Corynebacterium diphtheriae* in South Dakota. *J. Clin. Microbiol.* **37**:1092–1099.
 11. **Sottnek, F. O., and J. M. Miller.** 1980. *Isolation and Identification of Corynebacterium diphtheriae*. Centers for Disease Control, Atlanta, Ga.

SUPPLEMENTAL READING AND WEBSITES

- Clarridge, J. E., T. Popovic, and T. J. Inzana.** 1998. Diphtheria and other corynebacterial and coryneform infections, p. 347–371. In W. J. Hausler and M. Sussman (ed.), *Topley and Wilson's Microbiology and Microbial Infections*, vol. 3. Oxford University Press, New York, N.Y.
- Efstratiou, A., and P. A. C. Maple.** 1994. *Manual for the Laboratory Diagnosis of Diphtheria*. Copenhagen: Expanded Programme on Immunization in the European Region of World Health Organization, ICP/EPI 038(C).
- Efstratiou, A., K. H. Engler, I. K. Mazurova, T. Glushkevich, J. Vuopio-Varkila, and T. Popovic.** 2000. Current approaches to the laboratory diagnosis of diphtheria. *J. Infect. Dis.* **181**:S138–S145.
<http://www.bu.edu/cohis/lnfxns/bacteria/cory.htm>
<http://www.cdc.gov>
<http://www.phls.co.uk/facts/Immunisation/Diphtheria/diptintro.htm>

APPENDIX 3.11.7–1

Media for Detection of *Corynebacterium diphtheriae*

Refer to manufacturers' package inserts for additional information.

- A. TIN agar: a peptone agar base enriched with bovine serum, cysteine hydrochloride, potassium tellurite, and sodium thiosulfate
 1. The TIN kit (Remel) includes one tube of TIN agar base along with one bottle of desiccated TIN enrichment containing tellurite and serum. One tube and one bottle make one plate. Prepare a sufficient number of plates for QC testing and patient specimens.
 2. Melt the agar in the bottle by boiling.
 3. Rehydrate the enrichment by adding 3.3 ml of sterile distilled water aseptically to the bottle. Allow to sit until completely hydrated.
 4. Cool the base to 47 to 50°C. Add the enrichment and pour one plate. Plate is good for 5 days after pouring. Perform QC.
- B. CTBA is a heart infusion agar supplemented with 5% rabbit blood, tellurite, and L-cystine; shelf life is 1 month. Deeps with a longer shelf life are available.
- C. Loeffler blood serum medium contains eggs and beef serum, rather than agar, to coagulate to produce a solid medium. It is best purchased commercially.
- D. Pai agar slant
 1. 666 ml of fresh eggs, 333 ml of distilled water, and 80 ml of glycerin
 2. Filter eggs and water through gauze and add glycerin.
 3. Tube in 3-ml amounts, slant, and autoclave for 1 h at 110°C.
 4. Tightly cap; shelf life is 1 year (1).

Reference

1. **Sottnek, F. O., and J. M. Miller.** 1980. *Isolation and Identification of Corynebacterium diphtheriae*. Centers for Disease Control, Atlanta, Ga.

3.11.8

Group A Streptococcus Culture and Direct Antigen Detection

[Updated March 2007]

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Group A beta-hemolytic streptococcus (GABHS) is an important pathogen that causes pharyngitis, cellulitis, and bacteremia. Serious sequelae, including scarlet fever, acute glomerulonephritis, toxic shock syndrome, and acute rheumatic fever, can result from infections with this microorganism.

The primary reason this microorganism is an effective pathogen is the variety of virulence factors it can produce. Numbering among them are somatic constituents like M protein and capsule, enzymes like streptolysin O, DNase B, and streptokinase, and toxins like erythrogenic toxins A to C.

GABHS accounts for 30% of pharyngitis cases in children aged 5 to 15 years but only 10% of adult cases. Other bacterial causes of pharyngitis include group C and G beta-hemolytic streptococci, *Neisseria gonorrhoeae*, *Corynebacterium diphtheriae*, and *Arcanobacterium haemolyticum*. However, most cases of pharyngitis have a viral etiology. In children, the most worrisome complications of GABHS infection are acute rheumatic fever, acute glomerulonephritis, and peritonsillar abscess. Antimicrobial therapy alleviates pharyngeal symptoms and prevents some of the sequelae of infection.

Since Breese et al. (5) first described throat culturing for GABHS, numerous methods of detection have been published. It is important to select diagnostic tests

that are highly sensitive for use in children, since low numbers of GABHS in specimens may still indicate infection. That is because collecting high-quality pharyngeal specimens from uncooperative children can be difficult. For this reason, the American Academy of Pediatrics continues to recommend that negative rapid antigen detection tests be confirmed by culture (1).

Guidelines were published recently for testing adults with pharyngitis who lacked the complications of heart disease, chronic lung disease, or a history of acute rheumatic fever (11). They state that these patients should be screened for presence of the Centor criteria (8): history of fever, tonsillar exudates, absence of cough, and tender anterior cervical lymphadenopathy (lymphadenitis). Patients with two or more criteria should have rapid GABHS antigen detection performed, with antimicrobial therapy prescribed only for patients with positive results. Alternatively, antimicrobial therapy can be administered to patients who meet three or four of the criteria without a need for diagnostic testing.

The Infectious Diseases Society of America (IDSA) does not support diagnosing GABHS pharyngitis on clinical grounds alone, which it feels could result in the unnecessary use of antimicrobial agents in uninfected patients. It recommends instead that adult patients suspected of being infected have their diag-

nosis confirmed by at least a positive antigen detection test prior to antimicrobial therapy (4).

Both sets of guidelines discourage pharyngeal cultures during the routine evaluation of pharyngitis in adults or for confirming negative antigen test results when test sensitivities exceed 80%. However, the IDSA guidelines leave the decision to the physician as to whether cultures should be performed instead of or in addition to antigen detection assays. Additional indications for pharyngeal cultures in adults are (i) investigations of outbreaks of GABHS disease, (ii) monitoring the appearance and spread of antimicrobial resistance, and (iii) examination for pathogens other than GABHS.

GABHS are important causes of bacteremia and skin and soft tissue infections as well. Recently appreciated is the role of GABHS as an etiologic agent of perianal dermatitis in children following swallowing or direct inoculation of infectious respiratory secretions (2, 17). Two other highly publicized GABHS diseases in recent years have been necrotizing fasciitis ("flesh-eating" bacterial disease) and streptococcal toxic shock syndrome.

This procedure describes laboratory options for detection and identification of GABHS by direct methods and by culture. The emphasis is on pharyngeal specimens, since they are often submitted specifically for culture of this organism.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING

NOTE: Refer to procedure 3.3.1 for additional details. The following can be copied for preparation of a specimen collection instruction manual for physicians and caregivers. It is the responsibility of the laboratory director or designee to educate physicians and caregivers on proper specimen collection.

A. Specimen collection

1. Pharynx (6)
 - a. Place gentle pressure on the tongue with a tongue depressor.
 - b. Extend one or two sterile swabs (one for the antigen test and one for culture if necessary) between the tonsillar pillars and behind the uvula, avoiding the tongue, buccal mucosa, and uvula.
 - c. Sweep the swabs back and forth across the posterior pharynx, tonsillar areas, and any inflamed or exudative areas.
 - d. Do not obtain specimens if the epiglottis is inflamed, since sampling may trigger life-threatening respiratory obstruction.
2. Perianal dermatitis (cellulitis)
 - a. Cleanse the sampling area with saline, followed by two applications of 70% alcohol.
 - b. Collect an aspirate from the leading edge of the inflamed area.
 - c. Order an aerobic (routine) wound culture (*see* procedure 3.13.1)
3. Blood culture (*see* procedure 3.4.1)
4. Wound culture (*see* procedure 3.13.1)

B. Specimen transport

1. Submit swabs for GABHS culture to the laboratory as follows.
 - a. Dry in a paper envelope *or*
 - b. Moistened or immersed in transport medium
2. Label specimens with the patient's demographic information, name of person requesting the test, date of specimen collection, the specimen collection site, and the test to be performed.

C. Rejection criteria

1. Pharyngeal cultures for GABHS are not recommended for confirmation of negative antigen detection results for adults when the antigen test sensitivity exceeds 80%.
2. Do not perform Gram stains of pharyngeal specimens for diagnosis of GABHS pharyngitis, since the results are not helpful.

III. MATERIALS

A. Primary tests: antigen detection tests for GABHS

NOTE: Many kits for detection of GABHS antigen are commercially available (12). Some of these products are Clinical Laboratory Improvement Amendments (CLIA) waived and are readily performed in physician offices. For an up-to-date list of CLIA-waived tests, please refer to the website <http://www.cms.hhs.gov/clia/waivetbl.pdf>. Almost all of the assays are based on nitrous acid extraction of group A streptococcal antigen from specimens followed by particle agglutination, staphylococcal coagglutination, EIA, immunochromatography, or optical immunoassay techniques for detection of antigen. Kits vary in their sensitiv-

ities in detection of GABHS, and reports of the same kit can differ depending on the culture media and conditions used for the comparison (13).

B. Culture

1. Inoculate one of the following.
 - a. BAP
 - b. NNA: BAP with 30 µg of neomycin per ml and 15 µg of nalidixic acid per ml (BD Diagnostic Systems, Sparks, Md.)
 - c. Selective streptococcal agar: BAP with neomycin and polymyxin B (Remel, Inc.; Hardy Diagnostics) or colistin and oxolinic acid (Remel, Inc.).
 - d. Selective group A streptococcus agar containing trimethoprim-

III. MATERIALS (continued)

sulfamethoxazole with or without colistin or crystal violet (available from most vendors)

☐ NOTE: Media with trimethoprim-sulfamethoxazole can be used for growth of GABHS and group B beta-hemolytic streptococci but not for group C, F, or G beta-hemolytic streptococci. Contact the vendor for information on the antimicrobial agents in their selective media, as they vary from vendor to vendor.

2. Identification methods
 - a. Gram stain (procedure 3.2.1)
 - b. Catalase test (procedure 3.17.10)
 - c. One or more of the following
 - (1) Pyrrolidonyl-β-naphthylamide (PYR) (procedure 3.17.41)
 - (2) AccuProbe STREP A DNA probe assay for RNA (Gen-Probe Inc.)
 - (3) Latex particle agglutination or staphylococcal protein A coagglutination tests for streptococcal grouping
 - (a) Directigen (BD Diagnostic Systems)

- (b) Meritec Strep (Meridian Diagnostics)
- (c) Oxoid Strep Grouping (Oxoid)
- (d) PathoDx (Diagnostic Products Corp., Los Angeles, Calif.)
- (e) Prolex (Pro-Lab Diagnostics, Austin, Tex.)
- (f) Slidex Strep (bio-Mérieux Vitek, Hazelwood, Mo.)
- (g) Streptex (Abbott Diagnostics, Abbott Park, Ill.)

3. Other supplies needed

- a. Incubator maintained at 35°C with provision for ambient, 5 to 10% CO₂, or anaerobic incubation
- b. Disposable cards for latex particle agglutination or staphylococcal protein A coagglutination identification tests

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

A. Antigen detection tests

1. Test a positive and negative control antigen with each new lot and at the start of each work shift.
- ☐ NOTE:** Use of some commercial kits, having an internal patient control, does not necessitate other daily QC. Consult the package insert.
2. Determine whether the antigen detection test is at least 80% sensitive compared to culture as a QA check.

B. Immunological identification tests

1. Test each new lot of antiserum with a corresponding positively and negatively reacting control organism prior to use and every 6 months thereafter.
2. Test each lot of extraction reagent with a known culture of GABHS to verify its ability to extract the antigen.

C. Culture media

1. Verify that culture media meet expiration date and QC parameters per current Clinical and Laboratory Standards Institute (CLSI; formerly NCCLS) M22 document. See procedures 14.2 and 3.3.1 for further procedures, especially for in-house-prepared media.
2. Perform growth checks on each new lot of selective blood agar that contains antimicrobial agents (see below).

Test organism	Result
<i>Proteus mirabilis</i> ATCC 12453	Partial to complete inhibition
<i>Streptococcus pyogenes</i> ATCC 19615	Growth of beta-hemolytic colonies

IV. QUALITY CONTROL (continued)

NOTE: CLSI QC standards (9) propose the elimination of user QC for commercially prepared group A selective agars. Consult with local regulatory agencies and CLSI documents prior to discontinuation of user QC.

V. PROCEDURE



Observe standard precautions.

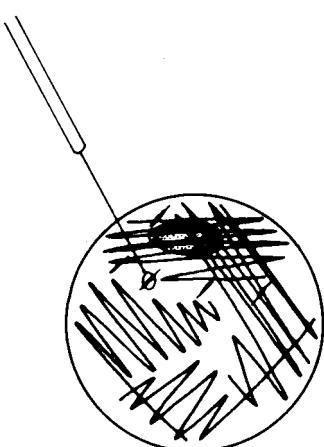


Figure 3.11.8–1 Method of streaking plate for throat culture with stabs in agar.

A. GABHS antigen detection tests

1. Perform antigen detection tests on pharyngeal specimens to provide rapid results.
 - a. Follow each step of the manufacturer's instructions carefully.
 - b. Include control steps, as indicated by the manufacturer.
 2. Confirm negative results from pediatric patients with culture, because current tests are unable to detect low numbers of GABHS in poorly collected specimens.
- NOTE:** Confirmation of negative test results for adult patients is generally unnecessary, unless the sensitivity of the test is less than 80%.
3. Do not confirm positive results for any patients, except during validation testing, to determine the test specificity, or to recover isolates for antimicrobial susceptibility testing (AST) (16).

B. Culture

1. Inoculation of culture media
 - a. Choose either BAP and/or one of the selective sheep blood agars

NOTE: Inoculating two swabs with both nonselective and selective media adds to the expense but will increase the sensitivity of culture (18).
 - b. Firmly roll a pharyngeal swab over one-sixth (no more) of the agar surface, and streak carefully for isolation in four quadrants to minimize overgrowth by other microorganisms. Carefully stab the agar several times with the same loop both in an area that has been streaked and in an area that has not been streaked, so as not to leave gaps, in order to improve detection of beta-hemolysis when plates are incubated aerobically (Fig. 3.11.8–1).
2. Incubate culture plates at 35 to 37°C for 48 h under one of the following conditions.
 - a. Aerobic with ambient air for BAP with agar stabs
 - b. Aerobic with 5 to 10% CO₂ for selective streptococcal agar with agar stabs (*This method is not acceptable for BAP without antimicrobials.*)
 - c. Anaerobic for either BAP or selective agars (3)

NOTE: Each of the methods described above has good sensitivity; in comparative studies no one method was consistently better for detection of GABHS (16, 18). However, aerobic incubation in CO₂ is helpful for isolating pathogens other than GABHS, such as group C and G streptococci. Incubation for 48 h consistently increases the yield of positive cultures by 5 to 46% depending on the study (16). Prolonged incubation is most important if selective media, which may somewhat inhibit the growth of GABHS, or if anaerobic incubation is used.
3. Examination of culture media (Fig. 3.11.8–2)
 - a. Observe culture media after 24 (and 48) h for small translucent or transparent colonies that are dome shaped, have an entire edge, and are surrounded by a relatively wide zone of complete (beta-) hemolysis.
 - b. Perform a catalase test on one or two colonies. Catalase-positive colonies are not streptococci.
 - c. Perform a Gram stain on catalase-negative colonies. Organisms other than gram-positive cocci in pairs or chains are not streptococci.

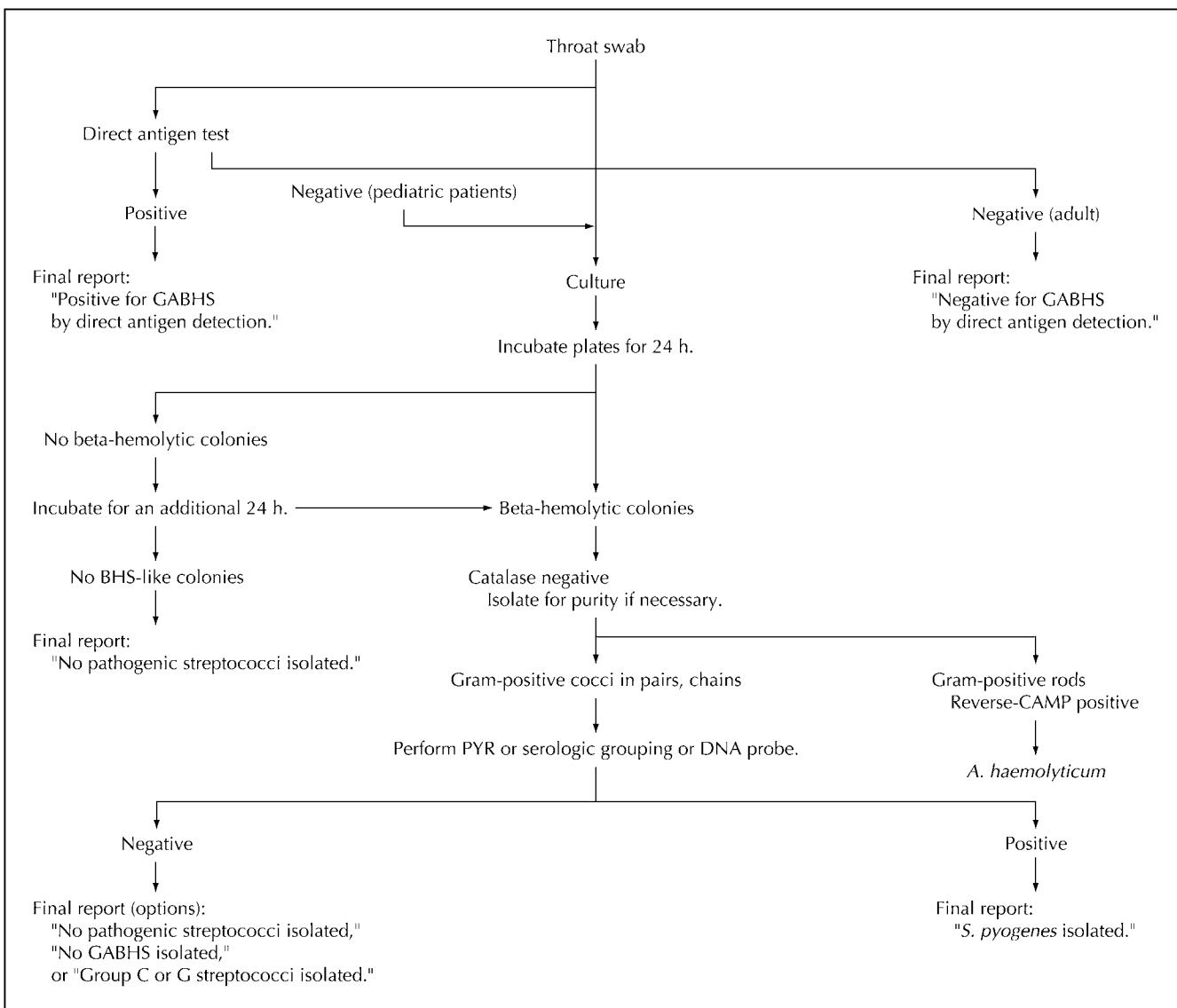


Figure 3.11.8–2 Algorithm for laboratory diagnosis of streptococcal pharyngitis.

V. PROCEDURE (continued)

4. Confirmatory tests

- a. Confirm beta-hemolytic, catalase-negative, gram-positive cocci in pairs and chains as *Streptococcus pyogenes* by one of the following.
 - (1) Positive PYR test (19). For specimens from nonpharyngeal sites, confirm with a negative bile-esculin or esculin test (procedure 3.17.5).
 - (2) Positive result for group A streptococcal antigen with an immunological grouping test
 - (3) Positive DNA probe result
- **NOTE:** The presence of a zone of growth inhibition around a bacitracin (Taxo A) disk to identify streptococci as GABHS is not reliable (20).
- b. If a catalase-negative, beta-hemolytic isolate is PYR negative, test it with immunological grouping reagents to identify it as group B, C, F, or G. Do not report GABHS that are PYR negative as *S. pyogenes* because they belong to the *Streptococcus anginosus* group of normal respiratory microbiota.

V. PROCEDURE (continued)

- c. If a colony Gram stain reveals gram-positive bacilli, perform the CAMP test (procedure 3.17.8). *A. haemolyticum* is catalase negative and reverse-CAMP test positive and forms slowly growing beta-hemolytic colonies on nonselective or selective sheep blood agar.
5. Do not perform AST as a routine, since all GABHS continue to exhibit penicillin susceptibility. Save positive culture plates (preferably for 7 days) in case AST is needed for determination of erythromycin or clindamycin resistance. See section 5 and reference 10 for methods.

POSTANALYTICAL CONSIDERATIONS**VI. REPORTING RESULTS****A. Negative results from pharyngeal specimens**

1. Report the antigen detection result as "Negative for group A streptococci by antigen detection assay."
2. Report the culture result as "No pathogenic streptococci isolated" if there are no beta-hemolytic colonies or if beta-hemolytic colonies are identified as group A (PYR negative), B, D, or F.

B. Positive results from pharyngeal specimens

1. Report the antigen detection result as "Positive for group A streptococcus by direct antigen test."
2. Report the culture results with a comment that indicates the specific pathogens for which the specimen was analyzed. For negative cultures include a comment such as "Negative for group A streptococcus" if there are no beta-hemolytic colonies.
3. If *A. haemolyticum* is identified, report the culture as positive for this microorganism.
4. If group C or G beta-hemolytic streptococci are identified, report or do not report according to the preferences of the ordering physician.

VII. INTERPRETATION

- A. A positive pharyngeal culture for GABHS indicates the presence of *S. pyogenes* but does not distinguish between infection and colonization (14, 15).
- B. All *S. pyogenes* organisms are susceptible to penicillin (the antimicrobial agent of choice for treating infection), negating the need for susceptibility testing. If testing is performed, penicillin resistance must be confirmed by a reference laboratory (10). For penicillin-allergic patients, erythromycin is the therapeutic agent of choice. Recurrent tonsillitis may require surgery and treatment with clindamycin (7).
- C. GABHS can be resistant to erythromycin and clindamycin; in some geographic areas the incidence is high (20), and testing may be indicated when penicillin therapy is contraindicated.

VIII. LIMITATIONS

- A. Falsely negative pharyngeal cultures can result from overgrowth of cultures by normal oral microorganisms or from the lack of beta-hemolysis in cultures incubated aerobically.
- B. Falsely positive pharyngeal culture reports can result from misinterpretation of identification tests. PYR-positive colonies may be enterococci, which are rarely beta-hemolytic and uncommonly found in the pharynx. Isolates from rectal swabs should be identified by immunological methods or the combination of PYR and esculin hydrolysis tests.
- C. PYR-negative GABHS are not *S. pyogenes* but members of the *S. anginosus* group, which is not a cause of pharyngitis.
- D. Group C and G beta-hemolytic streptococci can cause pharyngitis and fever, but they do not place patients at risk of acute rheumatic fever.

REFERENCES

1. American Academy of Pediatrics. 2003. Group A streptococcal infections, p. 573–584. In L. K. Pickering (ed.), *2003 Red Book: Report of the Committee on Infectious Diseases*, 26th ed. American Academy of Pediatrics, Elk Grove Village, Ill.
2. Amren, D. P., A. S. Anderson, and L. W. Wannamaker. 1966. Perianal cellulitis associated with group A streptococci. *Am. J. Dis. Child.* **112**:546–548.
3. Belli, D. C., R. Auckenthaler, L. Paunier, and P. E. Ferrier. 1984. Throat cultures for group A beta-hemolytic *Streptococcus*. Importance of anaerobic incubation. *Am. J. Dis. Child.* **138**:274–276.
4. Bisno, A. L., M. A. Gerber, J. M. Gwaltney, Jr., E. L. Kaplan, and R. H. Schwartz. 2002. Practice guidelines for the diagnosis and management of group A streptococcal pharyngitis. Infectious Diseases Society of America. *Clin. Infect. Dis.* **35**:113–125.
5. Breese, B. B., F. A. Disney, and W. Talpey. 1966. The nature of a small pediatric group practice. II. The incidence of beta hemolytic streptococcal illness in a private pediatric practice. *Pediatrics* **38**:277–285.
6. Brien, J. H., and J. W. Bass. 1985. Streptococcal pharyngitis: optimal site for throat culture. *J. Pediatr.* **106**:781–783.
7. Capper, R., and R. J. Canter. 2001. Is there agreement among general practitioners, paediatricians and otolaryngologists about the management of children with recurrent tonsillitis? *Clin. Otolaryngol.* **26**:371–378.
8. Centor, R. M., J. M. Witherspoon, H. P. Dalton, C. E. Brody, and K. Link. 1981. The diagnosis of strep throat in adults in the emergency room. *Med. Decis. Making* **1**:239–246.
9. Clinical and Laboratory Standards Institute. 2004. *Quality Assurance for Commercially Prepared Microbiological Culture Media*, 3rd ed. Approved standard M22-A3. Clinical and Laboratory Standards Institute, Wayne, Pa.
10. Clinical and Laboratory Standards Institute. 2006. *Performance Standards for Antimicrobial Susceptibility Testing*. Sixteenth informational supplement. Approved standard M100-S16. Clinical and Laboratory Standards Institute, Wayne, Pa.
11. Cooper, R. J., J. R. Hoffman, J. G. Bartlett, R. E. Besser, R. Gonzales, J. M. Hickner, and M. A. Sande. 2001. Principles of appropriate antibiotic use for acute pharyngitis in adults: background. *Ann. Intern. Med.* **134**:509–517.
12. Evangelista, A. T., A. L. Truant, and P. P. Bourbeau. 2002. Rapid systems and instruments for the identification of bacteria, p. 22–49. In A. L. Truant (ed.), *Manual of Commercial Methods in Microbiology*. ASM Press, Washington, D.C.
13. Graham, L., Jr., F. A. Meier, R. M. Centor, B. K. Garner, and H. P. Dalton. 1986. Effect of medium and cultivation conditions on comparisons between latex agglutination and culture detection of group A streptococci. *J. Clin. Microbiol.* **24**:644–646.
14. Gunnarsson, R. K., S. E. Holm, and M. Soderstrom. 1997. The prevalence of beta-hemolytic streptococci in throat specimens from healthy children and adults. Implications for the clinical value of throat cultures. *Scand. J. Prim. Health Care* **15**:149–155.
15. Kaplan, E. L., F. H. Top, Jr., B. A. Dudding, and L. W. Wannamaker. 1971. Diagnosis of streptococcal pharyngitis: differentiation of active infection from the carrier state in the symptomatic child. *J. Infect. Dis.* **123**:490–501.
16. Kellogg, J. A. 1990. Suitability of throat culture procedures for detection of group A streptococci and as reference standards for evaluation of streptococcal antigen detection kits. *J. Clin. Microbiol.* **28**:165–169.
17. Kokx, N. P., J. A. Comstock, and R. R. Facklam. 1987. Streptococcal perianal disease in children. *Pediatrics* **80**:659–663.
18. Kurtz, B., M. Kurtz, M. Roe, and J. Todd. 2000. Importance of inoculum size and sampling effect in rapid antigen detection for diagnosis of *Streptococcus pyogenes* pharyngitis. *J. Clin. Microbiol.* **38**:279–281.
19. NCCLS. 2002. *Abbreviated Identification of Bacteria and Yeast*. Approved guideline M35-A. NCCLS, Wayne, Pa.
20. York, M. K., L. Gibbs, F. Perdreau-Remington, and G. F. Brooks. 1999. Characterization of antimicrobial resistance in *Streptococcus pyogenes* isolates from the San Francisco Bay Area of Northern California. *J. Clin. Microbiol.* **37**:1727–1731.

SUPPLEMENTAL READING

- Bannatyne, R. M., C. Clausen, and L. R. McCarthy. 1979. *Cumitech 10, Laboratory Diagnosis of Upper Respiratory Tract Infections*. Coordinating ed., I. B. R. Duncan. American Society for Microbiology, Washington, D.C.
- Bisno, A. L., G. S. Peter, and E. L. Kaplan. 2002. Diagnosis of strep throat in adults: are clinical criteria really good enough? *Clin. Infect. Dis.* **35**:126–129.

3.11.9

Nasal Sinus Cultures

[Updated March 2007]

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Acute rhinosinusitis, an infection of one or both of the paranasal sinuses, is among the most common health problems (7). It is manifest by an inflammatory response of the mucous membranes of the nasal cavity, seen as edema and hypersecretion of mucus following a common upper respiratory viral infection (6, 9). The disease is mild but results in considerable medical cost to relieve facial pain and swelling, nasal discharge, fatigue, and symptoms related to the inflammatory process (9). Therapies to alleviate the symptoms of congestion and improve nasal drainage are recommended early in the viral infection and can prevent progression to bacterial infection (8). Antimicrobial agents are important, if the disease progresses beyond 7 to 10 days, to reduce time to recovery, aid

in symptom resolution, and prevent complications from progression of the disease (3, 8). Antimicrobial therapy is usually targeted to the most common agents, *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Moraxella catarrhalis*, and *Streptococcus pyogenes* (2, 3). Some authors include anaerobes and *Staphylococcus aureus* as possible agents of disease, but these bacteria are less often associated with acute inflammation (11). In 40% of cases, no agent is cultured, which has suggested that *Mycoplasma pneumoniae* or *Chlamydia pneumoniae* is involved (6). Sinus infections can become chronic, although the etiology of this presentation has been controversial. For chronic infections, particularly in diabetic patients and

in tropical countries, fungi can invade the sinuses and cause progressive, life-threatening infections. Anaerobes have also been implicated, and therapy usually includes agents, such as metronidazole or clindamycin, which are active against anaerobes (1).

This procedure deals only with invasively collected specimens for diagnosis of acute sinusitis. Refer to other procedures in this handbook for culture for viruses, fungi, and mycobacteria and for nasal carriage of contagious pathogens or *Bordetella pertussis* (see Table 3.11.1–1). Collection of specimens from patients with sinusitis is performed by otolaryngologists who perform nasal endoscopy or sinus puncture and aspiration.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING



Observe standard precautions.

NOTE: Refer to procedure 3.3.1 for additional details. The following can be copied for preparation of a specimen collection instruction manual for physicians and caregivers. It is the responsibility of the laboratory director or designee to educate physicians and caregivers on proper specimen collection.

- A. Specimen collection is performed by an otolaryngologist.
 1. Rigid endoscopy (11)
 - a. Provide patient with an intranasal decongestant and then a topical anesthetic.
 - b. Identify the middle meatus adjacent to the maxillary sinus ostium ipsilateral to the side to be aspirated.
 - c. Collect drainage from the middle meatus with a small swab on a wire.
 2. Maxillary sinus puncture and aspiration
 - a. Clean the anterior nares with antiseptic solution.
 - b. Apply topical anesthetic.
 - c. Puncture the maxillary antrum and aspirate secretions with a needle and syringe.
 - d. If no material is aspirated, irrigate with 2 ml of nonbacteriostatic saline.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING (continued)

- B. Specimen transport**
 1. Submission to the laboratory
 - a. Submit swabs in tube of transport medium or in BD Culturette EZ.
 - b. Aspirates should be transferred to a sterile container for submission to the laboratory. If an anaerobic culture is required, submit a portion of the sample in a sterile tube containing prereduced anaerobically sterilized (PRAS) media. Syringes with the needle attached should never be sent to the laboratory because of the sharps and biohazard risk to staff. Syringes with a Luer-Lok are also not ideal for culture since the specimen may become congealed in transport in the device and become contaminated during handling.
 2. Label specimens with demographic information, date and time of collection, and site of collection.
 3. List the diagnosis and whether it is chronic or acute.
 4. Order anaerobic culture if appropriate to the diagnosis.
- C. Rejection criteria**
 1. Reject requests for routine bacterial culture of nasal washes and nasal aspirates not collected by otolaryngologists as inappropriate and lacking specificity to diagnose either sinusitis or lower respiratory disease (5, 10). These specimens are acceptable for *B. pertussis* and for viral culture.
 2. Nasal swabs not collected by otolaryngologists are acceptable only for specific culture (e.g., *B. pertussis* or *Corynebacterium diphtheriae*) or staphylococcal carriage, which should be indicated along with the culture request. They are unacceptable to diagnose acute sinusitis (5, 10). They may, however, be acceptable for diagnosis of fungal invasion of the sinuses (see section 8).

III. MATERIALS

- | | | |
|-------------------------------------------------------------------------------|----------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------|
| A. Primary media | B. Identification methods | C. Other supplies |
| 1. BAP
2. CHOC
3. Anaerobic plate media (on request).
See section 4. | 1. Gram stain (procedure 3.2.1) | 1. Incubator at 35°C with 5% CO ₂ or a CO ₂ -generating system
2. Inoculating sticks or loops
3. Petri dishes and filter paper |

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

- A. Verify that media meet expiration date and QC parameters per current Clinical and Laboratory Standards Institute (CLSI; formerly NCCLS) M22 document. See procedures 14.2 and 3.3.1 for further procedures, especially for in-house-prepared media.
- B. Test each lot of CHOC per procedure 3.3.1.

V. PROCEDURE



Observe standard precautions.

A. Inoculation

1. Inoculate specimen to BAP and CHOC.
2. Firmly roll swab over one-sixth (no more) of the agar surface, or deposit 3 or 4 drops of fluid onto agar. Streak carefully for isolation in four quadrants to minimize overgrowth by other microorganisms.

B. Incubation

1. Incubate plate at 35 to 37°C in 5% CO₂ for a minimum of 48 h.
2. For special requests in cases of chronic infections, extend incubation to 4 days.

V. PROCEDURE (continued)**C. Gram stain**

1. Perform a gram stain from the swab or fluid (procedure 3.2.1)
2. Note the presence of inflammatory cells and bacteria.

D. Culture examination

1. Observe for growth of *S. pneumoniae*, *H. influenzae*, *M. catarrhalis*, and *S. pyogenes*.
 - a. Refer to Table 3.3.2–5 for rapid identification tests.
 - b. *S. pneumoniae*
 - (1) Confirm bile solubility with optochin disk, if questionable or resistant.
 - (2) Perform antimicrobial susceptibility testing (AST), using standardized methods (4), per laboratory protocol and physician policy.
 - c. Perform beta-lactamase test on *H. influenzae*.
 - d. Since greater than 90% of *M. catarrhalis* organisms are beta-lactamase positive, testing is not helpful for treatment.
 2. Observe plates at 24 and 48 h for growth of enteric gram-negative rods and *S. aureus*.
 - a. These organisms are usually considered contaminants in sinus specimens and are not identified unless they are the predominant species in the culture.
 - b. Perform AST only if the Gram stain suggests that they are involved in an inflammatory process and no other pathogens are isolated.
- NOTE:** Normal skin microbiota (coagulase-negative staphylococci and corynebacteria) are not identified to the species level.
3. Identify any molds that are present. Yeasts need not be identified to the species level, as they have not been implicated in sinusitis.
 4. Identify any other predominant organism that is not part of the normal respiratory microbiota.
 5. If anaerobic cultures were ordered, refer to section 4 for identification; gram-positive anaerobes are more likely to be present in sinus infections than gram-negative rods.

POSTANALYTICAL CONSIDERATIONS**VI. REPORTING RESULTS****A. Gram stain: report smear as indicated in procedure 3.2.1.****B. Negative culture results**

1. Report preliminary and final reports as “No growth.”
2. Indicate the number of days the culture was incubated.

C. Positive results

1. Report all pathogens and susceptibility tests performed, using preliminary reports as indicated in procedure 3.3.2 and CLSI guidelines (4).
 2. Indicate presence of aerobic skin microbiota, without species identification.
 3. If the culture is mixed, with no predominating pathogen, indicate the genera and do not report further, e.g., “Mixed microbiota present, consisting of three morphologies of gram-negative rods and skin bacteria.”
- NOTE:** Mixed microbiota without a predominant pathogen generally indicates that the specimen was not collected appropriately. Exception is made if the Gram stain demonstrates inflammation by the presence of PMNs.

VII. INTERPRETATION

- A. A positive culture with *S. pneumoniae*, *H. influenzae*, *M. catarrhalis*, and *S. pyogenes* generally indicates infection with that organism.
- B. A positive culture with a predominant gram-negative rod or *S. aureus* may or may not indicate infection with that agent.

VII. INTERPRETATION (continued)

- C. A negative culture cannot rule out sinusitis. In fact, often a pathogen is not isolated.
- D. Most physicians agree on the benefits of antimicrobial therapy for acute sinus infections. Generally a beta-lactam agent, such as amoxicillin, amoxicillin-clavulanate, or a cephalosporin, is used, negating the need for culture (3). However, for persistent chronic infection in the face of therapy, cultural evaluation and antimicrobial profiling are indicated.

VIII. LIMITATIONS

- A. Inaccurate culture reporting can result from contamination of the specimen with normal oral microbiota.
- B. False-negative cultures can be caused by delays in processing.
- C. False-positive cultures can be caused by overinterpretation of the culture results.

REFERENCES

1. Brook, I., P. Yocum, and K. Shah. 2000. Aerobic and anaerobic bacteriology of concurrent chronic otitis media with effusion and chronic sinusitis in children. *Arch. Otolaryngol. Head Neck Surg.* **126**:174–176.
2. Brook, I., W. M. Gooch III, S. G. Jenkins, M. E. Pichichero, S. A. Reiner, L. Sher, and T. Yamauchi. 2000. Medical management of acute bacterial sinusitis. Recommendations of a clinical advisory committee on pediatric and adult sinusitis. *Ann. Otol. Rhinol. Laryngol. Suppl.* **182**:2–20.
3. Chow, A. W., C. B. Hall, J. Klein, R. B. Kammer, R. D. Meyer, and J. S. Remington. 1992. General guidelines for the evaluation of new anti-infective drugs for the treatment of respiratory tract infections. *Clin. Infect. Dis.* **15**(Suppl. 1):S62–S88.
4. Clinical and Laboratory Standards Institute. 2006. *Performance Standards for Antimicrobial Susceptibility Testing*. Sixteenth informational supplement. Approved standard M100-S16. Clinical and Laboratory Standards Institute, Wayne, Pa.
5. Dagan, R., R. Melamed, M. Muallem, L. Piglansky, and P. Yagupsky. 1996. Nasopharyngeal colonization in southern Israel with antibiotic-resistant pneumococci during the first 2 years of life: relation to serotypes likely to be included in pneumococcal conjugate vaccines. *J. Infect. Dis.* **174**:1352–1355.
6. Gwaltney, J. M. 2000. Sinusitis, p. 676–686. In G. L. Mandell, J. E. Bennett, and R. Dolin (ed.), *Principles and Practice of Infectious Diseases*, 5th ed. Churchill Livingstone, New York, N.Y.
7. Gwaltney, J. M. 1996. Acute community-acquired sinusitis. *Clin. Infect. Dis.* **23**:1209–1225.
8. Hadley, J. A. 2001. The microbiology and management of acute and chronic rhinosinusitis. *Curr. Infect. Dis. Rep.* **3**:209–216.
9. Lanza, D. C., and D. W. Kennedy. 1997. Adult rhinosinusitis defined. *Otolaryngol. Head Neck Surg.* **117**:S1–S7.
10. Robinson, D. A., K. M. Edwards, K. B. Waites, D. E. Briles, M. J. Crain, and S. K. Hollingshead. 2001. Clones of *Streptococcus pneumoniae* isolated from nasopharyngeal carriage and invasive disease in young children in central Tennessee. *J. Infect. Dis.* **183**:1501–1507.
11. Talbot, G. H., D. W. Kennedy, W. M. Scheld, and K. Granitol for the Endoscopy Study Group. 2001. Rigid nasal endoscopy versus sinus puncture and aspiration for microbiologic documentation of acute bacterial maxillary sinusitis. *Clin. Infect. Dis.* **33**:1668–1675.

[Updated March 2007]

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Urinary tract infections (UTI) account for seven million visits to physicians' offices and over one million hospital admissions per year. Epidemiologic studies by E. H. Kass (8) have shown that bacterial counts of $\geq 10^5$ CFU/ml for a pure culture of gram-negative bacilli were found to be associated with acute bacterial infections of the urinary tract. In females with dysuria and acute UTI, other investigators reported that $> 10^2$ CFU/ml can be significant (6, 7, 15). For infants and catheterized patients, low counts also have been shown to be significant (4, 16, 17). Because of the importance of colony counts for infection, urine cultures are always performed with an accompanying colony count (2).

Urine is normally a sterile body fluid. However, it is easily contaminated with

microbiota from the perineum, prostate, urethra, or vagina. The microbiologist must provide detailed instructions to ensure proper specimen collection, preservation, labeling, and transport of urine for culture.

The etiologic agents of urinary tract infection are generally limited to the patient's own intestinal microbiota, with *Escherichia coli*, *Enterococcus* spp., *Klebsiella-Enterobacter* spp., and *Proteus* spp. representing a majority of isolates from both hospitalized patients and outpatients. Refer to Table 3.12-1 for a list of usual microbiota of contaminated and infected urine.

Several terms may be encountered with the submission of specimens for culture. These are defined in Table 3.12-2. Since the significance of bacterial counts depends on the disease presentation, different criteria are often used to determine if the count of bacteria in the urine is significant and requires treatment. Because the laboratory rarely knows details other than patient age and sex and type of specimen, the significance of bacteria in the urine is usually based only on these criteria. Pyuria, in addition to bacteruria, is also an important factor in establishing the presence of a UTI (see procedure 3.2.3 and Appendix 3.12-1).

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING



Observe standard precautions.

NOTE: Refer to procedure 3.3.1 for additional details. The following can be copied for preparation of a specimen collection instruction manual for physicians and other caregivers. It is the responsibility of the laboratory director or designee to educate physicians and other caregivers on proper specimen collection.

A. Specimen collection

1. Clean-voided midstream urine collection

a. Preparation

(1) Females

NOTE: Collection of midstream urine specimens should be avoided during menses.

(a) While the labia are held apart with the aid of a pair of sponges, wash the vulva thoroughly from front to back with two successive cotton pledges or sponges soaked in soap. Special attention should be paid to the urethral meatus (benzalkonium or hexachlorophene should not be used, as a single drop of residual can sterilize the urine before the specimen reaches the laboratory).

Table 3.12-1 Urinary microbiota^a

Microbiota	Organism	Extent of workup if count is appropriate per Table 3.12-4
Urogenital	Viridans group streptococci, <i>Neisseria</i> spp., diphtheroids, <i>Lactobacillus</i> spp., anaerobes	Report as urogenital microbiota.
Skin	Diphtheroids, <i>Staphylococcus</i> spp.	Report as skin or with urogenital microbiota unless present in amounts >10-fold more than other microbiota. Then treat as uropathogen.
Uropathogens	Gram-negative bacilli <i>Staphylococcus</i>	ID to species level and AST ID and AST of <i>S. aureus</i> ; ID of <i>Staphylococcus saprophyticus</i> with novobiocin disk for females of childbearing age; AST generally not needed for <i>S. saprophyticus</i> or other coagulase-negative staphylococci.
	Yeast	ID of <i>C. albicans</i> and <i>Candida glabrata</i> ; ID of others to species level only on request
	Beta-hemolytic <i>Streptococcus</i> <i>Enterococcus</i> spp.	ID, especially of group B in women in childbearing years Check for VRE on inpatients; ID to species level and AST only if VRE and on request
	<i>G. vaginalis</i>	ID only if number is 10 times greater than that of all other microbiota
	<i>Aerococcus urinae</i>	ID only if number is 10 times greater than that of all other microbiota (20) (see Table 3.18.1-4b for tests to identify)
	<i>Corynebacterium</i> (urease positive)	ID and AST, if number is 10 times greater than that of all other microbiota and ≥100,000 CFU.
Bacteremia	All pathogens	Full identification and AST

^a Abbreviations: AST, antimicrobial susceptibility testing; ID, identification; VRE, vancomycin-resistant enterococcus.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING (continued)

- (b) Then with two additional sponges and sterile water or saline, rinse the vulva.
 - (2) Circumcised males: no preparation for midstream specimen.
 - (3) Uncircumcised males: the process is similar to that described above for females.
 - (a) Retract the foreskin, and wash the glans penis thoroughly with two successive cotton pledges or sponges soaked in soap, paying special attention to the urethral meatus.
 - (b) Then rinse the glans with additional successive pledges with sterile water or saline.
 - b. Have the patient collect voided urine directly into a disposable leakproof container, instructing the patient to not halt and restart the urinary stream for a “midstream” collection but preferably move the container into the path of the already voiding urine.
- NOTE:** Never collect urine from a bedpan or urinal.
- NOTE:** Most laboratories still recommend that patients follow the above instructions for cleansing the genital area before collection of a

Table 3.12–2 Definitions

Term	Definition
Bacteriuria	Presence of uropathogenic bacteria in the urine
Cystitis	Inflammation of the bladder
Cystostomy	Surgical procedure of inserting tube directly in the bladder through the suprapubic area to drain urine
Dysuria	Pain or burning on urination, a common complaint on presentation of UTI
Nephrostomy	Surgical procedure leaving tubing directly in the kidney
Prostatitis	Infection of the prostate gland; patient may present with fever or be asymptomatic
Pyelonephritis	Acute infection of the kidney and renal pelvis usually with fever, chills, and flank pain; chronic; may be without symptoms
Pyuria	Urine WBC count of 8–10/ μ l (or >5 /high-power field in a conventional urinalysis), which correlates with WBC excretion rate of $>400,000$ WBC/h
Urethritis	Inflammation of the urethra; may be caused by sexually transmitted diseases or uropathogens
Urosepsis	Pyelonephritis with accompanying bacteremia and septic shock
Urostomy	Surgical procedure leaving an external opening in the abdominal wall, usually made of intestine, for the egress of urine
Suprapubic aspirate	Specimen of urine collected with a syringe and needle inserted directly through the skin into the bladder
Straight catheter urine	Specimen collected by the insertion of a catheter through the urethra into the bladder
Uncomplicated UTI	Infection primarily in otherwise healthy women who have no structural or functional abnormalities of the urinary tract
Complicated UTI	Infection in a patient with structural and/or functional abnormalities of the urinary tract (e.g., spinal cord injury, neurogenic bladder, urinary tract obstruction, etc.)
Asymptomatic UTI	Infection with $>10^5$ bacteria/ml with no symptoms of infection

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING (continued)

urine culture specimen. Limited studies have so far shown that this practice may not be warranted. One study found that cleansing by both asymptomatic and symptomatic women did not decrease contamination of specimens (9). Cleansing of the male genitalia did not improve the detection of bacteriuria (11). No difference in contamination rates was seen when a clean but nonsterile container was used for urine collection.

2. Catheter urine

- a. Using a needle and syringe, collect urine through the catheter port, after cleaning with alcohol. Alternatively, collect the sample directly into a Vacutainer tube without anticoagulant, using a Vacutainer holder and needle. *Do not send urine obtained from a catheter bag.*
- b. A straight catheter (in and out) is used by a physician or trained health care worker (HCW) to obtain urine directly from the bladder.
 - (1) This procedure must be carried out with aseptic technique, to avoid the risk of introducing microorganisms into the bladder.
 - (2) Discard the initial 15 to 30 ml of urine and submit the next flow of urine for culture.

3. Ileal conduit

- a. Remove the external device.
- b. Cleanse the stoma with 70% alcohol followed by iodine.
- c. Remove the iodine with alcohol.
- d. Insert a double catheter into the cleansed stoma, to a depth beyond the fascial level, and collect the urine.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING (continued)

4. Urine collection by suprapubic needle aspiration directly into the bladder is performed by a physician or trained HCW. This method is the preferred method for infants, for patients for whom the interpretation of results of voided urine is difficult, and when anaerobic bacteria are suspected as the cause of infection.
 - a. Bladder should be full and palpable before aspiration.
 - b. Shave and disinfect the skin over the bladder.
 - c. Make a small lance wound through the epidermis above the symphysis pubis.
 - d. Aspirate using a needle and syringe. Place the urine specimen in a sterile container before submission to the laboratory. Syringes with the needle attached should not be accepted because of the sharps and biohazard risk to staff. Syringes with a Luer-Lok may leak during transport, resulting in contamination of the specimen.
5. Lower UTI in men is localized to the prostate by collection of sequential urine samples and expressed prostatic secretions (see section 3.9.1 and Appendix 3.9.1–2). Uropathogenic bacteria are the most common cause of acute and chronic prostatitis, but skin microbiota such as coagulase-negative *Staphylococcus* and *Corynebacterium* spp. may be found in low numbers in prostatic secretions from chronic prostatitis patients. *Neisseria gonorrhoeae* is found infrequently but has been reported as a cause of acute prostatitis.
6. Cystoscopy is a bilateral ureteral catheterization to determine the site of infection in the urinary tract. This procedure is usually performed in specially designated areas such as operating rooms or specialty clinics.
 - a. Clean the urethral area (and vaginal vestibule in females) with soapy water, and rinse the area well with water.
 - b. Insert a cystoscope (obturator in place) into the bladder.
 - c. With sterile technique, collect approximately 5 to 10 ml of urine from open stopcock into a sterile container.
 - d. Label this sample “CB,” for catheterized bladder urine, and refrigerate it. Then irrigate the bladder. (Use sterile nonbacteriostatic 0.85% NaCl to irrigate the bladder.)
 - e. After irrigation of the bladder and insertion of the ureteral catheters, collect irrigating fluid passing from the bladder through the ureteral catheters by holding the ends of both catheters over an opened sterile container.
 - f. Label this sample “WB,” for washed bladder urine, and refrigerate it.
 - g. Pass the ureteral catheters to each midureter or renal pelvis without introducing additional irrigating fluid. Open both stopcocks of the cystoscope to empty the bladder.
 - h. Discard the first 5 to 10 ml of urine from each ureteral catheter.
 - i. Collect four consecutive paired cultures (5 to 10 ml each) directly into opened sterile containers.
 - j. Label these specimens “LK-1,” “RK-1,” “LK-2,” and “RK-2” (LK for left kidney and RK for right kidney). Submit all samples for culture.

B. Timing of specimen collection

1. Obtain early-morning specimens whenever possible. Allowing urine to remain in the bladder overnight or for at least 4 h will decrease the number of false-negative results.
2. Do not force fluids in order to have the patient void urine. Excessive fluid intake will dilute the urine and may decrease the colony count to $<10^5$ CFU/ml.

C. Specimen transport

1. Transport urine to the laboratory after collection or, if urine cannot be delivered to the laboratory within 2 h after collection, refrigerate for up to 24 h both during the holding period and during transport. *Do not freeze.*

**II. SPECIMEN COLLECTION,
TRANSPORT, AND HANDLING**
(continued)

2. If refrigeration is not possible and specimens are delayed in transport, collect in transport tubes with preservatives.
 - a. Examples: 0.5 ml of freeze-dried boric acid-glycerol or boric acid-sodium formate (B-D urine tubes, Becton Dickinson, Rutherford, N.J.).
 - b. Place at least 3 ml of urine into the transport tube to avoid an inhibiting or diluting effect on the microorganisms.
- D. Specimen labeling and request submission**
 1. Label the urine container with demographic information of the patient and the time of collection. Using the laboratory's protocol, ensure that the collection method is communicated to the laboratory.
 2. Be alert to the fact that the person ordering the test may desire a Gram stain, may request only a colony count screen without preliminary identification, or may request omission of antimicrobial susceptibility testing (AST) in cases of uncomplicated UTI. Perform only those tests ordered.
■ NOTE: Fungal cultures of urine usually are requests to detect the presence of yeasts. It is rarely necessary to inoculate these to selective fungal media with centrifugation and prolonged incubation. Notify the physician that yeast cultures are included as part of the routine urine culture and yeasts will be cultured and reported if found. However, when yeast cultures are requested, culture at least 0.01 ml per plate and hold cultures for 48 to 72 h to detect yeasts in low numbers.
- E. Rejection criteria:**
 1. Request a repeat urine specimen when there is no evidence of refrigeration and the specimen is >2 h old.
 2. Request a repeat specimen or obtain the information when the collection time and method of collection have not been provided.
 3. Reject 24-h urine collections.
 4. Reject dipslide specimens that have been improperly collected (e.g., urine left in the container, or paddle not fully inoculated on both sides or only partially inoculated).
 5. Reject urine specimens obtained with the same collection method within 48 h of receipt of first specimen. Call this a duplicate specimen.
 6. Neonates, infants, and young toddlers cannot cooperate to provide a voided sample. Although collection of urine by in-and-out straight catheterization is the recommended method to obtain an accurate urine culture sample, catheterization is not always possible, particularly in neonates. Collection of urine by pedibag may be the only way to obtain a urine sample in this age group. Although the laboratory should discourage submission of bagged specimens as the routine for urine culture, this type of sample should be analyzed if it is the only sample that can be obtained.
 7. Reject Foley catheter tips as unacceptable for culture; they are unsuitable for the diagnosis of urinary infection (3).
 8. Reject urine from the bag of a catheterized patient.
 9. Reject specimens that arrive in leaky containers.
 10. Except for suprapubic bladder aspirates, reject specimen requests for anaerobic culture.
 11. If an improperly collected, transported, or handled specimen cannot be replaced, document in the final report that specimen quality may have been compromised and who was notified. Generally, voided urine from inpatients is easily recollected.
 12. For rapid urine screens for voided urine specimens from outpatients to eliminate those that do not warrant culture, see Appendix 3.12–2 and reference 12.

III. MATERIALS

A. Media

1. BAP
2. MAC or EMB
3. Columbia colistin-nalidixic acid agar (CNA) or phenylethyl alcohol agar (PEA) (optional)
- NOTE:** The advantage of adding CNA to the culture is that it allows detection of gram-positive microbiota when overgrown with gram-negative microbiota. In addition, the Columbia base allows growth of some bacteria that are inhibited on tryptic soy-based agars. The added expense may allow more accurate and often less time-consuming evaluation of cultures.
4. CHOC: use for surgically collected kidney urine or specimens collected by cystoscopy or after prostatic message. These can be labeled “EPS” or “VB3.”
5. Other media: see Table 3.12–3 for commercial systems. These systems work best for specimens from outpatients or those likely to have only one pathogen. When mixed microbiota are expected (e.g., specimens from patients with indwelling catheters or from geriatric pa-

tients), these systems are contraindicated.

B. Stain reagents

See Gram stain procedure (procedure 3.2.1).

C. Supplies

1. Loop method
 - a. Use either platinum or sterile plastic disposable loops.
 - b. Sizes
 - (1) 0.001-ml (1-µl) loop to detect colony counts greater than 1,000 CFU/ml
 - (2) 0.01-ml (10-µl) loop to detect colony counts between 100 and 1,000 CFU/ml.
 - (3) Disposable loops are color coded, according to volume delivery.
2. Pipettor method: Sterile pipette tips and pipettor to deliver 10 or 1 µl.
3. Sterile bent glass or plastic disposable sterile rod or “spreader,” if desired, to spread inoculum (Excel Scientific, Wrightwood, Calif., [760] 249-6371).
4. For commercial systems in Table 3.12–3, follow manufacturers instructions.

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

- A. Unless calibrated pipettors are used for the inoculum and spreaders are used to spread the inoculum, colony counts are only approximations and can be off by as much as a hundredfold (1). Especially at higher counts, one colony does not represent 1 CFU, nor is this accuracy necessary for urine culturing. Choose the method from Fig. 3.12–1 that is most efficient, depending on the number of specimens received and the expertise of the staff. When the method of colony count has been decided, validate the method with the following protocol.

1. Make a 0.5 McFarland suspension of *E. coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, and *Enterococcus faecalis* ATCC 29212 in broth or saline.
2. Using a sterile pipette tip and calibrated pipettor, dilute each 1:100 by placing 0.05 ml (50 µl) of culture into 5 ml of saline. Vortex. This should represent 10^6 CFU/ml.
3. Prepare a 1:10 dilution from the 1:100 dilution by inoculating 0.5 ml of suspension to 4.5 ml of saline (equals 10^5 CFU/ml). Vortex and repeat to prepare two more 1:10 dilutions.
4. Label the dilutions “ 10^6 ,” “ 10^5 ,” “ 10^4 ,” and “ 10^3 ,” respectively, beginning with the 1:100 dilution (the labeling may have to be adjusted after the final counts are calculated from the growth).
5. Inoculate each dilution to a BAP by the chosen colony count method for use in the laboratory, using both the 0.01- and 0.001-ml loops or pipettes. Label the plates with organism name, the expected CFU per milliliter (10^6 , 10^5 , 10^4 , and 10^3), and the dilution used (10^{-2} and 10^{-3} for 0.01 and 0.001 ml, respectively).

Table 3.12-3 Summary of bacteriological culture systems^a

Culture system	Principle	Comment(s)
Bactercult (Carter-Wallace/Wampole Division, Cranbury, N.J.)	Sterile, disposable tube coated with culture medium; contains phenol red indicator for presumptive ID of common uropathogens	Colonies counted
Bullseye Urine Plate (HealthLink Diagnostics, Jacksonville, Fla.)	Five-chambered plate containing media for isolation and identification of common uropathogens and their AST patterns	Colonies counted; direct AST
CPS ID 2 (bioMérieux Vitek, Inc., Hazelwood, Mo.)	Enumeration and presumptive ID of common urinary pathogens	Identifies <i>E. coli</i> , <i>Proteus mirabilis</i> , <i>Enterococcus</i> spp., and indole-positive <i>Proteus</i> ; presumptive ID of <i>Klebsiella pneumoniae</i> , <i>P. aeruginosa</i> , <i>S. aureus</i> , <i>S. agalactiae</i> , and <i>Candida</i> spp.
Diaslide (Diatech Diagnostics Inc., Boston, Mass.)	Transparent hinged plastic casing contains CLED and MAC for detection and presumptive identification of common UTI pathogens	Growth is compared to reference photographs for quantitation; morphology and color determine presumptive ID.
DIP N COUNT (Starplex Scientific, Etobicoke, Ontario, Canada)	Paddle contains CLED and MAC or EMB	Colonies are compared to a color density chart; color chart is used for ID.
Rainbow (Biolog, Inc., Hayward, Calif.)	Rainbow agar CP-8 used with eight confirmation spot tests for ID of eight microorganisms causing UTI; medium contains chromogenic substrates which color microorganisms	Interpretation may occur within 3 to 6 h for rapidly growing organisms.
URI-CHECK (Troy Biologicals, Inc., Troy, Mich.)	Dipslide culture for the enumeration and ID of uropathogens; dipslide contains CLED and MAC or EMB; similar to DIP N COUNT	Growth density is compared to chart.
Uri-Kit/Uri-Three (Culture Kits Inc., Norwich, N.Y.)	Agar plate systems used to detect common uropathogens; Uri-Kit is plastic hinged case containing CLED medium; Uri-Three triplate contains BAP and MAC and CLED	Growth density is compared to a colony density chart.
Uricult Trio (Orion Diagnostica, Espoo, Finland)	Three-medium dipslide containing CLED, MAC, and a β -glucuronidase substrate for enumeration of microorganisms and presumptive ID of <i>E. coli</i>	<i>E. coli</i> appears as brown colonies.

^a From reference 2. Abbreviations: ID, identification; CLED, cystine lactose electrolyte-deficient agar.

IV. QUALITY CONTROL (continued)

6. Also inoculate 0.05 ml (50 μ l) of each of the last three 1:10 dilutions to a BAP, using a sterile pipette tip and calibrated pipettor. Spread these plates with a spreader for accurate colony counting. Label these plates with the organism names, “10⁵,” “10⁴,” or “10³,” and the words “1:20 dil.” Do this in duplicate.

■ NOTE: Spreading with a loop will not give an accurate count.

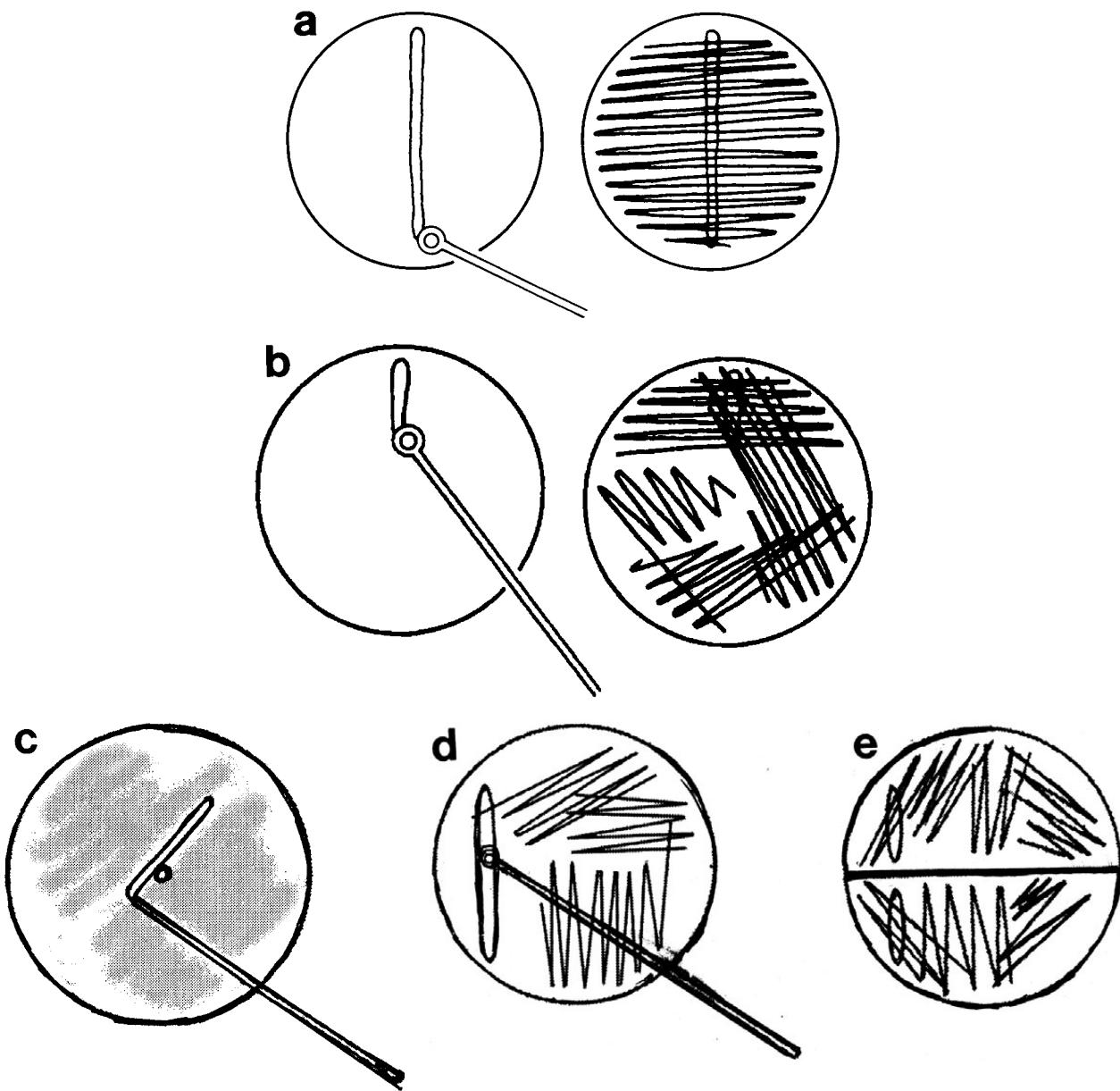


Figure 3.12-1 (a) Method of streaking urine for colony count using a 0.001-ml loop; (b) method of streaking urine for colony count using either a 0.01- or 0.001-ml loop; (c) spreader method of streaking urine for colony count after inoculation with either a 0.01- or 0.001-ml volume; (d) method of streaking 0.01 ml of urine using drip method for count and quadrant streaking for isolation; (e) MAC-CNA biplate with 0.01 ml of urine streaked in quadrants (EMB and PEA are other options).

IV. QUALITY CONTROL (continued)

7. Incubate plates for 18 h, and find the lowest dilution made with the 50- μ l pipette (1:20 dilution) that has 30 to 300 colonies. Multiply the count by 20 to determine the CFU per milliliter in that dilution of the culture. Repeat with the duplicate plate of that dilution and determine the average count of the two plates. Use this accurate count to adjust the final counts for each of the four dilutions (10^6 , 10^5 , 10^4 , and 10^3) based on this calculation.

IV. QUALITY CONTROL (continued)

Example: If the 1:20 dilution of the 10^4 plates has an average of 50 colonies, the true count is 50×20 , or 10^3 . All plates counts should then be reduced by a factor of 10 (e.g., the 10^4 is 10^3 , the 10^3 is 10^2).

8. Relabel each of the plates inoculated by the urine count method, to match the actual counts determined by the calibrated method.
9. Display plates for the laboratory staff to educate them on the appearance of each colony count. Photographs can be helpful. Prepare definitions of colony counts, using the appearance of each plate and enter as part of this procedure.

Example: “ 10^5 CFU/ml shows confluent growth in the initial drop of urine with the 0.01-ml inoculum but shows approximately 50 colonies with the 0.001- μ l loop.”

- B. Inspect nondisposable calibrated loops regularly to confirm that they remain round and are free of bends, dents, corrosion, or incinerated material. Refer to Appendix 3.12–3 for procedures to QC microbiological loops.
- C. For disposable loops, check for certificate of count validation from the manufacturer with each lot. Verify calibration for each of the first few lots when changing manufacturers, by following Appendix 3.12–3.
- D. Calibrate pipettors at regular intervals, if used (see Appendix 3.12–4).
- E. Verify that media meet expiration date and QC parameters per current Clinical and Laboratory Standards Institute (CLSI; formerly NCCLS) M22 document. See procedures 14.2 and 3.3.1 for further procedures, especially for in-house-prepared media.

V. PROCEDURE



Observe standard precautions.

A. Microscopic and other direct methods

■ **NOTE:** The Gram stain is useful in rapidly determining the type and count of bacteria and cells in urine and should be performed on request (14, 19). It is particularly helpful if results can be made available while the patient is in the clinic. For inpatients, the urine Gram stain can help diagnose the cause of sepsis before blood cultures are positive.

1. Gram stain: refer to procedure 3.2.1.
 - a. Place 10 μ l of well-mixed, *uncentrifuged* urine onto a glass slide, and allow it to air dry without spreading. Fix with methanol.
 - b. Determine the number of organisms per oil immersion field. One organism seen per $\times 1,000$ correlates to a colony count of 10^5 /ml of urine (2).
 - c. The presence of many squamous epithelial cells and different microbial morphotypes suggests contamination.
2. Detection of pyuria

■ **NOTE:** Pyuria with a WBC count of $>10/\mu\text{l}$ (or >5 per high-power field [HPF] in a conventional urinalysis) has a specificity of 90% for predicting catheter-associated UTI with greater than 10^5 CFU/ml but a sensitivity of only 37% (18).

 - a. Detect by either Gram stain or urinalysis (procedure 3.2.3), from examination of freshly collected uncentrifuged urine.
 - b. Use enhanced urinalysis for pediatric patients less than 2 years old. Place uncentrifuged urine in a hemacytometer and count WBCs (5, 13, 14). This method is reported to have a sensitivity of 84% and specificity of 90% for infants (10).
 - c. For commercial methods, see Appendix 3.12–1 and reference 13.

B. Culture methods

1. For voided urine specimens and those from indwelling catheters, culture at least 0.001 ml. For other urine specimens, culture 0.01 ml. See Table 3.12–4.
- **NOTE:** Only streak the blood plate for colony count. Other plates should be streaked in quadrants for isolation of colonies (Fig. 3.12–1b and e), rather

V. PROCEDURE (continued)

than for colony count, to minimize delays in obtaining isolated colonies and false-negative culture results due to antimicrobial inhibition. If colony count cannot be performed due to overwhelming spreading *Proteus*, an estimate of the count can be made from the isolation plates.

2. Inoculation methods for colony count (*choose one method*)
 - a. Calibrated-loop method
 - (1) Using the disposable or flamed and cooled calibrated loop, hold the loop vertically, and immerse it just below the surface of a well-mixed, uncentrifuged urine specimen.
 - (2) Deliver a loopful of well-mixed urine onto the blood agar plate and spread using one of the three methods below.
 - (a) Using the loop, make a straight line down the center of the plate and streak the urine by making a series of passes at 90° angles through the inoculum (method for 0.001 ml only). See Fig. 3.12-1a.
 - (b) Deliver the loopful onto one quadrant of plate by making a straight line or a V-shaped line. Streak urine onto the first quadrant, and proceed by streaking onto all four quadrants. See Fig. 3.12-1b.
 - (c) Use a sterile spreader or bent rod ("hockey stick") to spread the loopful of urine over the surface of the entire plate in three directions (Fig. 3.12-1c). An accurate colony count will not be obtained if the calibrated loop is used to inoculate the plates.
 - b. Pipette inoculation methods
 - (1) With a sterile pipette tip and calibrated pipettor, aspirate 0.01 ml of well-mixed urine onto one side of plate and allow to drip down plate. Streak urine in quadrants on rest of plate with sterile loop. See Fig. 3.12-1d.
 - (2) Alternatively, drop onto plate and spread by one of the methods described above.
 - c. For bacteriological culture systems, see Table 3.12-3. For each method, a measured amount of urine is placed on agar surfaces, and after incubation, colonies are counted. The sensitivities of these systems vary and are high when only one pathogen is likely.
 3. Take an additional loopful or pipette full of well-mixed urine for selective agar plates to be inoculated and streak in quadrants for isolation as indicated in Fig. 3.12-1e and Fig. 3.3.1-1 and 3.3.1-2 in procedure 3.3.1.
 4. Incubate in ambient air overnight at 35 to 37°C.
 - a. Anaerobic cultures of the urine should be done on request on suprapubic aspirates, when a vesticuloenteric fistula is suspected, and when bacterial morphotypes are seen in the direct smear but fail to grow on aerobic culture.
 - b. If convenient, incubate BAP and CNA in 5% CO₂ to enhance growth of gram-positive organisms.
 5. Examination of culture media
 - a. Examine cultures that have been incubated overnight but make final reading at 18 h unless the specimen fits the criteria below.
 - b. Reincubate until culture has incubated for 48 h if one of the following is true.
 - (1) The specimen was collected by an invasive technique, such as suprapubic bladder aspiration or straight catheter method.
 - (2) Tiny or scant colonies are present that are barely discernible.
 - (3) Culture results do not correlate with Gram stain findings or clinical conditions (e.g., the patient has sterile pyuria or symptoms without a positive culture).

Table 3.12-4 Protocol for workup of urine cultures

Type of urine	Inoculation	No. of isolates			Time to final report
		1 isolate ^a	2 uropathogens ^a	≥3 uropathogens	
Voided midstream; obtained in outpatient clinics and doctors' offices from patients <65 yr old	Streak 0.001 ml to BAP for count. Streak MAC (or EMB) in quadrants (CNA or PEA optional).	<10,000 CFU/ml, minimal ID ^b ≥10,000 CFU/ml (or ≥1,000 CFU of uropathogen/ml in females 14–30 yr old), ^c definitive ID ^d and AST	For each <100,000 CFU/ml, minimal ID For each that is ≥100,000 CFU/ml, definitive ID and AST	Report count plus “Multiple bacterial morphotypes present. Suggest appropriate recollection with timely delivery to the laboratory, if clinically indicated.”	Day 1 or 2 (minimum incubation, 18 h) ^e
Indwelling catheter; voided from geriatric (≥65 yr old) and all inpatients	Streak 0.001 ml to BAP for count. Streak CNA (or PEA) and MAC (or EMB) in quadrants.	<10,000 CFU/ml, minimal ID ≥10,000 CFU/ml, definitive ID and AST	For each <100,000 CFU/ml, minimal ID For each that is ≥100,000 CFU/ml, definitive ID and AST	If voided urine, or if catheter collected and urinalysis WBCs or leukocyte esterase is available and normal, report as for voided outpatient urine. <i>or</i> Contact caregiver to determine if specimen was collected through catheter port and if patient is febrile or symptomatic. If so, follow protocol as for two uropathogens. <i>or</i> Minimal ID of each uropathogen, with note: “Contact laboratory if definitive identifications are clinically indicated.”	Day 2 or 3 (minimum incubation, 36 h)
Straight catheter; pediatric catheterized, suprapubic, kidney, cystoscopy yeast cultures	Streak 0.01 ml to BAP for count. Streak CNA (or PEA) and MAC (or EMB) in quadrants. Add CHOC for surgically collected or prostate specimens.	100 to 1,000 CFU/ml with normal urogenital or skin microbiota, minimal ID ≥1,000 CFU/ml or any pure culture of lower count of uropathogen, definitive ID and AST	For each <1,000 CFU/ml, minimal ID For each uropathogen that is ≥1,000 CFU/ml, definitive ID and AST <i>or</i> Contact physician to determine extent of workup.	For each <10,000 CFU/ml, minimal ID For each that is ≥10,000 CFU/ml, definitive ID and AST <i>or</i>	Day 2 or 3 (minimum incubation, 48 h)

^a Urogenital or skin microbiota that is at least 10 times less than the uropathogens is ignored for purposes of workup. If this microbiota is equal to the uropathogens, report “Multiple bacterial morphotypes present. Suggest appropriate recollection with timely delivery to the laboratory, if clinically indicated.” For list of uropathogens and skin microbiota, see Table 3.12-1.

^b For minimal identification (ID), see Table 3.12-5.

^c Report any amount of group B streptococci (*S. agalactiae*) in this age group. Check staphylococci with novobiocin disk for *Staphylococcus saprophyticus*, a uropathogen in this age group.

^d For definitive ID, see Table 3.12-1.

^e See text (item V.B.5.b) for exceptions.

V. PROCEDURE (continued)

- (4) The patient is immunocompromised, including patients who have transplanted organs.
- (5) Yeast or fungal culture is requested or appropriate (e.g., neonatal intensive care unit cultures).
- NOTE:** Incubation may need to be up to 72 h if CNA or EMB is not used. Many yeasts grow well on EMB.
- c. For positive cultures, examine culture media for the quantity and morphological type of organisms present.
 - (1) With a 0.001-ml loop, one colony equals 1,000 CFU/ml.
 - (2) With a 0.01-ml loop, one colony equals 100 CFU/ml.
 - (3) When the colonies are too numerous to count
 - (a) The maximum readable using the 0.001-ml loop is $>10^5$ CFU/ml.
 - (b) The maximum readable on the 0.01-ml loop is $>10^4$ CFU/ml.
 - (4) Use the procedure in item IV to estimate counts between the maximum and minimum.
- d. An accurate colony count may be difficult to establish for all morphotypes on dipslide paddles. In order to determine the clinical significance of potential uropathogens if growth is mixed, morphotypes should be subcultured to plated media to establish an accurate colony count.
- 6. Further workup of positive cultures
 - a. Determine the colony count of each morphotype in the culture separately by examining BAP.
 - b. Using Tables 3.12–1 and 3.12–4, determine the extent of workup of each organism.
 - c. For testing for minimal identification guidelines, refer to Table 3.12–5.
 - d. Do not identify normal urogenital microbiota to the genus or species level.
 - e. *Streptococcus agalactiae* should be reported from women in childbearing years and from known diabetics, regardless of the count.
 - f. Because *E. coli* represents 80% of the pathogens in voided urine cultures, use rapid identification methods to identify this species. See Table 3.3.2–5.
 - g. For definitive identifications, refer to Table 3.3.2–5 and procedures 3.18.1 and 3.18.2.
 - h. Perform AST as indicated in the tables and described in section 5.
 - i. Always identify to the species level if oxidase positive and indole positive, since such organisms are pathogens regardless of count (e.g., *Aeromonas* and *Vibrio* spp.).
- 7. Hold positive culture plates at room temperature for at least 2 to 3 days for possible further workup if requested by the patient's physician.
- 8. Hold urine specimens at refrigeration temperatures for 24 h to resolve any problems with the specimen or culture results.

POSTANALYTICAL CONSIDERATIONS

VI. REPORTING RESULTS

- A. Report Gram stain results for bacteria and cells per Gram stain procedure (3.2.1).
- B. Negative results
 - 1. If no growth is observed on all media, report "Urine culture negative" or "No growth of uropathogens."
 - 2. If 0.001 ml was cultured, report "No growth of $\geq 10^3$ CFU/ml at xx."

Table 3.12–5 Reporting of isolates with minimal testing^a

Report	Minimal testing
Presumptive <i>E. coli</i>	Grows typically on MAC or EMB and appears lactose positive or spreading on any medium or is indole positive and oxidase negative.
Presumptive <i>P. aeruginosa</i>	Appears lactose negative on MAC or EMB and is oxidase positive and indole negative or has odor of <i>Pseudomonas</i> or <i>Alcaligenes</i> . If desired, inoculation and incubation of KIA or TSI can be used to distinguish from enteric rods.
Gram-negative rod	Grows well on EMB or MAC but does not fit either of the above criteria for gram-negative rods.
Yeast	Morphology consistent with yeast in wet mount or “feet” present on plate.
<i>S. aureus</i>	Catalase-positive, gram-positive cocci and slide coagulase positive. <i>Caution:</i> <i>S. saprophyticus</i> can be positive in latex or coagglutination serologic coagulase tests.
Coagulase-negative staphylococci	Catalase-positive, gram-positive cocci and slide coagulase negative
<i>Enterococcus</i>	Catalase-negative, PYR-positive, nonhemolytic, and gram-positive cocci
Viridans group streptococci	Gram-positive cocci in pairs and chains, catalase negative, alpha-hemolytic, or PYR negative. Rule out <i>A. urinae</i> (differs by a Gram stain showing tetrads and clusters) prior to reporting, if numbers are significant (20).
<i>Lactobacillus</i> spp.	Gram-positive rods, catalase negative, alpha-hemolytic
<i>Corynebacterium</i> spp.	Gram-positive rods, catalase positive. Rule out uropathogenic <i>Corynebacterium</i> with rapid urease test (procedure 3.17.48), if numbers are significant.

^a See Table 3.12–4 for indications. Abbreviations: KIA, Kligler’s iron agar; TSI, triple sugar iron agar; PYR, pyrrolidonyl-β-naphthylamide.

VI. REPORTING RESULTS (continued)

C. Positive results

1. If only urogenital or skin microbiota is observed, report as such.
Example: “10,000 CFU/ml normal urogenital microbiota.”
2. As specified in Table 3.12–4, mixed cultures are reported with the count in CFU per milliliter, followed by “Multiple bacterial morphotypes present; possible contamination; suggest appropriate recollection, with timely delivery to the laboratory, if clinically indicated.”
3. Otherwise, report the colony count of each pathogen separately, followed by the presumptive, minimal, or definitive identification and susceptibility test results, as indicated in Tables 3.12–4 and 3.12–5.
NOTE: For general principles of reporting preliminary and final results, refer to procedure 3.3.2.
4. When antimicrobial inhibition is observed (i.e., no growth in the primary area of the plate but growth in the area where the inoculum is diluted), do not report the count but report “Colony count unreliable due to antimicrobial inhibition.”
5. Notify physician of unusual positive findings (e.g., *Salmonella typhi* or *Burkholderia pseudomallei*).

- D. Document all testing in a hard copy or computerized work card.

VII. INTERPRETATION

- A. The criterion of $\geq 10^5$ CFU/ml for significance can be applied to a majority of specimens submitted for culture. However, the following are true.
 1. A mixed culture in an uncomplicated outpatient population likely indicates contamination.

VII. INTERPRETATION (continued)

2. Low levels ($<10^4$ /ml) of organisms commonly found on the skin and external and internal genitalia are considered to be contaminants, but in selected circumstances, a count of *Enterobacteriaceae* of 10^2 CFU/ml or more, especially for *Salmonella*, can be considered significant.
 3. Colony counts of $<10^5$ CFU/ml in voided specimens in the presence of dysuria and symptoms of UTI may be significant.
- B.** AST should not be performed directly from urine specimens. These methods are not Food and Drug Administration approved and are not standardized. Refer to *Cumitech 2B* for further discussion (2).

VIII. LIMITATIONS

- A. Women with uncomplicated UTI for which the microbial etiology is established (e.g., *E. coli* or *S. saprophyticus*) may be treated empirically with a single high dose or a short course (3 days) of antimicrobial agents. Urine culture for identification and AST of the causative organisms should be done in cases where there is treatment failure and relapsing infection, suspected subclinical pyelonephritis, or recurrent infection.
- B. In cases of sterile pyuria, the Gram stain is important. If organisms are seen but not cultured and findings persist, an anaerobic culture may be indicated.
- C. False-negative results are due, in part, to interfering substances, diluted urine, low urine pH, and subjective interpretation of the criteria for further workup of the culture.
- D. Acute urethral syndrome due to urethritis should be suspected in sexually active women who have dysuria and pyuria but sterile urine cultures. Genital testing for *N. gonorrhoeae*, *Chlamydia trachomatis*, and *Ureaplasma urealyticum* should be done. Dysuria also occurs in patients with vulvovaginitis, and vaginal swabs should be submitted for detection of bacterial vaginosis, *Candida* overgrowth, and *Trichomonas vaginalis* (see procedure 3.9.1).
- E. For sterile pyuria, mycobacterial infection should be considered (see section 7). The specimen for culture should be the first voided urine of the day, three days in a row.

REFERENCES

1. Albers, A. C., and R. D. Fletcher. 1983. Accuracy of calibrated-loop transfer. *J. Clin. Microbiol.* **18**:40–42.
2. Clarridge, J. E., J. R. Johnson, and M. T. Pezzlo. 1998. *Cumitech 2B, Laboratory Diagnosis of Urinary Tract Infections*. Coordinating ed., A. L. Weissfeld. American Society for Microbiology, Washington, D.C.
3. Gross, P. A., L. M. Harkay, G. E. Barden, and M. Kerstein. 1974. The fallacy of cultures of the tips of Foley catheters. *Surg. Gynecol. Obstet.* **139**:597–598.
4. Heldrich, F. J., M. A. Barone, and E. Spieglar. 2000. UTI: diagnosis and evaluation in symptomatic pediatric patients. *Clin. Pediatr.* (Philadelphia) **39**:461–472.
5. Hoberman, A., E. R. Wald, L. Penchansky, E. A. Reynolds, and S. Young. 1993. Enhanced urinalysis as a screening test for urinary tract infection. *Pediatrics* **91**:1196–1199.
6. Hooton, T. M., and W. E. Stamm. 1997. Diagnosis and treatment of uncomplicated urinary tract infection. *Infect. Dis. Clin. N. Am.* **11**:551–581.
7. Johnson, J. R., and W. E. Stamm. 1989. Urinary tract infections in women: diagnosis and treatment. *Ann. Intern. Med.* **111**:906–917.
8. Kass, E. H. 1956. Asymptomatic infections of the urinary tract. *Trans. Assoc. Am. Phys.* **69**:56–64.
9. Lifshitz, E., and L. Kramer. 2000. Outpatient urine culture: does collection technique matter? *Arch. Intern. Med.* **160**:2537–2540.
10. Lin, D. S., F. Y. Huang, N. C. Chiu, H. A. Koa, H. Y. Hung, C. H. Hsu, W. S. Hsieh, and D. I. Yang. 2000. Comparison of hemocytometer leukocyte counts and standard urinalyses for predicting urinary tract infections in febrile infants. *Pediatr. Infect. Dis. J.* **19**:223–227.
11. Lipsky, B. A., R. C. Ireton, S. D. Fihn, R. Hackett, and R. E. Berger. 1987. Diagnosis of bacteriuria in men: specimen collection and culture interpretation. *J. Infect. Dis.* **155**:847–854.

REFERENCES (continued)

12. Pezzlo, M. T., D. Amsterdam, J. P. Anhalt, T. Lawrence, N. J. Stratton, E. A. Vetter, E. M. Peterson, and L. M. de la Maza. 1992. Detection of bacteriuria and pyuria by URIS-CREEN, a rapid enzymatic screening test. *J. Clin. Microbiol.* **30**:680–684.
13. Pfaller, M. A., B. Ringenberg, L. Rames, J. Hegeman, and F. Koontz. 1987. The usefulness of screening tests for pyuria in combination with culture in the diagnosis of urinary tract infection. *Diagn. Microbiol. Infect. Dis.* **6**:207–215.
14. Shaw, K. N., K. L. McGowan, M. H. Gorlick, and J. S. Schwartz. 1998. Screening for urinary tract infection in infants in the emergency department: which test is best? *Pediatrics* **101**:1–5.
15. Stamm, W. E., G. W. Counts, K. R. Running, S. Fihn, M. Turck, and K. K. Holmes. 1982. Diagnosis of coliform infection in acute dysuric women. *N. Engl. J. Med.* **307**:463–468.
16. Stark, R. P., and D. G. Maki. 1984. Bacteriuria in the catheterized patient: what quantitative level of bacteriuria is relevant? *N. Engl. J. Med.* **311**:560–564.
17. Tambyah, P. A., and D. G. Maki. 2000. Catheter-associated urinary tract infection is rarely symptomatic: a prospective study of 1,497 catheterized patients. *Arch. Intern. Med.* **160**:678–682.
18. Tambyah, P. A., and D. G. Maki. 2000. The relationship between pyuria and infection in patients with indwelling urinary catheters: a prospective study of 761 patients. *Arch. Intern. Med.* **160**:673–677.
19. Washington, J. A., II, C. M. White, M. Laganiere, and L. H. Smith. 1981. Detection of significant bacteriuria by microscopic examination of urine. *Lab. Med.* **12**:294–296.
20. Zhang, Q., C. Kwoh, S. Attorri, and J. E. Clarridge III. 2000. *Aerococcus urinae* in urinary tract infections. *J. Clin. Microbiol.* **38**:1703–1705.

APPENDIX 3.12-1

Commercial Systems for Evaluation of Pyuria Prior to Culture

- A. The KOVA Glasstic Slide 10 (Hycor Biomedical, Inc.) is an optically clear, plastic, disposable slide chamber with grids that holds up to 10 specimens and allows the user to perform quantitative microscopic counts of somatic and bacterial cells. Each of the 10 chambers will hold a standardized 6.6 μ l of sample and has a depth of 0.1 mm.
 1. Add 6.6 μ l of uncentrifuged urine to one grid of the KOVA Slide.
 2. Allow grid to fill by capillary action.
 3. Quantitate cells under $\times 400$ magnification.
 4. A positive urine sample contains >10 WBCs/ μ l.
- B. Yellow IRIS (International Remote Imaging Systems) is an automated urinalysis instrument for use on midstream urine specimens from immunocompetent adults. It incorporates a flow microscope, video camera, and digitalized picture image-processing computer.
- C. Dipstick method for detection of bacteria and WBCs
 1. Specimen: fresh voided urine from immunocompetent patient
 2. Materials: Chemstrip LN (Bio-Dynamics, Division of Boehringer Mannheim Diagnostics, Indianapolis, Ind.)
 3. Procedure
 - a. Dip the strip into the urine specimen for 1 s.
 - b. Withdraw the strip over the specimen container rim to remove excess urine.
 - c. Read results within 2 min.
 - d. Compare the color intensity with the color guide provided by the manufacturer.
 4. Interpretation
 - a. Nitrate reductase
 - (1) Many, but not all, gram-negative bacilli reduce nitrate to nitrite.
 - (2) Low urine pH (<6) may yield false-negative test results.
 - b. WBC esterase in WBCs produces a positive reaction.
 5. Limitations
 - a. Vitamin C, phenazopyridine, and high protein levels may interfere with test results.
 - b. The leukocyte esterase is a sensitive test to determine pyuria, but the sensitivity of test results is higher when both parameters are used.
 - c. Some non-glucose-fermenting gram-negative bacilli, e.g., *Acinetobacter*, are nitrate negative. All gram-positive microorganisms are nitrate negative. False-negative results will occur with infections with these organisms, which may be common in nosocomial UTI.
 - d. Neutropenic immunocompromised patients, having very few if any WBCs in urine, have false-negative leukocyte esterase tests.

APPENDIX 3.12-2

Rapid Urine Screens Used To Reject Voided Urine Specimens

- A. Enzyme tube test: URISCREEN (Bard Patient Care Division, Murray Hill, N.J.) (1)
 - 1. Add 1.5 to 2.0 ml of well-mixed urine to a tube containing dehydrated reagent.
 - 2. Add 4 drops of 10% hydrogen peroxide.
 - 3. Gently mix the contents of the tube, avoiding formation of bubbles.
 - 4. Observe the tube for the formation of foam above the surface of the liquid.
 - 5. A positive reaction is indicated by the release of catalase and occurs within 2 min.
- B. Nonautomated filtration device: FiltraCheck-UTI (Meridian Diagnostics, Cincinnati, Ohio)
 - 1. Add 2 drops of well-mixed urine and 6 drops of diluted hydrochloric acid to a capsule.
 - 2. Gently mix, and pour into a disposable filter disk with a conical well.
 - 3. Add safranin O dye, and wash twice with decolorizer.
 - 4. Compare residual pink with a color guide.
- C. The semiautomated Coral UTI Screen and the Wave 180 Automated luminometer system (Coral Biotechnology, San Diego, Calif.) constitute an automated screening system that will detect the presence of organisms in urine. The Coral UTI Screen uses a somatic cell release agent (SRA) to release and destroy the ATP present in somatic cells (bacterial ATP is protected within bacterial cells). Bacterial ATP is then liberated from cells and detected as follows. A reduced form of luciferin reacts with bacterial (or yeast) ATP in the presence of the enzyme luciferase to produce oxidized luciferin, AMP, and light. The light produced is measured by a photomultiplier tube detector luminometer, and the signal is converted to relative light units (RLU). The RLU produced is proportional to the amount of bacterial ATP present in the urine sample. A 25- μ l aliquot of urine is tested in the Coral UTI Screen collected with or without preservative. After addition of the sample to the SRA tube and incubation for a minimum of 15 min, the test result is generated within seconds of loading the Wave 180 luminometer system. Two UTI-cheq calibration standards supplied by the manufacturer are tested during each run as part of the quality control procedure. The Wave 180 luminometer reports patient results as negative, positive, or OLV (sample counts greater than the response range), which are calculated as a percentage of the calibrator RLU for each sample. The Coral UTI Screen system has been shown to be an accurate, sensitive, and cost-effective method for screening of urine samples (2).

References

- 1. Pezzlo, M. T., D. Amsterdam, J. P. Anhalt, T. Lawrence, N. J. Stratton, E. A. Vetter, E. M. Peterson, and L. M. de la Maza. 1992. Detection of bacteriuria and pyuria by URISCREEN, a rapid enzymatic screening test. *J. Clin. Microbiol.* **30**:680–684.
- 2. Semeniuk, H., J. Noonan, H. Gill, and D. Church. 2002. Evaluation of the Coral UTI Screen™ system for rapid automated screening of significant bacteriuria in a regional centralized laboratory. *Diagn. Microbiol. Infect. Dis.* **44**:7–10.

APPENDIX 3.12-3

Use and Calibration of Microbiological Loops**I. PRINCIPLE**

The volume of fluid picked up by a calibrated loop depends on the volume and shape of the container holding the liquid and the direction and depth the loop is immersed. Variability results from surface tension and wetted surface of the loop. Liquids in containers with small diameters (<1 cm) have high surface tensions, which result in less loop pickup since glass-liquid and plastic-liquid (adhesive) forces are greater than liquid-liquid (cohesive) forces. When the wire above the loop is wetted by deep immersion into the fluid, excess liquid drains down the wire and enlarges the volume transferred (1).

Quantitative loops are commonly used to set up quantitative cultures and check inocula for antimicrobial tests. Quantitative loops are less accurate than pipettors yet are an excellent way to set up a semiquantitative culture or dilution. Quantitative loops are used when $\leq 20\%$ error is acceptable.

Loops are calibrated either by the dye method or by the drill bit method. Another method, the pour plate method, is rarely used because it is cumbersome.

II. TYPES OF LOOPS

- A. Volume is either 1 or 10 μ l.
- B. Reusable loops are made of wire, usually platinum or nichrome.
- C. Disposable loops are made of plastic.

APPENDIX 3.12–3 (continued)**III. MATERIALS**

- A. Drill bit method (drill bits can be purchased at a hardware store)
 1. Twist drill bits (no. 53 and 54) for 0.001-ml loop
 2. Twist drill bits (no. 21 and 22) for 0.01-ml loop
 3. For disposable loops, some manufacturers supply a “go-no go” bar to calibrate their loops. Drill bits do not work for these loops.
- B. Dye method
 1. Distilled water, either type I or type II
 2. Evans blue dye solution (EBD)
 - a. Stock
 - (1) Add 0.75 g of EBD to 100 ml of distilled water.
 - (2) Filter solution through no. 40 Whatman filter paper.
 - (3) Store at room temperature in a dark bottle for 6 months. Label bottle with contents, expiration date, and initials of preparer. Note lot number and expiration date in the work record.
 - b. Working solutions
 - (1) Prepare dilutions of the EBD stock solution in distilled water to equal to 1:500, 1:1,000, 1:2,000, and 1:4,000 by diluting 1 ml of stock dye in 49 ml of distilled water.
 - (2) Dilute 1 ml of this 1:50 dilution in 9 ml of distilled water. This is a 1:500 dilution.
 - (3) Perform twofold serial dilutions, starting with 5 ml of the 1:500 solution and 5 ml of distilled water, to prepare the remaining dilutions.
 - (4) Store the four dilutions for up to 6 months, but prepare new dilutions if the reading of any one dilution differs by 3% from previous readings.
 3. Supplies
 - a. Square glass cuvettes with a 1-cm light path. Use the same cuvette for all measurements.
 - b. Glassware: several clean 15-ml test tubes, several class A 1- and 10-ml pipettes
 - c. Good-quality spectrophotometer with the following specifications: linear through 2.0 absorbance units at 350 nm; spectral band width, ≤ 2 nm (348 to 352 nm); precision (drift) of $\leq 0.001 \text{ Å/h}$; accuracy of wavelength selected, ≤ 1 nm

IV. QUALITY CONTROL

- A. General considerations
 1. Reusable loops
 - a. Inspect calibrated loops regularly to confirm that they remain round and are free of bends, dents, corrosion, or incinerated material.
 - b. Check the loops to ensure that the delivery volume is accurate on a monthly basis (2).
 2. Check delivery volumes of disposable loops upon receipt of a new lot number. Satisfactory performance of multiple lots and manufacturer's certificate of in-house QC eliminate the need for routine calibration.
- B. Methods for calibration of loops
 1. Food and Drug Administration (FDA) drill bit method

NOTE: The FDA uses the standard 0.001-ml platinum-rhodium loop in plate counts on milk. The drill bit method of QC is simple and does not require a spectrophotometer.

 - a. Calibration of 0.001-ml loop (inside diameter of 1.45 ± 0.06 mm)
 - (1) Obtain two twist drill bits (no. 53 and 54).
 - (2) Carefully slip the 0.001-ml loop over the end of the no. 54 bit. If the loop is calibrated, it will fit over the bit.
 - (3) Repeat the procedure with the no. 53 bit. If the loop is calibrated, it will not fit over the end.
 - (4) If the loop fits over the no. 53 bit, it is inaccurate. Follow corrective action.
 - b. Calibration of 0.01-ml loop
 - (1) Obtain two twist drill bits (no. 21 and 22).
 - (2) Repeat the procedure described above for the 0.001-ml loop.
 - (3) A calibrated 0.01-ml loop will fit over the end of a no. 22 drill bit and will not fit over a no. 21 bit.

APPENDIX 3.12–3 (continued)

- c. For disposable loops, the drill bits will not work but the manufacturer may supply a calibration bar that is similar to the drill bits.
- 2. Colorimetric dye calibration procedure
 - a. Set spectrophotometer to absorbance mode, using a wavelength of 600 nm.
 - b. Zero spectrophotometer with distilled water.
 - c. Measure and record the absorbance of each dye dilution (1:500, 1:1,000, 1:2,000, 1:4,000).
 - d. Plot the optical density (vertical axis) against each of the concentrations of the dilutions (horizontal axis) on worksheet 1 (see p. 3.12.28). Draw a single line that most closely fits all four points to construct the calibration curve.
 - e. Using the 0.001-ml loop, transfer 10 loopfuls of the EBD stock dye solution to 10 ml of distilled water. After thorough mixing, measure and record the absorbance of this solution. The absorbance should correspond to that of the 1:1,000 dilution on the calibration curve.
 - f. Prepare and evaluate three additional test solutions for a total of four readings. Record the readings on the worksheet and determine the average. Then calculate the percent inaccuracy by following the instructions on the worksheet.
 - g. If the average reading is more than $\pm 20\%$ of the 1:1,000 stock solution dilution, the loop is inaccurate. Follow with corrective action.
 - h. To calibrate the 0.01-ml loop, transfer 10 loopfuls of the EBD stock solution to 100 ml of distilled water using the 0.01-ml loop. After thorough mixing, measure and record the absorbance of this solution. Prepare and evaluate three additional test solutions for a total of four readings. Then calculate the percent inaccuracy by following the instructions on the worksheet. The final reading should be the same as that of the 0.001 loop, i.e., $\pm 20\%$ of the 1:1,000 stock solution dilution.
- C. Corrective action
 - 1. If delivery of loop does not fall within the specified tolerance limit, the loop can be discarded and replaced or repaired and retested.
 - 2. Repair by dipping in sand to remove deposits, and clean by wetting with alcohol and igniting.
 - 3. Loops can be returned to their calibrated diameter by using standard, circular gauges (Remel, Inc.). Reconditioned loops must be calibrated before being put back in service.
 - 4. For inaccurate disposable loops, contact manufacturer for replacement.
- V. PROCEDURE FOR LOOP USE
 - A. For reusable loops, flame and cool before the first and after each transfer. Use a new, unwetted disposable loop for each transfer.
 - B. Fluid should be in a widemouthed container (diameter, >1 cm).
 - C. Swirl the specimen to mix the bacterial suspension evenly.
 - D. Hold the loop vertically, and immerse it to just below the surface of the liquid. Avoid any bubbles on the meniscus of the liquid.
 - E. Check to ensure that no bubbles are within the loop.
 - F. Move loop straight up and down only.
 - G. Transfer contents of loop to the plate by pressing liquid to the plate until the liquid is no longer visible in the loop. Spread with loop or, for greater accuracy, spread with a bent rod.
- VI. LIMITATIONS

Quantitative loops, both disposable and reusable, will be grossly inaccurate if bubbles are present in the film of fluid transferred. Avoid bubbles by not shaking liquid. Inspect loopful carefully for bubbles. Remove bubbles by flaming (reusable loop), replacing (disposable loop), or redipping (either loop).

References

- 1. Albers, A. C., and R. D. Fletcher. 1983. Accuracy of calibrated-loop transfer. *J. Clin. Microbiol.* **18**:40–42.
- 2. Clarridge, J. E., M. T. Pezzlo, and K. L. Vosti. 1987. *Cumitech 2A, Laboratory Diagnosis of Urinary Tract Infections*. Coordinating ed., A. S. Weissfeld. American Society for Microbiology, Washington, D.C.

APPENDIX 3.12–4

Use and Calibration of Pipettors

I. PRINCIPLE AND DESCRIPTION

A. Principle

Pipettors aspirate and expel by an air or positive-displacement mechanism. The former mechanism uses air to push out the liquid, and the latter uses a plunger. Positive-displacement pipettors are generally thought to be more accurate for smaller volumes and, in theory, do not need the pipettor tip replaced between samples. Air displacement pipettors are more commonly used in microbiology.

B. Description

The term “pipettor” describes dispensing, diluting, and pipetting equipment. The pipettor may be manual or automated. There are repeater, adjustable-volume, and multichannel models.

Manual pipettors are lightweight cylindrical tools consisting of a handheld mechanical handle for volume adjustment and calibration and a delivery shaft that fits to disposable tips. Pipettors are approximately 10 in. long and deliver microliter to milliliter volumes. On automated pipettors, the mechanical handle is replaced with instrumentation.

C. Pipetting technique

1. Forward pipetting

- a. Refer to Fig. 3.12–A1.
- b. When prerinsing by forward pipetting, follow procedure for forward pipetting but dispense aliquot back into original container or discard. Proceed with deliveries for calibration.
- c. Top and bottom stop positions do not include eject stroke position for ejecting pipettor tips.

2. Reverse pipetting

- a. Refer to Fig. 3.12–A2.
- b. When prerinsing by reverse pipetting, follow procedure for reverse pipetting. Dispense liquid back into original vessel or discard. Proceed with deliveries for calibration.
- c. Top and bottom stop positions do not include eject stroke position for ejecting pipettor tips.

D. Accuracy and precision

1. Definition of accuracy

“Accuracy” is the closeness of agreement between the stated volume of the pipettor and the mean volume obtained during repeated, controlled deliveries. Accuracy is numerically expressed as inaccuracy, given as a percentage. Inaccuracy can be thought of as the difference between the expected or theoretical result and the calculated result.

2. Definition of precision

“Precision” is the agreement between replicate measurements. Precision is numerically expressed as imprecision, given as the coefficient of variation. Imprecision can be thought of as the range of values in which 95% of the replicate measurements fall.

3. Use of inaccuracy and imprecision as they relate to calibration of liquid delivery instruments

- a. Proper calibration of pipettors requires calculation of both inaccuracy and imprecision.
- b. Inaccuracy and imprecision must be calculated for each volume of an adjustable pipettor and each channel of a multichannel pipettor unless the manufacturer’s procedure instructs otherwise.

E. Uses for liquid delivery systems in microbiology

Pipettors are used to dilute sera, set up quantitative cultures, prepare inocula for antimicrobial tests, add ingredients to media and reagents, and add exact amounts of reagents or specimen during a test procedure. Pipettors are used because of their excellent accuracy and precision and because they expeditiously dispense small volumes repeatedly.

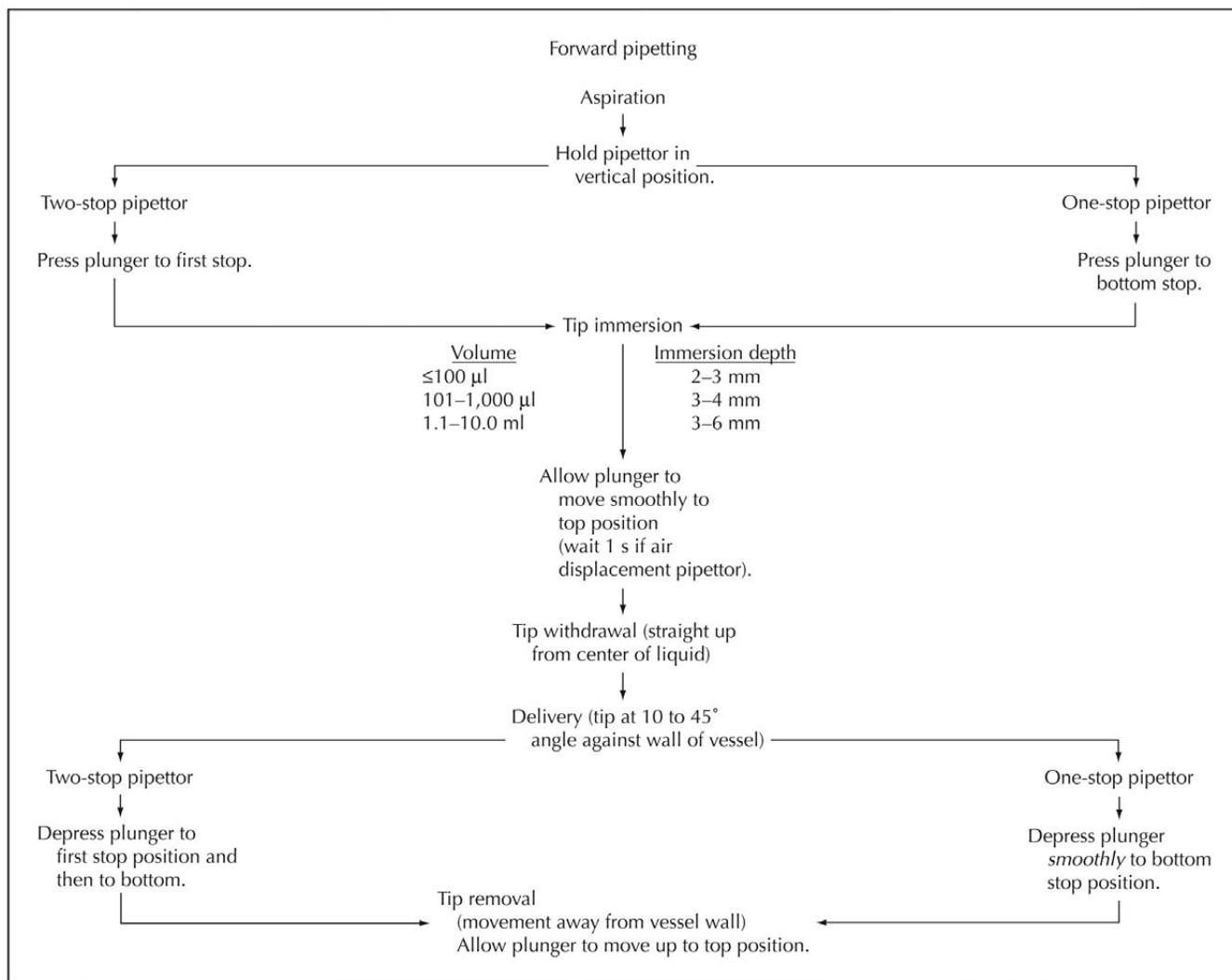


Figure 3.12-A1 Flowchart for forward pipetting (adapted from reference 7 with permission).

APPENDIX 3.12-4 (continued)

F. Calibration factors

1. Importance of calibration

Volumetric measurement using pipettors is a potential source of error in the clinical laboratory. A small error in pipetting can cause a large error in the final result.

2. Requirement for pipettor calibration

Laboratory inspecting agencies such as the CAP and the JCAHO require periodic testing of pipetting equipment to ensure accurate delivery.

3. Proposed standard for pipettor calibration

Guidelines for calibration of volumetric pipettors are found in many sources (1–10).

4. Calibration methods available (Table 3.12-A1)

Numerous methods for calibration are available. The gravimetric, spectrophotometric, and colorimetric methods are the most convenient and commonly used. Radioisotopic, enzymatic, and acid-base titration methods are less commonly recommended and are not discussed further. Commercially available pipettor calibration kits designed for in-house or send-out measurement and calculation are available (see Table 3.12-A2).

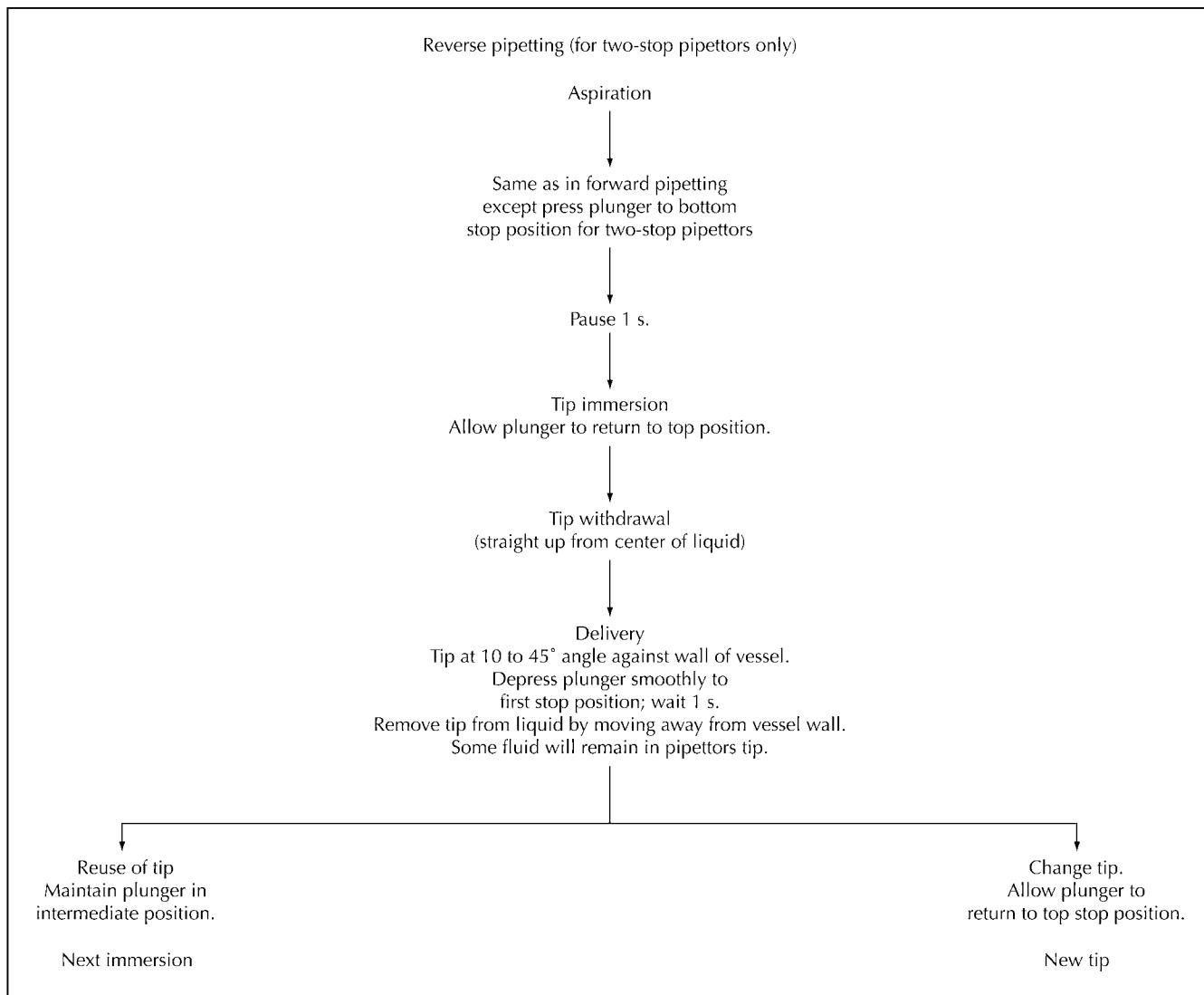


Figure 3.12–A2 Flowchart for reverse pipetting (adapted from reference 7 with permission).

APPENDIX 3.12–4 (continued)

II. SPECIAL PRECAUTIONS AND ENVIRONMENTAL CONCERNS

A. Special precautions for pipettor calibration

1. Changing pipettor tips during calibration procedure

Use the same pipettor tip for all deliveries during the calibration procedure, whether the pipettor is used for repetitive dispensing of several aliquots of the same liquid (e.g., buffers, reagents) or for transferring single aliquots of different liquids (e.g., serum). *Note:* During actual pipetting for routine use, a different tip must be used for each different liquid.

2. Prerinsing pipettor tips

“Prerinsing” is the precoating of the inside of the tip with the liquid being dispensed. Prerinse by aspirating an aliquot of the liquid into the tip and then dispensing it back into the original container or discarding it. Prerinsing improves uniformity and precision by providing identical contact surfaces for all aliquots.

- a. If the pipettor is normally used for repetitive dispensing of several aliquots of the same liquid, prerinse pipettor tip *at the beginning*, before dispensing the first aliquot.

APPENDIX 3.12-4 (continued)**Table 3.12-A1** Calibration methods for volume-dispensing instruments

Method	Basis of system	Limitation
Gravimetric	1 ml of water = 1 g (adjusted for temp and pressure)	Vol dispensed must be >0.002 ml
Spectrophotometric	Absorbances of potassium dichromate used to create calibration curve	Vol dispensed must be >0.01 ml

Table 3.12-A2 Commercial pipettor calibration systems and related products

Supplier	Product(s)
Brinkman Instruments, Inc.	Autoclavable pipettors
Calibrite Corp.	Pipette calibration service
Laboratory Equipment Support Service, Inc.	Audit systems, reference service for pipettor calibration
Medical Laboratory Automation, Inc.	Automatic pipettors, pipette calibration kits
SMI Scientific Manufacturing Industries, Inc.	Positive-displacement pipettors
Streck Laboratories	VC-100, automated acid-base titration system

b. If the pipettor is normally used for transferring single aliquots of different liquids, prerinising may not be necessary.

B. Environmental concerns for pipettor calibration

1. Temperature control
 - a. Temperatures of pipettors to be calibrated, room air, test liquid (water), and other equipment should be identical ($\pm 0.5^{\circ}\text{C}$).
 - b. Temperature should be as close as possible to temperature at which pipettor is used.
 - c. Keep temperature stable throughout procedure.
2. Miscellaneous environmental concerns
 - a. Maintain relative humidity at 45 to 75%. This reduces evaporation and limits buildup of static electricity.
 - b. Prepare aqueous test liquids from CLSI (NCCLS) type I or II water (see procedure 14.4), which prevents impurities from affecting water density.
 - c. Use water with no visible bubbles. Air bubbles alter measured volume.
 - d. Complete weighing steps quickly. Use a lid on the weighing vessel to decrease evaporation. These precautions obviate an evaporation factor in the calculations.

III. CALIBRATION OF PIPETTORS

A. Frequency of calibration

Microbiology laboratory guidelines for frequency of pipettor calibration (Table 3.12-A3)

In general, pipettors used in microbiology and serology laboratories are not used for critical-volume delivery and may not require a rigorous calibration schedule. Guidelines for testing frequency do not exist for pipettors that are used less frequently or that are not used for critical delivery.

1. Determine frequency of calibration for each pipettor on the basis of its use. Pipettors used daily require more frequent calibration than those used weekly or monthly.
2. Pipettors used for critical-volume delivery need more frequent testing than those used to deliver approximate volumes.
3. Carefully label pipettors for approximate delivery so that they are not used for more exact purposes.

APPENDIX 3.12–4 (continued)

Table 3.12–A3 Suggested calibration schedule for microbiology laboratory pipettors

Pipettor use	Calibration schedule ^a			
	New or after maintenance	Monthly	Quarterly	Annually
Critical-vol delivery required ^b				
Weekly or monthly use	A, P		A	A, P
Daily use	A, P	A		P
Critical-vol delivery not required ^c				
Weekly or monthly use	A, P		A	A, P
Daily use	A, P	A		P

^a A, test for accuracy; P, test for precision.^b Critical-volume delivery may be required for reagent delivery in some assays.^c Critical-volume delivery generally not required for serum dilutions (including Venereal Disease Research Laboratory and rapid plasma reagins tests), quantitative culture, inocula for antimicrobial tests, adding ingredients to media and reagents, adding reagents to test procedure, or adding specimen to slide for fluorescent staining.

B. Calibration procedures: gravimetric method

1. Materials and supplies

a. Reagent

Distilled water: CLSI (NCCLS) type I or II (*see* procedure 14.4)

b. Equipment

(1) Analytic balance: balance capable of measuring to 0.001 mg (1.0 µg)

(2) Thermometer: NBS calibrated to 0.1°C

(3) Weighing vessel: nonporous glass, plastic, or metal weighing container

Open surface area should be as small as possible to control evaporation. Use a loose-fitting lid to prevent evaporation of the small volumes of distilled water that will be weighed. Handle weighing vessel with forceps or washed gloves.

2. Procedure

a. Record temperature (rounded to the closest 0.5°C) before test procedure.

b. Tare the weighing vessel.

c. Prerinse the pipettor tip, if necessary, with distilled water.

d. Deliver one aliquot of distilled water into weighing vessel by using standard operating procedure for that pipettor (i.e., forward or reverse pipetting). The emphasis throughout is on smoothness, uniform timing, and uniform motion.

e. Replace lid of weighing vessel.

f. Record results of weighings on worksheet 2 (*see* p. 3.12.29).

g. Deliver additional aliquots (4 for inaccuracy determination or 10 for imprecision determination) with uniform timing. Record weight of each aliquot. After all weighings are completed, calculate the net weight of each aliquot.

3. Calculations

a. Use worksheet 2 (*see* p. 3.12.29).b. Calculate the mean weight (\bar{W}) by using the individual weights (W_i) and the number of weighings (n).

$$\bar{W} = \frac{\sum W_i}{n}$$

c. Calculate the mean volume (\bar{V}) by using the mean weight (\bar{W}) and factor Z, which controls for water density variation resulting from temperature, atmospheric pressure, and relative humidity changes.

$$\bar{V} = (\bar{W})(Z)$$

Factor Z is calculated by using Table 3.12–A4. A standard Z value, using an average room temperature and barometric pressure, can be used in calculations

APPENDIX 3.12-4 (continued)

Table 3.12-A4 Z value for distilled water as a function of temperature and pressure^a

Temp (°C)	Z at air pressure (mm Hg) ^b of:					
	600	640	680	720	760	800
15	1.0018	1.0018	1.0019	1.0019	1.0020	1.0020
15.5	1.0018	1.0019	1.0019	1.0020	1.0020	1.0021
16	1.0019	1.0020	1.0020	1.0021	1.0021	1.0022
16.5	1.0020	1.0020	1.0021	1.0022	1.0022	1.0023
17	1.0021	1.0021	1.0022	1.0022	1.0023	1.0023
17.5	1.0022	1.0022	1.0023	1.0023	1.0024	1.0024
18	1.0022	1.0023	1.0024	1.0024	1.0025	1.0025
18.5	1.0023	1.0024	1.0025	1.0025	1.0026	1.0026
19	1.0024	1.0025	1.0025	1.0026	1.0027	1.0027
19.5	1.0025	1.0026	1.0026	1.0027	1.0028	1.0028
20	1.0026	1.0027	1.0027	1.0028	1.0029	1.0029
20.5	1.0027	1.0028	1.0028	1.0029	1.0030	1.0030
21	1.0028	1.0029	1.0030	1.0030	1.0031	1.0031
21.5	1.0030	1.0030	1.0031	1.0031	1.0032	1.0032
22	1.0031	1.0031	1.0032	1.0032	1.0033	1.0033
22.5	1.0032	1.0032	1.0033	1.0033	1.0034	1.0035
23	1.0033	1.0033	1.0034	1.0035	1.0035	1.0036
23.5	1.0034	1.0035	1.0035	1.0036	1.0036	1.0037
24	1.0035	1.0036	1.0036	1.0037	1.0038	1.0038
24.5	1.0037	1.0037	1.0038	1.0038	1.0039	1.0039
25	1.0038	1.0038	1.0039	1.0039	1.0040	1.0041
25.5	1.0039	1.0040	1.0040	1.0041	1.0041	1.0042
26	1.0040	1.0041	1.0042	1.0042	1.0043	1.0043
26.5	1.0042	1.0042	1.0043	1.0043	1.0044	1.0045
27	1.0043	1.0044	1.0044	1.0045	1.0045	1.0046
27.5	1.0044	1.0045	1.0046	1.0046	1.0047	1.0047
28	1.0046	1.0046	1.0047	1.0048	1.0048	1.0049
28.5	1.0047	1.0048	1.0048	1.0049	1.0050	1.0050
29	1.0049	1.0049	1.0050	1.0050	1.0051	1.0052
29.5	1.0050	1.0051	1.0051	1.0052	1.0052	1.0053
30	1.0052	1.0052	1.0053	1.0053	1.0054	1.0055

^a Adapted from reference 7 with permission.^b 1 mm Hg = 133.322 Pa.

to calibrate pipettors that are not used to deliver critical volumes. Calculate the actual Z value (by using current temperature and atmospheric pressure) when calibrating pipettors used to deliver critical volumes.

- d. Calculate inaccuracy ($\bar{E}\%$) by using mean volume (\bar{V}) and nominal volume (V_0). Use four weighings generally.

$$\bar{E}\% = \frac{\bar{V} - V_0}{V_0} \times 100$$

- e. Calculate imprecision (CV%) by using individual weights (W_i), mean weight (\bar{W}), and number of weighings (n). Use 10 weighings generally.

$$CV\% = 100 \times \sqrt{\frac{\sum(W_i - \bar{W})^2}{n - 1}} / \bar{W}$$

APPENDIX 3.12–4 (continued)

- C. Calibration procedures: spectrophotometric method
1. Materials
 - a. Reagents
 - (1) Potassium dichromate: $K_2Cr_2O_7$, analytical reagent grade
 - (2) Perchloric acid diluent: $HClO_4$, analytical reagent grade (0.001 N)
 - (3) Distilled water: CLSI (NCCLS) type I or II (*see* procedure 14.4)
 - (4) Stock solutions

Stock solutions are stable for 1 year if protected from evaporation.

 - (a) Stock solution I

Weigh approximately 10 g of $K_2Cr_2O_7$, and record weight to nearest 0.001 g. Transfer chemical to clean, 1,000-ml volumetric flask. Dilute to 1,000 ml with 0.001 N $HClO_4$.
 - (b) Stock solution II

Using a volumetric pipettor, transfer 100 ml of stock solution I to another clean, 1,000-ml volumetric flask. Again, dilute to 1,000 ml with 0.001 N $HClO_4$.
 - b. Equipment
 - (1) Spectrophotometer

Good-quality instrument is needed. Specifications include the following: linear through 2.0 absorbance units at 350 nm; spectral band width, ≤ 2 nm (348 to 352 nm); precision (drift) of $\leq 0.001 \text{ \AA/h}$ ($\leq 0.0001 \text{ nm/h}$); accuracy of selected wavelength, ≤ 1 nm.
 - (2) Cuvettes: square glass cuvettes with a 1-cm light path

Use the same cuvette for all measurements.
 - (3) Volumetric glassware: two class A 1,000-ml volumetric flasks; one class A 100-ml volumetric pipette; several each class A 1-, 2-, and 10-ml pipettes
 - (4) Analytic balance: balance capable of measuring to 1.0 mg (0.001 g)
 2. Procedure
 - a. Set spectrophotometer to absorbance mode, using a wavelength of 350 nm.
 - b. Zero spectrophotometer with diluent (0.001 N $HClO_4$).
 - c. Consult Table 3.12–A5 to determine appropriate stock solution and diluent to use based on sample volume and cuvette size.
 - d. Record absorbance values on worksheet 3 (*see* p. 3.12.30).
 - e. Prerinse the pipettor tip if necessary with distilled water.
 - f. Aspirate sample by using standard operating procedure for that pipettor (i.e., forward or reverse pipetting). The emphasis throughout is on smoothness, uniform timing, and uniform motion.
 - g. Deliver appropriate volume of sample (stock solution) into cuvette.
 - h. Deliver the specified volume of diluent (0.001 N $HClO_4$) into cuvette.
 - i. Cap the cuvette with Parafilm. Mix solution by inverting cuvette at least three times.
 - j. Place cuvette in spectrophotometer. Close door.
 - k. Read absorbance value, and record on worksheet 3 (*see* p. 3.12.30).

Table 3.12–A5 Theoretical absorbance values for test solutions

Vol (μl) of pipettor to be tested	Stock solution to use	Vol (ml) of diluent to use	Theoretical absorbance of test solution ^a
10	I	2	0.532
20	I	10	0.213
50	II	2	0.261
100	II	2	0.510
200	II	10	0.210
250	II	10	0.261
500	II	10	0.509
1,000	II	10	0.972

^a Adapted from reference 7 (with permission).

APPENDIX 3.12-4 (continued)

Table 3.12-A6 Suggested tolerance limits for microbiology pipettors

Pipettor use ^a	Inaccuracy (%)	Imprecision (%)
Critical-vol delivery required		
Delivers <50 µl	± 2	± 2
Delivers >50 µl	± 1.5	± 1
Critical-vol delivery not required		
Delivering specimen to slide for FA staining	± 10	Not necessary
Other uses ^b	± 2	± 2

^a See Table 3.12-A3, footnotes *b* and *c*. FA, fluorescent antibody.^b Includes Venereal Disease Research Laboratory and rapid plasma reagent tests.

- l. Discard test solution from cuvette.
- m. Repeat steps III.C.2.f through 1 until 4 (inaccuracy determination) or 10 (imprecision determination) samples have been measured.
- n. Rezero spectrophotometer with diluent (0.001 N HClO₄). Difference should be <0.001 Å.
3. Calculations
 - a. Use worksheet 3 (*see* p. 3.12.30).
 - b. Calculate the mean absorbance (\bar{A}) by using the individual absorbances (A_i) and the number of absorbance measurements made (n).

$$\bar{A} = \frac{\sum A_i}{n}$$

- c. Calculate inaccuracy ($\bar{E}\%$) by using the mean absorbance (\bar{A}) and the theoretical absorbance (A_0).

$$\bar{E}\% = \frac{\bar{A} - A_0}{A_0} \times 100$$

- d. Calculate imprecision (CV%) by using the individual absorbance (A_i), the mean absorbance (\bar{A}), and the number of absorbance measurements (n).

$$CV\% = 100 \times \sqrt{\frac{\sum(A_i - \bar{A})^2}{n - 1}} / \bar{A}$$

D. Tolerance

1. Tolerance

- a. Different tolerance limits for inaccuracy and imprecision are used for various laboratory applications. Most recommended tolerance limits are designed for pipettors that must deliver critical volumes, such as those used in clinical chemistry laboratories.
- b. Tolerance limits for pipettors and quantitative loops tailored to specific laboratory uses can be determined by each user. Table 3.12-A6 is a list of suggested tolerance limits for common microbiology laboratory uses.

2. Corrective action

- a. If delivery of pipettor does not fall within the specified tolerance limits, the pipettor must be adjusted (either in-house or by manufacturer), used for less critical delivery, or discarded.
- b. Following pipettor adjustment, 10 weighings must be performed to evaluate both accuracy and precision.

IV. TROUBLESHOOTING

Pipettors that do not deliver volumes within the acceptable tolerance levels even after adjustment may have been calibrated incorrectly or may have the wrong pipettor tips.

APPENDIX 3.12–4 (continued)

- A. To verify calibration procedure, review carefully, and if necessary, calibrate by a second procedure (in-house or sent out).
- B. Different brands of micropipettor tips vary in quality and may contribute to pipetting error. Poorly fitting tips can affect volume of sample aspirated or dispensed. Different brands of tips can be tested for accuracy and precision.

References

1. **Bermes, E. W., and D. T. Forman.** 1976. Basic laboratory principles and procedures, p. 6–15. In N. W. Teitz (ed.), *Fundamentals of Clinical Chemistry*, 2nd ed. The W. B. Saunders Co., Philadelphia, Pa.
2. **Bio-Rad Laboratories.** 1983. Procedure for comparing precision of pipet tips. *Clin. Lab. Prod.* **12**:15.
3. **Bray, W.** 1995. Software for the gravimetric calibration testing of pipets. *Am. Clin. Lab.* **14**:14–15. (Available on the internet at http://www.labtronics.com/ptM_art.htm)
4. **Curtis, R. H.** 1994. Performance verification of manual action pipets. Part I. *Am. Clin. Lab.* **12**:8–9.
5. **Curtis, R. H.** 1994. Performance verification of manual action pipets. Part II. *Am. Clin. Lab.* **12**:16–17.
6. **Johnson, B.** 1999. Calibration to dye for: Artel's new pipette calibration system. *Scientist* **13**:14.
7. **NCCLS.** 1984. *Determining Performance of Volumetric Equipment*. Proposed guideline 18-P. NCCLS, Villanova, Pa.
8. **Connors, M., and R. Curtis.** 1999. Pipetting error: a real problem with a simple solution. Parts I and II. *Am. Lab. News.* **31**:20–22.
9. **Skeen, G. A., and E. R. Ashwood.** 2000. Using spectrophotometry to evaluate volumetric devices. *Lab. Med.* **31**:478–479.
10. **Steiner, P.** 1989. Basic laboratory principles and calculations, p. 19. In L. A. Kaplan and A. J. Pesce (ed.), *Clinical Chemistry*, 2nd ed. The C. V. Mosby Co., St. Louis, Mo.

Worksheet 1
Colorimetric Quantitative Loop Calibration

Loop size: _____ Date: _____ Scientist initials _____

Loop manufacturer and Lot No. _____ Date put into use _____

Sample No.	Absorbance	Average minus the 1:1000 absorbance std. (a) _____
1	_____	Divide (a) by the 1:1000 absorbance std. (b) _____
2	_____	% inaccuracy: Multiply (b) by 100 (c) _____ %
3	_____	Value (c) must be \pm 20%: Acceptable? Yes/No
4	_____	

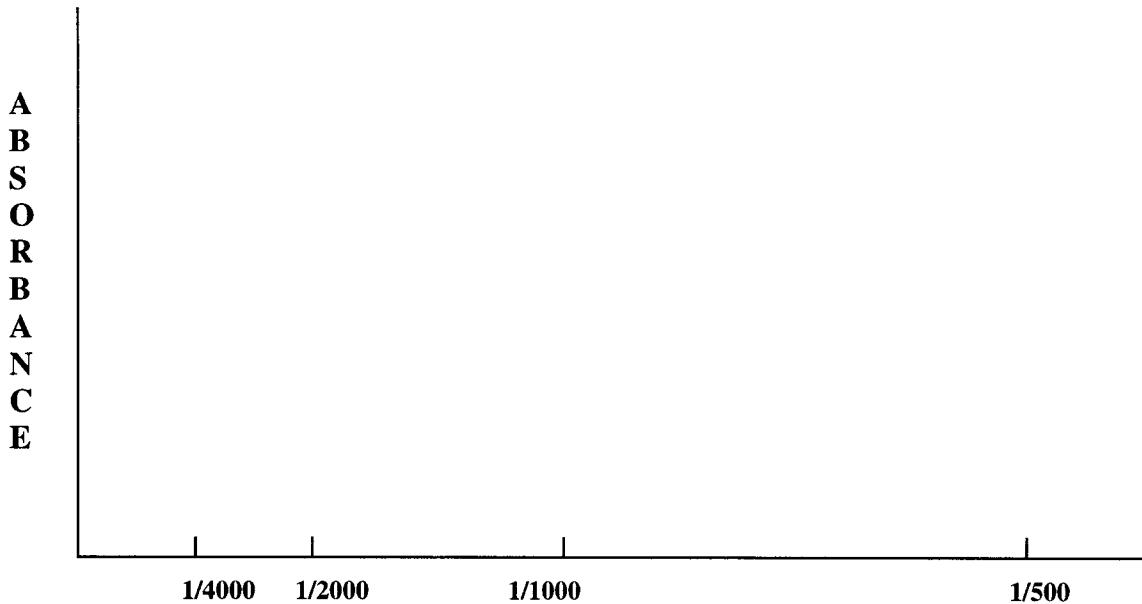
Sum of 4 readings _____

Average (divide by 4) _____

Corrective action:

Reviewed by: _____ Date: _____

Calibration curve:



Worksheet 2
Pipette Calibration—Gravimetric

Circle frequency:

Initial / Weekly / Monthly / Quarterly

Circle reason for calibration:

Accuracy check / Precision check

Date: _____

Technologist Initials: _____

Pipettor Type: _____

Pipettor Number: _____

Pipettor Size (μ l): _____

Z Value: _____

Record statistics below (see
page 3.12.31 for calculations):

<u>Sample No.</u>	<u>Sample Weight (mg)</u>
1	_____
2	_____
3	_____
4	_____
5	_____
6	_____
7	_____
8	_____
9	_____
10	_____

	<u>Result</u>	<u>Tolerance Limit</u>
Inaccuracy	_____	_____
Imprecision	_____	_____
Acceptable	Yes / No	_____

Corrective Action: _____

Reviewed by: _____ Date: _____

Worksheet 3
Pipette Calibration—Spectrophotometric

Circle frequency:

Initial / Weekly / Monthly / Quarterly

Circle reason for calibration:

Accuracy check / Precision check

Date: _____

Technologist Initials: _____

Pipettor Type: _____

Pipettor Number: _____

Pipettor Size (μ l): _____

Record statistics below (see
page 3.12.31 for calculations):

<u>Sample No.</u>	<u>Absorbance</u>	<u>Tolerance Limit</u>	
		<u>Result</u>	<u>Inaccuracy</u>
1	_____		_____
2	_____		_____
3	_____		Acceptable
4	_____		Yes / No
5	_____		
6	_____		
7	_____		
8	_____		
9	_____		
10	_____		

Corrective Action: _____

Reviewed by: _____ Date: _____

Calculations for worksheet 2

W_i = individual weights
 \bar{W} = mean weight
 n = number of weighings
 \bar{V} = mean volume
 V_0 = nominal volume

Mean weight (\bar{W})

$$\bar{W} = \frac{\sum W_i}{n}$$

Mean volume (\bar{V})

$$\bar{V} = (\bar{W})(Z)$$

Inaccuracy ($\bar{E}\%$): use four weighings.

$$\bar{E}\% = \frac{\bar{V} - V_0}{V_0} \times 100$$

Imprecision (CV%): use 10 weighings.

$$CV\% = 100 \times \sqrt{\frac{\sum (W_i - \bar{W})^2}{n - 1}} / \bar{W}$$

Calculations for worksheet 3

A_i = individual absorbance
 n = number of absorbance measurements
 \bar{A} = mean absorbance
 A_0 = theoretical absorbance

Mean absorbance (\bar{A})

$$\bar{A} = \frac{\sum A_i}{n}$$

Inaccuracy ($\bar{E}\%$): use four readings.

$$\bar{E}\% = \frac{\bar{A} - A_0}{A_0} \times 100$$

Imprecision (CV%): use 10 readings.

$$CV\% = 100 \times \sqrt{\frac{\sum (A_i - \bar{A})^2}{n - 1}} / \bar{A}$$

3.13.1

Wound/Abscess and Soft Tissue Cultures

[Updated March 2007]

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

A wide variety of microorganisms that reside on the skin and mucous membranes of the body, as well as those found in the environment, can cause skin and soft tissue infections. These organisms enter the body through breaks in the skin or mucous membranes, through wounds made by trauma or bites (exogenous) or as a complication of surgery or foreign-body implants (endogenous), or they can be spread through the vascular system (hematogenous).

Laboratories may distinguish superficial wound, abscess/fluid, or tissue specimens from those collected from deep body sites. Superficial wound and abscess specimens usually grow primary pathogens causing skin and soft tissue infections. A much broader microbial diversity is usually recovered from deep wound and invasively collected abscess/fluid and tissue specimens. A variety of aerobic organisms and anaerobes may be recovered if the appropriate media and cultures are inoculated (see Tables 3.13.1–1 and 3.13.1–2).

Acute wound infections are normally caused by external damage to intact skin, such as those produced during surgery or by trauma and bites. Conversely, chronic infections, such as decubiti or foot and leg ulcers, are normally due to complications related to impaired vascular flow or metabolic disease (e.g., diabetes mellitus). Wound colonization and/or infection is often polymicrobial, with both aerobes and anaerobes (see section 4) involved.

Tissues collected during surgery or aspirates obtained through intact skin by needle and syringe or by fine-needle biopsy are the best types of specimen to obtain for microbiology culture. If the skin

surface and surgical areas are properly disinfected prior to specimen collection, the organisms present can be assumed to be the cause of infection. Interpretation of microbial cultures taken from open skin or abscesses may be compromised, due to the fact that these lesions are often colonized with a large number of indigenous microbiota. Such cultures are indicated only if there are clear signs of infection or if a wound is failing to heal. Proper preparation of the wound prior to specimen collection can minimize contamination. After appropriate debridement and cleansing of the wound, the specimen should be obtained by biopsy from the leading edge of the lesion, where pathogens should be present and colonizing organisms are less likely to occur. Bacterial cultures of purulent material obtained by needle and syringe aspiration can also provide meaningful results. If an aspirate or tissue sample cannot be obtained, swab collections of exudate from the deep portion of lesions can be submitted. Swabs are the least appropriate specimen for microbiology analysis, as the organisms isolated may only be colonizing the area and not involved in the infective process.

The primary agents of skin and tissue infections are *Staphylococcus aureus*, *Pseudomonas aeruginosa*, members of the *Enterobacteriaceae*, beta-hemolytic streptococci, and a variety of anaerobes. In appropriately collected specimens, the presence of one of these organisms may indicate the need for antimicrobial therapy. Since wound infections can be polymicrobial, treatment may initially need to be broad in spectrum, and there is little need to identify and perform antimicrobial

susceptibility testing (AST) on all isolates. Tissues and aspirates are acceptable for anaerobic culture, as anaerobes can account for 38 to 48% of the total number of microbial isolates in wound specimens (Table 3.13.1–1 and reference 1). It must be emphasized that wound specimens collected on swabs will be less appropriate than tissues or aspirates for anaerobic culture, provided that the tissues and aspirates are submitted under anaerobic transport conditions.

The accumulation of inflammatory cells and the resultant collection of pus within an abscess or a sinus tract is a hallmark of local infection. Evidence of this process can be documented by the presence of PMNs in the Gram-stained smear. Therefore, the quality of a wound specimen can be assessed by Gram stain, which should be used to guide the extent of microbiology testing. The presence of epithelial cells indicates contamination of the specimen with skin or mucous membrane microbiota and may compromise the significance of the culture results. Quantitative cultures of tissue specimens have been shown to be useful in evaluation of wound healing related to skin grafting (procedure 3.13.2); however, the presence of organisms in the Gram stain of an appropriately collected specimen from an infected wound correlates with a clinically significant count of bacteria (2). In addition, many publications have shown that for acute wounds, a swab culture with enumeration of the organisms present correlates well with quantitative tissue cultures (see review by Bowler et al. [1] and refer to procedure 3.13.2 for quantitative methods).

3.13.1.1

Table 3.13.1-1 Aerobic and anaerobic isolates from acute and chronic infections^a

Aerobic and facultative microorganisms	Anaerobic bacteria	Aerobic microorganisms from unusual, specialized, and zoonotic infections	Yeasts
Coagulase-negative staphylococci	<i>Peptostreptococcus</i> spp.	<i>Actinobacillus actinomycetemcomitans</i>	<i>Candida albicans</i>
<i>Staphylococcus aureus</i>	<i>Clostridium</i> spp.	<i>Aeromonas</i> spp.	<i>Candida krusei</i>
Beta-hemolytic streptococci	<i>Eubacterium limosum</i>	<i>Bacillus anthracis</i>	<i>Candida parapsilosis</i>
<i>Enterococcus</i> spp.	<i>Propionibacterium acnes</i>	<i>Bergeyella zoohelcum</i>	
<i>Streptococcus</i> spp. (viridans group)	<i>Bacteroides fragilis</i> group	<i>Capnocytophaga</i> spp. and EF-4	
<i>Streptococcus anginosus</i>	<i>Prevotella</i> spp.	<i>Chromobacterium violaceum</i>	
<i>Corynebacterium</i> spp.	<i>Porphyromonas asaccharolytica</i>	<i>Eikenella corrodens</i>	
<i>Bacillus cereus</i>	<i>Fusobacterium necrophorum</i>	<i>Erysipelothrix rhusiopathiae</i>	
<i>Escherichia coli</i>	<i>Veillonella</i> spp.	<i>Francisella tularensis</i>	
<i>Serratia</i> spp.		<i>Haemophilus</i> spp.	
<i>Klebsiella</i> spp.		<i>Kingella kingae</i>	
<i>Enterobacter</i> spp.		NO-1	
<i>Citrobacter</i> spp.		<i>Pasteurella multocida</i>	
<i>Morganella morganii</i>		<i>Streptobacillus moniliformis</i>	
<i>Providencia stuartii</i>		<i>Vibrio vulnificus</i>	
<i>Proteus</i> spp.			
<i>Acinetobacter baumannii</i>			
<i>Pseudomonas aeruginosa</i>			
<i>Stenotrophomonas maltophilia</i>			
<i>Sphingobacterium multivorum</i>			

^a Data revised from Bowler et al. (1). Wounds include cutaneous abscesses, postsurgical wounds, bites, ulcers, and pressure sores.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING

 **NOTE:** Refer to procedure 3.3.1 for additional details. The following can be copied for preparation of a specimen collection instruction manual for caregivers. It is the responsibility of the laboratory director or designee to educate physicians and other caregivers on proper wound specimen collection.



Observe standard precautions.

A. General considerations

1. Preferably collect specimen prior to initiation of therapy and only from wounds that are clinically infected or deteriorating or that fail to heal over a long period.
2. Cleanse skin or mucosal surfaces.
 - a. For closed wounds and aspirates, disinfect as for a blood culture collection with 2% chlorhexidine or 70% alcohol followed by an iodine solution (1 to 2% tincture of iodine or a 10% solution of povidone-iodine [1% free iodine]). Remove iodine with alcohol prior to specimen collection.
 - b. For open wounds, debride, if appropriate, and thoroughly rinse with sterile saline prior to collection.
3. Sample viable infected tissue, rather than superficial debris.
4. Avoid swab collection if aspirates or biopsy samples can be obtained.
5. Containers
 - a. Anaerobe transport vial for small tissues
 - b. Sterile cup for large tissues with nonbacteriostatic saline on a gauze pad to keep moist
 - c. Wound or abscess aspirates
 - (1) Samples collected by using a syringe and needle should be placed in a sterile container or blood collection tube without anticoagulant (e.g., Vacutainer or similar type) for submission to the laboratory.
 - (2) A portion of the samples should also be placed in a sterile tube containing prereduced anaerobically sterilized (PRAS) medium if an anaerobic culture is required.

Table 3.13.1–2 Commonly encountered superficial and deep-wound/abscess, drainage, and tissue infections

Clinical condition	Location of infection	Primary pathogen(s)	Comments ^a
Superficial infections Boils/carbuncles Cellulitis Cysts Folliculitis Injury (abrasions, first-degree burns, cuts) Subcutaneous abscesses Ulcers	Skin and soft tissues anywhere on the body	<i>S. aureus</i> /MRSA Beta-hemolytic streptococci (A, B, C, G) Coagulase-negative staphylococci <i>Corynebacterium</i> spp. <i>P. acnes</i> Herpes simplex virus	<i>B. anthracis</i> may cause a skin eschar.
Deep-wound infections Bites—human Bites—animal Second- or third-degree burns Episiotomy Injury Surgical wounds Ulcer (decubiti, diabetic foot, etc.)	Skin and deeper tissues below the dermis, fascia, and muscle	Animal bites <i>Pasteurella multocida</i> <i>Capnocytophaga</i> spp. <i>Eikenella corrodens</i> Human bites and other deep wounds Mixed aerobes and facultative organisms Anaerobes can also usually be recovered from deep wounds	<i>Aeromonas</i> spp., <i>Vibrio</i> spp., or <i>C. violaceum</i> may be found in wounds exposed to water. Anaerobic culture should be performed on request or when the Gram stain suggests the presence of anaerobes.
Abscesses/fluids	Aspirated from deep body spaces or tissues	Mixed aerobes and facultative organisms Anaerobes can also usually be recovered from deep abscesses.	Anaerobic culture should be performed on request or when the Gram stain suggests the presence of anaerobes.
Drainages	Fluid freshly aspirated from disinfected tubing or area being drained	Mixed aerobes and facultative organisms Anaerobes can also usually be recovered from drainages from deep body spaces or tissues.	Anaerobic culture should be performed on request or when the Gram stain suggests the presence of anaerobes. After broad-spectrum antimicrobial agent treatment, recovery of resistant organisms and yeasts increases.
Tissues	Invasively collected biopsy samples of deep organs and tissues	Mixed aerobes and facultative organisms Anaerobes can also usually be recovered from deep abscesses.	Anaerobic culture should be performed on request or when the Gram stain suggests the presence of anaerobes.

^aSee Table 3.13.1–1 for a complete listing of aerobic and anaerobic isolates from acute and chronic wound/abscess and tissue infections.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING (continued)

- (3) Syringes with the needle attached should not be accepted due to the sharps and biohazard risk to staff. Syringes capped with a Luer-Lok are also not acceptable because the specimen may leak during transport and the samples may also be contaminated during handling.
- d. Broth culture medium in small sterile snap-top microcentrifuge tubes for fine-needle aspirates (FNA). These tubes are ideal for this type of specimen, because the specimen is easily visible and can be minced with a sterile glass rod in the laboratory, if necessary.
- e. Swabs (*ideally, submit two, one for Gram stain and one for culture*) in either the CultureSwab EZ II system (BD Diagnostic Systems) or a swab transport system with Stuart's or Amies medium to preserve specimen and to neutralize inhibitory effects of swabs. EZ II and synthetic swab systems are best for anaerobic preservation (6).

**II. SPECIMEN COLLECTION,
TRANSPORT, AND HANDLING**
(continued)

- B. Specimen collection after proper disinfection**
- NOTE:** Refer to procedure 3.13.2 for quantitative culture methods.
- 1. Closed abscesses**
 - a.** Aspirate infected material with needle and syringe.
 - b.** If the initial aspiration fails to obtain material, inject sterile, nonbacteriostatic saline subcutaneously. Repeat the aspiration attempt.
 - c.** Wound or abscess aspirates collected by using a syringe and needle should be placed in a sterile container or blood collection tube without anticoagulant (e.g., Vacutainer or similar type) for submission to the laboratory. A portion of the samples should also be placed in a sterile tube containing PRAS medium if an anaerobic culture is required.

■ NOTE: Syringes with the needle attached should not be accepted due to the sharps and biohazard risk to staff. Syringes capped with a Luer-Lok are also not acceptable because the specimen may leak during transport and the samples may also be contaminated during handling.
 - 2. FNA**
 - a.** Insert the needle into the tissue, using various directions, if possible.
 - b.** If the volume of aspirate is large, remove the needle and submit with Luer-Lok on the syringe.
 - c.** If the volume is small, aspirate the specimen into the sterile locking microcentrifuge tube containing broth by drawing up and down to release the specimen from the syringe.

■ NOTE: Always use a safety device on the needle. Do not submit needle to the laboratory.
 - 3. Open wounds**
 - a.** Cleanse the superficial area thoroughly with sterile saline, changing sponges with each application. Remove all superficial exudates.
 - b.** Remove overlying debris with scalpel and swabs or sponges.
 - c.** Collect biopsy or curette sample *from base or advancing margin of lesion*.
 - 4. Pus**
 - a.** Aspirate the deepest portion of the lesion or exudate with a syringe and needle.
 - b.** Collect a biopsy sample of the advancing margin or base of the infected lesion after excision and drainage.
 - c.** For bite wounds, aspirate pus from the wound, or obtain it at the time of incision, drainage, or debridement of infected wound. (Do not culture fresh bite wounds, as there is generally not yet evidence of infection. These wounds will harbor the resident respiratory microbiota introduced from the bite, but cultures cannot predict if they will cause infection.)
 - d.** Submit as for closed abscesses.
 - 5. Drainage tube specimens**

Drainage tube devices should not be cultured. Drainage fluids for culture should never be collected from the bag due to organism overgrowth in fluid samples that are not freshly collected, and the concerns about contamination by skin and other normal microbiota in the area being drained. Drainage fluid should be freshly collected by direct aspiration of fluid from the area being drained or by aspiration of fresh fluid in the drainage tube after decontamination of the surface of the device. Abdominal, chest tube, and biliary t-tube drainages are the most commonly submitted drainages. Cultures of surgical drainage from clean surgical procedures are not indicated if there are no signs of infection (3).

 - a.** Disinfect the collection tubing, and aseptically aspirate fresh fluid from the tubing.
 - b.** Submit the drainage fluid in a sterile, leakproof container.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING (continued)

- c. Do not inoculate blood culture bottles with drainage fluids, because a Gram stain and culture correlation is needed to determine the clinical significance of drainage fluid isolates.
 - NOTE:** Drainage tubes and tubing should be rejected for culture. Physicians may be educated about the proper collection of drainage fluid samples if a comment is included on the report such as "Drainage tubes of this type are not acceptable for culture because of the high rate of contamination by normal skin flora. Drainage fluid is acceptable for culture if a fresh sample is aseptically collected by aspiration into a sterile container after disinfecting the collection tubing."
- 6. Tissues and biopsy samples
 - a. Tissue biopsy samples should be collected from areas within and adjacent to the area of infection. Large enough tissue samples should be collected to perform all of the tests required (i.e., 3- to 4-mm biopsy samples).
 - b. If anaerobic culture is required, a separate piece of tissue should be submitted in a sterile tube containing PRAS media.
- 7. Collect swabs only when tissue or aspirate cannot be obtained.
 - a. Limit swab sampling to wounds that are clinically infected or those that are chronic and not healing.
 - b. Remove superficial debris by thorough irrigation and cleansing with non-bacteriostatic sterile saline. If wound is relatively dry, collect with two cotton-tipped swabs moistened with sterile saline.
 - c. Gently roll swab over the surface of the wound approximately five times, focusing on area where there is evidence of pus or inflamed tissue.
 - d. If anaerobic and aerobic culture is indicated, transfer swabs immediately to an anaerobic transport tube or submit in CultureSwab EZ II system. For aerobic culture, submit in aerobic transport tube or CultureSwab EZ II system.
- NOTE:** Organisms may not be distributed evenly in a burn wound, so sampling different areas of the burn is recommended. Blood cultures should be used to monitor patient status.
- C. Label specimen and requisition.
 - 1. List demographic information on the patient.
 - 2. Describe the type of specimen (deep tissue, superficial tissue, decubitus, catheter site, boil, abscess, cellulitis, aspirate, pus, drainage, surgical incision site, etc.)
 - 3. State anatomic location (arm, leg, etc.)
 - 4. Record collection time and date.
 - 5. List diagnosis or ICD9 code, including cause and clinical signs of infection.
 - 6. List antimicrobial therapy prior to specimen collection.
 - 7. Choose tests requested, including anaerobic culture, if appropriate.
- NOTE:** To avoid the overuse of full fungal cultures that require incubation periods of greater than 1 week, the laboratory can offer a fungal culture with a shorter (2- to 4-day) incubation period. Such cultures are useful and cost-effective for the diagnosis of nosocomial, foreign-body, and postoperative infections, where the likely pathogen is either bacteria, *Candida* species, or *Aspergillus* species. *Candida* or *Aspergillus* species will grow on routine bacterial culture media within 1 week; however, a selective fungal medium may be indicated for cultures expected to contain mixed microbiota. Full fungal cultures should be reserved for diagnosis of chronic infections, particularly those caused by dematiaceous and biphasic molds, and should be performed only from specimens not submitted on swabs.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING (continued)

- D. Deliver aspirates and tissues to the laboratory within 30 min for best recovery.
1. Keep tissues moist to preserve organism viability.
 2. Do not refrigerate or incubate before or during transport. If there is a delay, keep sample at room temperature, because at lower temperature there is likely to be more dissolved oxygen, which could be detrimental to anaerobes.
- E. Rejection criteria
1. Do not accept specimens for microbiological analysis in container with formalin.
 2. If numerous squamous epithelial cells are present on the Gram stain, especially from swab specimens, request a recollection if there is evidence of infection.
 3. Discourage submission of specimens to determine *if* an infection is present.
 4. Reject swabs that have been delayed in transit more than 1 h if they are not in some transport system (either CultureSwab EZ II system or one with preservative).
 5. For multiple requests (acid-fast bacilli, fungal, bacterial, and viral) but little specimen, contact the physician to determine which assays are most important and reject the others as “Quantity not sufficient.”

III. MATERIALS

A. Media

1. BAP
2. CHOC for surgical tissues, closed aspirates, biopsy samples, and FNA specimens or specimens from genital sites to culture for *Neisseria* or *Haemophilus* spp.
3. MAC or EMB, except for clean surgical specimens (e.g., orthopedic cultures). When in doubt, examine Gram stain to determine the likelihood of a mixed culture.
4. Phenylethyl alcohol agar (PEA) or Columbia colistin-nalidixic acid agar (CNA), if source (e.g., gastrointestinal) or *Gram stain indicates that the culture contains gram-negative rods*, which may inhibit gram-positive organisms.
5. Thayer-Martin or similar selective media for genital specimens or for other specimens if *Neisseria gonorrhoeae* is suspected.
6. Anaerobic culture media, if appropriate for site of collection and transport conditions. See section 4.
7. Special media for recovery of certain fastidious or unusual organisms. Refer to the table of contents of this section for procedures listed by specific microorganism names.

8. Tissue broth cultures

Broth culture of deep tissues may increase the detection of small numbers of organisms. Biopsy samples from deep tissues (e.g., lung, brain, liver, bone, etc.) and other FNA samples may be inoculated to enrichment broth. Common broth media include the following.

- a. Anaerobic BHI or TSB with 0.1% agar with or without yeast extract
- b. Fastidious anaerobic broth (Quebec Laboratories, Inc., Montreal, Quebec, Canada)
- c. Although THIO is an excellent medium for anaerobe recovery, it is the least suitable for recovery of low numbers of aerobic organisms, including yeasts (5).

NOTE: Broth cultures inoculated as an adjunct to direct plating of wounds and fluid samples have been shown to seldom yield results that would alter patient management and could be omitted for most specimens without compromising patient care (4). Broth cultures should also not be done on swabs obtained from superficial or deep wound sites (7).

III. MATERIALS (continued)**B. Tissue-homogenizing apparatus**

1. Scalpels and petri dishes (Fig. 3.13.1–1)
2. Mortars and pestles. Use only in an anaerobic chamber if anaerobes are suspected, as these devices aerate the specimen (Fig. 3.13.1–2).
3. Automated pummeling instrument (stomacher [Tekmar Co., Cincinnati, Ohio]; MiniMix [Interscience

Laboratories, Inc., Hingham, Mass.]) (Fig. 3.13.1–3)

4. Commercially available disposable plastic grinding devices (BD Diagnostic Systems; Sage Products Inc., Crystal Lake, Ill.). These are safer than glass handheld grinders (Fig. 3.13.1–4)

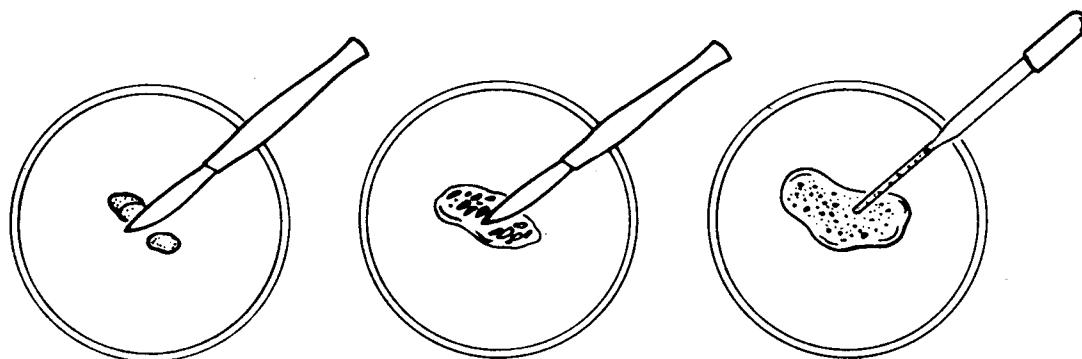
C. Gram stain reagents

Figure 3.13.1–1 Illustration of sterile-scalpel method of homogenization of tissue.

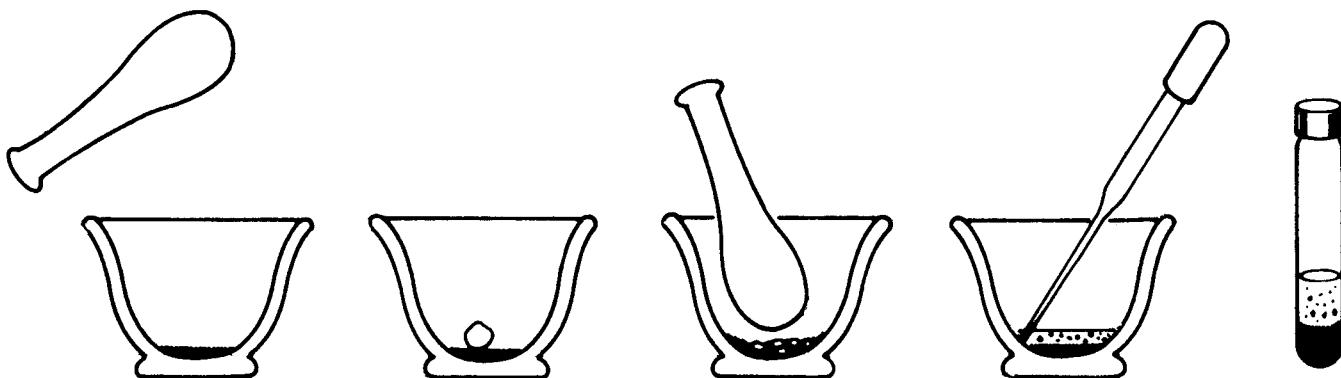


Figure 3.13.1–2 Illustration of mortar-and-pestle method of homogenization of tissue.

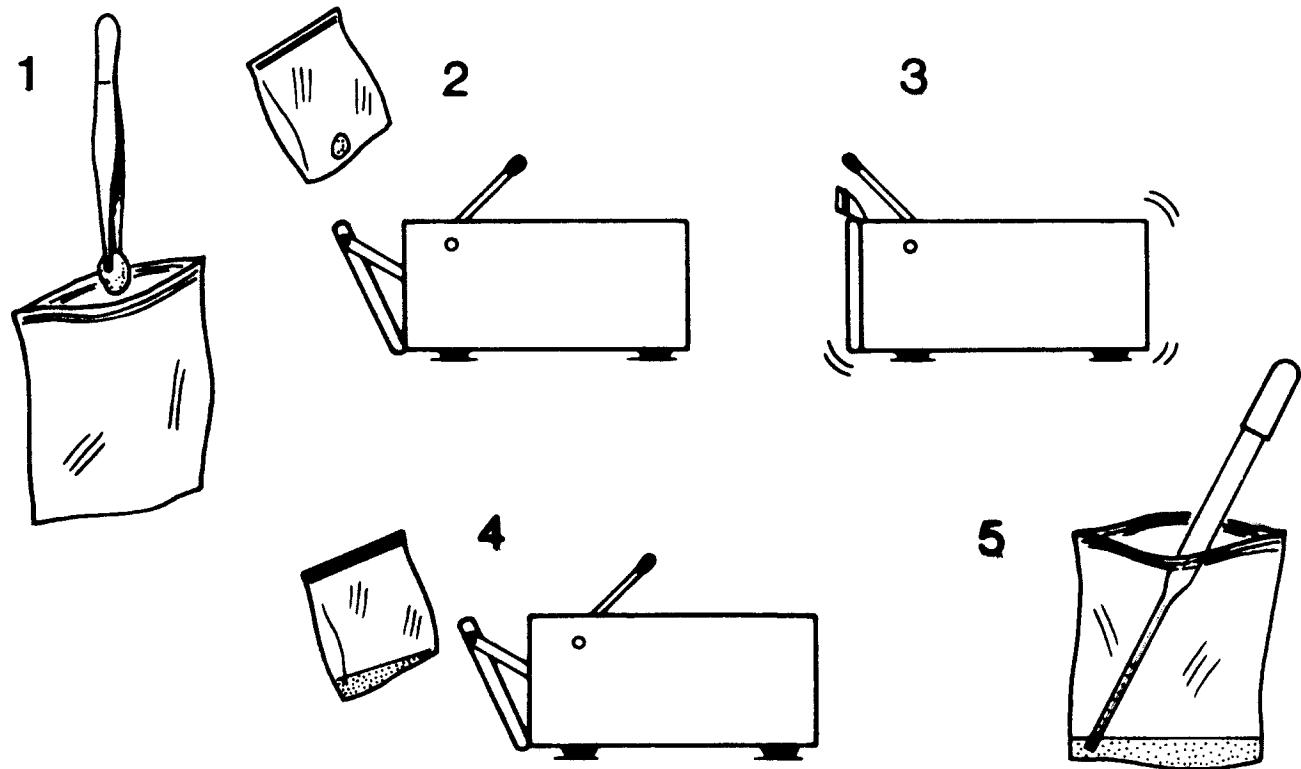


Figure 3.13.1-3 Illustration of stomacher method of homogenization of tissue.

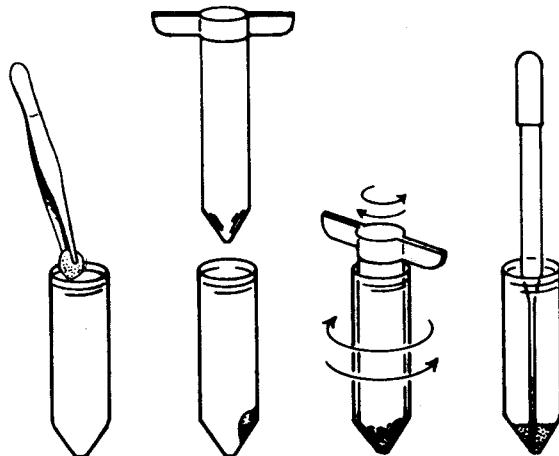


Figure 3.13.1-4 Illustration of tissue-grinding kit method of homogenization of tissue.

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

- A. Verify that media meet expiration date and QC parameters per current Clinical and Laboratory Standards Institute (CLSI; formerly NCCLS) M22 document. See procedures 14.2 and 3.3.1 for further procedures, especially for in-house-prepared media.
- B. QC each lot of CHOC (procedure 3.3.1) and *N. gonorrhoeae* selective agar (procedure 3.9.3) using appropriate microorganisms listed in these procedures.
- C. Perform other user QC per current CLSI document M22 and section 14 of this handbook.

V. PROCEDURE



Observe standard precautions.

- A. For safety reasons, as with all microbiological specimens, and to protect the specimen from environmental contamination, place specimen and media in a biological safety cabinet or anaerobic chamber to perform specimen preparation and inoculations.
- B. Inoculation
 - 1. Tissues
 - a. Select a portion of the tissue biopsy sample for culture that is bordering and within the area of infection (i.e., necrotic tissue is usually at the center of infected tissue areas).
 - b. Perform anaerobic culture first, preferably in an anaerobic chamber.
 - (1) If the tissue is large enough to be safely handled, place the tissue in a petri dish or specimen cup and cut it in half with a knife. Cut a smaller piece from the half and immediately touch the cut surface to the inoculum area of the anaerobe plates, place the tissue into broth culture medium, and streak the anaerobe plates for isolation.
 - (2) Alternatively, and for smaller tissue specimens, grind or homogenize tissues in THIO or other reduced broth (Fig. 3.13.1–1 to 3.13.1–4).
 - (3) Incubate immediately.
 - c. If the tissue can be easily teased apart (e.g., lung, kidney, brain tissue), cut a portion of the tissue into several pieces (use a sterile blade and stick or scissors) or gently tease it apart with sterile sticks (Fig. 3.13.1–1). (Save one piece of cut tissue without teasing for the smears.) Inoculate a piece of tissue onto each of the culture plates.
 - d. If the tissue is hard (e.g., bone, skin)
 - (1) A sterile scalpel may be used to chip off small bone pieces for culture from a larger specimen. This procedure, however, creates a safety issue for staff, and manipulation of the sample may cause contamination. Alternatively, viable tissue may be removed from the bone biopsy sample, and the tissue and bone may be plated to the same plates after the bone is sonicated.
 - (2) Alternatively, place some of the tissue in the grinding apparatus (Fig. 3.13.1–2 and 3.13.1–4) and grind with about 0.5 ml of fluid from broth culture medium.
 - (3) After homogenization, remove the homogenized specimen using a sterile pipette. Inoculate plates and place the rest into broth culture medium.
 - e. Use an automated pummeling instrument to grind tissues that cannot be easily teased, if available (Fig. 3.13.1–3).
 - (1) Place a portion of the specimen into a sterile bag along with a small amount of sterile broth. This aids the recovery of organisms from samples that remain intact after pummeling. The tissue and broth should both be cultured.
 - (2) Insert sample bag between door and paddles in blender, allowing 4 cm of bag to project above top of door.
 - (3) Pull handle forward to firmly close door, and switch machine on for 1 to 5 min.
 - (4) Switch machine off, hold bag, open door by lifting handle, and remove bag.
 - (5) Remove sample from bag by using sterile pipette, and inoculate media.

NOTE: Always examine these specimens for the presence of any soft tissue. If you find any such material, carefully remove it with a sterile surgical scalpel. This tissue may be processed separately.

V. PROCEDURE (continued)**f. For Gram stain**

(1) *Touch preps of the tissue sample are made onto sterile glass slides before the culture is inoculated. If sterile slides are not used for the touch prep smears, then these should be made after the culture has been inoculated to avoid contamination.*

(2) Make a fresh cut of tissue and prepare the smear by touching the tissue to the slide. If the tissue is hard and does not stick to the slide, place the tissue between the two slides and press the slides together. Then separate by drawing the slides against each other (see Fig. 3.2.1–1 and 3.2.1–2).

g. If the tissue is large enough, save an intact piece in the refrigerator for up to 7 days, or in the freezer for extended storage.

2. Aspirates and pus

a. Mix the specimen thoroughly. Place a drop of the specimen onto each piece of the medium.

b. If sufficient specimen is submitted, inoculate invasively collected aspirates to broth culture medium to make a 1:10 dilution. If the volume is small, omit broth culture.

c. Prepare smear for Gram stain by placing a drop of specimen on a slide and spreading it to make a thin preparation. If the aspirate fluid is clear, use the cyt centrifuge to concentrate the specimen for the smear.

d. If sufficient specimen is available, save a portion in the refrigerator for up to 7 days for further testing, if indicated.

3. Swabs

a. If an anaerobic culture is to be performed, inoculate anaerobic plates first.

b. Then place swab in 1 to 2 ml of broth and vortex.

c. Squeeze the swab against the side of the broth tube to express remaining fluid and then discard.

d. Inoculate aerobic plates and prepare smear for Gram stain as described for aspirates and pus.

e. Alternatively, the swab can be used for direct specimen plating. Always inoculate media from the least inhibitory to the most inhibitory.

f. Save broth in the refrigerator for up to 7 days for further testing, if indicated.

■ **NOTE:** Do not culture swabs from superficial wounds or abscesses in broth medium.

C. Aerobic incubation conditions (see section 4 for anaerobic incubation conditions and workup of anaerobic culture)

1. Incubate BAP, CNA or PEA, and CHOC in humidified incubator at 35 to 37°C with 5% CO₂. Incubate for a minimum of 48 h for open wound cultures and for 3 to 4 days for invasively collected specimens with no initial growth. Incubation may be extended to 7 days for invasive specimens (i.e., aspirated fluids and tissues) that remain culture negative after 3 to 4 days of aerobic incubation.

2. Critical deep-wound, abscess, and tissue samples should have anaerobic cultures requested (see Table 3.13.1–2 and section 4) in order to recover all of the primary pathogen(s) causing infection in specific clinical conditions (e.g., brain abscesses, brain, lung, liver tissue, deep wounds, abscesses, etc.). The laboratory should also routinely do anaerobic cultures on these types of samples when the specimen Gram stain demonstrates purulence (i.e., PMNs) and one or more bacterial morphotypes suggestive of anaerobes.

3. Incubate MAC or EMB plates in ambient air at 35 to 37°C, unless it is inconvenient to keep them separate from the rest of the culture in 5% CO₂.

V. PROCEDURE (continued)

4. Incubate broth in ambient air at 35 to 37°C for 3 to 4 days. If possible, hold for 1 week at room temperature to ensure that some specimen is available if further testing is indicated.
- D. Perform a Gram stain on all specimens and use in the evaluation of culture.
 1. Refer to procedure 3.2.1 for details on smear preparation and staining.
 2. Record the relative numbers of WBCs, epithelial cells, and bacterial and fungal morphotypes.
 - a. If clinically important organisms are recognized or suspected (e.g., from a normally sterile site) based on the Gram stain interpretation, telephone or report results to the appropriate caregiver immediately. Report any bacteria seen in a surgically collected specimen from a normally sterile site. Some examples of significant bacteria include the following.
 - (1) Clostridium-like gram-positive rods seen on specimens from soft tissue infections or aspirates even in the absence of numerous intact PMNs.

NOTE: Clostridia and other anaerobes produce phospholipases and lipases, and some aerobes (e.g., *S. aureus*, group A *Streptococcus*, *Listeria*, and *Corynebacterium* spp.) produce phospholipases that can destroy host cells, so a reduced or absent number of PMNs may be found in the direct specimen Gram smear.
 - (2) Numerous PMNs and gram-positive cocci in clusters resembling *Staphylococcus* in an abscess or tissue.
 - (3) Bacteria seen from brain abscess specimens.
 - (4) Gram-positive cocci in chains suggestive of streptococci from endometrial tissue may be found in group A streptococcal puerperal sepsis.
 - b. If multiple morphologies are seen on the smear and the culture was not inoculated onto selective agar, go back to the specimen and inoculate it to CNA or PEA and EMB or MAC.
- E. Culture workup
 1. Read plates and broth daily. Refer to procedure 3.3.2 for description of colony types, initial reading, testing, and reporting preliminary and final cultures. For identifications refer to Table 3.3.2–5 and procedures 3.18.1 and 3.18.2.
 2. For cultures of lymph nodes, work in a biological safety cabinet, since pathogens can be found in these specimens that are hazardous, e.g., *Francisella*, *Mycobacterium*, and *Brucella*.
 3. Refer to Table 3.13.1–1 for the list of the most common pathogenic organisms associated with wound infections and Fig. 3.13.1–5 for algorithm for extent of workup of cultures. Follow Fig. 3.13.1–6 and identify any number of the organisms listed.
 4. Generally identify up to three microorganisms listed in Table 3.13.1–1 if any of the following is true.
 - a. PMNs were present on direct smear.
 - b. The specimen was collected from a normally sterile site.
 - c. The specimen was of good quality (e.g., no or few epithelial cells present).
 - d. The organism was seen on the direct smear.



It is imperative that these cultures be handled in a biosafety hood.

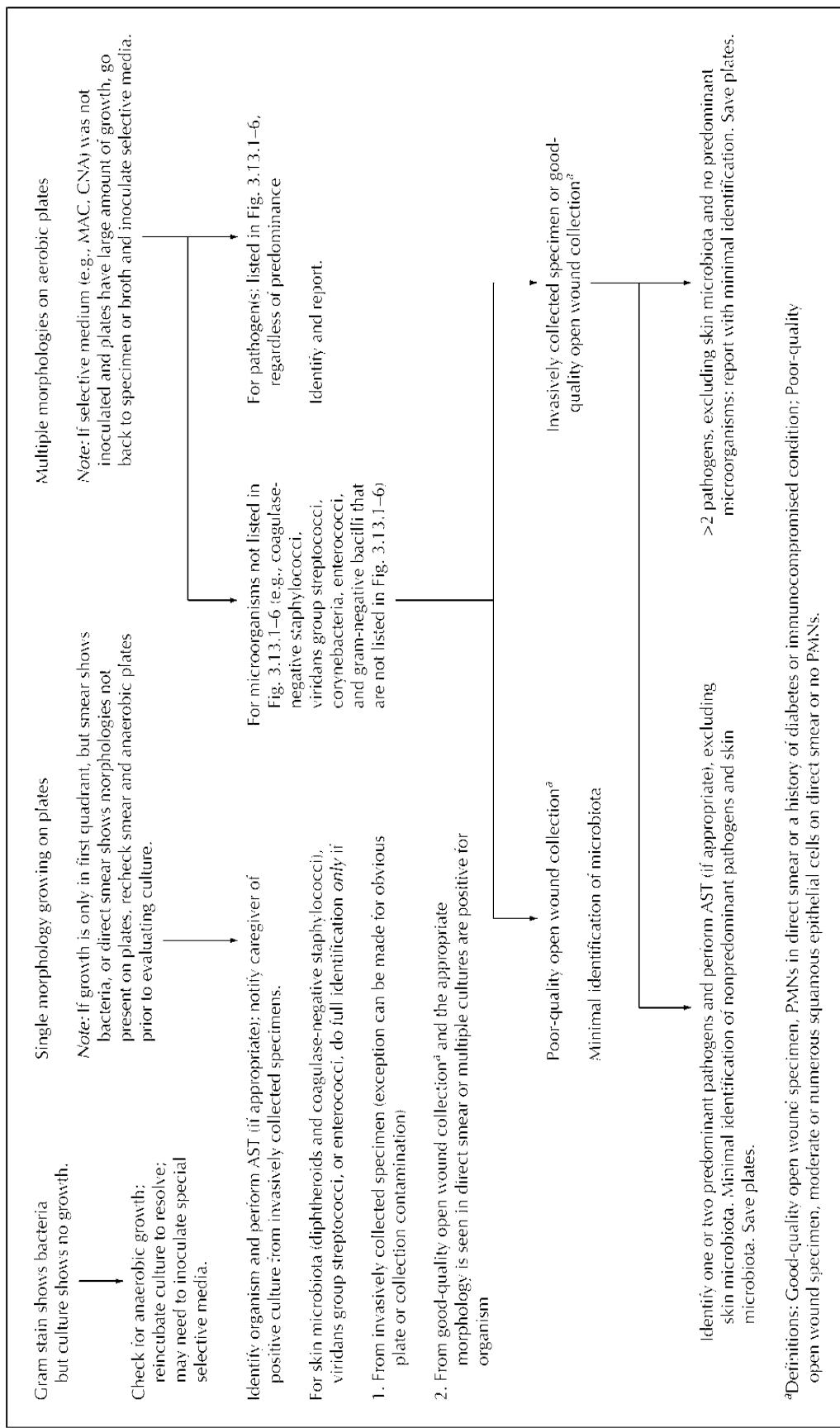


Figure 3.13.1–5 Initial evaluation of positive wound cultures for organisms growing aerobically. **Note:** For lymph nodes, perform all work using biological safety cabinet.

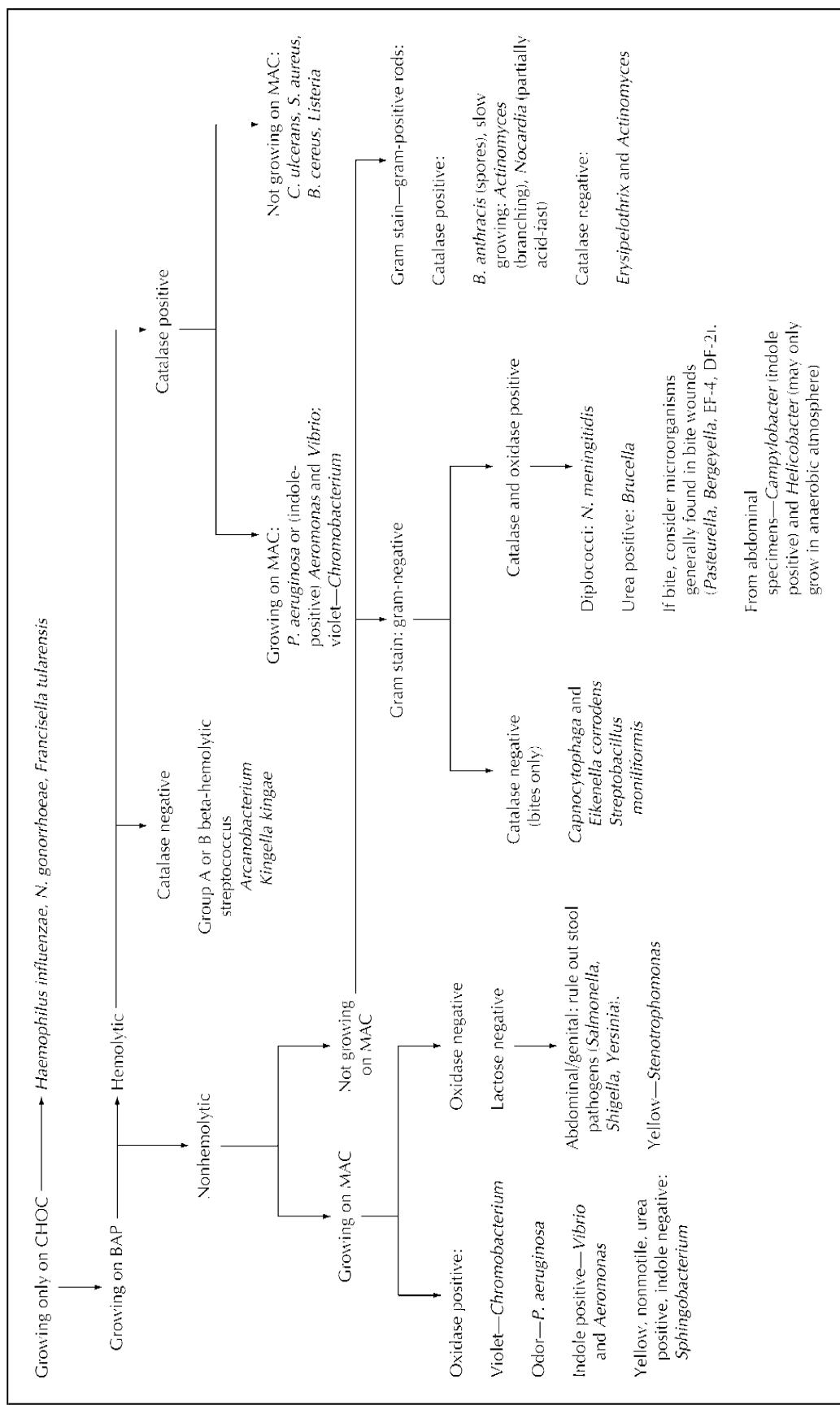


Figure 3.13.1–6 Algorithm to rapidly detect aerobic and facultatively aerobic microorganisms usually considered significant, even in low numbers or in mixed cultures. See procedures 3.18.1 and 3.18.2 for other tests needed to confirm suspected identifications.

V. PROCEDURE (continued)

5. Perform only minimal testing to indicate the type of microbiota present for noninvasively collected specimens with any of the following.
 - a. Moderate or numerous epithelial cells present on the smear
 - b. No evidence of infection on the smear (no PMNs) and no clinical information accompanying the specimen to indicate an infection
 - c. ≥ 3 Organisms growing in the culture. See exceptions for specific organisms in Fig. 3.13.1–6, which are generally always reported.

■ **NOTE:** Save all culture plates with growth for several days in case further work is requested by the physician. Seven days is usually sufficient, but if space is a problem, transfer isolates to culture tubes for storage or save for a shorter period.
6. Identify any number of microorganisms that only grow on CHOC, and not on BAP (*N. gonorrhoeae*, *Haemophilus*, and *Francisella*). Identify *Neisseria meningitidis*.
7. Identify *Streptococcus pyogenes* or *Streptococcus agalactiae*.

■ **NOTE:** Notify the physician of the isolation of *S. pyogenes*, as it may represent a life-threatening case of necrotizing fasciitis.
8. *Staphylococcus*
 - a. *S. aureus*
 - (1) Perform AST from invasively collected specimens and from others, if the Gram stain indicates a good-quality specimen and an infectious process with this organism (e.g., PMNs with few or no squamous epithelial cells and staphylococci seen on specimen Gram stain).
 - (2) If an infectious process is not apparent but it is the hospital policy to track nosocomial methicillin-resistant *S. aureus* (MRSA) infections, rule out MRSA on inpatient specimens, unless the patient has a prior positive culture with MRSA. Notify infection control practitioner if MRSA is present, per hospital policy.
 - b. When *coagulase-negative staphylococci* are present, perform AST only if they are the only organisms isolated from invasively collected specimens, if they are associated with PMNs in the direct smear, or if they are isolated from multiple cultures. Report as normal cutaneous microbiota if found in mixed cultures in any amount from superficial wound specimens or if numerous epithelial cells are present in the specimen.
9. For *viridans group streptococci* or *enterococci*
 - a. Identify at least to the genus level from surgically, invasively collected specimens where the organism is the single or predominant pathogen and the Gram stain indicates infection (the presence of PMNs).
 - b. Include in normal microbiota if found in mixed cultures and not predominant.
 - c. If determined to be a significant isolate or if indicated by infection control policies, perform a vancomycin screen on enterococci from inpatients and from transplant and oncology outpatients. Perform AST only if isolate is from normally sterile site (e.g., bone, brain) in pure or almost pure culture.
10. For *gram-positive rods*, if specimen is from a normally sterile site or biopsy sample, rule out *Listeria*, *Erysipelothrix*, *Bacillus cereus*, *Bacillus anthracis*, *Arcanobacterium*, *Corynebacterium ulcerans*, *Nocardia*, and *Actinomyces*. Identify other gram-positive rods if numerous or seen as predominant in smear. Otherwise include these in skin microbiota.
11. Include *yeasts* as part of normal microbiota unless predominant or numerous. Except for specimens from normally sterile sites, generally identify only *Candida albicans* to the species level.

V. PROCEDURE (continued)

12. For predominant or moderate to numerous amounts of *enteric gram-negative rods*
 - a. If only one or two species are present or predominant and *an indication of infection is seen on smear*, identify and perform AST. For specimens from the abdominal cavity, the aerobic plates may contain only a few *Escherichia coli* organisms but the smear appears to represent mixed morphologies. In such cases, do not set up AST on the *E. coli* until the results of the anaerobic culture can be evaluated. Potentially the anaerobic microbiota may be the significant, predominant pathogen(s).
 - b. If enteric bacilli are few in amount or not predominant, or if >2 species are present with no predominant strain, report as “mixed GI [for gastrointestinal] microbiota.”
 - (1) Rule out fecal pathogens (*Salmonella*, *Shigella*, *Campylobacter*, and *Yersinia* spp.) for specimens from abdominal abscesses.
 - (2) Generally save a representative plate for up to 7 days in case further work is requested.
 - c. Identify and perform susceptibility tests on multiple morphologies of enteric gram-negative rods only on special request after consultation with the laboratory director, designee, or physician.

■ NOTE: When cultures contain a variety of enteric rods, treatment must include a combination of antimicrobial agents which are known to eradicate normal intestinal microbiota. Examination of a culture with fecal contamination to detect and separate each species is futile and not helpful for overall treatment decisions.
13. For gram-negative rods that are not of the *Enterobacteriaceae* family
 - a. Rule out organisms which are always considered pathogenic (e.g., *Bacillus*, *Haemophilus*, *Pasteurella*, *Francisella*). Generally these organisms are recognized because they do not grow on MAC or EMB. These organisms are not uncommon in dog and cat bite wounds. Work in a biological safety cabinet, and see procedure 3.18.2 for identification flowcharts and tables.

■ *Francisella* can be found in lymph node biopsy samples and is extremely infectious. It is a tiny coccobacillus that grows slowly and is catalase positive or weak and oxidase negative. It can ferment glucose, but it is negative for other biochemical tests. It is beta-lactamase positive. Refer to procedure 16.8 on bioterrorism for other details.
 - b. Identify obvious *P. aeruginosa* (characteristic odor and beta-hemolytic colonies) and *Stenotrophomonas maltophilia* (yellow and oxidase negative). If in pure culture or significant amounts and the Gram stain suggests an infective process, perform AST.
 - c. Identify oxidase-positive, indole-positive organisms (Table 3.18.2–8) which are likely to be *Aeromonas* or *Vibrio*. Also examine for the pigmented gram-negative rods *Chromobacterium violaceum* and *Sphingobacterium*.
 - d. Identify and perform AST on other gram-negative rods (*Pseudomonaceae*, *Acinetobacter*, and related non-glucose-fermenting rods) by following the algorithm in Fig. 3.13.1–5.
14. Refer to procedure 3.3.2 for details on handling of broth cultures.
- F. Hold positive culture plates at room temperature or in the refrigerator for several days (generally 7 days) after the culture is completed for additional work if requested by the physician.



It is imperative that these cultures be handled in a biosafety hood.

POSTANALYTICAL CONSIDERATIONS

VI. REPORTING RESULTS

- A. Report Gram stain results as soon as possible, generally within 1 h for specimens from critical sites.
- B. Report all negative cultures as "No growth in _____ days."
- C. Report individually those organisms that are always considered pathogenic (Fig. 3.13.1–6) with enumeration, using a preliminary identification initially and the genus and species (if applicable) as the final identification, if applicable.
- D. Due to their known virulence factors, indicate the presence of the following species.
 - 1. Beta-hemolytic streptococci
 - 2. *S. aureus*
 - 3. *P. aeruginosa*
 - 4. *Clostridium perfringens*
 - 5. Report "Pigmented anaerobes," *Bacteroides* spp., and "Mixed anaerobes" without further identification. (See section 4 for identification methods.)

Table 3.13.1–2 Examples of mixed wound culture reporting^a

Microbiological observation	Examples of specimen submissions			
	Example 1	Example 2	Example 3	Example 4
PMNs in Gram stain ^b	—	2+	3+	1+
Bacteria in Gram stain	3+ gram-negative rods	3+ gram-negative rods	3+ gram-positive and -negative rods	No organisms seen
Bacteria in culture				
<i>S. aureus</i>	1+	2+	1+	—
<i>P. aeruginosa</i>	—	3+	—	—
Beta-hemolytic streptococci	—	—	—	1+ group A
<i>Enterobacteriaceae</i> (agents of gastroenteritis ruled out)	3+ pure	1+	2+	1+
<i>Peptostreptococcus</i> spp.	—	1+	1+	1+
<i>Clostridium</i> spp.	1+	—	3+	—
Pigmented gram-negative anaerobes	—	—	3+	—
Nonpigmented gram-negative anaerobes	1+	—	1+	—
Information provided on microbiology report	3+ <i>E. coli</i> ; 1+ anaerobic GI microbiota; 1+ <i>S. aureus</i>	2+ <i>S. aureus</i> (AST); 3+ <i>P. aeruginosa</i> (AST); 1+ mixed skin and enteric microbiota	4+ mixed aerobic and anaerobic GI microbiota; 1+ <i>S. aureus</i>	1+ <i>S. pyogenes</i> ; 1+ mixed skin and enteric microbiota
Notes	May rule out MRSA for infection control purposes. Save plates; may need to do AST on <i>E. coli</i> if diabetic, etc.		May rule out MRSA for infection control purposes.	

^a Revised from Bowler et al. (1).^b Caution must be used in using the presence or absence of PMNs to report the presence of certain pathogens from mixed wound cultures. Clostridia and other anaerobes produce phospholipases and lipases, and some aerobes (e.g., *S. aureus*, group A *Streptococcus*, *Listeria*, and *Corynebacterium* spp.) produce phospholipases that can destroy host cells, so a reduced or absent number of PMNs may be found in the direct specimen Gram smear.

VI. REPORTING RESULTS

(continued)

- E. Report other pathogens, as indicated in Fig. 3.13.1–5, with either definitive or minimal identification, depending on quantitation, number of species present, and Gram stain results. (See Table 3.13.1–2 for examples of reports.)
- F. Report AST on gram-negative rods, enterococci, or *S. aureus*, using the flowchart in Fig. 3.13.1–5. Generally do not perform AST on microorganisms that are not predominant, are in mixed cultures, or are skin microbiota or if culture does not show evidence of an infectious process. Make exceptions to this general policy if requested to do so by physician caring for the patient or for infection control purposes.
- G. When multiple morphologies are present, report with minimal identification.
Example: “Culture yields growth of >3 colony types of enteric gram-negative bacilli. Consult microbiology laboratory if more definitive studies are clinically indicated.”
See Table 3.13.1–2 for other examples.
- H. Additionally, if mixed microbiota are cultured with no predominant microorganism, report as GI, oronasal, skin, or genital microbiota. Use of selective media is helpful in evaluation of polymicrobial nature of culture.
- I. For further details on reporting, refer to procedure 3.3.2.

VII. INTERPRETATION

- A. Continuous dialogue between the clinician or nurse and the microbiology laboratory should be encouraged for proper interpretation of results.
- B. The results of wound cultures will only be as valuable as the quality of the specimen submitted, its transport, and expedient processing.
- C. Reporting selected organisms in mixed cultures can lead to erroneous interpretation of the number and variety of infecting pathogens.
- D. Performance of AST is not indicated in cases of mixed microbiota indicative of infection of the abdominal cavity with bowel contents. Treatment should include broad-spectrum coverage for normal intestinal microbiota.
- E. *Use of the Gram stain can improve the accuracy of evaluating the importance of each potential pathogen. Organisms present in the Gram stain of an appropriately collected specimen correlate with $\geq 10^5$ organisms per g of tissue (2, 3).*
- F. Clinical studies have demonstrated that the microbial load in an acute wound can predict delayed healing or infection. The more numerous the organisms, the more likely they are to be indicative of infection (1).
- G. Many wound infections are polymicrobial, and the isolation of an organism in culture may or may not correlate with infection of the wound.

VIII. LIMITATIONS

- A. The microbiologist plays a critical role in the treatment of wound infections because practitioners often consider the report from the laboratory as definitive proof of infection. Providing inappropriate identifications and susceptibility results can prompt unnecessary treatment.
- B. The presence of PMNs is an indication of an inflammatory or infectious process, while the presence of epithelial cells indicates surface contamination of the specimen. Specimens containing numerous epithelial cells yield culture results of questionable accuracy in the diagnosis of the infectious process, and one can consider rejection of these specimens for culture.
- C. If a patient is immunocompromised or has poor vascular supply, inflammatory cells may not be present in the specimen as a guide to the extent of workup of the culture.
- D. Low levels of organisms or fastidious organisms that grow poorly on the direct plates may be missed in culture.

VIII. LIMITATIONS (continued)

- E. Unusual treatment considerations may alter the usual policies of the laboratory in reporting AST.
- F. The lack of isolation of a pathogen does not necessarily mean that the laboratory was unable to detect the agent. Other inflammatory diseases can have the same presentations as infectious diseases, including the presence of PMNs on the Gram stain.

REFERENCES

1. Bowler, P. G., B. I. Duerden, and D. G. Armstrong. 2001. Wound microbiology and associated approaches to wound management. *Clin. Microbiol. Rev.* **14**:244–269.
2. Levine, N. S., R. B. Lindberg, A. D. Mason, Jr., and B. A. Pruitt, Jr. 1976. The quantitative swab culture and smear: a quick, simple method for determining the number of viable aerobic bacteria on open wounds. *J. Trauma* **16**:89–94.
3. Magee, C., B. Haury, G. Rodeheaver, J. Fox, M. T. Edgerton, and R. F. Edlieh. 1977. A rapid technique for quantitating wound bacterial count. *Am. J. Surg.* **133**:760–762.
4. Morris, A. J., S. J. Wilson, C. E. Marx, M. L. Wilson, S. Mirrett, and L. B. Reller. 1995. Clinical impact of bacteria and fungi recovered only from broth cultures. *J. Clin. Microbiol.* **33**:161–165.
5. Rinehold, C. E., D. J. Nickolai, T. E. Piccinni, B. A. Byford, M. K. York, and G. F. Brooks. 1988. Evaluation of broth media for routine culture of cerebrospinal and joint fluid specimens. *Am. J. Clin. Pathol.* **89**:671–674.
6. Roelofsen, E., M. van Leeuwen, G. J. Meijer-Severs, M. H. Wilkinson, and J. E. De gener. 1999. Evaluation of the effects of storage in two different swab fabrics and under three different transport conditions on recovery of aerobic and anaerobic bacteria. *J. Clin. Microbiol.* **37**:3041–3043.
7. Silleiti, R. P., E. Ailey, S. Sun, and D. Tang. 1997. Microbiologic and clinical value of primary broth cultures of wound specimens collected with swabs. *J. Clin. Microbiol.* **35**:2003–2006.

SUPPLEMENTAL READING

- Bartlett, R. C. 1974. *Medical Microbiology: Quality Cost and Clinical Relevance*. John Wiley & Sons, Inc., New York, N.Y.
- Bartlett, R. C., M. Mazens-Sullivan, J. Z. Treteault, S. Lobel, and J. Nivard. 1994. Evolving approaches to management of quality in clinical microbiology. *Clin. Microbiol. Rev.* **7**:55–88.
- Hindiyeh, M., V. Acevedo, and K. C. Carroll. 2001. Comparison of three transport systems (Starplex StarSwab II, the new Copan Vi-Pak Amies agar gel collection and transport swabs, and BBL Port-A-Cul) for maintenance of anaerobic and fastidious aerobic organisms. *J. Clin. Microbiol.* **39**:377–380.
- Miller, J. M. 1996. *A Guide to Specimen Management in Clinical Microbiology*, 2nd ed. ASM Press, Washington, D.C.
- Perry, J. L. 1997. Assessment of swab transport systems for aerobic and anaerobic organism recovery. *J. Clin. Microbiol.* **35**:1269–1271.
- Sharp, S. E. 1999. Algorithms for wound specimens. *Clin. Microbiol. News.* **21**:118–120.
- Sharp, S. E. 1999. Commensal and pathogenic microorganisms in humans, p. 23–32. In P. R. Murray, E. J. Baron, M. A. Pfaffer, F. C. Tenover, and R. H. Yolken (ed.), *Manual of Clinical Microbiology*, 7th ed. ASM Press, Washington, D.C.

3.13.2

Quantitative Cultures of Wound Tissues

[Updated March 2007]

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Quantitative culturing is a patient management tool that can be used with a limited variety of specimen types. However, as indicated in the review by Bowler et al. (2), several publications have demonstrated a correlation between quantitative tissue biopsy cultures and the semiquantitative method of enumeration of organism growth (see Table 3.3.2–2) in a qualitative swab culture. Tissues from acute wounds, such as those from trauma and burn patients, and duodenal aspirates are the specimen types that may be used for quantitative microbiological analysis. This pro-

cedure describes collection and processing of tissue specimens and determination of bacterial counts. The presence of bacteria in tissue in significant amounts is one of a number of factors which have been associated with delayed healing and has also been correlated with infection. When tissue is not readily available, a swab sample may be a convenient substitute for a tissue biopsy sample, and in a quantitative culture, it may similarly be an indicator of an infectious process. However, semiquantitative swab culture is generally sufficient for patient management.

Quantitative cultures for anaerobic bacteria are problematic and thus less meaningful. Anaerobic microorganisms tend to live in microbial synergy with other organisms in the culture and do not grow well when diluted.

Quantitation of bacteria in duodenal aspirates can predict defects in mobility of the intestines. See procedure 13.15 for details. For quantitative culture of specimens from bronchoscopy, refer to the respiratory procedure (Appendix 3.11.2–1).

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING

A. Specimen collection

1. Tissues

- a. Submit 2- by 1-cm or larger tissue sample collected after cleansing and/or surgical debridement to yield the most useful information. (Refer to procedure 3.13.1 for collection details). This will yield approximately 500 mg of tissue, depending on the density (3).
- b. Submit as soon as possible without transport medium, but keep moist in a sealed, sterile container.

2. Swab collection

- a. Refer to procedure 3.13.1 for collection.
- b. *Use only alginate swabs for quantitative culture.*

B. Rejection criteria

1. If insufficient specimen is received for quantitation, process the specimen for qualitative culture only.
2. Do not process dry swabs.

III. MATERIALS

- A. Media**
 1. BAP or CHOC
 2. EMB or MAC
 3. THIO or saline for dilutions
- B. Reagents**
 1. Stain reagents for Gram stain
 2. 0.85% sterile NaCl
 3. Sterile Ringer's solution (available from suppliers of intravenous solutions) containing the following per liter
 - a. 8.5 g of NaCl
 - b. 0.3 g of KCl
 - c. 0.33 g of CaCl₂
- C. Other supplies**
 1. Loop method: use either platinum or sterile plastic disposable loops to deliver 0.001 ml (1 µl) or 0.01 ml (10 µl).
 2. Pipettor method: sterile pipette tips and pipettor to deliver 10 or 1 µl
 3. Sterile bent glass or plastic disposable sterile rods to spread inoculum (Excel Scientific, Wrightwood, Calif.; [760] 249-6371)
 4. Sterile pipettes
 5. Analytical balance
 6. Polytron omnimixer (Brinkman Instruments, Westburg, N.Y.) or automated pummeling instrument (stomacher; Tekmar Co., Cincinnati, Ohio)
 7. CO₂ incubator at 35 to 37°C
 8. Anaerobic atmosphere (optional)

ANALYTICAL CONSIDERATIONS**IV. QUALITY CONTROL**

- A. Verify that media meet expiration date and QC parameters per current Clinical and Laboratory Standards Institute (formerly NCCLS) M22 document. See procedures 14.2 and 3.3.1 for further procedures, especially for in-house-prepared media.
- B. Validate the method of quantitation using known cultures of various colony counts. Refer to Appendixes 3.12–3 and 3.12–4 for QC for loops and pipettors, respectively.

V. PROCEDURE

Observe standard precautions.

A. Specimen processing

- 1. Tissues**
 - a. Weigh the tube containing the tissue on an analytical balance.
 - b. Remove the tissue by using aseptic technique, and place it in 5 ml of sterile 0.85% NaCl or THIO (4, 5, 7). Use THIO only if anaerobic culture is performed. This is a 1:5 dilution of tissue.
 - c. Reweigh the now empty original specimen tube, and subtract to determine the weight of the tissue in grams or milligrams.
 - d. Homogenize the tissue for 15 to 30 s. Keep the homogenate cool during processing. See procedure 3.13.1 for detailed tissue homogenization methods.
 - e. Plate 0.1 ml of the original homogenate. Label plate “10⁻¹” for amount of dilution of original homogenate (which is a 1:5 dilution of the specimen).
 - f. Make one to three serial 1:10 dilutions of the homogenate with 0.5-ml aliquots and 4.5 ml of sterile 0.85% NaCl per aliquot.
 - g. Plate 0.1 ml of each dilution onto BAP or CHOC and EMB or MAC. Evenly distribute the inoculum with a sterile bent rod. Label plates “10⁻²,” “10⁻³,” and “10⁻⁴.” Alternatively, for the 10⁻³ and 10⁻⁴ dilutions, inoculate 0.01 and 0.001 ml of the first dilution with 10- and 1-µl loops, respectively.
 - h. Repeat inoculations onto anaerobic BAP, if desired.

V. PROCEDURE (continued)

- i. Incubate aerobic plates at 35°C in 5% CO₂ for 18 to 24 h. Use an anaerobic atmosphere for anaerobic plates.
2. Swabs
 - a. Place swab in 5 ml of Ringer's solution. Vortex and remove remaining swab material. (Swab should dissolve.)
 - b. Beginning with item V.A.1.e above, serially make 10-fold dilutions in 0.85% NaCl and process as for tissue.
- B. Gram stains
 1. Prepare Gram stain of tissue specimens by spreading a 0.01-ml aliquot of the homogenate on a 1- by 1-cm area of a glass slide (6).
 2. Allow the slide to dry, fix with methanol, and Gram stain.
 3. Examine 10 fields under a 100× oil immersion objective (see procedure 3.2.1 for reporting guidelines).
 4. Organisms are visible in Gram stains when at least 10⁵ organisms per g of tissue are present in the specimen (6).
- C. Examination of cultures: tissue and swabs
 1. Determine the number of organisms per gram of tissue by counting the colonies on the plate that grew between 30 and 300 colonies.

NOTE: Only use MAC or EMB if overgrowth on BAP or CHOC precludes accurate counting of gram-negative bacilli.
 2. Calculate the total number of organisms by using the colony count times the dilution factor (use 5 as the factor for the homogenate dilution and the dilutions labeled on the plates for subsequent dilutions) divided by the original weight of the tissue. There is no weight of the tissue for swab specimens. Use only dilution factors.

Example: Tissue weighed 0.3 g.
Count was 50 on plate labeled "10⁻³."

$$\frac{50 \text{ CFU} \times 5 \text{ (homogenate dilution)} \times 10^3 \text{ (plate dilution)}}{0.3 \text{ g}} = \frac{2.5 \times 10^5 \text{ organisms}}{0.3 \text{ g}} = 8.3 \times 10^5 \text{ CFU/g}$$

3. Report total count "per gram of tissue" or "per swab."
4. Work up pathogenic organisms for identification and antimicrobial susceptibility testing (AST) according to procedure 3.13.1 when count is >10⁵ organisms per g. Special consideration must be made case by case for counts of <10⁵ organisms per g, depending especially on the identity of the isolate(s) and the type of disease of the patient.

POSTANALYTICAL CONSIDERATIONS

VI. REPORTING RESULTS

- A. Report direct results of smear of tissues with enumeration of each morphotype.
- B. Report wound cultures as total number of organisms per gram of tissue.
- C. See procedure 3.13.1 for reporting predominant species and AST.

VII. INTERPRETATION

- A. The probability that tissue from a traumatic wound or burn is infected or will fail to heal can be predicted from cultures showing >10⁵ organisms per g (2–5).
- B. Colony counts of approximately 10⁵ CFU/g of tissue were found to be equivalent to colony counts of 10³ bacteria/ml of specimen obtained on a moist *alginated* swab (1).

VIII. LIMITATIONS

- A. These tests should be used only under certain circumstances and after consultation with the physician or clinical service.
- B. Loops are not as accurate as pipettes to deliver the inoculum.
- C. Spreading with a loop is not as accurate as spreading with a bent rod.

REFERENCES

1. **Bornside, G. H., and B. B. Bornside.** 1979. Comparison between moist swab and tissue biopsy methods for quantitation of bacteria in experimental incisional wounds. *J. Trauma* **19**:103–105.
2. **Bowler, P. G., B. I. Duerden, and D. G. Armstrong.** 2001. Wound microbiology and associated approaches to wound management. *Clin. Microbiol. Rev.* **14**:244–269.
3. **Edlich, R. F., G. T. Rodeheaver, T. R. Stevenson, C. M. Magee, J. G. Thaaker, and M. T. Edgerton.** 1977. Management of the contaminated wound. *Compr. Ther.* **3**:67–74.
4. **Lawrence, J. C., and H. A. Lilly.** 1972. A quantitative method for investigating the bacteriology of skin: its application to burns. *Br. J. Exp. Pathol.* **53**:550–558.
5. **Loebl, E. C., J. A. Marvin, E. L. Heek, P. W. Curreri, and C. R. Baxter.** 1974. The use of quantitative biopsy cultures in bacteriologic monitoring of burn patients. *J. Surg. Res.* **16**:1–5.
6. **Magee, C., B. Haury, G. Rodeheaver, J. Fox, M. T. Edgerton, and R. F. Edlich.** 1977. A rapid technique for quantitating wound bacterial count. *Am. J. Surg.* **133**:760–762.
7. **Rodeheaver, G. T., J. Hiebert, R. F. Edlich, and M. Spengler.** 1980. Practical bacteriologic monitoring of the burn patient. *Curr. Concepts Trauma Care* **3**:8–15.

SUPPLEMENTAL READING

Heggers, J. R., and M. C. Robson (ed.). 1991. *Quantitative Bacteriology: Its Role in the Armamentarium of the Surgeon*. CRC Press, Boca Raton, Fla.

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Leptospirosis is a spirochetal zoonosis caused by the genus *Leptospira*. Human-pathogenic *Leptospira* infects about 160 wild and domesticated mammalian species worldwide, which excrete the organism in their urine. The human disease is more commonly associated with occupations or recreational activities associated with direct skin or mucous membrane contact either with the animal reservoir or with water, soil, or sewage contaminated with the animal urine. Although most reports consider human infections to be caused by the species *Leptospira interrogans*, DNA relatedness studies have demonstrated that *L. interrogans* probably consists of at least seven species, including *L. interrogans*, *Leptospira noguchii*, *Leptospira weilii*, *Leptospira santarosai*, *Leptospira borgpetersenii*, *Leptospira kirschneri*, and *Leptospira inadai* (9). Serologic studies indicate that there are more than 218 serovars of pathogenic *Leptospira*, but the serovar cannot be used to predict an isolate's molecular identification. Species identification is thus limited to special reference laboratories, such as the CDC.

The disease can present as a febrile illness with or without meningitis or as

Weil's syndrome, a potentially fatal illness that presents as hemorrhage, renal failure, and jaundice. Symptoms in milder disease include fever, malaise, muscle aches, and headache. Clinical recognition of leptospirosis is difficult because leptospires can affect many different organ systems, resulting in a nonspecific clinical presentation. Consequently, leptospirosis is often misdiagnosed as influenza, aseptic meningitis, encephalitis, dengue fever, hepatitis, or gastroenteritis. Effective treatment and preventive measures are available, if the disease is diagnosed in a timely manner.

Diagnosis is generally made by serologic testing, which is available from specialized reference laboratories, such as the CDC. The microscopic agglutination test (MAT) is the "gold standard" serologic test to which others are compared. Because a fourfold rise in titer between acute- and convalescent-phase samples is necessary for serologic confirmation, the MAT is not useful for guiding clinical management early in the course of the patient's illness.

Several serologic tests have recently been evaluated by the CDC, including a

microplate immunoglobulin M (IgM) ELISA (PanBio, Windsor, Brisbane, Australia; 61-7-3357-1177), an indirect hemagglutination assay (MRL Diagnostics, Cypress, Calif.), and an IgM dot ELISA dipstick test (DST; Integrated Diagnostics Inc., Baltimore, Md.), as rapid alternatives to the MAT (1). The CDC conclude that the ease of use and significantly high sensitivity and specificity of DST and ELISA make these good choices for diagnostic testing.

Leptospires are thin, spiral-shaped bacteria requiring special techniques for isolation. They are aerobic bacteria that can be cultivated at 30°C in media containing either rabbit serum (Fletcher's medium) or bovine serum albumin and fatty acids (*Leptospira* or Ellinghausen-McCullough-Johnson-Harris [EMJH] medium [4, 7]). See Appendix 3.14-1 for further descriptions of media. Growth is observed in broth in a few days to 4 weeks. Culture of spinal fluid and blood during the first week of illness and of urine after the first week can be helpful in confirming the diagnosis. However, rapid methods, such as PCR, are more helpful since culture is not timely for treatment of serious infections.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING

A. Specimen collection

1. CSF (collect during first week of illness)
2. Urine (collect during second week of illness)
 - a. Inoculate into culture medium within 1 h of collection *or*
 - b. Dilute 1:10 in 1% bovine serum albumin and store at 5 to 20°C for longer (a few days) stability (9).
3. Dialysis fluid

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING (continued)

4. Blood
 - a. Collect with sodium polyamethol sulfonate (SPS) or inoculate directly into culture medium.
 - b. In SPS, leptospires are stable for at least 6 days (9).
 - c. Protect from excessive heat or cold (5).
- B. Specimen timing
 1. Alert laboratory that specimen is being submitted for *Leptospira* culture, so that media can be available prior to collection and/or arrangements for PCR testing can be made.
 2. Collect blood and CSF only in the first week of illness, prior to initiation of treatment.
 3. Collect urine during the second week of illness or later. Organisms can be present for up to several months.
 4. Culture multiple specimens taken at least 1 day apart to increase likelihood of a positive result (6).
- C. Rejection criteria
 1. Urine received greater than 1 h after collection unless it has been diluted in albumin
 2. Blood or CSF from patients who have been symptomatic for greater than 10 days
 3. Direct dark-field smears are not recommended since protoplasmic extrusions are too easily mistaken for spirochetes (6).

III. MATERIALS

- A. Media
 1. Leptospira EMJH base and enrichment medium
 - a. Purchase dehydrated powdered media from BD Diagnostic Systems (3). Follow manufacturer's instructions.
 - b. Other manufacturers may refer to medium as bovine serum albumin-Tween 80 medium.
 - c. EMJH medium can be prepared in-house; see Appendix 3.14-1 (4, 7).
NOTE: Bovine serum albumin-Tween 80 medium is recommended (6).
 2. Fletcher's medium (3) (beef extract and peptone with rabbit serum and 1.5% agar) with and without 5-fluorouracil can be purchased in prepared tubes from Remel, Inc., or BD Diagnostic Systems.
 3. Dispense medium in 5-ml amounts into sterile screw-cap tubes.
 4. For urine specimens, add 5-fluorouracil to the medium to inhibit contaminants (2, 8).
 - a. Preparation of 5-fluorouracil
 - (1) Add 1.0 g of 5-fluorouracil to 50 ml of sterile distilled water.
 - (2) Add 1 to 2 ml of 2 N NaOH, and heat gently (less than 56°C) for 1 to 2 h to dissolve.
 - (3) Adjust to pH 7.4 with 1 N HCl, and bring volume to 100 ml with sterile distilled water.
 - (4) Filter sterilize, and store at 2 to 8°C.
 - b. Descriptions of this reagent do not give a shelf life (8). Keep for no longer than 1 year. Write expiration date on the label.
 - c. Add 0.1 ml of solution described above to 5 ml of medium for a final concentration of 200 µg/ml.
 5. Store medium at 2 to 8°C, or add 1.5 g of agar per liter to the base to make medium semisolid. Then store at room temperature.
 6. Shelf life is as long as 36 months for EMJH medium (5).
- B. Other supplies
 1. Fluorescent antiserum to *Leptospira* (Animal and Plant Health Inspection Service, U.S. Department of Agriculture, Ames, Iowa)
 2. Dark-field microscope
 3. Fluorescent microscope
 4. Incubator at 28 to 30°C

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

A. Organisms needed

1. *Leptospira interrogans* subsp. *canicola* (ATCC 23470)
2. *Leptospira interrogans* subsp. *grippotyphosa* (ATCC 23604)

B. Procedure

1. Inoculate *L. interrogans* subsp. *canicola* into one tube of medium and *L. interrogans* subsp. *grippotyphosa* into another tube of medium.
2. Incubate at 28 to 30°C (or at room temperature). Medium should give good growth with typical microscopic morphology by 7 days (3).
3. Check the performance of the medium every 6 months, or keep strains viable by subculturing 0.5 ml monthly to new media.

V. PROCEDURE



Observe standard precautions.

A. Inoculation

1. Process specimen as soon as received.

NOTE: Use of biosafety cabinet will avoid contamination of the culture or specimen as well as protect laboratory processing personnel.

2. Inoculate media.

a. Blood

- (1) Inoculate four tubes of bovine serum albumin-Tween 80 medium with 1 or 2 drops of fluid per tube (two tubes with 1 drop and two tubes with 2 drops).
- (2) If it is not possible to inoculate with freshly drawn blood, the blood sample can be taken with sodium oxalate or heparin as well as with SPS. Citrate may be inhibitory (10).

b. CSF or dialysis fluid

- (1) Inoculate up to 0.5 ml per 5-ml tube.
- (2) Inoculate several tubes if enough specimen is available, using various amounts of inoculum per tube.

c. Urine

- (1) Prepare 1:10 and 1:100 dilutions of urine with bovine serum albumin-Tween 80 medium. This will dilute out growth-inhibiting substances that may be present in the urine (9). It may also dilute out contaminating organisms.
- (2) For each of the following dilutions, inoculate two tubes of bovine serum albumin-Tween 80 medium.
 - (a) Urine undiluted, 1 drop per tube
 - (b) Urine diluted 1:10, 1 drop per tube
 - (c) Urine diluted 1:100, 1 drop per tube
- (3) Duplicate these inoculations with medium containing 5-fluorouracil.
- (4) Using this protocol, inoculate a total of 12 tubes for one urine specimen.

B. Incubate the tubes in the dark at 28 to 30°C (or at room temperature).

C. Using a fluorescent microscope, directly examine specimen with specific fluorescent antiserum, if available (Animal and Plant Health Inspection Service, U.S. Department of Agriculture).

D. Examination of cultures

1. Examine all culture tubes weekly for signs of growth (turbidity, haze, or a ring of growth).

V. PROCEDURE (continued)

2. Examine all tubes microscopically each week.
 - a. Take a small drop from 1 cm below the surface, and examine it with dark-field illumination. Use $\times 400$ magnification. Leptospires will be seen as tightly coiled spirochetes about 1 μm wide and 6 to 20 μm long. They rotate rapidly on their long axes and usually have hooked ends.
 - b. Positive culture tubes generally have a ring of growth (Dinger's ring) several centimeters below the surface of the semisolid medium.
3. If the specimen is positive, subculture one of the tubes to two tubes of fresh medium. Transfer about 0.5 ml taken from the area of growth.
 - a. Cultures can remain viable for at least 8 weeks in semisolid medium (9).
 - b. Submit positive cultures to a reference laboratory for confirmation and possible serologic typing.
 - c. Securely seal in tube of bovine serum albumin-Tween 80 medium for submission of culture.
4. Incubate cultures for 6 weeks before reporting them as negative.

POSTANALYTICAL CONSIDERATIONS**VI. REPORTING RESULTS**

- A. Report negative cultures as "No *Leptospira* isolated after 6 weeks."
- B. If all tubes inoculated with a specimen become contaminated before 6 weeks, report as "Culture contaminated; no further examination possible."
- C. Report positive cultures as "Leptospires isolated."
- D. Document notification of physician of positive findings.

VII. INTERPRETATION

- A. A positive culture indicates infection with the organism.
- B. A negative result does not rule out leptospirosis.
- C. Susceptibility testing is rarely performed. The treatments of choice are penicillin and doxycycline.

VIII. LIMITATIONS

- A. False-negative cultures can result from contamination of the urine with other microbiota.
- B. Culture is the definitive test but has a low sensitivity and may take up to 6 weeks for a positive result.

REFERENCES

1. Bajani, M. D., D. A. Ashford, S. L. Bragg, C. W. Woods, T. Aye, R. A. Spiegel, B. D. Plikaytis, B. A. Perkins, M. Phelan, P. N. Levett, and R. S. Weyant. 2003. Evaluation of four commercially available rapid serologic tests for diagnosis of leptospirosis. *J. Clin. Microbiol.* **41**:803–809.
2. Blair, E. B. 1970. Media, test procedures and chemical reagents, p. 791–857. In H. L. Bodily, E. L. Updyke, and J. O. Mason (ed.), *Diagnostic Procedures for Bacterial, Mycotic and Parasitic Infections*, 5th ed. American Public Health Association, New York, N.Y.
3. Difco Laboratories. 1984. *Difco Manual*, 10th ed., p. 513. Difco Laboratories, Detroit, Mich.
4. Ellinghausen, H. C., Jr., and W. G. McCullough. 1965. Nutrition of *Leptospira pomona* and growth of 13 other serotypes: fractionation of oleic albumin complex (OAC) and a medium of bovine albumin and polysorbate 80. *Am. J. Vet. Res.* **26**:45–51.
5. Ellinghausen, H. C., Jr., A. B. Thiermann, and C. R. I. Sulzer. 1981. Leptospirosis, p. 463–499. In A. Balows and W. J. Hausler, Jr. (ed.), *Diagnostic Procedures for Bacterial, Mycotic and Parasitic Infections*, 6th ed. American Public Health Association, Washington, D.C.
6. Johnson, R. C. 1982. Leptospirosis. *Clin. Microbiol. Newslet.* **4**:113–116.
7. Johnson, R. C., and V. G. Harris. 1967. Differentiation of pathogenic and saprophytic leptospires. 1. Growth at low temperatures. *J. Bacteriol.* **94**:27–31.
8. Johnson, R. C., and P. Rogers. 1964. 5-Fluorouracil as a selective agent for growth of leptospires. *J. Bacteriol.* **87**:422–426.
9. Weyant, R. S., S. L. Bragg, and A. F. Kauffmann. 1999. *Leptospira* and *Leptonema*, p. 739–745. In P. R. Murray, E. J. Baron, M. A. Pfaffer, F. C. Tenover, and R. H. Yolken (ed.), *Manual of Clinical Microbiology*, 7th ed. ASM Press, Washington, D.C.
10. Wolff, J. W. 1954. *The Laboratory Diagnosis of Leptospirosis*. Charles C Thomas, Publisher, Springfield, Ill.

SUPPLEMENTAL READING

- Binder, W. D., and L. A. Mermel.** 1998. Leptospirosis in an urban setting: case report and review of an emerging infectious disease. *J. Emerg. Med.* **16**:851–856.
- Farr, R. W.** 1995. Leptospirosis. *Clin. Infect. Dis.* **21**:1–6.
- Levett, P. N.** 2001. Leptospirosis. *Clin. Microbiol. Rev.* **14**:296–326.

APPENDIX 3.14-1***Leptospira* Ellinghausen-McCullough-Johnson-Harris Base and Enrichment Medium (1, 2)****A. Basal medium****1. Ingredients**

Na ₂ HPO ₄ , anhydrous	1.0 g
KH ₂ PO ₄ , anhydrous	0.3 g
NaCl	1.0 g
NH ₄ Cl, 25% aqueous	1.0 ml
thiamine hydrochloride, 0.5% aqueous	1.0 ml
sodium pyruvate, 10% aqueous	1.0 ml
glycerol, 10% aqueous	1.0 ml
distilled water	996.0 ml

2. Procedure

- Dissolve salts in 996 ml of distilled water, add stock solutions (ammonium chloride, thiamine hydrochloride, sodium pyruvate, and glycerol), and adjust to pH 7.4.
- Autoclave at 121°C for 20 min.

B. Albumin-fatty acid supplement**1. Ingredients**

bovine albumin fraction V	20.0 g
CaCl ₂ ·2H ₂ O, 1.0% aqueous	2.0 ml
MgCl ₂ ·6H ₂ O, 1.0% aqueous	2.0 ml
ZnSO ₄ ·7H ₂ O, 0.4% aqueous	2.0 ml
CuSO ₄ ·5H ₂ O, 0.3% aqueous	0.2 ml
FeSO ₄ ·7H ₂ O, 0.5% aqueous	20.0 ml
vitamin B ₁₂ , 0.2% aqueous	2.0 ml
Polysorbate (Tween) 80, 10% aqueous ...	25.0 ml
distilled water	to 200.0 ml

2. Procedure

- NOTE:** Aqueous stock solutions must be prepared prior to formulation of medium.
- Add the bovine albumin slowly, with careful stirring to avoid foaming, to 100 ml of water.
 - Slowly add the remaining ingredients, stirring constantly.
 - Adjust the pH to 7.4, and add water to make a 200-ml final volume.
 - Filter sterilize through a Seitz or membrane filter (0.2- to 0.3-μm porosity).

C. Prepare the complete medium by combining 1 part supplement with 9 parts basal medium.**References**

- Ellinghausen, H. C., Jr., and W. G. McCullough.** 1965. Nutrition of *Leptospira pomona* and growth of 13 other serotypes: fractionation of oleic albumin complex (OAC) and a medium of bovine albumin and polysorbate 80. *Am. J. Vet. Res.* **26**:45–51.
- Johnson, R. C., and V. G. Harris.** 1967. Differentiation of pathogenic and saprophytic leptospires. 1. Growth at low temperatures. *J. Bacteriol.* **94**:27–31.

Mycoplasma pneumoniae, *Mycoplasma hominis*, and *Ureaplasma* Cultures from Clinical Specimens

[Updated March 2007]

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Mycoplasma pneumoniae is a common cause of upper and lower respiratory infections in persons of all ages. Tracheobronchitis is the most common clinical syndrome, but pneumonia commonly occurs and extragenital infections have been described. A review of human disease caused by *M. pneumoniae* (2) contains more detailed information about clinical aspects of infections due to this organism.

Specimens obtained from the upper and lower respiratory tract are appropriate to culture for the presence of *M. pneumoniae*. Since the organism has also been known to cause invasive disease in other body sites, it may also be appropriate in some circumstances to culture sterile body fluids such as CSF, synovial fluid, or other types of clinical specimen, depending on the type of infection suspected. Growth and presumptive identification of *M. pneumoniae* can be accomplished by demonstration of glucose hydrolysis in SP-4 broth and development of spherical 10- to 100-µm colonies on SP-4 agar media after 4 to 20 days or more of incubation. Definitive organism identification has been accomplished using a variety of tests such as hemadsorption, tetrazolium reduction, or growth inhibition in the presence of specific antiserum. More recently, the PCR assay has been used to distinguish *M. pneumoniae* from several species of commensal mycoplasmas that often inhabit the human respiratory tract. Since culture may not detect *M. pneumoniae* in some specimens, alternative methods such as the

PCR assay and serologic testing should be considered for optimum detection, even if culture is attempted.

Mycoplasma hominis and *Ureaplasma* species are the predominant mycoplasmal species recoverable by culture from the human urogenital tract. Even though these organisms may occur as commensals, they have been associated with a number of human urogenital infections in adults and can also cause extragenital infections involving the lungs and sterile body sites such as synovial fluid or CSF, most commonly in immunosuppressed children or adults or in preterm neonates. Refer to recent reviews (3) and reference texts (4) for more detailed descriptions of specific diseases that may be caused by these organisms. Although other potentially pathogenic species such as *Mycoplasma fermentans* and *Mycoplasma genitalium* may occur in some settings, their cultivation conditions are not well established and their detection is more readily achieved by non-culture-based methods such as the PCR assay. Therefore, cultivation techniques for them are not described in this handbook. The species *Ureaplasma urealyticum* (the only pathogenic *Ureaplasma* of humans) was recently divided into two separate species, *Ureaplasma urealyticum* and *Ureaplasma parvum* sp. nov. However, it is not practical or necessary to distinguish between them for diagnostic purposes.

A single liquid growth medium such as 10B broth can be used for cultivation of

M. hominis and *Ureaplasma* species. Evidence of growth in liquid medium is based on an alkaline shift due to arginine hydrolysis by *M. hominis* or urea by *Ureaplasma* species. Definitive identification of *Ureaplasma* spp. can be established based on characteristic 15- to 60-µm brown granular colonies that develop in 1 to 3 days. Presumptive identification of *M. hominis* can be determined by typical 200- to 300-µm “fried-egg” colonies that develop in 2 to 4 days, but definitive species identification requires additional tests such as growth inhibition with homologous antiserum or the PCR assay (see procedure 12.2.3, part 10).

A variety of serologic assays are commercially available to test for the presence of antibodies against *M. pneumoniae*. Due to the organism’s slow growth in culture and the limited availability of PCR in diagnostic laboratories, demonstration of seroconversion (fourfold change in titer) using quantitative assays for immunoglobulin M (IgM) and IgG antibodies has been the primary means for diagnosis of *M. pneumoniae* respiratory infections. Commercially available serologic tests for *M. pneumoniae* include both qualitative and quantitative assays and utilize EIA, immunofluorescence, and particle agglutination technologies. These products are discussed in greater detail elsewhere (5, 6). Evaluation of commercial diagnostic kits and media can be found in Cumitech 34 (5).

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING



Observe standard precautions.

■ **NOTE:** Refer to procedure 3.3.1 for additional details. The following can be copied for preparation of a specimen collection instruction manual for physicians and caregivers. It is the responsibility of the laboratory director or designee to educate physicians and caregivers on proper specimen collection.

A. Specimen collection

1. Fluid specimens (e.g., pleural fluid, bronchoalveolar lavage fluid, sputum, endotracheal aspirates, CSF, synovial fluid, amniotic fluid, pouch of Douglas fluid, semen, prostatic secretions, urine)

■ **NOTE:** Urethral swabs from men and vaginal or cervical swabs from women are preferred over urine specimens for detection of genital mycoplasmas.

- a. Collect in a sterile container.
- b. If greater than a 1-h delay in inoculation is anticipated, and/or volume of fluid obtained is very small and susceptible to drying, as in the case of neonatal endotracheal aspirates, place fluid in 0.3 to 1.5 ml of transport medium, described below and in Appendix 3.15–1.
- c. Collect urine specimens from women by urethral catheterization, for more meaningful result. A first-void urine specimen should be collected from men with nongonococcal urethritis.

2. Blood

- a. Collect in a vial of mycoplasma broth culture medium without anticoagulant in at least a 1:5 or 1:10 ratio.
- b. Collect at least 10 ml for adults, but smaller volumes comparable to those obtained for conventional bacterial blood cultures are acceptable for neonates and children.
- c. Do not use any medium containing sodium polyanetholsulfonate (SPS) anticoagulant, which is present in most commercial blood culture bottles, since it is inhibitory to mycoplasmas and ureaplasmas.

3. Tissues (e.g., lung, placenta, endometrium, fallopian tube, bone chips, and urinary calculi)

Collect in a sterile container with sufficient transport medium to prevent drying.

4. Swab specimens (e.g., nasopharynx, throat, urethra, vagina, cervix, wound)

- a. Use only Dacron or polyester swabs with aluminum or plastic shafts.

(1) Do not use cotton swabs because the fibers and wooden sticks are inhibitory to mycoplasmas.

(2) Do not use vaginal lubricants or antiseptics prior to collection of urogenital specimens, as they are inhibitory to mycoplasmas.

- b. Collect throat, nasopharyngeal, vaginal, or wound specimens by swabbing back and forth over the mucosa or surface of wound to maximize recovery of cells.

■ **NOTE:** Vaginal swabs are somewhat more likely to contain genital mycoplasmas and ureaplasmas than cervical or urethral swabs from women.

- c. Collect specimens from the male or female urethra or female cervix by inserting a urethral swab at least 1 cm into the urethra and rotating it 360°.

- d. After collection of swab specimen, swirl swab in a vial containing transport medium, express excess fluid by pressing the swab against the inside of the vial, and then discard swab.

B. Timing of specimen collection

1. Collect early-morning specimens of expectorated sputum.
2. Obtain all specimens prior to initiation of antimicrobial therapy when possible.

**II. SPECIMEN COLLECTION,
TRANSPORT, AND HANDLING**
*(continued)***C. Specimen transport**

1. Transport specimens to the laboratory as soon as possible because of the fastidious nature of mycoplasmas and potential loss of mycoplasmal viability if specimens are allowed to dry out.
 - a. Add 0.3 to 1.5 ml of transport medium to original fluid specimens if more than 1 h is likely to elapse before a specimen can be transported to the laboratory. Always collect swab specimens, blood, and tissues in transport medium.
 - b. Refrigerate specimen in transport medium at 4°C if delay in transportation to the laboratory is anticipated, but this should be kept to a minimum.
 - c. Freeze the original specimen in transport medium at -70°C or in liquid nitrogen if specimen will be shipped to a reference laboratory for processing or if storage will exceed 24 h.
 - d. Ship frozen specimens in transport medium on dry ice to a reference laboratory for processing.
 - e. Do not store at -20°C for even short periods, since this will result in loss of viability.
2. Transport media
 - a. Broths used for myoplasmal cultivation are most desirable for use as transport media. Refer to Appendix 3.15-1 for instructions for preparing these media.
 - (1) 10B or SP-4 (Remel, Inc.) for genital mycoplasmas
 - (2) SP-4 (Remel, Inc.) for *M. pneumoniae*
 - b. Other commercially available media for genital mycoplasmas
 - (1) Mycotrans (Irvine Scientific, Santa Ana, Calif., [800] 437-5706, <http://www.irvinesci.com>)
 - (2) A3B (Remel, Inc.)
 - (3) Arginine broth (Remel, Inc.)
 - c. For transport of culture specimens and for sample preparation for PCR assays
 - (1) 2-SP with 10% (vol/vol) heat-inactivated fetal calf serum with 0.2 M sucrose in 0.02 M phosphate buffer (pH 7.2)
 - (2) TSB with 0.5% (vol/vol) bovine serum albumin
3. Limits at receiving site
 - (1) Process all specimens immediately after receipt in the laboratory.
 - (2) If this is not possible, add transport medium to the specimen, if not already present, and store as described above.

D. Request submission

In addition to the usual essential information, including identifying information for the patient and body site cultured, clearly specify which organisms are to be sought (e.g., *M. pneumoniae* or genital mycoplasmas), since cultivation procedures differ.

E. Rejection criteria

1. Perform diagnostic cultures for mycoplasmas for patients who are suspected of having a condition known to be associated with or caused by these organisms, but not in circumstances in which there has been no such association. Screening the lower urogenital tract of asymptomatic adults for genital mycoplasmas is not recommended.
2. Reject specimens received in the laboratory >24 h after collection that have not been frozen at -70°C in transport medium, because the likelihood of finding viable mycoplasmas is significantly reduced.
3. If time of specimen collection and/or storage conditions cannot be documented after consultation with the ordering physician or clinic from which the specimen was obtained, do not process the specimen.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING

(continued)

4. If an improperly collected, transported, or handled specimen cannot be replaced, and it is deemed critical for patient management, perform the culture and document in the final report that specimen quality may have been compromised in the event that no organisms are detected.
5. Do not process blood, tissues, or swab specimens that are not received in transport medium.

III. MATERIALS

- A. Media (from Remel, Inc., or see instructions for medium preparation and formulations in Appendix 3.15-1)**
1. SP-4 broth and agar—a glucose-based medium appropriate for cultivation of *M. pneumoniae* and *M. hominis*
 - **NOTE:** This medium can also be used for cultivation of the more fastidious species, e.g., *M. genitalium* and *M. fermentans*, but as stated earlier, non-culture-based techniques such as the PCR assay are recommended for detection of these organisms.
 - a. Add agar to SP-4 broth for preparation of solid medium.
 - b. Add arginine as a metabolic substrate if *M. hominis* is being sought.
 2. 10B broth—an enriched urea- and arginine-containing medium for cultivation of ureaplasmas and *M. hominis*
 - a. Add clindamycin or lincomycin (10 µg/ml) to make selective for ureaplasmas.
 - b. Add erythromycin (10 µg/ml) to select for *M. hominis*.
 3. A8 agar—a differential agar medium useful for isolating *M. hominis* and ureaplasmas
 - **NOTE:** Urea and CaCl₂ are provided to allow differentiation of ureaplasmas from non-urea-hydrolyzing mycoplasmas.
- B. Diagnostic kits (Irvine Scientific)**
1. Mycoscreen GU for genital mycoplasmas
 2. Mycotrim GU triphasic flask system for genital mycoplasmas
 3. Mycotrim RS for *M. pneumoniae*
 - **NOTE:** Consult the *Manual of Commercial Methods in Clinical Microbiology* (6) and *Cumitech 34* (5) for a more comprehensive discussion of these products.
- C. Stain reagents**
1. Direct specimens
 - **NOTE:** Mycoplasmas lack a cell wall and are not visualized by Gram stain.
- a. Giemsa stain for examination of body fluids. Results can be difficult to interpret because debris and artifacts can be confused with mycoplasmas because of their very small size.**
- b. DNA fluorochrome stains, such as Hoechst 33258 (ICN Biomedicals, Inc., Costa Mesa, Calif., [800] 854-0530), for clinical specimens or culture do not distinguish mycoplasmas from other bacteria. Refer to procedure 3.2.2 for acridine orange stain.**
- 2. Stains to distinguish mycoplasmal colonies on agar from artifacts (Sigma Diagnostics, St. Louis, Mo.)**
- a. Diene's methylene blue stain—10% in distilled water or ethanol
 - b. Neutral red stain
- 3. Urease stain for ureaplasmas**
- a. Add 0.2 ml of CaCl₂ solution to isolated colonies.
 - b. Observe whether colonies change from colorless to dark brown within 15 min.
 - **NOTE:** Incorporation of CaCl₂ directly into A8 agar eliminates the need for a separate test to detect urease production to identify colonies as *Ureaplasma* spp.
- D. Strains**
- Purdue University Mollicutes Collection contains a large variety of ATCC type strains, clinical isolates, and diagnostic reagents for use in mycoplasma culture.
- Contact:
Maureen Davidson, Ph.D.,
Associate Scientist
Department of Veterinary
Pathobiology
School of Veterinary Medicine
Purdue University
725 Harrison St.
Room VPRB B 13
West Lafayette, IN 47907-2027
Phone: (765) 496-6753
Fax: (765) 496-2627
E-mail: mkdAVIDS@purdue.edu

III. MATERIALS (continued)**E. Immunological reagents**

Plain and fluorescein isothiocyanate-conjugated antisera for the identification of mollicutes can be obtained from the University of Florida Mollicutes Collection.

F. Other supplies

1. Pipetting device and sterile pipette tips
2. Sterile 2-ml screw-cap vials suitable for freezing organisms isolated in culture

3. Racks for broth tubes

4. Parafilm, American National Can
5. Candle jar or anaerobe jar with GasPak (BD Diagnostic Systems, Sparks, Md.) if CO₂ incubator is unavailable

6. Fluorescent microscope for fluorescent stains

ANALYTICAL CONSIDERATIONS**IV. QUALITY CONTROL****A. Shelf life**

1. Store broths and agars containing inhibitory antimicrobials at 4°C, where they should usually provide satisfactory growth parameters over a 4-week period.
 2. Store broths in tubes that are tightly capped.
 3. Store agar plates in sealed plastic bags.
- **NOTE:** Some commercial broth media are available in small lyophilized vials designed to be used for individual cultures that can be rehydrated as needed. This can substantially prolong shelf life.

B. Perform sterility checks on all new batches or lots of broth and agar by overnight incubation of an aliquot of broth and an agar plate at 37°C. Note and record the appearance of agar plates and verify clarity, surface, and color of media.**C. If any of the commercial media or kits are used for diagnostic purposes**

1. Confirm with the manufacturer the extent of QC performed prior to shipment.
2. Perform rigorous QC on-site, since many products have not been subjected to extensive comparative studies to document their abilities to detect mycoplasmas in clinical specimens.

D. QC testing of a new batch of broth

1. Include type strains of each species to be cultivated in the broth, a low-passage clinical isolate of each species, and a minimum of two strains of *Ureaplasma* species. Recommended choices are serotypes 3 and either 5, 7, or 8 since some ureaplasmas are more fastidious than others. Some choices include the following.
 - a. *Ureaplasma* serotype 3 ATCC 27815
 - b. *Ureaplasma* serotype 5 ATCC 27817
 - c. *Ureaplasma* serotype 7 ATCC 27819
 - d. *Ureaplasma* serotype 8 ATCC 27618
 - e. *M. hominis* ATCC 23114
 - f. *M. pneumoniae* ATCC 15531
2. Thaw frozen stock cultures of the desired organisms, mix on a vortex mixer, and serially dilute each strain 1:10 (0.1 ml of culture into 0.9 ml of broth) in the reference and the new batch out to 10⁻⁹.
3. Incubate broths at 37°C under atmospheric conditions for up to 72 h for *M. hominis* and *Ureaplasma* spp. and 7 days for *M. pneumoniae*.
4. Compare the number of tube dilutions with color change (color changing units) between the reference broth and the new batch.
5. Consider new lots or batches of broth satisfactory if the numbers of all strains tested grow within 10-fold (1 dilution) of the number in the reference batch.

IV. QUALITY CONTROL (continued)

6. If growth in the new broth is >1 dilution less than the reference batch, repeat the test. If results are still outside the acceptable range, do not use the broth for specimens.
7. Maintain records of all media QC tests (refer to section 14 for general QC principles).
- E. QC testing of a new batch of agar
 1. Include the same assortment of mycoplasmas and ureaplasmas and record keeping as described above for testing broths.
 2. Use one new and one reference plate for each group of three strains to be tested.
 3. Prewarm agar plates bottom side up for 5 to 10 min in a 37°C incubator prior to testing.
 4. Thaw organisms as described above and serially dilute (0.1 ml of culture into 0.9 ml of broth) in the appropriate broth (e.g., 10B when checking A8 agar and SP-4 when checking SP-4 agar) so that there will be approximately 30 to 300 colonies on each plate after incubation. The number of dilutions necessary to yield plates with this colony density will depend on the number of organisms in the original culture. Plating the 10^{-2} , 10^{-3} , and 10^{-4} dilutions will usually yield this number of colonies.
 5. Plate 0.02 ml of the 10^{-2} to 10^{-4} dilutions of each strain onto the test agar and the reference agar, allow to dry, and incubate at 37°C under 5% CO₂ in air. Colonies of *M. hominis* and *Ureaplasma* spp. may be visible in 48 to 72 h, whereas *M. pneumoniae* will take 7 days or more.
 6. Count colonies and determine the number of CFU per milliliter.
 7. Calculate the percentage of growth on new medium compared to old medium by dividing the CFU per milliliter of the new test plate by that of the old test plate. Agar plates should differ by not more than 1/2 log in the number of colonies from the reference batch.
 8. If >1/2-log difference occurs for any of the test strains, repeat the QC test. If a second test is still out of the acceptable range, do not use the agar for diagnostic purposes.

V. PROCEDURES

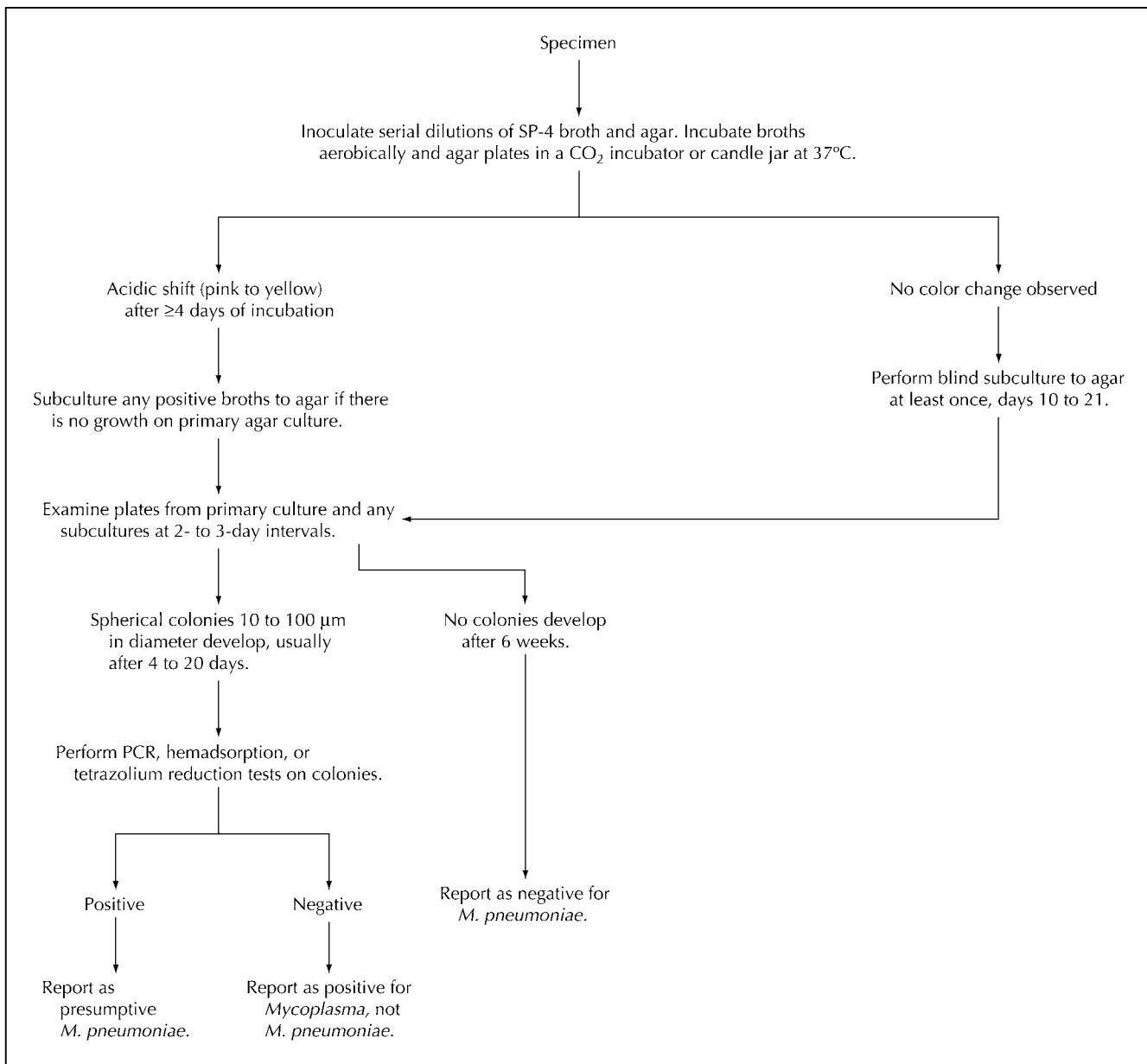
■ NOTE: Refer to Fig. 3.15–1 for flowchart descriptions of culture techniques for *M. pneumoniae* and Fig. 3.15–2 for *M. hominis* and *Ureaplasma* spp.

- A. Direct nucleic acid amplification by the PCR assay is important for detection of *M. pneumoniae* in clinical specimens, since culture may fail to detect the organism in some instances even when done properly.

■ NOTE: Laboratories may perform in-house PCR testing for *M. pneumoniae*, and procedures using available primers have been described and are included in procedure 12.2.3, part 10. Alternatively, commercial multiplex PCR assays have recently become available for the detection of *M. pneumoniae* and *Chlamydia pneumoniae* (ProPneumono-1) (Prodesse Inc.). The Pneumoplex (Prodesse Inc.) PCR assay is also available and detects the previous respiratory pathogens as well as *Legionella pneumophila*, *Legionella micdadei*, and *Bordetella pertussis* (1). Another multiplex PCR assay will also become available in 2006 (ProPneumo-2), which detects *M. pneumoniae*, *C. pneumoniae*, and *L. pneumophila*. Nonamplified direct antigen detection kits for mycoplasmas or ureaplasmas are no longer available commercially in the United States.
- B. Culture methods
 1. Inoculation
 - a. Mix fluid specimens by gently rotating the container and then centrifuge at 600 × g for 15 min. Use the pellet for medium inoculation.



It is imperative that these cultures be handled in a biosafety hood.

Figure 3.15-1 Algorithm for isolation of *M. pneumoniae* from clinical specimens.

V. PROCEDURES (continued)

- b. Mince tissues with a sterile scalpel blade prior to inoculation. Mincing is preferable to grinding since it minimizes release of tissue inhibitors that may affect growth.
- c. Perform serial 10-fold dilutions (0.1 ml of specimen into 0.9 ml of appropriate broth) out to at least 10^{-3} in order to overcome inhibitors that may be present in original specimen.
- d. Label each plate with patient's name and laboratory accession number.
- e. Mark the bottom of the appropriate agar plate with a felt tip pen into at least four sections: one for the original specimen and three for the three 10-fold dilutions.
- f. Inoculate agar plate with 0.02 ml of the original specimen and each dilution.
- g. Seal agar plates with Parafilm to prevent desiccation.

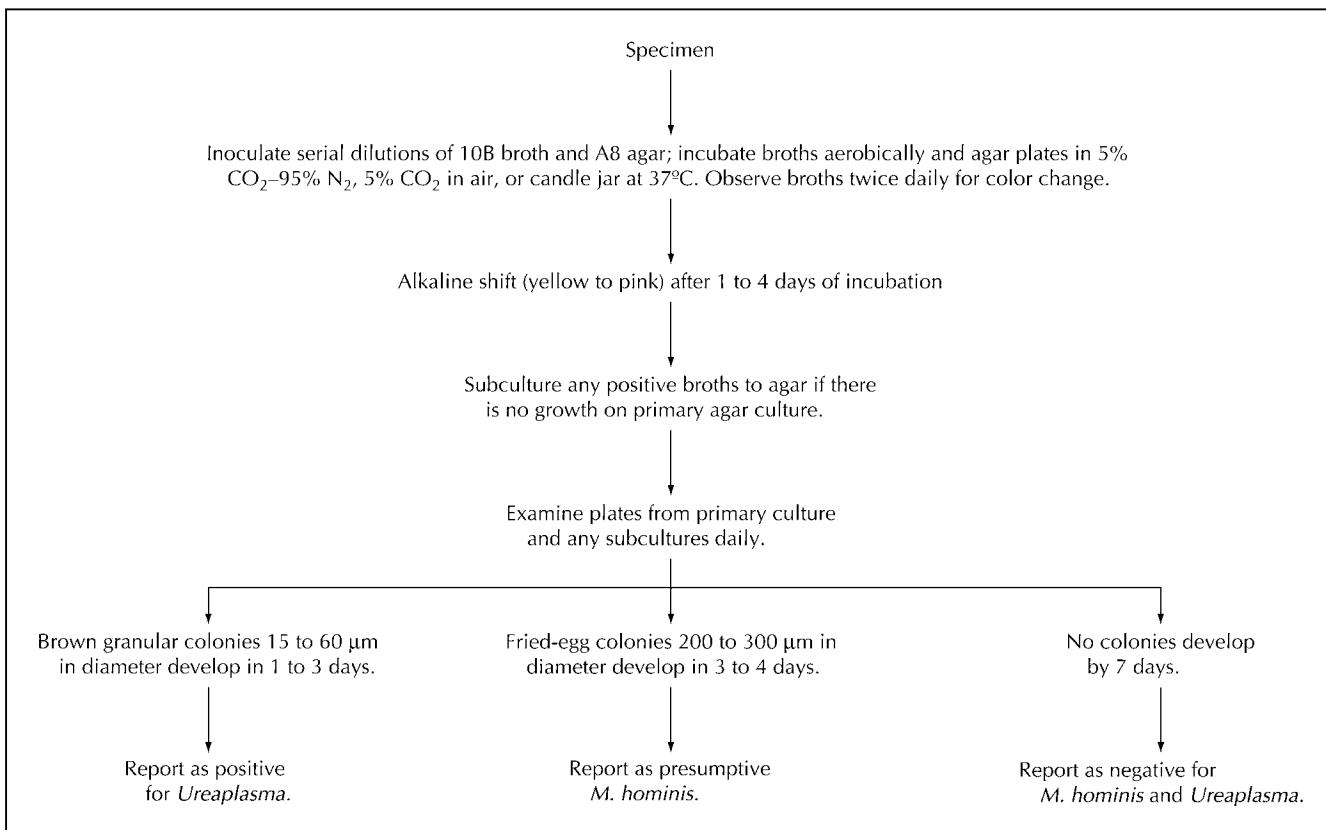


Figure 3.15–2 Algorithm for isolation of *M. hominis* and *Ureaplasma* spp. from clinical specimens.

V. PROCEDURES (continued)

2. Incubation

- a. Incubate broths at 37°C under atmospheric conditions in a humidified incubator. Agitate broths on a mechanical rotator to shorten time by about 1 day for detection of *M. pneumoniae*.
- b. Incubate agar plates for genital mycoplasmas at 37°C in an atmosphere of 95% N₂-5% CO₂ or room air plus 5% CO₂. Incubate agar plates for *M. pneumoniae* in room air plus 5% CO₂. Use candle jars or anaerobe jars with a CO₂-generating system, if a CO₂ incubator is not available.

3. Examination of culture media

- a. *M. pneumoniae* cultures
 - (1) Visually inspect SP-4 broth cultures daily for acidic shift (pink to yellow).
 - (2) Subculture 0.02 ml of positive SP-4 broths to SP-4 agar if there has been no growth on primary agar culture, and incubate as described above.
 - (3) Perform blind subculture of broth to agar at least once after 10 to 21 days of incubation if no color change has occurred.
 - (4) Using a stereomicroscope, examine agar plates from primary culture and any subcultures at 2- to 3-day intervals for the presence of spherical colonies of *M. pneumoniae* that are 10 to 100 µm in diameter (Fig. 3.15–3).
 - (5) Incubate specimens for 6 weeks before discarding.
- b. *M. hominis* and *Ureaplasma* cultures
 - (1) Visually inspect 10B broths daily for basic shift (yellow to pink).

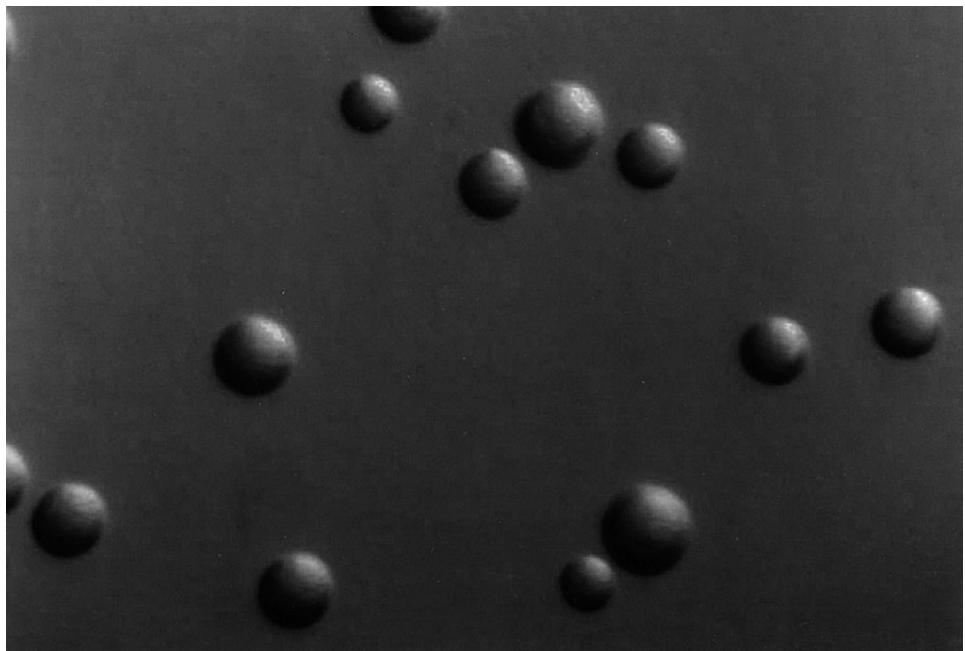


Figure 3.15–3 Spherical colonies of *M. pneumoniae* up to 100 μm in diameter growing on SP-4 agar. Magnification, $\times 126$.

V. PROCEDURES (continued)

- (2) Subculture 0.02 ml of positive broths to A8 agar if there is no growth on primary agar culture.
- (3) Using a stereomicroscope, examine agar plates from primary culture and any subcultures daily.
 - (a) Brown granular colonies 15 to 60 μm in diameter characteristic of *Ureaplasma* spp. develop in 1 to 3 days (Fig. 3.15–4).
 - (b) Fried-egg colonies 200 to 300 μm in diameter typical of *M. hominis* develop in 2 to 4 days (Fig. 3.15–4).
- (4) Incubate plates for 7 days before discarding.
4. Further workup of positive cultures
 - a. Enumerate each type of mycoplasma or ureaplasma present by colony counts, and record results.
 - b. Perform procedures as described below if definitive species identification is desired for large-colony mycoplasmas.
5. Freeze an aliquot of the original specimen at -70°C in case additional procedures are necessary.
6. Freeze any positive mycoplasma broths detected by culture at -70°C in case further workup is required or antimicrobial susceptibility tests are to be performed.

NOTE: It is important to freeze genital mycoplasmas soon after color change occurs in broth due to a rapid death phase in liquid medium.

 - a. To remove mycoplasmal colonies from agar for subculture or freezing, use a sterile scalpel or Pasteur pipette to cut out a block of agar that contains the colonies.
 - b. Immerse block in a small vial containing approximately 1 to 1.5 ml of broth, and mix on a vortex mixer.
 - c. Incubate or freeze the broth, depending on the need.
7. If broth is turbid, indicating bacterial contamination, filter original liquid specimens through a 0.45- μm -pore-size filter to remove bacteria and allow the smaller mycoplasmas to pass through. Inoculate media and repeat culture.

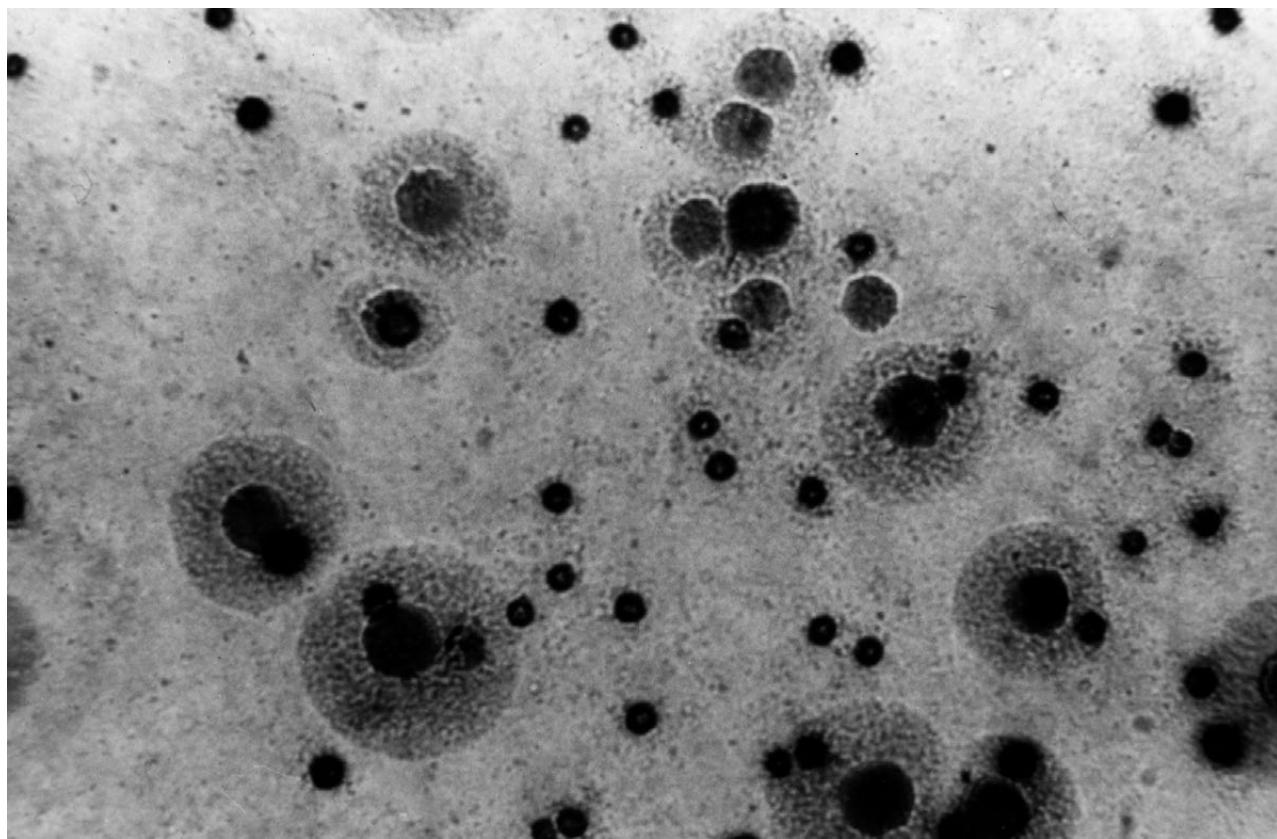


Figure 3.15-4 Small colonies typical of *Ureaplasma* spp. and larger fried-egg colonies of *M. hominis*. Diene's stain; magnification, $\times 100$. Reprinted from M. G. Gabridge. 1981. *Pathogenic Mycoplasma*, part 2, no. 6702 (teaching set). American Society for Microbiology, Washington, D.C.

POSTANALYTICAL CONSIDERATIONS

VI. REPORTING RESULTS

- A. Report presumptive *M. pneumoniae* based on spherical colonies that will be evident on SP-4 agar after 4 to 20 days or more of incubation and on glucose utilization. Definitive species identification requires supplemental tests.
 1. Perform additional procedures, such as tetrazolium reduction, hemadsorption, or PCR assay, for definitive identification of *M. pneumoniae* to distinguish it from commensal mycoplasmas that commonly inhabit the respiratory tract.
 2. Refer isolate to a reference laboratory or see procedures for PCR assay (procedure 12.2.3, part 10) and hemadsorption activity (Appendix 3.15-2).
- B. Report at the genus level for *Ureaplasma* spp. Definitive identification sufficient for clinical purposes is based on characteristic granular brown colonies on A8 agar within 1 to 3 days of incubation, due to urease production in the presence of the CaCl_2 indicator in A8 agar.
- C. Report presumptive *M. hominis* based on fried-egg colonies on A8 agar within 2 to 4 days of incubation and on arginine utilization. Definitive identification requires additional procedures such as PCR, agar growth inhibition in the presence of homologous antisera, or colony epiimmunofluorescence. Refer to procedure 12.2.3, part 10, or submit to reference laboratory for confirmation.

VI. REPORTING RESULTS (continued)

- D. Identification to the species level of large-colony mycoplasmas is generally not clinically indicated, unless the infection occurs in a normally sterile site such as CSF and/or the patient is immunocompromised. Submit organisms to a reference laboratory, such as the UAB Diagnostic Mycoplasma Laboratory (University of Alabama at Birmingham, Birmingham, Ala., [205] 934-9142) unless a laboratory has molecular diagnostic capabilities for performance of the PCR assay.
- E. Document all testing in hard copy and computerized work card, according to the laboratory information system in use.

VII. INTERPRETATION

- A. Isolation of *M. pneumoniae* from respiratory tract specimens is clinically significant in most instances, but it should be correlated with the presence of clinical respiratory disease since a small portion of asymptomatic carriers may exist. Detection of *M. pneumoniae* in normally sterile body fluids is always clinically significant.
- B. Isolation of ureaplasmas in any quantity in normally sterile body fluids or tissue is significantly associated with disease. The presence of fewer than 10^4 organisms in the male urethra is less likely to be significant.
- C. Isolation of *M. hominis* in any quantity in normally sterile body fluids or tissue is significantly associated with disease, but reporting the quantities of organisms present in nonsterile body sites may be of value in interpreting the significance of its presence.
- D. Treatment considerations (see Appendix 3.15–3 for methods)
 - 1. *M. pneumoniae*: macrolides, including erythromycin, azithromycin, and clarithromycin; tetracyclines; fluoroquinolones, including levofloxacin, moxifloxacin, and gatifloxacin
 - 2. *M. hominis*: clindamycin; fluoroquinolones, including levofloxacin, moxifloxacin, and gatifloxacin; tetracyclines (some strains may be resistant)
 - 3. *Ureaplasma* spp.: macrolides, including erythromycin, azithromycin, and clarithromycin; tetracyclines (some strains may be resistant); fluoroquinolones, including levofloxacin, moxifloxacin, and gatifloxacin

VIII. LIMITATIONS

- A. Culture is the method of choice for detection of organisms such as *M. hominis* and *Ureaplasma* spp. that can be isolated rapidly and easily from clinical specimens, and it has the advantage of being able to provide quantitative results and an isolate for antimicrobial susceptibility testing.
- B. Culture alone is not optimum for fastidious and/or slow-growing species such as *M. genitalium*, *M. fermentans*, and, to some degree, *M. pneumoniae*. Serology has been used for many years for detection of *M. pneumoniae* infection, and more recently PCR assays have been developed for human mycoplasmal species of clinical importance.
- C. In some circumstances, particularly in systemic infections of sterile sites such as CSF or synovial fluid, for immunocompromised hosts or neonates it may be appropriate to collect a follow-up specimen after treatment has been initiated or completed to determine whether the mycoplasma is still present. This may be particularly relevant in the event that the patient has not responded to antimicrobial therapy in a satisfactory manner and because mycoplasmas tend to produce chronic infections that can be difficult to eradicate.
- D. Due to the fastidious nature of mycoplasmas, it is not uncommon for batches of culture medium to fail to support the growth of some strains. If this occurs, it is necessary to systematically evaluate each individual component of the medium, if self-prepared, and to choose a different lot if medium is commercially obtained.

REFERENCES

1. Khanna M., J. Fan, K. Pehler-Harrington, C. Waters, P. Douglass, J. Stallock, S. Kehl, and K. J. Henrickson. 2005. The Pneumoplex assays, a multiplex PCR-enzyme hybridization assay that allows simultaneous detection of five organisms, *Mycoplasma pneumoniae*, *Chlamydia pneumoniae*, *Legionella pneumophila*, *Legionella micdadei*, and *Bordetella pertussis*, and its real-time counterpart. *J. Clin. Microbiol.* **43**:565–571.
 2. Talkington, D. F., K. B. Waites, S. B. Schwartz, and R. E. Besser. 2002. Emerging from obscurity: understanding pulmonary and extrapulmonary syndromes, pathogenesis, and epidemiology of human *Mycoplasma pneumoniae* infections, p. 57–84. In W. M. Scheld, W. A. Craig, and J. M. Hughes (ed.), *Emerging Infections 5*. ASM Press, Washington, D.C.
 3. Taylor-Robinson, D. 1996. Infections due to species of *Mycoplasma* and *Ureaplasma*: an update. *Clin. Infect. Dis.* **23**:671–684.
 4. Waites, K. B., Y. Rikihisa, and D. Taylor-Robinson. 2003. *Mycoplasma* and *Ureaplasma*, p. 972–990. In P. R. Murray, E. J. Baron, J. H. Jorgensen, M. A. Pfaller, and R. H. Yolken (ed.), *Manual of Clinical Microbiology*, 8th ed. ASM Press, Washington, D.C.
 5. Waites, K. B., C. M. Bébéar, J. A. Robertson, D. F. Talkington, and G. E. Kenny. 2001. *Cumitech 34, Laboratory Diagnosis of Mycoplasmal Infections*. Coordinating ed., F. S. Nolte. American Society for Microbiology, Washington, D.C.
 6. Waites, K. B., D. F. Talkington, and C. M. Bébéar. 2002. Mycoplasmas, p. 201–224. In A. L. Truant (ed.), *Manual of Commercial Methods in Clinical Microbiology*. ASM Press, Washington, D.C.
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APPENDIX 3.15-1
Medium Formulations for Cultivation of Mycoplasmas from Humans

- A. A8 agar
 1. Purpose. A8 is a differential agar medium useful for isolating genital mycoplasmas. Urea is included in this medium to enhance differentiation of *Ureaplasma* spp. from non-urea-hydrolyzing mycoplasmas.
 2. Ingredients
 - a. Base
 - (1) 825 ml of ultrapurified water (Milli-Q; Millipore, Inc.)
 - (2) 0.15 g of CaCl₂ dihydrate (dissolve before adding other ingredients)
 - (3) 24 g of TSB (BD Diagnostic Systems)
 - (4) 2 g of yeast extract (Difco, BD Diagnostic Systems)
 - (5) 1.7 g of putrescine dihydrochloride
 - (6) 0.2 g of DNA
 - (7) 10.5 g of Select Agar (BD Diagnostic Systems)
 - b. Supplements
 Filter sterilize (0.2-μm-pore-size filter) each supplement separately, if not received sterile from manufacturer.
 - (1) 200 ml of horse serum (HyClone, Logan, Utah)
 - (2) 5 ml of IsoVitaleX (BD Diagnostic Systems)
 - (3) 10 ml of 10% urea
 - (4) 1 ml of GHL tripeptide solution (Calbiochem-Novabiochem International, Inc., La Jolla, Calif.)
 - (5) 5 ml of 2% L-cysteine (prepare fresh on day of use)
 - (6) 1,000 IU of penicillin per ml to prevent bacterial overgrowth
 3. Preparation
 - a. Mix the base ingredients in a flask in the order specified above to prepare 1 liter.
 - b. Adjust pH to 5.5 with 2 N HCl.
 - c. Autoclave for 15 min at 121°C. Cool in a 56°C water bath before adding supplements.
 - d. After preparing and filter sterilizing each supplement separately, mix together and add supplements to autoclaved and cooled base agar.
 - e. Adjust pH to 6.0.
 - f. Pour plates, and after 2 h, invert and keep at room temperature overnight. (Small petri plates [60 mm] that can be used for agar require 10 ml, whereas standard 100-mm plates require 20 ml.)
 - g. Place plates in sealed plastic bags and refrigerate at 4°C.
 4. Limitation
 Plates will usually perform in a satisfactory manner for at least 4 weeks. Loss of inhibitory effect of antimicrobial agents when plates older than 4 weeks are used may result in bacterial overgrowth and inability to detect mycoplasmas when culturing specimens from nonsterile sites.

APPENDIX 3.15–1 (continued)

- B. 10B broth
1. Purpose
10B is an enriched broth medium useful for cultivation of *Ureaplasma* spp. and *Mycoplasma hominis*.
 2. Ingredients
 - a. Base
 - (1) 825 ml of ultrapurified water (Milli-Q; Millipore Inc.)
 - (2) 14 g of mycoplasma broth base without crystal violet (BD Diagnostic Systems)
 - (3) 2 g of arginine
 - (4) 0.2 g of DNA
 - (5) 1 ml of 1% phenol red (prepare fresh monthly)
 - b. Supplements
Filter sterilize (0.2-μm-pore-size filter) each supplement separately, if not received sterile from manufacturer.
 - (1) 200 ml of horse serum (HyClone) (heat-inactivated fetal bovine serum can also be used instead of horse serum)
 - (2) 100 ml of 25% yeast extract (Difco, BD Diagnostic Systems)
 - (3) 5 ml of IsoVitaleX (BD Diagnostic Systems)
 - (4) 4 ml of 10% urea
 - (5) 2.5 ml of 4% L-cysteine (prepare fresh on day of use)
 - (6) 1,000 IU of penicillin per ml to prevent bacterial overgrowth
 3. Preparation
 - a. Mix the base ingredients in a flask in the order specified above to prepare 1 liter.
 - b. Adjust the pH to 5.5 with 2 N HCl.
 - c. Autoclave for 15 min at 121°C. Cool in a 56°C water bath before adding supplements.
 - d. After preparing and filter sterilizing each supplement separately, mix together and add supplements to autoclaved and cooled base agar.
 - e. Adjust the pH to 5.9 to 6.1.
 - f. Dispense broth aseptically into sterile tubes each containing 0.9 to 1.0 ml.
 4. Limitation
10B broth will usually perform in a satisfactory manner for at least 4 weeks when stored at 4°C. Loss of inhibitory effect of antimicrobial agents when broths older than 4 weeks are used may result in bacterial overgrowth and inability to detect mycoplasmas.
- C. SP-4 broth and agar
1. Purpose
SP-4 broth is an enriched growth medium used for cultivation of many *Mycoplasma* species, including *Mycoplasma pneumoniae*. Agar may be added to SP-4 broth for preparation of solid medium and glucose and/or arginine and urea may be added as metabolic substrates, depending on which mycoplasmas are being sought.
 2. Ingredients
 - a. Base
 - (1) 643 ml of ultrapurified water (Milli-Q; Millipore, Inc.)
 - (2) 3.5 g of mycoplasma broth base without crystal violet (BD Diagnostic Systems)
 - (3) 10 g of tryptone (Difco; BD Diagnostic Systems)
 - (4) 5.3 g of peptone (Difco; BD Diagnostic Systems)
 - (5) 2 g of arginine (only if medium is to be used to isolate *M. hominis*) (Sigma)
 - (6) 2 ml of 1% phenol red (prepare fresh monthly)
 - (7) 0.2 g of DNA
 - (8) 15 g of Noble agar (only if preparing SP-4 agar) (Difco, BD Diagnostic Systems)
 - b. Supplements
 - (1) 50 ml of 10× CMRL 1066 (formula no. 01-0127DJ, in 10-liter volumes; Invitrogen Life Technologies, Carlsbad, Calif.)
 - (2) 35 ml of 25% yeast extract (Difco, BD Diagnostic Systems)
 - (3) 100 ml of 2% yeastolate (Difco, BD Diagnostic Systems)
 - (4) 170 ml of fetal bovine serum (HyClone) (heat inactivate at 56°C for 30 min)
 - (5) 10 ml of 50% glucose
 - (6) 1,000 IU of penicillin per ml to prevent bacterial overgrowth
 3. Preparation
 - a. Mix the base ingredients in a flask in the order specified above to prepare 1 liter.
 - b. Autoclave for 15 min at 121°C and cool before adding supplements.

APPENDIX 3.15–1 (continued)

- c. For agar, cool in a 56°C water bath. For broth, cool to room temperature.
- d. After preparing and filter sterilizing each supplement separately, mix together and add supplements to autoclaved and cooled base.
- e. Adjust the pH to 7.4 to 7.6.
- f. If agar is added, pour plates, and after 2 h, invert and keep at room temperature overnight. (Small petri plates [60 mm] that can be used for agar require 7.5 ml, whereas standard 100-mm plates require 10 ml.) Place plates in plastic bags and refrigerate at 4°C.
- g. Dispense broth in sterile tubes. (The volume of broth required for individual cultures is dictated by the length of incubation required, e.g., 1.8 to 4.5 ml for *M. pneumoniae* and 0.9 to 1 ml for the genital mycoplasmas.)
- 4. Limitation
Plates will usually perform in a satisfactory manner for at least 4 weeks. Loss of inhibitory effect of antimicrobial agents when broths or agar plates older than 4 weeks are used may result in bacterial overgrowth and inability to detect mycoplasmas when culturing specimens from nonsterile anatomic sites.
- D. Contact information for manufacturers not listed in procedure 3.1
 - 1. Calbiochem-Novabiochem International, Inc., La Jolla, Calif., (800) 854-3417, <http://www.calbiochem.com>
 - 2. Cyclone, Logan, Utah, (800) 492-5663, <http://www.hyclone.com>
 - 3. Millipore, Inc., Billerica, Mass., (978) 715-4321

APPENDIX 3.15–2**Hemadsorption Test for Identification of *Mycoplasma pneumoniae*****I. PRINCIPLE**

This procedure involves flooding colonies on agar with a dilute suspension of washed guinea pig erythrocytes, incubating and washing them, and examining them microscopically for adherent erythrocytes. Hemadsorption is unique to *Mycoplasma pneumoniae* and *Mycoplasma genitalium* among mycoplasmas isolated from humans. Since *M. genitalium* is much more fastidious and very slow-growing and has not been reliably isolated from humans using culture methods described for *M. pneumoniae*, a positive hemadsorption test provides a strong basis for an organism's identification as *M. pneumoniae*.

II. MICROORGANISMS TESTED

Unidentified *Mycoplasma* colonies <10 days old on SP-4 agar

III. SUPPLIES

- A. Guinea pig erythrocytes (Biowhittaker, Walkersville, Md.)
- B. Sterile phosphate-buffered saline (PBS) (pH 7.2)
- C. 15-ml conical centrifuge tubes
- D. Pipetting device and sterile disposable pipette tips

IV. QUALITY CONTROL

- A. Use *M. pneumoniae* ATCC 15531 (positive control) and *Mycoplasma hominis* ATCC 23114 (negative control) grown for <10 days on SP-4 agar.
- B. The *M. pneumoniae* positive control should show hemadsorption. The *M. hominis* negative control should not show any hemadsorption.

V. PROCEDURE

- A. Prepare a 10% working suspension of washed guinea pig erythrocytes by transferring 5 to 8 ml of the blood and an equal volume of sterile PBS (pH 7.2) aseptically into a 15-ml conical centrifuge tube.
- B. Centrifuge at 900 × g for 5 min at room temperature. Discard supernatant, and resuspend cell pellet in 10 ml of PBS by gently pipetting cells up and down several times.
- C. Centrifuge at 900 × g for 5 min at room temperature, and repeat washing procedure.
- D. Discard supernatant and measure packed-cell volume. Add a volume of PBS equal to nine times the packed-cell volume to yield a 10% suspension.
- E. Store the suspension at 4°C and use within 7 days. Do not use if there is evidence of hemolysis or bacterial contamination.
- F. Use this stock solution to prepare a 0.5% working solution by combining 1 part 10% suspension to 19 parts sterile PBS.
- G. Flood agar plates containing the unknown mycoplasma and positive and negative controls with 0.5% guinea pig erythrocytes.

APPENDIX 3.15–2 (continued)

- H. Cover plates and incubate at room temperature for 30 min.
- I. Tilt plates and aspirate the cell suspension from the agar surface.
- J. Flood plate with PBS to wash the agar surface, rocking the plate back and forth.
- K. Aspirate and discard the PBS.
- L. Observe the plate macroscopically and microscopically under a stereomicroscope for absorption of erythrocytes to the surface of the colonies.
- M. *M. pneumoniae* will hemadsorb; *M. hominis* and other large-colony mycoplasmas that are cultivable on SP-4 agar in 4 to 20 days will not do so.

APPENDIX 3.15–3**Performance of Antimicrobial Susceptibility Testing for Mycoplasmas****I. PRINCIPLE**

Susceptibility testing is useful mainly for systemic infections, particularly in immunosuppressed hosts, caused by *Mycoplasma hominis* or *Ureaplasma* spp. Testing is limited to antimicrobial agents that are commonly used to treat such infections, such as the tetracyclines, to which the organism may be resistant. Broth microdilution, agar dilution, and agar gradient diffusion (Etest technique) have been described for testing of antimicrobial agents against mycoplasmas and ureaplasmas in vitro (3). Broth microdilution is the most widely used method and the one most amenable to use in diagnostic laboratories for testing multiple antimicrobials. At present, there are no Clinical and Laboratory Standards Institute (CLSI; formerly NCCLS) standardized methods or approved MIC breakpoints for testing mycoplasmas. However, the broth microdilution method described below has been endorsed by the Chemotherapy Working Group of the International Research Program for Comparative Mycoplasmology (2).

II. SUPPLIES

- A. Reference powders of antimicrobial agents of known purity
- B. Conical centrifuge tubes (10 and 50 ml)
- C. 96-well microtiter plates with covers
- D. Acetate sealers for microtiter plates
- E. Multichannel pipetting device and sterile pipette tips
- F. Plastic bags
- G. Paper towels
- H. SP-4 broth for testing *Mycoplasma* spp. or 10B broth for testing *Ureaplasma* spp. without antimicrobials added (Remel, Inc., or Appendix 3.15–1)
- I. SP-4 or A8 agar plates (Remel, Inc., or Appendix 3.15–1)

III. QUALITY CONTROL

- A. Results are valid only if the inoculum is 10^4 to 10^5 CFU/ml based on the assay performed at the time the microtiter plate is inoculated.
- B. Results are invalid if the positive control does not show color change, the drug or broth controls show color change, or there is turbidity indicating bacterial contamination.
- C. Read MICs when the positive control first shows evidence of color change, because the value tends to shift over time because of nonspecific color change after prolonged incubation.
- D. There are no type strains with expected MIC ranges for specific drugs that have been recommended for universal QC by the CLSI. Always include an isolate for which the MICs of the drugs being used are reproducible to ensure validity of results for the test organisms, until reliable type strains have been designated for this purpose.
- E. When testing a new antimicrobial agent, test a type strain of a reference bacterium such as *Staphylococcus aureus* against the drug simultaneously in cation-adjusted Mueller-Hinton broth according to CLSI standards (1) and in 10B or SP-4 broth to determine whether there is any interaction between the enriched mycoplasmal media and the antimicrobial being tested. The MICs should agree within ± 1 two-fold dilution. Commonly tested drugs that are used for testing against mycoplasmas and ureaplasmas do not show such an interaction.

APPENDIX 3.15–3 (continued)**IV. PROCEDURE**

- A. Test the following drugs.
 1. A fluoroquinolone such as levofloxacin, gatifloxacin, or moxifloxacin
 2. Tetracycline or doxycycline
 3. A macrolide such as erythromycin, azithromycin, or clarithromycin (for *Mycoplasma pneumoniae* and *Ureaplasma* spp.)
 4. Clindamycin (for *M. hominis*)
 5. Optional: test chloramphenicol on isolates from CSF.
- B. Preparation of stocks
 1. Weigh out an appropriate amount of powdered drug to prepare 10 ml of a stock solution containing 2,048 µg/ml.
 2. Dissolve antimicrobial agents according to manufacturer's instructions and in accordance with CLSI guidelines (1).
 3. Depending on drug stability, store most stock solutions at –70°C. Some agents work best if prepared on the day of assay.
- C. Prepare a stock strain of known titer by incubating a stock culture of each strain to be tested, including controls, in the appropriate broth medium (10B for ureaplasmas, SP-4 for mycoplasmas) until color change occurs, and then freeze immediately at –70°C in ≤1-ml volumes.
 1. The approximate number of organisms in the frozen vials is determined by serially diluting a thawed vial.
 - a. Place 0.1 ml of culture into 0.9 ml of appropriate broth.
 - b. Make dilutions to at least 10⁻⁸ to achieve an endpoint titer.
 - c. Incubate tubes and count the dilutions that exhibit color change, indicative of mycoplasmal growth.
 2. Thaw vials of the organisms to be tested on the day the assay is to be performed and dilute in appropriate media to yield concentrations of 10⁴ to 10⁵ CFU/ml, based on the original predetermined inoculum.
 3. Prepare a total of 40 ml of each isolate for testing up to six drugs.
 4. Incubate the inoculated broth at 37°C for 2 h prior to use to allow organisms to become metabolically active prior to inoculating microtiter plates.
- D. Prepare two microtiter plates for every three drugs tested to allow testing a full range of drug concentrations from 0.008 to 256 µg/ml.
 1. If fewer concentrations are required, all testing for an individual drug can be performed in a single microtiter plate and the concentrations adjusted accordingly.
 2. Test each drug in duplicate, skipping a row between drugs. (Drug one, rows A and B; drug two, rows D and E; drug three, rows G and H.)
 3. Add 0.025 ml of appropriate medium in rows 2 to 12 of the first microtiter plate and rows 1 to 4, 6, and 10 of the second microtiter plate.
 4. Add 0.175 ml of appropriate medium to well 10 (total of 0.2 ml) for the broth control and row 8 (total of 0.175 ml) for the drug control.
 5. Add 0.025 ml of drug 1 to wells 1 and 2 in rows A and B in the first microtiter plate and well 8 in rows A and B of the second microtiter plate for drug control. (The other drugs to be tested will be added the same way in their respective rows.)
 6. Dilute the antimicrobial agent through all wells in the first microtiter plate using a 0.025-ml multichannel pipette, beginning at the second well and continuing through wells 1 to 4 in the second microtiter plate. Discard the final 0.025 ml.
 7. Add 0.175 ml of inoculated medium that has been prewarmed for 2 h to each well in rows 1 to 12 in the first microtiter plate and rows 1 to 4 and to the positive organism control (well 6) in the second microtiter plate as soon as the dilutions are completed for each drug to be tested. Start with row 6, move to row 4, and work backwards to row 1 in first microtiter plate to prevent drug carryover.
 8. Repeat the above procedure for each drug and organism to be tested.
- E. Perform a final determination of color changing units (CCU) by serial dilution of the cultures, as described above, at this time to check that a proper dilution was made and that the inoculum contains 10⁴ to 10⁵ CCU/ml.

APPENDIX 3.15–3 (continued)

- F. Place plastic covers over plates being tested against *M. pneumoniae*, and seal them in plastic bags with a piece of paper towel moistened with water to prevent desiccation of the broth. Cover plates being tested for *Ureaplasma* spp. and *M. hominis* with adhesive-back acetate sealers before placing lid to prevent urea by-products escaping to other wells and causing color change. Incubate aerobically at 37°C.
- G. Read the microtiter plates daily for color change in the organism control wells.
 1. Ureaplasmas will normally grow after 12 to 18 h of incubation. A positive reaction for *Ureaplasma* in 10B broth will be evidenced by a color change from yellow to pink.
 2. *M. hominis* may take 1 to 3 days. A positive reaction for *M. hominis* in SP-4 broth will be evidenced by a color change from pink to deeper red.
 3. *M. pneumoniae* may take 5 to 10 days for completion of the assay. A positive reaction for *M. pneumoniae* in SP-4 broth will be evidenced by a color change from pink to yellow.
- H. Record the MIC as the concentration of antimicrobial agent inhibiting color change in broth medium at the time when the organism control well first shows color change.
- I. Determine minimal bactericidal concentrations (MBC), if desired, by subculturing 0.3 ml of fluid from wells of the microbroth dilution system that do not show color change into a volume of fresh broth sufficient to dilute the antimicrobial beyond the MIC. Usually a 1:100 dilution is sufficient.
 1. Incubate broths and examine for color change.
 2. Subculture to agar if color change occurs.
 3. Subculture fluid from the growth control to make certain organisms have remained viable in the absence of antimicrobials.
 4. Determine the MBC as the lowest concentration of antimicrobial at which there is no evidence of color change and no colonies formed on subculture to agar.

V. REPORTING ANTIMICROBIAL SUSCEPTIBILITY RESULTS

- A. Report actual MICs since there are no breakpoints specified for mycoplasmas or ureaplasmas.
- B. In addition, interpretive breakpoints developed for conventional bacteria may be reported for mycoplasmas, but *add a comment* to the report indicating that breakpoints used for interpretation of results have not been developed specifically for mycoplasmas and may not be valid.
- C. Ureaplasmas must be tested at an acidic pH (6.0 to 6.5) in order for organisms to grow adequately. MICs for some macrolides such as erythromycin will be 2 to 3 dilutions higher at this pH than at neutral pH, making these organisms appear less susceptible *in vitro* than they may be *in vivo*. Include an explanatory comment with all MIC reports for macrolides when testing ureaplasmas.
- D. Correlation of MICs for tetracycline with the presence or absence of the *tetM* transposon, which mediates resistance to this drug, has indicated that the MICs for *tetM*-containing strains of *M. hominis* or *Ureaplasma* spp. will be $\geq 8 \mu\text{g/ml}$, whereas the MICs for strains that do not contain this resistance determinant will be $\leq 2 \mu\text{g/ml}$, with no overlap between these two distinct populations.

References

1. Clinical and Laboratory Standards Institute. 2006. *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically*, 7th ed. Approved standard M7-A7. Clinical and Laboratory Standards Institute, Wayne, Pa.
2. Waites, K. B., C. M. Bébéar, J. A. Robertson, D. F. Talkington, and G. E. Kenny. 2001. *Cumitech 34, Laboratory Diagnosis of Mycoplasmal Infections*. Coordinating ed., F. S. Nolte. American Society for Microbiology, Washington, D.C.
3. Waites, K. B., K. C. Canupp, and G. E. Kenny. 1999. In vitro susceptibilities of *Mycoplasma hominis* to six fluoroquinolones determined by Etest. *Antimicrob. Agents Chemother.* **43**:2571–2573.

3.16

Guidelines for Biochemical Identification of Aerobic Bacteria

[Updated March 2007]

On the following pages are 48 biochemical procedures in alphabetical order, including both simple, rapid test procedures and standard conventional methods, for the identification of gram-positive and gram-negative bacteria. The biochemical tests have been selected that are most useful to laboratories, with emphasis on rapid testing. The list of tests includes the generally accepted tests that all laboratories should be able to perform to identify the clinically important microorganisms encountered in the laboratory, as well as some for use by reference or referral laboratories. Smaller laboratories may choose to perform fewer tests and refer cultures when less common microorganisms are found in culture. When identification to the species level is not clinically important, tests to separate these species are not included. When there is a choice of different tests that can be performed, both are presented and the user can choose which is preferable for use in the laboratory.

Rapid and overnight commercial identification systems, containing many tests, provide very accurate identifications (Tables 3.16–1 to 3.16–4) (1, 2) with little hands-on time. The tests listed here are intended to supplement those systems, not replace them. For more detailed instructions on the performance of those test systems, refer to the instruction manual received with the product. If individual tests are available on a kit system, then the separate purchase of that biochemical test

may not be needed. However, commercial systems can be expensive, and rapid spot tests can often be a substitute or can be used to screen in order to suggest which commercial system might yield an identification. Serologic tests are not listed and should be a part of the identification methods used in most laboratories for various bacteria. See section 11.

As a policy, manufacturers are not listed if the product is available from most vendors. When a test has unique names depending on the manufacturer, attempts are made to list each of the product names. Product suppliers, addresses, and websites are listed in the introduction to this section, procedure 3.1. Often useful, detailed procedural information can be found on the vendor's website.

Generally, the formulas are presented, if it is likely that a laboratory might make the media or reagents in-house or the ingredients are of interest for the purchase or use of the test. Otherwise, only the key ingredients are listed for informational purposes, since good-quality commercial products are readily available and preparation of media in most laboratories is not cost-effective.

Below are a few reminders for performing the biochemical tests described in the following procedures. (i) Use only fresh cultures (18 to 24 h) as inoculum. (ii) Most reactions are aerobic. Keep caps loose, and incubate at 35°C without CO₂,

unless otherwise indicated. (iii) Perform the oxidase, indole, and sometimes catalase tests on gram-negative bacteria before selecting additional tests. Perform a catalase and Gram stain on gram-positive bacteria prior to inoculation of other tests. (iv) Properly identify all material, prepared or purchased, with a legible label. Include the expiration date on the label and also log in a work record or on a receipt record. (v) Perform QC testing to ensure proper functioning of each reagent and of the test procedure with a negatively and a positively reacting microorganism prior to use of each new lot and possibly more often, depending on local regulations and on the frequency of usage. (vi) Although specific ATCC strains are listed for each procedure, any strain that will give the same result is acceptable. (vii) Record on each package, tube, or vial or in the QC records the date the reagent or test medium was initially put into use. (viii) Inoculum can be important in the use of medium and kit identifications. When appropriate, this is indicated with the appropriate McFarland standard. Refer to Appendix 3.16–1 for details on McFarland standards. (ix) Perform spot tests only from BAP or CHOC; *do not use selective media with dyes or biochemical tube agars for spot testing.*

Information presented in the following procedures was taken in part from HUGO, the product information manual from Hardy Diagnostics, with permission.

Table 3.16–1 Summary of bacterial identification systems^a

System	Manufacturer	Database	Storage temp (°C)	No. of tests	Length of incubation (h)	Automated
<i>Enterobacteriaceae</i>						
API 20E	bioMérieux	See Table 3.16–2	2–8	21	24–48 ^b	No
CRYSTAL E/NF	BD Diagnostic Systems	See Table 3.16–2	2–8	30	18–20	No
Enterotube II	BD Diagnostic Systems	84 taxa of <i>Enterobacteriaceae</i>	2–8	15	18–24	No
GN Microplate	Biolog	See Table 3.16–2	2–8	95	4–24	Reader only
GNI	bioMérieux	See Table 3.16–2	2–8	29	2–18	Yes
GNI+	bioMérieux	See Table 3.16–2	2–8	28	2–12	Yes
Micro-ID	Remel	32 taxa of <i>Enterobacteriaceae</i>	2–8	15	4	No
NEG ID Type 2	Dade Behring MicroScan	See Table 3.16–2	2–8	33	15–42	Yes
Rapid NEG ID Type 3	Dade Behring MicroScan	See Table 3.16–2	2–8	36	2.5	Yes
RapID ONE	Remel	See Table 3.16–2	2–8	19	4	No
r/b Enteric Differential System	Remel	37 taxa of <i>Enterobacteriaceae</i>	2–8	15	18–24	No
Sensititre AP 80	Trek Diagnostic Systems, Inc.	84 taxa of <i>Enterobacteriaceae</i>	RT	32	5–18	Yes
Nonfermenting or oxidase-positive gram-negative rods						
API 20 NE	bioMérieux	45 nonfermenting and 14 oxidase-positive fermenting taxa	2–8	20	24–48	No
CRYSTAL E/NF	BD Diagnostic Systems	24 nonfermenting and 18 oxidase-positive fermenting taxa	2–8	30	18–20	No
GNI+	bioMérieux	35 nonfermenting and 11 oxidase-positive fermenting taxa	2–8	28	2–12	Yes
NEG ID Type 2	Dade Behring MicroScan	31 nonfermenting and 14 oxidase-positive fermenting taxa	2–8	33	15–42	Yes
Rapid NEG ID Type 3	Dade Behring MicroScan	34 nonfermenting and 19 oxidase-positive fermenting taxa; does not include <i>Burkholderia pseudomallei</i>	2–8	36	2.5	Yes
OxiIIerm II	BD Diagnostic Systems	17 nonfermenting and 5 oxidase-positive fermenting taxa; does not include <i>B. pseudomallei</i>	2–8	9	24–48	No
RapID NF Plus	Remel	54 nonfermenting and 18 oxidase-positive fermenting taxa	2–8	17	4	No
Sensititre AP 80	Trek Diagnostic Systems, Inc.	44 nonfermenting and 24 oxidase-positive fermenting taxa; does not include <i>B. pseudomallei</i>	RT	32	5–18	Yes
Uni-N/F-Tek	Remel	47 nonfermenting taxa; does not include <i>B. pseudomallei</i>	2–8	18	24–48	No
Fastidious gram-negative microorganisms in addition to <i>Haemophilus</i> and <i>Neisseria</i> ^c						
CRYSTAL N/H	BD Diagnostic Systems	19 taxa	2–8	29	4	No
NHI	bioMérieux	16 taxa	2–8	15	4	No
RapID NH	Remel	19 taxa	2–8	13	4	No
Staphylococci and related catalase-positive cocci						
API Staph	bioMérieux	23 taxa (Table 3.16–3)	2–8	20	24	No
CRYSTAL Gram-Positive	BD Diagnostic Systems	35 taxa (Table 3.16–4)	2–8	29	18–24	No
CRYSTAL Rapid Gram-Positive	BD Diagnostic Systems	19 taxa (Table 3.16–4)	2–8	29	4	No
GPI	bioMérieux	15 taxa (Table 3.16–3)	2–8	29	2–15	Yes

(continued)

Table 3.16–1 Summary of bacterial identification systems^a (*continued*)

System	Manufacturer	Database	Storage temp (°C)	No. of tests	Length of incubation (h)	Automated
Pos ID 2	Dade Behring MicroScan	20 taxa (Table 3.16–3)	2–30	27	18–48	Yes
Rapid POS ID	Dade Behring MicroScan	27 taxa (Table 3.16–3)	2–8	34	2	Yes
Sensititre AP 90	Trek Diagnostic Systems, Inc.	13 taxa (Table 3.16–4)	RT	32	24	Yes
Streptococci and related catalase-negative genera						
API 20 Strep	bioMérieux	39 streptococcal and related taxa and 5 enterococci (Table 3.16–3)	2–8	20	4–24	No
CRYSTAL Gram-Positive	BD Diagnostic Systems	56 streptococcal and related taxa and 8 enterococci (Table 3.16–4)	2–8	29	18–24	No
CRYSTAL Rapid Gram-Positive	BD Diagnostic Systems	41 streptococcal and related taxa and 6 enterococci (Table 3.16–4)	2–8	29	4	No
GPI	bioMérieux	22 streptococcal taxa and 8 enterococci (<i>also see</i> Table 3.16–3)	2–8	29	2–15	Yes
Pos ID 2	Dade Behring MicroScan	13 streptococcal and related taxa and 7 enterococci (<i>also see</i> Table 3.16–3)	2–30	27	18–48	Yes
Rapid POS ID	Dade Behring MicroScan	15 streptococcal and related taxa and 4 enterococci (<i>also see</i> Table 3.16–3)	2–8	34	2	Yes
RapID STR	Remel	24 streptococcal and related taxa and 8 enterococci (<i>also see</i> Table 3.16–4)	2–8	14	4	No
Sensititre AP 90	Trek Diagnostic Systems, Inc.	15 streptococcal and related taxa and 4 enterococci (<i>also see</i> Table 3.16–4)	RT	32	24	Yes
<i>Corynebacterium</i> and related genera ^d						
API Coryne	bioMérieux	49 coryneform taxa; published studies show 90.5% correct to species level	2–8	20	24	No
CRYSTAL Gram-Positive	BD Diagnostic Systems	26 coryneform taxa plus <i>Bacillus</i> species; no published comparisons	2–8	29	18–24	No
GP Microplate	Biolog	Published data show 60% correct to genus level	2–8	95	4–24	Reader only
RapID CB Plus	Remel	52 coryneform taxa; published studies show 93% correct to genus level and 81% correct to species level	2–8	18	4	No
Sensititre AP 90	Trek Diagnostic Systems, Inc.	19 coryneform taxa; no published comparisons	RT	32	24	Yes

^a Data extrapolated in part from reference 2. Not all systems are included (*see* reference 2). Database for Biolog includes extensive taxa of gram-positive and gram-negative bacteria but was included only for *Enterobacteriaceae* and gram-positive rods; see Table 3.16–4 for other gram-positive microorganisms in the Biolog system as well as the MIDI system. For vendor contact information, see procedure 3.1. Abbreviation: RT, room temperature.

^b The API 20E can be heavily inoculated and read at 4 h with a different database.

^c See procedure 3.9.3 for *Neisseria* identification options.

^d Data for accuracy from reference 1.

Table 3.16-2 Database entries of the *Enterobacteriaceae* (human isolates)^a

Organism(s)	API 20E, version 4.0	BBL CRYSTAL, version 4.0	ID5 RapID onE, version 1.93	Vitek		MicroScan		Biolog ^b , ver- sion 6.01	MIDI, ver- sion 4.0
				GNI+, ver- sion R8.03	ID-GNB, version R02.03	ID 32E, ver- sion 1.0	Conven- tional, ver- sion 22.28		
<i>Buchneria</i>									
<i>aquatica</i>									
<i>Burkholderia</i>	×								
<i>agrestis</i>									
<i>Cedecea da-</i>			×						
<i>viseae</i>				×					
<i>Cedecea la-</i>			×						
<i>pagei</i>				×					
<i>Cedecea ne-</i>			×						
<i>teri</i>									
<i>Cedecea</i> sp.									
strain 3									
<i>Cedecea</i> sp.									
strain 5									
<i>Citrobacter</i>									
<i>amalonati-</i>									
<i>cus</i>									
<i>Citrobacter</i>									
<i>braakii</i>	×								
<i>Citrobacter</i>									
<i>(C. ko-</i>									
<i>farmeri</i>	×								
<i>Citrobacter</i>									
<i>freundi</i>	×								
<i>Citrobacter</i>									
<i>gillenii</i>									
<i>Citrobacter</i>									
<i>koseri</i>									
<i>Citrobacter</i>									
<i>murliniae</i>									
<i>Citrobacter</i>									
<i>sedlakii</i>									
<i>Citrobacter</i>									
<i>werkmanii</i>									
<i>Citrobacter</i>									
<i>youngiae</i>	×								

(continued)

Table 3.16-2 Database entries of the *Enterobacteriaceae* (human isolates)^a (continued)

Organism(s)	Vitek			MicroScan		
	API 20E, version 4.0	BBL CRYSTAL, version 4.0	ID5 RapID onE, version 1.93	GNI +, ver- sion R8.03	ID-GNB, version R02.03	ID 32E, ver- sion 1.0
<i>Edwardsiella</i> <i>hoshiniae</i>	×	×	×	×	×	×
<i>Edwardsiella</i> <i>tarda</i>	×	×	×	×	×	×
<i>Enterobacter</i> <i>aerogenes</i>	×	×	×	×	×	×
<i>Enterobacter</i> <i>agglomer-</i> <i>ans</i> group	×	–	–	–	–	–
<i>Enterobacter</i> <i>amnigenus</i> group 1	×	–	–	–	–	–
<i>Enterobacter</i> <i>amnigenus</i> group 2	–	–	–	–	–	–
<i>Enterobacter</i> <i>ashburiae</i>	–	–	–	–	–	–
<i>Enterobacter</i> <i>cancerogene-</i> <i>nus</i>	–	–	–	–	–	–
<i>Enterobacter</i> <i>cloacae</i>	–	–	–	–	–	–
<i>Enterobacter</i> <i>ergusoniae</i>	–	–	–	–	–	–
<i>Enterobacter</i> <i>hommae-</i> <i>chei</i>	–	–	–	–	–	–
<i>Enterobacter</i> <i>interme-</i> <i>dius</i>	–	–	–	–	–	–
<i>Enterobacter</i> <i>sakazakii</i>	–	–	–	–	–	–
<i>Escherichia</i> <i>coli</i>	–	–	–	–	–	–
<i>Escherichia</i> <i>ergusonii</i>	–	–	–	–	–	–
<i>Escherichia</i> <i>hermannii</i>	–	–	–	–	–	–
<i>Escherichia</i> <i>vulnalis</i>	–	–	–	–	–	–
						MIDI, ver- sion 4.0
						Biolog ^b , ver- sion 6.01
						MicroScan
						Conven- tional, ver- sion 22.28
						Rapid, ver- sion 22.28

Table 3.16-2 Database entries of the *Enterobacteriaceae* (human isolates)^a (*continued*)

<i>Serratia</i>	×	×	×	×	× (groups 1 and 2)	×	×	× (groups 1 and 2)	×	×	×	×
<i>Serratia</i> group 2	×	×	×	×	×	×	×	×	×	×	×	×
<i>Serratia pylmuthica</i>	×	×	×	×	×	×	×	×	×	×	×	×
<i>Serratia rubidaea</i>	×	×	×	×	×	×	×	×	×	×	×	×
<i>Shigella</i> spp.	×	(two spp.)	×	(three groups)	×	(two groups)	×	(four spp.)	×	(two groups)	×	(four spp.)
<i>Tatumella</i>												
<i>physeos</i>												
<i>Trabulsiella</i>												
<i>guamensis</i>												
<i>Yersinia enterocolitica</i>	×	×	(group) ^b	×	×	×	×	× (group)	×	×	× (group)	×
<i>Yersinia frederiksenii</i>	×	(<i>Y. intermedia</i>)	×	(group)	×	×	×	×	×	×	× (group)	×
<i>Yersinia intermedia</i>	×	(<i>Y. fred-eriksenii</i>)	×	(group)	×	×	×	×	×	×	× (group)	×
<i>Yersinia kristensenii</i>	×	×	(group)	×	×	×	×	×	×	×	× (group)	×
<i>Yersinia pestis</i>	×											
<i>Yersinia pseudotuberculosis</i>	×											
<i>Yersinia ruckeri</i>												
<i>Yokenella regensbergaei</i>	×											

^a Reproduced from Reference 2. Some products give a choice between two species; the alternate species is indicated in parentheses.^b Database also includes 19 additional organisms not listed here.^c Reported as *Enterobacter taylorae*.^d Includes the ability to differentiate between serogroups O111 and O157.^e Recently assigned to the genus *Raoultella*.^f #, genus-only designation.^g System does not separate *Proteus penneri* from *Proteus vulgaris*.^h "Group" indicates *Yersinia* group.

Table 3.16-3 Database entries of the gram-positive organisms (human isolates) for bioMérieux and Dade MicroScan products^a

Organism(s)	API			Vitek			MicroScan	
	Staph, version 4.0	Rapidec Staph, version 4/99	20 Strep, version 6.0	GPI, version R7.01	ID-GPC, version R02.03	ID32 Staph, version 2.0	Conventional Pos ID 2, version 22.28	Rapid Pos ID, version 22.28
<i>Listeria monocytogenes</i>			×	<i>Listeria</i> species			×	×
<i>Micrococcaceae</i>								
<i>Micrococcus (Kocuria) kristinae</i>	×					×	<i>Micrococcus</i> species	×
<i>Micrococcus luteus</i>	<i>Micrococcus</i> species				×	×		
<i>Micrococcus lyliae</i>						×		
<i>Micrococcus (Kocuria) roseus</i>	×	(<i>M. varians</i>)			×	×	<i>Micrococcus</i> species	×
<i>Kocuria varians</i>	×	(<i>M. rosea</i>)			×	×		
<i>Micrococcus sedentarius</i>								
<i>Staphylococcus arlettae</i>	×					×		×
<i>Staphylococcus aureus</i>	×	×		×	×	×	×	×
<i>Staphylococcus auricularis</i>	×			×	×	×	×	×
<i>Staphylococcus capitis</i> subsp. <i>capitis</i>	×	×		×	×	×	×	×
<i>Staphylococcus capitis</i> subsp. <i>ureolyticus</i>							×	
<i>Staphylococcus caprae</i>	×					×		×
<i>Staphylococcus carnosus</i>	×					×		×
<i>Staphylococcus caseolyticus</i>								×
<i>Staphylococcus chromogenes</i>	×					×		
<i>Staphylococcus cohnii</i> subsp. <i>cohnii</i>	×			×	×	×	×	×
<i>Staphylococcus cohnii</i> subsp. <i>urealyticum</i>	×				×	×	×	
<i>Staphylococcus epidermidis</i>	×	×		×	×	×	×	×
<i>Staphylococcus equorum</i>						×		×
<i>Staphylococcus felis</i>								
<i>Staphylococcus gallinarum</i>						×		×
<i>Staphylococcus haemolyticus</i>	×	×		×	×	×	×	×
<i>Staphylococcus hominis</i> subsp. <i>hominis</i>	×			×	×	×	×	×
<i>Staphylococcus hominis</i> subsp. <i>novobiosepticus</i>							×	
<i>Staphylococcus hyicus</i>	×			×	×	×	×	×
<i>Staphylococcus intermedius</i>				×	×	×	×	×
<i>Staphylococcus kloosii</i>					×	×		×
<i>Staphylococcus latus</i>	×			×	×	×		×

Table 3.16-3 (continued)

Organism(s)	API			Vitek			MicroScan	
	Staph, version 4.0	Rapidec Staph, version 4/99	20 Strep, version 6.0	GPI, version R7.01	ID-GPC, version R02.03	ID32 Staph, version 2.0	Conventional Pos ID 2, version 22.28	Rapid Pos ID, version 22.28
<i>Staphylococcus lugdenensis</i>	×				×	×	×	×
<i>Staphylococcus pasteuri</i>								
<i>Staphylococcus saccharolyticus</i>								
<i>Staphylococcus saprophyticus</i>	×	×		×	×	×	×	×
<i>Staphylococcus schleiferi</i>	×				×	×	×	×
<i>Staphylococcus sciuri</i>	×			×	×	×	×	×
<i>Staphylococcus simulans</i>	×			×	×	×	×	×
<i>Staphylococcus vitulinus</i>								
<i>Staphylococcus warneri</i>	×			×	×	×	×	×
<i>Staphylococcus xylosus</i>	×	×		×	×	×	×	×
<i>Stomatococcus mucilaginosus</i> (<i>Rothia mucilaginosa</i>)						×		
<i>Streptococcaceae</i>								
<i>Aerococcus viridans</i>			×	<i>Aerococcus</i> species	×	×	×	×
<i>Alloiococcus otitis</i>						×		
<i>Dermacoccus nishinomiyaensis</i>								
<i>Enterococcus avium</i>			×	×	×		×	×
<i>Enterococcus casseliflavus</i>				×	×		×	×
<i>Enterococcus durans</i>			×	×	×		×	×
<i>Enterococcus faecalis</i>			×	×	×		×	×
<i>Enterococcus faecium</i>			×	×	×		×	×
<i>Enterococcus gallinarum</i>			×	×	×		×	
<i>Enterococcus hirae</i>								
<i>Enterococcus malodoratus</i>							×	
<i>Enterococcus raffinosus</i>								×
<i>Enterococcus solitarius</i>								
<i>Gemella haemolysans</i>			×					
<i>Gemella morbillorum</i>			×	×	×			
<i>Globicatella sanguinis</i>								
<i>Helcococcus kunzii</i>								
<i>Lactococcus</i> species							×	
<i>Leuconostoc</i> species							×	
<i>Pediococcus</i> species								
<i>Streptococcus acidominimus</i>			×	×	×			
<i>Streptococcus agalactiae</i>			×	×	×		×	×
<i>Streptococcus anginosus</i>				×	×			
<i>Streptococcus bovis</i>			×	×	×		×	×
<i>Streptococcus constellatus</i>				×	×			

(continued)

Table 3.16-3 Database entries of the gram-positive organisms (human isolates) for bioMérieux and Dade MicroScan products^a (continued)

Organism(s)	API		Vitek		MicroScan			
	Staph, version 4.0	Rapidec Staph, version 4/99	20 Strep, version 6.0	GPI, version R7.01	ID-GPC, version R02.03	ID32 Staph, version 2.0	Conventional Pos ID 2, version 22.28	Rapid Pos ID, version 22.28
<i>Streptococcus criceti</i>								
<i>Streptococcus cre-moris/thermophilus</i>			×					
<i>Streptococcus crista</i>								
<i>Streptococcus dysga-lactiae/equisimilis</i>			×	×	×			
<i>Streptococcus equi</i>			×	×	×			×
<i>Streptococcus equinus</i>			×	×	×			×
<i>Streptococcus equisimi-lis</i>			×				×	×
<i>Streptococcus gordonii</i>				× (S. san-guis)	×			
<i>Streptococcus</i> groups E, G, L, P, and U			×					
<i>Streptococcus interme-dius</i>				×	×			
<i>Streptococcus lactis/ diacetylactis</i>			×					
<i>Streptococcus milleri</i> group			×	×			×	×
<i>Streptococcus mitis</i> group			×	×	×		×	×
<i>Streptococcus mutans</i>			×	×	×		×	×
<i>Streptococcus oralis</i>				×				
<i>Streptococcus parasan-guis</i>								
<i>Streptococcus pneumo-niae</i>			×	×	×		×	×
<i>Streptococcus porcinus</i>								
<i>Streptococcus pyogenes</i>			×	×	×		×	×
<i>Streptococcus salivar-ius</i>				×	×		×	×
<i>Streptococcus san-guinis</i>				×				
<i>Streptococcus sanguis</i>			×	× (S. gor-donii)	×		×	×
<i>Streptococcus sobrinus</i>								
<i>Streptococcus uberis</i>			×	×	×			
<i>Streptococcus vestibu-laris</i>					×			
<i>Streptococcus zooepi-demicus</i>			×	×				×
<i>Weissella confusa</i>								

^a Reproduced from reference 2.

Table 3.16–4 Database entries of the gram-positive organisms (human isolates) for BD, IDS, Sensititre, Biolog, and MIDI products^a

Organism(s)	BBL CRYSTAL		IDS RapID STR, ^b version 1.3.97	Pasco Gram-Positive ID, ^c version 4.6	Sensititre AP90, version 2.2	Biolog ^d version 6.01	MIDI, version 4.0
	Gram-Pos, version 4.0	Rapid Gram-Pos, version 4.0					
<i>Listeria</i> species	× (four species)	× (two species)	<i>L. monocytogenes</i>	<i>L. monocytogenes</i>	× (two species)	× (seven species)	× (six species)
<i>Micrococcaceae</i>							
<i>Micrococcus (Kocuria) kristinae</i>	×	×				×	×
<i>Micrococcus luteus</i>	×	×		<i>Micrococcus</i> species	×	×	×
<i>Micrococcus lyliae</i>	×					×	×
<i>Micrococcus (Kocuria) roseus</i>	×	×			×	×	×
<i>Kokuria varians</i>						×	×
<i>Micrococcus (Kyto-coccus) sedentarius</i>	×					×	×
<i>Staphylococcus arlettae</i>						×	×
<i>Staphylococcus aureus</i>	×	×		×	×	× (two subspecies)	×
<i>Staphylococcus auricularis</i>	×			×		×	×
<i>Staphylococcus capitis</i> subsp. <i>capitis</i>	×			×	×	×	×
<i>Staphylococcus capitis</i> subsp. <i>ureolyticus</i>	×						×
<i>Staphylococcus caprae</i>	×					×	
<i>Staphylococcus carnosus</i>	×					×	×
<i>Staphylococcus caseolyticus</i>						×	×
<i>Staphylococcus chromogenes</i>						×	×
<i>Staphylococcus cohnii</i> subsp. <i>cohnii</i>	×			×		×	×
<i>Staphylococcus cohnii</i> subsp. <i>urealyticum</i>	×			×			×
<i>Staphylococcus epidermidis</i>	×	×		×	×	×	×
<i>Staphylococcus equorum</i>	×					×	×
<i>Staphylococcus felis</i>	×					×	
<i>Staphylococcus galinarum</i>	×	×				×	×
<i>Staphylococcus haemolyticus</i>	×	×		×	×	×	×
<i>Staphylococcus hominis</i> subsp. <i>hominis</i>	×	×		×	×	×	×
<i>Staphylococcus hominis</i> subsp. <i>novobiosepticus</i>						×	
<i>Staphylococcus hyicus</i>						×	×

(continued)

Table 3.16-4 Database entries of the gram-positive organisms (human isolates) for BD, IDS, Sensititre, Biolog, and MIDI products^a (continued)

Organism(s)	BBL CRYSTAL		IDS RapID STR, ^b version 1.3.97	Pasco Gram-Positive ID, ^c version 4.6	Sensititre AP90, version 2.2	Biolog ^d version 6.01	MIDI, version 4.0
	Gram-Pos, version 4.0	Rapid Gram-Pos, version 4.0					
<i>Staphylococcus intermedius</i>	×	×				×	×
<i>Staphylococcus kloosii</i>	×					×	×
<i>Staphylococcus lentus</i>	×	×				×	×
<i>Staphylococcus lugdunensis</i>		×				×	×
<i>Staphylococcus muscae</i>						×	×
<i>Staphylococcus pasteurii</i>	×					×	
<i>Staphylococcus saccharolyticus</i>	×	×					
<i>Staphylococcus saprophyticus</i>	×	×		×	×	×	×
<i>Staphylococcus schleiferi</i>	× (two subspecies)					×	× (two subspecies)
<i>Staphylococcus sciuri</i>	×	×				× (two subspecies)	×
<i>Staphylococcus simulans</i>	×	×		×		×	×
<i>Staphylococcus vitulinus</i>	×						×
<i>Staphylococcus warneri</i>		×		×	×	×	×
<i>Staphylococcus xylosus</i>		×		×		×	×
<i>Stomatococcus mucilaginosus</i> (<i>Rotthia mucilaginosa</i>)	×	×					×
<i>Streptococcaceae</i>							
<i>Aerococcus viridans</i>	× (two species)	×	<i>Aerococcus</i> species	<i>Aerococcus</i> species	×	× (three species)	×
<i>Alloioococcus otitis</i>	×					×	
<i>Dermacoccus nishinomiyaensis</i>						×	×
<i>Enterococcus avium</i>	×	×	×	×	×	×	×
<i>Enterococcus casseliflavus</i>	<i>E. gallinarum</i>	<i>E. gallinarum</i>	<i>E. mundtii</i>		×	×	×
<i>Enterococcus cecorum</i>						×	
<i>Enterococcus columbae</i>						×	×
<i>Enterococcus durans</i>	×	×	×	×	×	×	×
<i>Enterococcus faecalis</i>	×	×	×	×	×	×	×
<i>Enterococcus faecium</i>	×	×	×	×	×	×	×
<i>Enterococcus gallinarum</i>			×			×	×
<i>Enterococcus hirae</i>	×		×			×	×
<i>Enterococcus malodoratus</i>						×	×

Table 3.16-4 (continued)

Organism(s)	BBL CRYSTAL		IDS RapID STR, ^b version 1.3.97	Pasco Gram-Positive ID, ^c version 4.6	Sensititre AP90, version 2.2	Biolog ^d version 6.01	MIDI, version 4.0
	Gram-Pos, version 4.0	Rapid Gram-Pos, version 4.0					
<i>Enterococcus mundtii</i>			<i>E. casseliflavus</i>			×	×
<i>Enterococcus raffinosus</i>	×	×		×		×	×
<i>Enterococcus solitarius</i>	×					×	×
<i>Gemella haemolysans</i>	×	×			×	× (G. morbillorum)	×
<i>Gemella morbillorum</i>	×	×		×		× (G. haemolysans)	
<i>Globicatella sanguinis</i>	×					×	
<i>Helcococcus kunzii</i>	×					×	
<i>Lactococcus</i> species	× (five species)	× (four species)				× (seven species)	× (six species)
<i>Leuconostoc</i> species	× (four species)	× (three species)	× (three species)			× (eight species)	× (five species)
<i>Pediococcus</i> species	× (three species)	×	× (two species)			× (five species)	× (five species)
<i>Streptococcus acidominimus</i>	×	×	×			×	
<i>Streptococcus agalactiae</i>	×	×	×	×	×	×	×
<i>Streptococcus anginosus</i>	×	×	×		×	×	×
<i>Streptococcus bovis</i>	×	×	×	×	×	×	×
<i>Streptococcus constellatus</i>	×	×	×		×	×	
<i>Streptococcus criceti</i>	×	×				×	
<i>Streptococcus crista</i>	×	×				×	
<i>Streptococcus dysgalactiae/Streptococcus equisimilis</i>						×	
<i>Streptococcus equi</i>	×	×				×	×
<i>Streptococcus equinus</i>	×	×	×			×	×
<i>Streptococcus equisimilis</i>					×	×	
<i>Streptococcus gordonii</i>	×	×	× (S. sanguis)			×	
<i>Streptococcus intermedius</i>	×	×		×		×	×
<i>Streptococcus milleri</i> group	×	×					
<i>Streptococcus mitis</i> group	×	×	×		×	×	×
<i>Streptococcus mutans</i>	×	×	×		×	×	×
<i>Streptococcus oralis</i>	×	×				×	×
<i>Streptococcus parasanguis</i>	×					×	×
<i>Streptococcus pneumoniae</i>	×	×	×	×	×	×	×
<i>Streptococcus porcinus</i>	×					×	×

(continued)

Table 3.16-4 Database entries of the gram-positive organisms (human isolates) for BD, IDS, Sensititre, Biolog, and MIDI products^a (continued)

Organism(s)	BBL CRYSTAL						
	Gram-Pos, version 4.0	Rapid Gram- Pos, version 4.0	IDS RapID STR, ^b ver- sion 1.3.97	Pasco Gram- Positive ID, ^c version 4.6	Sensititre AP90, ver- sion 2.2	Biolog ^d ver- sion 6.01	MIDI, ver- sion 4.0
<i>Streptococcus pyogenes</i>	×	×	×	×	×	×	×
<i>Streptococcus salivarius</i>	×	×	×		×	×	×
<i>Streptococcus sanguinis</i>						×	
<i>Streptococcus sanguis</i>	×	×	× (<i>S. gordoni</i>)		×		×
<i>Streptococcus sobrinus</i>	×	×				×	×
<i>Streptococcus uberis</i>	×	×			×	×	×
<i>Streptococcus vestibularis</i>	×	×				×	×
<i>Streptococcus viridans</i> group				×			
<i>Streptococcus zooepidemicus</i>	×	×				×	×
<i>Weissella confusa</i>			×				× (five species)

^a Reproduced from reference 2. Some products give a choice between two species; the alternate species is indicated in parentheses.

^b Manufactured by Remel.

^c Manufactured by Becton Dickinson Biosciences.

^d Database also includes 36 additional organisms not listed here.

REFERENCES

- Funke, G., and K. A. Bernard. 2003. Co-cyaneform gram-positive rods, p. 480. In P. R. Murray, E. J. Baron, J. H. Jorgensen, M. A. Pfaller, and R. H. Yolken (ed.), *Manual of Clinical Microbiology*, 8th ed. ASM Press, Washington, D.C.
- O'Hara, C. M., M. P. Weinstein, and J. M. Miller. 2003. Manual and automated systems for detection and identification of microorganisms, p. 185–207. In P. R. Murray, E. J. Baron, J. H. Jorgensen, M. A. Pfaller, and R. H. Yolken (ed.), *Manual of Clinical Microbiology*, 8th ed. ASM Press, Washington, D.C.

APPENDIX 3.16-1

Preparation of McFarland Turbidity Standards

I. PRINCIPLE

A chemically induced precipitation reaction between barium chloride and sulfuric acid to form barium sulfate (BaSO_4) can be used to approximate the turbidity of a bacterial suspension (3). Alternatively, a latex particle suspension can be purchased as an optical equivalent to the chemical standard (Remel, Inc.; Hardy Diagnostics), or a nephelometer can be used directly on the bacterial suspension. Varying BaSO_4 turbidities are referred to as McFarland turbidity standards and range between 0.5 and 10 (3).

II. REAGENTS AND SUPPLIES

- The following reagents are prepared in sterile distilled water (2).
 - Solution A: 0.048 mol of BaCl_2 per liter (1.175% [wt/vol] $\text{BaCl}_2 \cdot 2\text{H}_2\text{O}$)
 - Solution B: 0.18 mol of H_2SO_4 , chemically pure grade, per liter (1% [vol/vol])
- Supplies
 - Nine thoroughly cleaned test tubes that hold 10 ml of reagent
 - Test tubes with screw caps that have the same diameter as broth or saline tubes that are used to prepare bacterial suspensions

APPENDIX 3.16-1 (continued)

Include QC information on reagent container and in QC records.

3. Parafilm, liquid paraffin, or airtight tape, such as duct tape.
4. White card with contrasting black lines (available from Remel, Inc., and Hardy Diagnostics).

III. QUALITY CONTROL

As a control of the ability to prepare appropriate turbidity standards, a colony count of the bacterial suspension can be determined. Generally the suspension will have to be diluted 1:100 or 1:10 several times in broth and vortexed until the expected concentration of the suspension is 10^8 /ml (see chart below for corresponding cell density). Such a suspension will yield 100 to 300 colonies on a plate when inoculated with a 0.001-ml loop. Inoculate a second plate with a 0.01-ml loop in case there is no growth on the first plate. Spread with a bent rod for greatest accuracy.

IV. PROCEDURE

- A. Procedure for preparation of standards (1)
 1. Label each test tube from 0.5 to 8.
 2. Dispense the appropriate aliquot of solution A *into* the appropriate aliquot of solution B with constant agitation to maintain a suspension, using the chart below.

Barium sulfate turbidity standard	Vol (ml) of:		Turbidity equivalent to cell density of <i>Escherichia coli</i> broth culture (10^8 /ml)
	Solution A, 0.048 mol of BaCl_2 /liter	Solution B, 0.18 mol of H_2SO_4 /liter	
0.5	0.05	9.95	1.5
1	0.1	9.9	3
2	0.2	9.8	6
3	0.3	9.7	9
4	0.4	9.6	12
5	0.5	9.5	15
6	0.6	9.4	18
7	0.7	9.3	21
8	0.8	9.2	24

3. Verify the correct density of the 0.5 turbidity standard by using a spectrophotometer with a 1-cm light path and matched cuvette to determine the absorbance. The absorbance at 625 nm should be 0.08 to 0.10 for the 0.5 McFarland standard (2).
4. Transfer the barium sulfate suspensions in aliquots into screw-cap tubes of the same size as those used in growing or diluting the bacterial inoculum. Label each tube with the appropriate turbidity equivalent.
5. Tighten tubes and seal with airtight tape, melted paraffin, or Parafilm, and store in the dark at room temperature.
6. Vigorously agitate the barium sulfate turbidity standard on a mechanical vortex mixer before each use, and inspect for a uniformly turbid appearance. If large particles appear, the standard should be replaced. Latex particle suspensions should be mixed by inverting gently, not on a vortex mixer.
7. Verify the density of the 0.5 standard monthly and replace the standards if the density falls outside of the acceptable limits (2).

B. Procedure for use of standards

1. Vigorously agitate the chosen standard on a mechanical vortex mixer before use.
2. Obtain a screw-cap tube with the same diameter as the standard containing either broth, saline, or sterile water, as appropriate for the use of the bacterial suspension.
3. While working in a biological safety cabinet prepare a suspension of the bacteria matching the turbidity of the standard as closely as possible. Recap tube. Vortex suspension.
4. Using adequate light, hold the standard next to the culture tube and display them both against a white card with contrasting black lines.
5. Add culture (if suspension is too light) or additional broth (if suspension is too heavy) to achieve a turbidity optically comparable to the standard.



It is imperative that these cultures be handled in a biosafety hood.

APPENDIX 3.16–1 (*continued*)**References**

1. Baron, E. J., L. Peterson, and S. M. Finegold. 1994. *Bailey and Scott's Diagnostic Microbiology*, 9th ed., p. 170–171. Mosby, St. Louis, Mo.
2. Clinical and Laboratory Standards Institute. 2006. *Performance Standards for Antimicrobial Disk Susceptibility Tests*, 9th ed. Approved standard M2-A9. Clinical and Laboratory Standards Institute, Wayne, Pa.
3. McFarland, J. 1907. Nephelometer: an instrument for estimating the number of bacteria in suspensions used for calculating the opsonic index and for vaccines. *JAMA* **49**:1176–1178.

3.17.1

Acetamide Utilization Test

*[Updated March 2007]***I. PRINCIPLE**

Acetamide agar is used to test an organism's ability to utilize acetamide by deamidation. The medium contains acetamide as the sole carbon source and inorganic ammonium salts as the sole source of nitrogen. Growth is indicative of a positive test for acetamide utilization. When the

bacterium metabolizes acetamide by the enzymatic action of an acylamidase, the ammonium salts are broken down to ammonia, which increases alkalinity. The shift in pH turns the bromthymol blue indicator in the medium from green to blue, indicative of a positive test. Assimilation

of acetamide will result in a yellow color and should not be mistaken for a positive result (2). In general, deamidation is limited to only a few organisms. This medium is recommended for differentiating *Pseudomonas aeruginosa* from other non-glucose-fermenting, gram-negative rods.

II. MICROORGANISMS TESTED

- A. Isolated colonies of non-glucose-fermenting, gram-negative rods that are suggestive of *P. aeruginosa* and produce a fluorescent pigment
- B. Unusual non-glucose-fermenting, gram-negative rods, as part of the identification

III. MEDIA, REAGENTS, AND SUPPLIES

- A. **Medium**
 - 1. Agar slant containing the following per liter
 - sodium chloride 5.0 g
 - magnesium sulfate 0.2 g
 - ammonium phosphate,
 - monobasic 1.0 g
 - dibasic 1.0 g
 - potassium phosphate,
 - acetamide 10.0 g
 - agar 15.0 g
 - bromthymol blue 0.08 g
 - 2. The final pH is 6.8.
 - 3. Store at 2 to 8°C.
 - **NOTE:** Tablets (Key Scientific) are also available. Tablet is dissolved in 1 ml of distilled water and boiled to destroy any vegetative bacteria present. It is then heavily inoculated and incubated at 35°C for up to 6 days. A color change from yellow to purple is a positive reaction.
- B. **Supplies/equipment**
 - 1. Sterile inoculating loops or sticks
 - 2. Incubator at 35°C

IV. QUALITY CONTROL

- A. Perform QC on each new lot or shipment of media prior to putting it into use.
- B. Inspect agar for evidence of prior freezing, contamination, cracks, dehydration, and bubbles prior to storage and before use. Discard any tubes that are blue.
- C. Organisms
 - 1. *P. aeruginosa* ATCC 27853—acetamide positive (growth; blue color)
 - 2. *Escherichia coli* ATCC 25922—acetamide negative (no growth; green color)

V. PROCEDURE

- A. Streak the slant back and forth with inoculum picked from the center of a well-isolated colony.
- B. Place cap loosely on tube.
- C. Incubate aerobically at 35 to 37°C for up to 4 days.
- D. Observe a color change from green to blue along the slant.

VI. INTERPRETATION

- A. A positive test is growth and change from green to intense blue color along the slant.
- B. A negative test is no growth, no color change, and slant remains green.

VII. REPORTING RESULTS

- A. Acetamide deamination is most actively accomplished by *Delftia* (*Comamonas acidovorans*), *P. aeruginosa*, and *Alcaligenes faecalis* (3).
- B. Of the fluorescent pseudomonads, *P. aeruginosa* is positive for acetamide. Unless the isolate is from a normally sterile site, the combination of fluorescent pigment, acetamide deamination, and oxidase positivity is sufficient to identify *P. aeruginosa* (1, 3).

VIII. LIMITATIONS

- A. Growth on the slant without an accompanying color change may indicate a positive test. However, if the agar does not turn blue with further incubation, the test should be repeated with less inoculum.
- B. A negative test does not rule out an identification of *P. aeruginosa*, and 6% of *Pseudomonas fluorescens* organisms have been reported to give a positive reaction (1, 3). Other fluorescent *Pseudomonas* spp. do not give positive reactions.
- C. Other tests should be performed, such as growth at 42°C, to separate *P. aeruginosa* from the other fluorescent pseudomonads if the isolate is from a blood culture, since the patient could have received blood products contaminated with *P. fluorescens*, which can grow at refrigeration temperatures (1).
- D. Tests with equivocal results should be repeated.
- E. Do not stab the slant, since the test requires an aerobic environment.
- F. Do not inoculate from broth cultures, due to carryover of media.
- G. To avoid false-positive reactions, use a light inoculum to prevent carryover of substances from previous media.

REFERENCES

1. Kiska, D. L., and P. H. Gilligan. 2003. *Pseudomonas*, p. 719–728. In P. R. Murray, E. J. Baron, J. H. Jorgensen, M. A. Pfaller, and R. H. Yolken (ed.), *Manual of Clinical Microbiology*, 8th ed. ASM Press, Washington, D.C.
2. MacFaddin, J. F. 1985. *Biochemical Tests for Identification of Medical Bacteria*, p. 15–17. Williams and Wilkins, Baltimore, Md.
3. Weyant, R. S., C. W. Moss, R. E. Weaver, D. G. Hollis, J. G. Jordan, E. C. Cook, and M. I. Daneshvar. 1995. *Identification of Unusual Pathogenic Gram-Negative Aerobic and Facultatively Anaerobic Bacteria*, 2nd ed., p. 5. Williams & Wilkins, Baltimore, Md.

3.17.2

Acetate Utilization Test

I. PRINCIPLE

Acetate agar is used to test an organism's ability to utilize acetate. The medium contains sodium acetate as the sole carbon source and inorganic ammonium salts as the sole source of nitrogen. Growth is indicative of a positive test for acetate util-

ization. When the bacteria metabolize acetate, the ammonium salts are broken down to ammonia, which increases alkalinity. The shift in pH turns the bromthymol blue indicator in the medium from green to blue (2). This medium is recommended in dif-

ferentiating *Shigella* spp. from *Escherichia coli* (1). Approximately 84% of *E. coli* strains utilize acetate, whereas the majority of *Shigella* species are incapable of acetate utilization.

II. MICROORGANISMS TESTED

- A. Non-lactose-fermenting, gram-negative rods that are oxidase negative, nonmotile, and anaerogenic, which are likely to be either *E. coli* or *Shigella*
- B. Nonfermentative, gram-negative rods

III. MEDIA, REAGENTS, AND SUPPLIES

A. Media

- 1. Sodium acetate agar slants contain the following ingredients per liter of solution.

sodium chloride	5.0 g
magnesium sulfate	0.1 g
ammonium phosphate, monobasic	1.0 g
potassium phosphate, dibasic	1.0 g
sodium acetate	2.0 g
agar	20.0 g
bromthymol blue	0.08 g

- 2. The final pH is 6.7.

- 3. Vendors include Hardy Diagnostics; Remel, Inc.; and BD Diagnostic Systems.

B. Supplies

- 1. Sterile inoculating loops or sticks
- 2. Sterile pipette
- 3. Incubator at 35°C
- 4. Sterile saline

IV. QUALITY CONTROL

- A. Perform QC on new lots of media prior to putting them into use.

- B. Inspect agar for evidence of prior freezing, contamination, cracks, dehydration, and bubbles prior to storage and before use. Discard any tubes that are blue.

C. Organisms

- 1. *E. coli* ATCC 25922—acetate positive (growth; blue color)
- 2. *Shigella flexneri* ATCC 12022—acetate negative (no growth or trace of growth)

V. PROCEDURE

- A. Using an 18- to 24-h culture from a noninhibitory culture plate, prepare a turbid saline suspension. Inoculate the slant with 1 drop of the suspension.
 - B. Alternatively, streak the slant back and forth with a light inoculum picked from the center of a well-isolated colony.
 - C. Place cap loosely on tube.
 - D. Incubate aerobically at 35 to 37°C for up to 5 days for *Enterobacteriaceae*; incubate at 30°C for nonfermenting, gram-negative rods for up to 7 days.
 - E. Observe a color change from green to blue along the slant.
-

VI. INTERPRETATION

- A. A positive test is growth and change from green to intense blue color along the slant.
 - B. A negative test is no growth, no color change, and slant remains green.
-

VII. REPORTING RESULTS

- A. Most *E. coli* organisms are positive for acetate utilization; most *Shigella* organisms are negative.
 - B. Some *S. flexneri* organisms are acetate positive. Lysine decarboxylase, citrate, and serologic tests (e.g., Alkalescens-Dispar [AD] antisera for *E. coli*) are also helpful to rule out *Shigella*, if any of these results are positive.
-

VIII. LIMITATIONS

- A. Growth on the slant without an accompanying color change may indicate a positive test. However, if the agar does not turn blue on further incubation, the test should be repeated with less inoculum.
 - B. Tests with equivocal results should be repeated.
 - C. Do not stab the slant, since the test requires an aerobic environment.
 - D. Do not inoculate from broth cultures, due to carryover of media.
 - E. To avoid false-positive reactions, use a light inoculum to prevent carryover of substances from previous media.
-

REFERENCES

1. Trabulsi, L. R., and W. H. Ewing. 1962. Sodium acetate medium for differentiation of *Shigella* and *Escherichia* cultures. *Public Health Lab.* **20**:137–140.
2. Weyant, R. S., C. W. Moss, R. E. Weaver, D. G. Hollis, J. G. Jordan, E. C. Cook, and M. I. Daneshvar. 1995. *Identification of Unusual Pathogenic Gram-Negative Aerobic and Facultatively Anaerobic Bacteria*, 2nd ed., p. 6–7. Williams & Wilkins, Baltimore, Md.

3.17.3

ALA (δ -Aminolevulinic Acid) Test for Porphyrin Synthesis

[Updated March 2007]

I. PRINCIPLE

The δ -aminolevulinic acid (ALA) test is a rapid test used to determine the growth requirement for hemin (X factor) in the identification of *Haemophilus* spp. (1). Organisms which do not require hemin can produce porphobilinogen from ALA by the enzyme porphobilinogen synthase. The porphobilinogen is then converted to protoporphyrin IV, the immediate precursor of the heme moiety, in a series of steps. The presence of porphyrins is detected by

the emission of red-orange fluorescence under UV light (360 nm). This method is more rapid than the X factor strip and is superior because it avoids erroneous results due to carryover (2). In the tube test, if no fluorescence is observed, indole reagent can be added to detect porphobilinogen, the precursor to porphyrin (3), which is considered a positive test for porphobilinogen synthase.

ALA has also been described for detecting cytochromes in catalase-negative, gram-positive cocci (4). A positive test for porphobilinogen in a gram-positive coccus indicates that the organism is a member of the family *Micrococcaceae*, even if it is catalase negative. This test is useful for identification of organisms that have the colony morphology of staphylococci but are catalase negative.

II. MICROORGANISMS TESTED

- A. Tiny gram-negative rods or coccobacilli growing only on CHOC with the typical *Haemophilus* colonial morphology (e.g., large size colony in 24 h) and which do not grow on BAP. They do grow on BAP by satelliting around *Staphylococcus* spp.
- B. Gram-positive cocci that have the colonial appearance of staphylococci but are catalase negative

III. REAGENTS AND SUPPLIES



Include QC information on reagent container and in QC records.

A. ALA reagent (3)

1. Purchase disks (BD Diagnostic Systems; Hardy Diagnostics; Remel, Inc.). Store at 4°C or
2. Prepare by combining the following.
 - a. 33.5 mg of ALA (Sigma) (stored at -20°C)
 - b. 19.72 mg of MgSO₄ · 7H₂O
 - c. 100 ml of 0.1 M Sorensen's phosphate buffer, pH 6.9
 - (1) 3.15 g of NaH₂PO₄ · H₂O and 7.4 g of Na₂HPO₄ · 7H₂O in 500 ml of deionized water
 - (2) Check pH; adjust with either 0.1 M HCl or 0.1 M NaOH.
 - (3) Store at 2 to 8°C. Reagent is stable for 6 months.
 - d. Reagent should appear colorless. Do not use if reagent is tan

to brown or particulate matter is present.

- e. Dispense in 0.5-ml aliquots into 13- by 100-mm tubes. Cover tightly and store at -20°C.
- f. Label rack of tubes with preparation date, expiration date (9 months from preparation), storage requirements, and preparer's initials.

B. Kovács' indole reagent

1. Prepare in isoamyl alcohol for porphobilinogen test.
2. See Appendix 3.17.23–1.

C. Other supplies

1. Filter paper or test tubes
2. Petri dish
3. Sterile wooden sticks, plastic or wire inoculating loops
4. Long-wave UV light (360 nm or Wood's lamp)
5. Incubator at 35°C

IV. QUALITY CONTROL

- A. Perform QC on each new lot or shipment of disks or reagent prior to putting it into use.
- B. Test positive control with use to verify length of incubation and color reaction (optional).
- C. Organisms
 - 1. *Haemophilus influenzae* ATCC 10211—ALA negative; no red fluorescence
 - 2. *Haemophilus parainfluenzae* ATCC 7901—ALA positive; red fluorescence

V. PROCEDURE

- A. Inoculation
 - 1. Filter paper method
 - a. Place piece of filter paper to fit flat in a petri dish.
 - b. With a pencil, make circles approximately 10 mm in diameter for each test, plus a circle for the positive control.
 - c. Inoculate center of each circle heavily with colony of test organism using a wooden stick or loop.
 - d. Slowly pour thawed ALA reagent over filter paper, covering all test circles.
 - 2. Disk method
 - a. Place ALA-containing disks in bottom of petri dish, marking the petri dish near each disk with culture identifying number.
 - b. Add 0.04 ml (one small drop) of saline to each disk.
 - c. Inoculate center of each disk heavily with test organism using wooden sticks; repeat for each control organism. Colony should be visible on disk. Alternatively, touch disk to colony and then place in petri dish.
 - d. Place a filter paper saturated with water on lid of dish. Cover.
 - 3. Tube method: add a heavy inoculum of organism to 0.5 ml of ALA reagent.
- B. Incubation and observation
 - 1. Incubate test in non-CO₂ incubator at 35°C for 2 h.
 - 2. Observe reactions for red fluorescence under Wood's lamp in darkened room.
 - 3. Reincubate for 2 to 4 h more if control is negative and reread.
 - 4. If the tube test is negative, incubate for 18 to 24 h, add 0.5 ml of Kovács' reagent to the tube, and shake vigorously. Observe for immediate color change.

VI. INTERPRETATION

- A. The presence of red fluorescence is a positive test, indicating that the organism does not require X factor or hemin and that the ALA has been utilized.
- B. The lack of red fluorescence is a negative test, indicating that the organism requires X factor and that the ALA has not been utilized.
- C. When the indole reagent is added, a red color in the lower aqueous phase indicates a positive test for porphobilinogen, which is also indicative that the organism does not require hemin for growth.

VII. REPORTING RESULTS

- A. A negative test or no fluorescence for a gram-negative rod that grows as large colonies in 24 h on CHOC but not on BAP indicates *H. influenzae*.
- B. A negative test or no fluorescence for a catalase-negative, oxidase-positive, gram-negative rod that takes several days to grow on CHOC, does not grow on BAP, even with a streak of staphylococci, and is sodium polyanethol sulfonate sensitive indicates *Haemophilus ducreyi*.
- C. A positive test for fluorescence or the red color in the lower aqueous phase after adding indole reagent is consistent with *H. parainfluenzae*, *Haemophilus parahaemolyticus*, or *Haemophilus paraphrophilus*. The last organism is lactose

VII. REPORTING RESULTS

(continued)

- positive. Further biochemical tests are needed to separate (2). See Table 3.18.2–2.
- D. A positive test for porphobilinogen in a gram-positive coccus indicates that the organism is a member of the family *Micrococcaceae*, even if it is catalase negative (4). *Aerococcus* and *Streptococcus* are porphobilinogen negative, and *Staphylococcus* and *Rothia mucilaginosa* (*Stomatococcus mucilaginosus*) are positive.

VIII. LIMITATIONS

- A. Many organisms will give a positive reaction. If test is performed only on gram-negative rod colonies that grow well on CHOC in 24 h and not on BAP, results are for *Haemophilus* species.
- B. The ALA test will not separate *H. influenzae* from *Haemophilus haemolyticus*; the latter is rare and not pathogenic. It will sometimes grow on BAP without the need for staphylococcal streak, if it is able to hemolyze the blood to supply it with V factor.
- C. Read test only in a darkened room.
- D. Make a heavy inoculum to avoid false-negative results.
- E. Test only cultures less than 24 h old.
- F. If test paper is not kept moist during the incubation, the reaction can be falsely negative.
- G. *Francisella tularensis* is ALA negative but does not grow in 24 h on CHOC and is oxidase negative. It does not satellite around staphylococci on BAP.

REFERENCES

1. Kilian, M. 1974. A rapid method for the differentiation of *Haemophilus* strains—the porphyrin test. *Acta Pathol. Microbiol. Scand. Sect. B* **82**:835–842.
2. Lund, M. S., and D. J. Blazevic. 1977. Rapid speciation of *Haemophilus* with the porphyrin production test vs. the satellite test for X. *J. Clin. Microbiol.* **5**:142–144.
3. Weyant, R. S., C. W. Moss, R. E. Weaver, D. G. Hollis, J. G. Jordan, E. C. Cook, and M. I. Daneshvar. 1995. *Identification of Unusual Pathogenic Gram-Negative Aerobic and Facultatively Anaerobic Bacteria*, 2nd ed., p. 18. Williams & Wilkins, Baltimore, Md.
4. Wong, J. D. 1987. Porphyrin test as an alternative to benzidine test for detecting cytochromes in catalase-negative gram-positive cocci. *J. Clin. Microbiol.* **25**:2006–2007.

3.17.4

Antimicrobial Disk Tests for Identification (Especially of Staphylococci)

[Updated March 2007]

I. PRINCIPLE

Identification of coagulase-negative staphylococci to the species level is often not clinically useful (14). The use of the pyrrolidonyl- β -naphthylamide (PYR) test in combination with the coagulase test, ornithine decarboxylase, and a number of disk susceptibility tests allows identification of clinically important species: *Staphylococcus saprophyticus* in urinary tract and genital infections, *S. lugdunensis* in blood and joint cultures, and *S. haemolyticus*, which is important only because it can be intermediately susceptible to vancomycin. For repeatedly isolated coagulase-negative staphylococci from blood and clinically significant or post-operative specimens, antimicrobial susceptibility profiles are required. Nosocomial strains may need to be typed by pulse-field gel electrophoresis to determine the spread of clonal strains in outbreak situations.

For urinary tract specimens, for which a report of either *S. saprophyticus* or coagulase-negative staphylococci is sufficient, only the novobiocin disk need be

tested, along with testing for any antimicrobials that are requested by caregivers (2, 11). *S. saprophyticus* is resistant to novobiocin (1, 7, 10); a few other staphylococcal species are also resistant to novobiocin, but they are rarely found in clinical specimens, especially in the urinary tract. Evaluations of the novobiocin disk method on both Mueller-Hinton (MH) agar (1, 7) and BAP (10, 12) have been published. Generally, zone sizes are larger for novobiocin on MH agar, and a 16-mm breakpoint has been recommended. Hébert et al. (10) tested 1,000 clinical isolates of coagulase-negative staphylococci on BAP and found that a lower zone size (<12 mm) for resistance increased the specificity of the test.

The bacitracin 0.04-U disk, previously considered useful for identification of *Streptococcus pyogenes*, is a very sensitive assay to separate staphylococci from *Rothia mucilaginosa* (formerly *Stomatococcus mucilaginosus* [3]) and *Micrococcus* (4, 10, 12). This separation is gener-

ally performed restrictively, usually on strains with pigment or sticky colony morphology from significant anatomic sites. Bacitracin differentiation may also be useful for penicillin-susceptible strains, since the great majority of *Micrococcus* and *Rothia* organisms are susceptible to penicillin and most coagulase-negative staphylococci are not (13). Alternatively, the furazolidone disk test (4), from BD Diagnostic Systems, or the modified oxidase test using a 6% solution of tetramethylphenylenediamine in dimethyl sulfoxide (5) sold as the Microdase test by Remel, Inc., can be used to separate these genera (see procedure 3.17.39). These tests are not covered in this procedure, since the bacitracin disk is easily obtained and does not require additional QC organism stocks.

The polymyxin B disks (or colistin disks) are useful to separate species of staphylococci and are also very useful in the identification of *Neisseria* and gram-negative rods (procedure 3.18.2).

II. MICROORGANISMS TESTED

A. Novobiocin

For gram-positive cocci in clusters which are catalase positive and coagulase negative isolated from urine specimens, usually from sexually active young women

B. Bacitracin

For penicillin-susceptible or “sticky” colonies of gram-positive cocci in clusters which are catalase positive and coagulase negative from invasive-site specimens to separate staphylococci from micrococci. Do not test lemon-yellow colonies, because they are presumed to be *Micrococcus*.

C. Polymyxin B

For differentiation of *R. mucilaginosa* from *Micrococcus* spp. and for separation of *S. aureus* and *S. intermedius* or characterization of other species of staphylococci, especially *S. epidermidis*, in unusual circumstances. This disk is also

II. MICROORGANISMS TESTED

(continued)

used as part of the identification of *Neisseria*, *Vibrio*, and non-glucose-fermenting, gram-negative rods, especially from cystic fibrosis patients.

D. Penicillin and vancomycin

For screening gram-positive cocci in clusters from positive blood cultures for confirmation of Gram stain, for induction for the beta-lactamase test, for detection of unusual resistance, and for *Pediococcus* and *Leuconostoc* identification

III. MEDIA, REAGENTS, AND SUPPLIES

A. Disks

Store a small supply at 4°C; store stock at -20°C.
 1. Novobiocin, 5 µg
 2. Bacitracin, 0.04 U
 3. Penicillin, 10 U
 4. Vancomycin, 30 µg
 5. Polymyxin B, 300 U (colistin [10-µg] disk can be substituted)

B. Media

1. BAP or
2. MH agar

C. Supplies

1. Swabs
2. Broth for inoculum

IV. QUALITY CONTROL

A. Perform QC of disks weekly or with each use for tests that are performed less often than weekly. Test on the medium that is used for test performance. For disks used for identification only (bacitracin, novobiocin), testing need not be performed weekly, but test each new lot or shipment of disks with both a susceptible and resistant strain prior to using for identification tests. Testing should be repeated monthly with a susceptible control organism for disks that are stored at 4°C rather than -20°C.

B. Strains, media, and zone size requirements

Antimicrobial agent	Test organism	Zone size (mm) with 6-mm disk
Novobiocin, 5 µg, on BAP or MH agar	<i>Staphylococcus aureus</i> ATCC 25923	≥22
	<i>Staphylococcus saprophyticus</i> ATCC 15305	≤15
Bacitracin, 0.04 U, on BAP	<i>Streptococcus pyogenes</i> ATCC 19615	≥12
	<i>Staphylococcus aureus</i> ATCC 25923	6
Polymyxin B, 300 U, on MH agar	<i>Pseudomonas aeruginosa</i> ATCC 27853	17–21 (6)
	<i>Escherichia coli</i> ATCC 25922	17–20 (6), 12–16 (BD package insert)
Colistin, 10 µg, on MH agar	<i>Pseudomonas aeruginosa</i> ATCC 27853	15–19 (6)
	<i>Escherichia coli</i> ATCC 25922	16–20 (6), 11–15 (BD package insert)
Penicillin, 10 U, on MH agar	<i>Staphylococcus aureus</i> ATCC 25923	26–37
Vancomycin, 30 µg, on MH agar	<i>Staphylococcus aureus</i> ATCC 25923	17–21

V. PROCEDURE**A. Hébert method (9, 10, 12) using BAP**

1. Prepare no. 1 McFarland suspension of organism from overnight growth.
2. Inoculate a section of a BAP in one direction; area of inoculation should allow a separation of 10 mm between each disk to be placed on the inoculum.
3. After allowing 10 min for drying, place the disks on agar.
4. Tap disk with sterile stick to ensure adherence.
5. Incubate for 24 h at 35°C in non-CO₂ incubator.

B. MH agar method (1, 7)

1. If other disk susceptibility testing is being performed to report susceptibility to antimicrobial agents on a urinary isolate, use MH agar for the novobiocin test (1, 7).
2. Prepare a no. 0.5 McFarland suspension of the organism and inoculate MH agar in three directions as for a disk susceptibility test (2).
3. Place novobiocin and other susceptibility test disks on agar.
4. Incubate for 18 h at 35°C in a non-CO₂ incubator.

C. For positive blood cultures

1. Place a drop from the positive culture onto BAP and spread with a loop over one-fourth of the plate.
2. Place penicillin and vancomycin disks on this area of the plate.
3. Use interpretations for MH agar only for identification purposes, not for susceptibility reporting.

VI. INTERPRETATION**A. Novobiocin**

1. Hébert method using BAP (10)
 - a. Zone of <12 mm is resistant.
 - b. Zone of ≥12 mm is susceptible.
2. NCCLS method using MH agar (1, 2, 7)
 - a. Zone of ≤16 mm is resistant.
 - b. Zone of >16 mm is susceptible.
3. Resistance corresponds to an MIC of ≥1.6 µg/ml.

B. Bacitracin, 0.04 U (4, 10, 12), on either BAP or MH agar

1. Zone of 6 mm is resistant.
2. Zone of ≥10 mm is susceptible.
3. Repeat tests with values between 7 and 10 mm; these are probably susceptible.

C. Polymyxin B (10) on either BAP or MH agar

1. Zone of <10 mm is resistant.
2. Zone of ≥10 mm is susceptible.
☒ NOTE: If colistin is substituted for polymyxin B, the zone sizes may be smaller for susceptible strains; for gram-negative rods, any zone is considered susceptible (M. York, personal observation).

D. Penicillin (2)

1. Zone of ≤28 mm is resistant.
2. Zone of ≥29 mm is susceptible.

E. Vancomycin (2)

1. Zone of <15 mm is resistant.
2. Zone of ≥15 mm is susceptible.

VII. REPORTING RESULTS

- A. For gram-positive cocci in clusters which are catalase positive and coagulase negative
 - 1. Novobiocin-resistant strains
 - a. In a urine culture, report as *S. saprophyticus* (11).
 - b. From other body sites, *do not use this result alone* for species identification. Generally refer to the organism as “coagulase-negative staphylococci.”
 - 2. If an isolate is penicillin susceptible and from a significant body site, use the bacitracin 0.04-U disk to separate coagulase-negative staphylococci (resistant) from *Micrococcus* (susceptible) and *Rothia* (susceptible) (4, 10, 12). The latter also adheres to the agar.
 - 3. Most staphylococcal species are susceptible to polymyxin B, but *S. aureus*, *S. lugdunensis*, and *S. epidermidis* (97.2% are resistant) are notable exceptions (10). Use this resistance as part of the identification of *S. epidermidis* and to separate *S. aureus* from *S. intermedius*. *Rothia* is also polymyxin B resistant. See Table 3.18.1–1.
 - B. Any gram-positive coccus that is resistant to vancomycin is either *Enterococcus*, *Pediococcus*, *Leuconostoc*, or possibly a vancomycin-resistant staphylococcus, which must be further tested to confirm the result (*see section 5 and procedure 3.18.1*).
 - C. Refer to procedure 3.18.2 for use of the polymyxin B disk in the identification of gram-negative rods.
-

VIII. LIMITATIONS

- A. Strains of staphylococci, other than *S. saprophyticus*, that are resistant to novobiocin include *S. xylosus*, *S. kloosi*, and *S. cohnii*. Any strain may become resistant to novobiocin, including *S. aureus*; reporting *S. saprophyticus* should be limited to urinary tract isolates unless further tests for identification to the species level are performed.
- B. While Goldstein et al. (7) recommend using <16 mm as the breakpoint for resistance to novobiocin for BAP and MH agar, their data and those of others demonstrate greater specificity without loss of sensitivity by using the <12-mm breakpoint for BAP (7, 10).
- C. Harrington and Gaydos (8) reported a rapid novobiocin test that is completed in 5 h.
- D. While *Micrococcus* and *R. mucilaginosa* can be resistant to penicillin, this is a very rare event (12, 13).
- E. Staphylococci should show no zone of inhibition around the bacitracin 0.04-U disk on BAP (10). Zone sizes of >7 mm but less than the 10-mm breakpoint may be obtained for *Micrococcus* if incubation is not a full 24 h or MH agar is used (10).
- F. Direct tests from blood cultures are not standardized but can work well to provide preliminary reports and guide subsequent identifications and more standardized antimicrobial susceptibility testing.

REFERENCES

1. Almeida, R. J., and J. H. Jorgensen. 1982. Use of Mueller-Hinton agar to determine novobiocin susceptibility of coagulase-negative staphylococci. *J. Clin. Microbiol.* **16**:1155–1156.
2. Clinical and Laboratory Standards Institute. 2006. *Performance Standards for Antimicrobial Disk Susceptibility Tests*, 9th ed. Approved standard M2-A9. Clinical and Laboratory Standards Institute, Wayne, Pa.
3. Collins, M. D., R. A. Hutson, V. Bäverud, and E. Falsen. 2000. Characterization of a *Rothia*-like organism from a mouse: description of *Rothia nasmurium* sp. nov. and reclassification of *Stomatococcus mucilaginosus* as *Rothia mucilaginosa* comb. nov. *Int. J. Syst. Evol. Microbiol.* **50**:1247–1251.
4. Falk, D., and S. J. Guering. 1983. Differentiation of *Staphylococcus* and *Micrococcus* spp. with the Taxo A bacitracin disk. *J. Clin. Microbiol.* **18**:719–721.
5. Faller, A., and K. H. Schleifer. 1981. Modified oxidase and benzidine tests for separation of staphylococci from micrococci. *J. Clin. Microbiol.* **13**:1031–1035.
6. Gales, A. C., A. O. Reis, and R. N. Jones. 2001. Contemporary assessment of antimicrobial susceptibility testing methods for polymyxin B and colistin: review of available interpretative criteria and quality control guidelines. *J. Clin. Microbiol.* **39**:183–190.
7. Goldstein, J., R. Schulman, E. Kelley, G. McKinley, and J. Fung. 1983. Effect of different media on determination of novobiocin resistance for differentiation of coagulase-negative staphylococci. *J. Clin. Microbiol.* **18**:592–595.
8. Harrington, B. J., and J. M. Gaydos. 1984. Five-hour novobiocin test for differentiation of coagulase-negative staphylococci. *J. Clin. Microbiol.* **19**:279–280.
9. Hébert, G. A. 1990. Hemolysins and other characteristics that help differentiate and biotype *Staphylococcus lugdunensis* and *Staphylococcus schleiferi*. *J. Clin. Microbiol.* **28**:2425–2431.
10. Hébert, G. A., C. G. Crowder, G. A. Hancock, W. R. Jarvis, and C. Thornsberry. 1988. Characteristics of coagulase-negative staphylococci that help differentiate these species and other members of the family *Micrococcaceae*. *J. Clin. Microbiol.* **26**:1939–1949.
11. Meers, P. D., W. Whyte, and G. Sandys. 1975. Coagulase-negative staphylococci and micrococci in urinary tract infections. *J. Clin. Pathol.* **28**:270–273.
12. Mitchell, P. S., B. J. Huston, R. N. Jones, L. Holcomb, and F. P. Koontz. 1990. *Stomatococcus mucilaginosus* bactemias. Typical case presentations, simplified diagnostic criteria, and a literature review. *Diagn. Microbiol. Infect. Dis.* **13**:521–525.
13. von Eiff, C., M. Herrmann, and G. Peters. 1995. Antimicrobial susceptibilities of *Stomatococcus mucilaginosus* and of *Micrococcus* spp. *Antimicrob. Agents Chemother.* **39**:268–270.
14. Weinstein, M. P., S. Mirrett, L. Van Pelt, M. McKinnon, B. L. Zimmer, W. Kloos, and L. B. Reller. 1998. Clinical importance of identifying coagulase-negative staphylococci isolated from blood cultures: evaluation of MicroScan Rapid and Dried Overnight gram-positive panels versus a conventional reference method. *J. Clin. Microbiol.* **36**:2089–2092.

3.17.5

Bile-Esculin and Esculin Tests

I. PRINCIPLE

The basis of the esculin test is the hydrolysis of esculin (a glucoside) into glucose and esculutin by a microorganism that has a constitutive (noninducible) β -glucosidase or esculinase enzyme. When esculutin is produced by the hydrolysis of esculin, it reacts with an iron salt in the

medium to form a phenolic iron complex which produces a dark brown or black color (3). Alternatively, esculin is a fluorescent compound and its hydrolysis can be observed by a loss of fluorescence. If bile is added to the medium, the microorganism must be able to grow in its pres-

ence in order to hydrolyze esculin. The 40% bile (equivalent to 4% oxgall) in bile-esculin medium inhibits most strains of streptococci, other than *Streptococcus bovis*, but does not inhibit enterococci or *Listeria* (1, 2).

II. MICROORGANISMS TESTED

- A. Gram-positive cocci in chains, which are catalase negative and morphologically identified as presumptive *S. bovis*
- B. Isolates of alpha- or gamma-hemolytic, gram-positive cocci as part of differentiation of enterococci from other pyrrolidonyl- β -naphthylamide (PYR)-positive organisms
- C. Non-spore-forming, hemolytic, gram-positive rods that are catalase positive and morphologically identified as presumptive *Listeria*
- D. Positive blood cultures with gram-positive cocci in chains or gram-positive rods, to rapidly (4 h) identify enterococci and *Listeria*
- E. Esculin without bile for the identification of oxidase-positive aerobic gram-negative rods, including *Aeromonas* spp. and yellow-pigmented non-glucose-fermenting rods (4)

III. MEDIA, REAGENTS, AND SUPPLIES

A. Media

Store at 2 to 8°C.

- 1. Bile-esculin agar slants with iron(III) citrate. Agar plate media, such as Enterococcose agar (BD Diagnostic Systems), have a similar formulation.
- 2. Bile-esculin-azide agar or broth with iron(III) citrate and azide. Azide will inhibit most gram-negative bacteria.
- 3. Peptose-yeast-esculin broth (usually in anaerobic atmosphere)
NOTE: This medium can be decanted into small volumes for use on aerobic and microaerobic organisms.
- 4. Esculin agar (0.1% esculin in heart infusion basal medium) without

bile or azide but with iron(III) citrate (Hardy Diagnostics)

NOTE: Color Spot (Remel, Inc.) and Wee-Tab (Key Scientific) incorporate PYR and esculin tests on filter paper cards in a rapid test. BactiCard Strep (Remel, Inc.) and StrepQuick (Hardy Diagnostics) contain PYR, leucine aminopeptidase (LAP), and a rapid esculin test. Enterococcus/Group A Screen (Remel, Inc.) is a rapid tube test for esculin and PYR.

B. Reagents and supplies

- 1. Long-wave (360 nm) UV light
- 2. 1% ferric [iron(III)] ammonium citrate if iron(III) is not incorporated into the medium

IV. QUALITY CONTROL

- A. Perform QC on new lots or shipments of media and ferric ammonium citrate reagent prior to putting them into use.
- B. Inspect agar for freezing, contamination, cracks, dehydration, and bubbles prior to storage and before use.
- C. Examine uninoculated broth for fluorescence.
- D. Organisms
 - 1. *Enterococcus faecalis* ATCC 29212—bile-esculin and esculin positive
 - 2. *Escherichia coli* ATCC 25923—bile-esculin and esculin negative
 - 3. *Streptococcus pyogenes* ATCC 19615—bile-esculin and esculin negative

V. PROCEDURE**A. Tube test**

- 1. Inoculate slant or broth with a single colony. For enterococcus and *S. bovis* identification, use only 40% bile and inoculate with a 10- μ l calibrated loopful of a 0.5 McFarland standard suspension prepared in sterile water (1).
- 2. Place cap loosely on tube.
- 3. Incubate for 24 h at 35°C (or up to 7 days for slow-growing gram-negative rods and anaerobes) and observe color change. For enterococci and *S. bovis*, read only at 18 to 24 h.
- 4. For esculin broth without iron(III) citrate, observe daily for loss of fluorescence. Add 2 or 3 drops of 1.0% ferric ammonium citrate to the esculin tube if no fluorescence is observed. Observe for a color change. Aliquot broth for daily testing with indicator, if desired.
- 5. When evaluating loss of fluorescence, use an uninoculated control tube for comparison.

B. Disk test

- 1. Moisten disk with a single drop of distilled or deionized water. Do not saturate.
- 2. Using a sterile loop, pick two or three well-isolated colonies from an overnight (18- to 24-h) culture.
- 3. Allow to react at room temperature for 10 min.
- 4. Observe the disk for the development of a dark brown or black color.

VI. INTERPRETATION

- A. A positive tube test in medium containing ferric ammonium citrate is demonstrated by blackening of the medium.
- B. A negative tube test is indicated by lack of color change. The medium will fluoresce under UV light (366 nm).
- C. For esculin broth without iron(III) citrate, a positive test is demonstrated either by blackening of the broth after addition of the ferric [iron(III)] reagent or by the loss of fluorescence of the medium.
- D. A negative test result also occurs in bile-esculin medium if the organism cannot grow in the presence of bile, regardless of the ability to hydrolyze esculin.
- E. A positive disk test is the development of a dark brown or black color.
- F. A negative disk test remains colorless.

VII. REPORTING RESULTS

- A. *Listeria monocytogenes* is a bile-esculin-positive, beta-hemolytic, CAMP-positive, catalase-positive, motile, nonpigmented, small gram-positive rod.
- B. Esculin- or bile-esculin-positive, catalase-negative, gram-positive cocci in chains may be *Enterococcus*. Confirmation includes large-diameter (1-mm) colonies that are PYR and LAP positive. *Lactococcus* organisms are not easily separated from enterococci.

VII. REPORTING RESULTS (continued)

- C. Esculin- or bile-esculin-negative, gamma- or alpha-hemolytic, gram-positive cocci in *chains* that are bile insoluble (procedure 3.17.6) and catalase negative and grow well on BAP are presumptively identified as viridans group streptococci.
- D. If the inoculum is controlled and the medium contains 40% bile, PYR-negative streptococci (catalase-negative, gram-positive cocci in chains) can be presumptively identified as *S. bovis* with a 97% sensitivity by a positive bile-esculin test (1).
- E. Positive esculin reactions can separate the species of yellow, non-glucose-fermenting, gram-negative rods (Tables 3.18.2–6 and 3.18.2–7) and *Aeromonas* species.

VIII. LIMITATIONS

- A. If the inoculum is too great or the concentration of bile is less than 40%, viridans group streptococci other than *S. bovis* can give a positive reaction on bile-esculin agar (1, 2). Esculin tests without bile do not separate *S. bovis* (previously referred to as group D streptococci, not enterococci) from other viridans group streptococci.
- B. H₂S, which is produced by several organisms during metabolism, also reacts with iron to produce a black complex, which interferes with the interpretation of the esculin hydrolysis test. Therefore, for gram-negative rods, check tubes showing darkening after the addition of the reagent under UV light; intact esculin fluoresces white-blue, whereas hydrolyzed esculin has lost its fluorescence.
- C. Some microorganisms, such as *E. coli*, have an inducible β-glucosidase and will react in this test only after prolonged incubation. Prolonged incubation should not be used if the test is being used to detect only constitutive β-glucosidase.

REFERENCES

1. Chuard, C., and L. B. Reller. 1998. Bile-esculin test for presumptive identification of enterococci and streptococci: effects of bile concentration, inoculation technique, and incubation time. *J. Clin. Microbiol.* **36**:1135–1136.
2. Facklam, R. R. 1973. Comparison of several laboratory media for presumptive identification of enterococci and group D streptococci. *Appl. Microbiol.* **26**:138–145.
3. MacFaddin, J. F. 2000. *Biochemical Tests for the Identification of Medical Bacteria*, 3rd ed., p. 8–26. The Lippincott, Williams & Wilkins Co., Philadelphia, Pa.
4. Sneath, P. H. A. 1956. Cultural and biochemical characteristics of the genus *Chromobacterium*. *J. Gen. Microbiol.* **15**:70–98.

3.17.6

Bile Solubility Test

I. PRINCIPLE

The bile solubility test is used to differentiate *Streptococcus pneumoniae* from alpha-hemolytic *Streptococcus* spp. It may be performed using a cell suspension on a slide or in a tube or by applying the reagent directly to the colony.

The bile solubility test is based on the observation that pneumococcal cells lyse

when sodium desoxycholate (bile salts) is applied to the colony under specific conditions of time and temperature, but other streptococci do not lyse (6). The pneumococcus has an intracellular autolytic enzyme, an amidase, that causes the organism to undergo rapid autolysis when cultivated on artificial medium. The bile

salts alter the surface tension of the medium and cause cell membrane rearrangement. The working mechanism of the test is not clearly understood; however, one theory is that the bile salts facilitate lysis of pneumococcal cells by activating the autolytic enzyme (2).

II. MICROORGANISMS TESTED

- A. Any alpha-hemolytic, catalase-negative, gram-positive cocci in chains, having the characteristic central depression (flattened center) or mucoid colony morphology suggestive of *S. pneumoniae*
- B. Any gram-positive cocci in lancet-shaped pairs from a positive blood culture

III. REAGENTS AND SUPPLIES



Include QC information on reagent container and in QC records.

- A. Reagents**
 - 1. Bile salts
 - a. Purchase or prepare 10% bile salt solution
 - sodium desoxycholate
(Sigma) 10 g
 - deionized water 100 ml
 - b. Dispense in small amounts to minimize contamination. Shelf life is 270 days.
 - c. Store at 15 to 30°C. Storage of the reagent at cool temperatures can cause it to thicken.
- 2. 0.85% NaCl (sterilized)
- 3. Broth culture medium (e.g., BHI)
- B. Supplies**
 - 1. Loops
 - 2. Test tubes or slide
 - 3. Pasteur pipettes

IV. QUALITY CONTROL

- A. Test each new lot of reagent with known positive and negative controls before putting it into use.
- B. Do not use if bile reagent is not a clear and very light amber.
- C. Organisms
 - 1. *S. pneumoniae* ATCC 49619—positive (bile soluble)
 - 2. *Enterococcus faecalis* ATCC 29212—negative (bile insoluble)

V. PROCEDURE**A. Test tube method**

1. Dispense 0.5 ml of sterile saline or suitable broth into a small test tube.
2. Prepare a heavy suspension of the organism in the saline (equivalent to no. 1 McFarland standard). Shake or vortex to form a uniform suspension.
3. Divide the suspension into two tubes, one labeled "TEST" and the other labeled "CONTROL."
4. Dispense 5 drops of bile reagent into the tube marked "TEST." Add 5 drops of saline to the tube marked "CONTROL." Gently mix each tube.
5. Incubate the tubes for 3 h at 35°C, checking hourly for clearing, or examine each tube by Gram stain or methylene blue wet mount for lysis of cells at 15 min.

B. Direct plate method

1. Place a drop of bile spot reagent near a suspected 18- to 24-h colony; gently roll the drop over several representative colonies by tilting the plate. Take care not to dislodge the colonies.
☒ **NOTE:** Do not touch the agar surface with the tip of the dropper of bile reagent.
2. Keep the plate right side up and incubate at 35°C for 15 to 30 min or until the drop has evaporated. Placing the plate on a heat block is a substitute for use of an incubator.
3. Observe for flattening of the colony. Be sure the colony did not simply float away.

C. Direct slide blood culture test (4)

1. Add 1 drop of blood culture broth to 1 drop of bile reagent on a glass slide and allow to dry.
2. As a control, add 1 drop of broth blood culture to 1 drop of water and allow to dry.
3. Gram stain and examine for cocci.

VI. INTERPRETATION**A. Test tube method**

Bile solubility is demonstrated as a clearing or loss of turbidity, relative to the "CONTROL" tube, within 3 h or lysis of cells observed microscopically.

B. Direct slide blood culture method

If all the cocci in the smear are completely lysed and the control smear shows intact bacteria, the organism is bile soluble.

C. Direct plate method

1. Bile solubility is demonstrated as a disintegration or flattening of the colony within 30 min, leaving an area of alpha-hemolysis where the colonies were located.
2. Insolubility is demonstrated when there is no change in the integrity of the colony within 30 min.

VII. REPORTING RESULTS

- A. If either the spot or tube test demonstrates bile solubility of an alpha-hemolytic colony from a catalase-negative, lancet-shaped, gram-positive coccus, definitively report as *Streptococcus pneumoniae* (5).
- B. If the test does not demonstrate bile solubility, the organism is likely a viridans group streptococcus, but a percentage of bile-resistant organisms may still be *S. pneumoniae*; further testing is indicated from typical pneumococcal colonies (3).

VIII. LIMITATIONS

- A. Some *S. pneumoniae* organisms will not lyse in the presence of bile, possibly due to the loss of virulence factor or capsule. If lysis is not present, the isolate may still be *S. pneumoniae*. Therefore, colonies resembling *S. pneumoniae* which are not bile soluble should be further identified using another method, such as optochin susceptibility and/or DNA probe (3).
- B. Use bile solubility only to differentiate *S. pneumoniae* from other alpha-hemolytic streptococci.
- C. In one study comparison with the DNA probe and Quellung test, using 529 presumed viridans group streptococci, the tube test had a 96% specificity (23 false positives) and the colony test had a 99.4% specificity (3). For greatest specificity, tube test-positive, spot test-negative strains should be confirmed with an optochin disk test or DNA probe.
- D. The colony test has a sensitivity of 99% and the tube test has a sensitivity of 99 to 100% for encapsulated strains of *S. pneumoniae* (1, 3). However, Mundy et al. (3) showed that the colony test was not very sensitive (three positive results) in the detection of 33 unencapsulated strains that were detected by DNA probe. The tube test detected 19 of the 33 unencapsulated strains positive by probe. All 33 had some zone of inhibition of optochin, although most were in the intermediate category. Whether DNA probe-positive, bile-negative strains are important pathogens or represent false-positive DNA probe tests is not known.
- E. Normal autolysis of *S. pneumoniae* may be inhibited by a high concentration of bile salts. Evaporation may cause the reagent to become more concentrated, thus affecting the test.
- F. The bile solubility test is not reliable with old cultures that have autolysed.
- G. When performing the bile solubility tube test using saline or unbuffered broth, it is essential to adjust the pH to neutral before adding the reagent, in order to avoid false-negative reactions.
- H. When testing using the plate method, care must be taken not to dislodge the colony being tested, thus leading to false-positive results. If the direct plate is difficult to interpret, the test should be repeated using the tube or slide method.
- I. Storage of the reagent at cool temperatures can cause it to thicken. Warm the reagent bottle in a 37°C incubator to liquefy the reagent before use.

REFERENCES

1. Kellogg, J. A., D. A. Bankert, C. J. Elder, J. L. Gibbs, and M. C. Smith. 2001. Identification of *Streptococcus pneumoniae* revisited. *J. Clin. Microbiol.* **39**:3373–3375.
2. MacFaddin, J. F. 2000. *Biochemical Tests for the Identification of Medical Bacteria*, 3rd ed., p. 27–34. The Lippincott, Williams & Wilkins Co., Philadelphia, Pa.
3. Mundy, L. S., E. N. Janoff, K. E. Schwebke, C. J. Shanholtzer, and K. E. Willard. 1998. Ambiguity in the identification of *Streptococcus pneumoniae*. Optochin, bile solubility, quellung, and the AccuProbe DNA probe tests. *Am. J. Clin. Pathol.* **109**:55–61.
4. Murray, P. R. 1979. Modification of the bile solubility test for rapid identification of *Streptococcus pneumoniae*. *J. Clin. Microbiol.* **9**:290–291.
5. NCCLS. 2002. *Abbreviated Identification of Bacteria and Yeast*. Approved guideline M35-A. NCCLS, Wayne, Pa.
6. Neufeld, F. 1900. Über eine spezifische bakteriolytische Wirkung der Galle. *Z. Hyg. Infektionskr.* **34**:454–464.

3.17.7

Butyrate Esterase Test

[Updated March 2007]

I. PRINCIPLE

The butyrate test is a rapid test for the detection of the enzyme butyrate esterase. When used in conjunction with characteristic morphology on BAP, typical Gram stain, and a positive oxidase test, the butyrate test is useful for the definitive identification of *Moraxella catarrhalis*.

The value of tributyrin hydrolysis for differentiating *M. catarrhalis* from *Neis-*

seria spp. was first reported by Berger in 1962 (1). Subsequently, several authors have reported the usefulness of butyrate esterase in differentiating *M. catarrhalis*, using various substrates. Both bromochloro-indolyl butyrate (IB) (2, 3) and 4-methylumbelliferyl butyrate (MUB) (4, 5, 6, 7) can serve as substrates. Hydrolysis of

the IB substrate by the butyrate esterase releases indoxyl, which in the presence of oxygen spontaneously forms indigo, a chromogenic compound which appears blue to blue-violet. Hydrolysis of the MUB substrate produces a fluorescent compound visible under UV light.

II. MICROORGANISMS TESTED

Gram-negative, oxidase-positive diplococci growing on BAP as white colonies that remain together when lifted with a loop or wire

III. REAGENTS AND SUPPLIES



Include QC information on reagent container and in QC records.

A. Disks

1. Disks impregnated with IB or MUB
2. Store at 2 to 8°C. Protect from light.
3. Vendors include Hardy Diagnostics; Remel, Inc.; and Key Scientific.

NOTE: The BactiCard *Neisseria* (Remel, Inc.) includes the IB substrate as one test in the kit.

B. MUB tube test

1. MUB stock solution

MUB (Sigma) 100 mg
dimethyl sulfoxide
(Sigma) 10 ml
Triton X-100 100 ml

2. 0.1 M citrate buffer, 90 ml; final pH, 5.0
 - a. 0.1 M citric acid
 - b. 0.05 M Na₂HPO₄ · 12H₂O
3. Dissolve the MUB powder in dimethyl sulfoxide-Triton X-100 solution.

4. Prepare a 1:10 dilution of the stock solution in the citrate buffer.

5. Dispense 250-μl aliquots into sterile snap-cap tubes.

6. The substrate is stable for at least 1 month.

7. Clearly label the substrate, indicating preparation and expiration dates.

8. Record the expiration date in a work record.

9. Store frozen at -20°C or below. Protect from light.

C. Supplies/equipment

1. Sterile wooden applicator sticks or bacteriologic loops

2. Distilled water

3. Petri dish, slide, or tube

4. Long-wave (360 nm) UV light (Wood's lamp) (MUB reagent only)

IV. QUALITY CONTROL

- A. Discard disks if they do not appear white with no visible color.
 - B. Perform QC on each new lot and shipment of disks or MUB reagent prior to putting them into use.
 - C. Organisms
 - 1. *M. catarrhalis* ATCC 25240—butyrate positive
 - 2. *Neisseria gonorrhoeae* ATCC 43069 or *Neisseria lactamica* ATCC 23970—
butyrate negative
-

V. PROCEDURE

- A. **Disk method**
 - 1. Remove disk from vial and place on a clean glass slide or petri dish.
 - 2. Add 1 drop of distilled or deionized water to moisten the disk.
 - 3. Obtain a heavy, visible inoculum with a sterile wooden applicator stick or loop from a 24- to 72-h culture and rub it onto the disk.
 - 4. Incubate at room temperature (15 to 30°C) for up to 5 min.
 - 5. *Incubation for slightly longer periods may yield false-positive results. Do not read after 5 min.*
 - B. **Tube method**
 - 1. Thaw tubes at room temperature.
 - 2. Inoculate substrate using several colonies to obtain a turbid suspension.
 - 3. Incubate at room temperature (15 to 30°C) for up to 5 min. *Incubation for slightly longer periods may yield false-positive results.*
 - 4. Observe fluorescence using long-wave UV light in a darkroom.
-

VI. INTERPRETATION

- A. A positive test results in a blue to blue-violet color (IB substrate) or fluorescence (MUB substrate) within 5 min, indicating the hydrolysis of IB or MUB, respectively, by butyrate esterase.
 - B. A negative test is indicated by no color change.
-

VII. REPORTING RESULTS

- A. Report as *Moraxella catarrhalis* if an oxidase-positive, gram-negative diplococcus meets the following criteria.
 - 1. Grows on BAP as colonies that remain together when sampled *and*
 - 2. Is butyrate positive
 - B. Test butyrate-negative colonies that are suggestive of *M. catarrhalis* by the above-listed criteria for DNase production. Report DNase-positive colonies as *Moraxella catarrhalis*.
-

VIII. LIMITATIONS

- A. Do not incubate test beyond 5 min, to avoid false-positive reactions.
- B. Many strains of other *Moraxella* species, as well as *Eikenella* and *Acinetobacter*, may give a positive or weakly positive reaction (2, 6). The organism must be an oxidase-positive, gram-negative diplococcus with typical morphology to be accurately identified as *M. catarrhalis*.
- C. Unrelated organisms such as staphylococci and pseudomonads may also give positive results.
- D. False-negative tests may result from using too small an inoculum. If the organism is suspected but the test is negative, repeat with a large inoculum or test for DNase production.

REFERENCES

1. Berger, U. V. 1962. Ueber die Spaltung von Tributyryl durch *Neisseria*. *Arch. Hyg. Bakteriol.* **146**:388–391.
2. Dealler, S. F., M. Abbott, M. J. Croughan, and P. M. Hawkey. 1989. Identification of *Branhamella catarrhalis* in 2.5 min with an indoxyl butyrate strip test. *J. Clin. Microbiol.* **27**:1390–1391.
3. Janda, W. M., and P. Ruther. 1989. B.CAT Confirm, a rapid test for confirmation of *Branhamella catarrhalis*. *J. Clin. Microbiol.* **27**:1130–1131.
4. Louie, M., E. G. Ongansoy, and K. R. Forward. 1990. Rapid identification of *Branhamella catarrhalis*. A comparison of five rapid methods. *Diagn. Microbiol. Infect. Dis.* **13**:205–208.
5. Perez, J. L., A. Pulido, F. Pantozzi, and R. Martin. 1990. Butyrate esterase (4-methylumbelliferyl butyrate) spot test, a simple method for immediate identification of *Moraxella (Branhamella) catarrhalis* [corrected]. *J. Clin. Microbiol.* **28**:2347–2348.
6. Speeleveld, E., J. M. Fossépré, B. Gordts, and H. W. Van Landuyt. 1994. Comparison of three rapid methods, tributyryne, 4-methylumbelliferyl butyrate, and indoxyl acetate, for rapid identification of *Moraxella catarrhalis*. *J. Clin. Microbiol.* **32**:1362–1363.
7. Vaneechoutte, M., G. Verschraegen, G. Claeys, and P. Flamen. 1988. Rapid identification of *Branhamella catarrhalis* with 4-methylumbelliferyl butyrate. *J. Clin. Microbiol.* **26**:1227–1228.

3.17.8

CAMP Factor Tests (Standard and Rapid) and the Reverse CAMP Test

[Updated March 2007]

I. PRINCIPLE

Streptococcus agalactiae produce a thermostable, extracellular, diffusible protein that acts synergistically with the beta-lisin produced by *Staphylococcus aureus* to produce a zone of enhanced lysis of sheep or bovine erythrocytes (1, 5). The protein was named CAMP factor for the initials of the authors of the manuscript that first described the phenomenon (3). The standard CAMP test relies on the elaboration of two toxins during growth to form a typical arrowhead or flame-shaped clearing at the juncture of the two organisms when they

are placed perpendicular to each other (Fig. 3.17.8–1). The rapid test utilizes an extract of staphylococcal beta-lisin that acts directly with the CAMP factor previously diffused in the medium around the *S. agalactiae* colony. A positive CAMP reaction is indicated by an enhanced hemolysis within 30 min of adding the CAMP factor reagent (6, 11). The test is useful in the identification of both *S. agalactiae* and many gram-positive rods, including *Listeria monocytogenes*.

A reverse CAMP reaction is a reaction whereby hemolysis by the beta-hemolysin of staphylococci is inhibited through the production of phospholipase C or D by some organisms (e.g., *S. agalactiae*, *Listeria*, *Corynebacterium* spp., and *Clostridium perfringens*). An arrow of no hemolysis is formed at the junction of the organism being tested with the staphylococci if the reverse CAMP test is positive (Fig. 3.17.8–1) (2, 7).

II. MICROORGANISMS TESTED

- A. Gram-positive cocci in pairs and chains that are catalase negative with medium-sized, smooth, whitish colonies with a small halo of beta-hemolysis. Nonhemolytic isolates also may be tested, since the CAMP factor can still be present.
- B. Gram-positive rods, as part of their identification
- C. Gram-positive cocci in clusters, as part of the identification of staphylococci to the species level, if *Staphylococcus intermedius* AB148 is used as the strain that supplies the hemolysin (8)

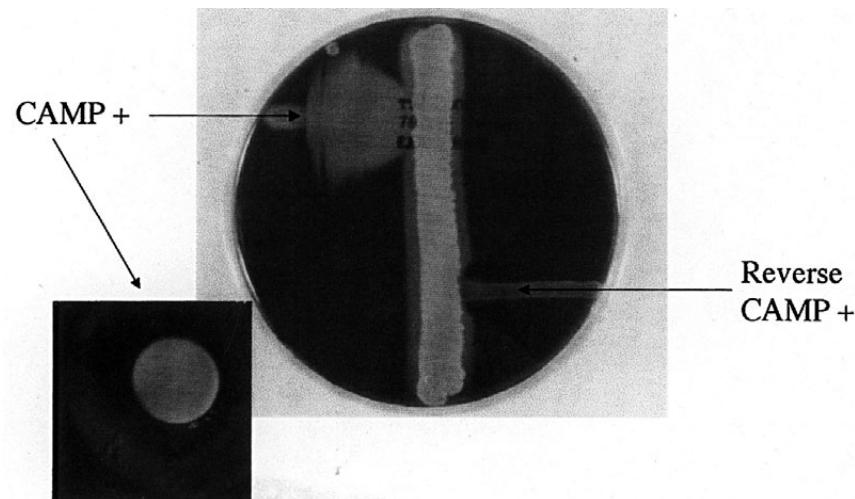


Figure 3.17.8–1 Demonstration of positive CAMP and reverse CAMP test, using *S. aureus* streak and CAMP disk.

III. MEDIA, REAGENTS, AND SUPPLIES



Include QC information on reagent container and in QC records.

- A. BAP
- B. Beta-lysin reagent
 - 1. Culture of *S. aureus* ATCC 25923
 - 2. Commercial reagents
 - a. Disks containing beta-lysin of *S. aureus* (catalog no. SS697; Remel, Inc.); store at 4°C (Fig. 3.17.8-1).
 - b. Spot CAMP liquid reagent (catalog no. Z206; Hardy Diagnostics)
 - 3. Preparation of spot CAMP reagent (10)
 - a. Inoculate two 5-ml tubes of BHI with a swab of growth from a fresh subculture of *S. aureus* ATCC 25923.
 - b. Incubate overnight (shaking is optional) at 35°C in air.
- c. Working with gloves in a laminar-flow safety cabinet, combine the two broth suspensions and filter sterilize them using a 0.45- μm -pore-size cellulose-acetate filter.
- d. Aliquot the filter-sterilized broth into small tubes of about 1 ml.
- e. Label with the name of reagent (CAMP), date of preparation, and expiration date of 6 months from preparation.
- f. Freeze at -20°C or lower.
- g. Store defrosted reagent at 4°C and use within 2 weeks. Do not refreeze.

IV. QUALITY CONTROL

- A. Test each lot of rapid beta-lysin reagent with a positively and negatively reacting organism in the same manner as patient isolates are tested prior to putting the reagent into use.
- B. Test each lot or shipment of commercial beta-lysin reagent or disks with a positively and negatively reacting organism in the same manner as patient isolates are tested prior to putting the reagent into use.
- C. Test each lot or shipment of BAP with a positively reacting organism. This can be performed on the same plate as the test organism when the test is performed, by streaking the positive control in a line parallel to the test organism. Alternatively, if BAP is purchased from a commercial medium vendor, omit testing if vendor supplies documentation that BAP has tested acceptably for the CAMP reaction (4).
- D. Organisms
 - 1. *S. agalactiae* ATCC 12386—positive
 - 2. *Streptococcus pyogenes* ATCC 19615—negative
 - 3. Periodically use an in-house laboratory strain of *Arcanobacterium haemolyticum* to demonstrate the reverse CAMP test for training purposes.

V. PROCEDURE

- A. Standard method
 - 1. Streak *S. aureus* ATCC 25923 in a straight line across the center of the plate.
 - 2. Streak the unknown microorganism in the same manner perpendicular to the staphylococcus, but avoid touching the previously streaked area.
 - 3. Streak the positive control organism parallel to and approximately 1 in. from the unknown organism.
 - 4. Label the location of each streak on the back of the plate.
 - 5. Incubate the plate overnight at 35°C in a CO₂ incubator.
- B. Disk method
 - 1. Place disk on warmed BAP.
 - 2. Streak microorganism 2 to 3 mm from the edge of the disk.
 - 3. Incubate the plate overnight at 35°C in a CO₂ incubator.
- C. Spot rapid method
 - 1. Place 1 drop or a 10- μl loopful of reagent next to a presumptive *S. agalactiae* colony growing on BAP. Do not worry if the liquid touches or even engulfs the colony.

V. PROCEDURE (continued)

2. Incubate the plate right side up, to prevent the spot CAMP reagent from running over the plate's surface, for 20 min at 35°C.
3. Examine with transmitted light for a zone of enhanced hemolysis next to the colony.
4. Reincubate for up to 30 min if reaction is initially negative. Use a hand lens if necessary for examining the plate.
5. Refrigeration may enhance reaction after incubation.

VI. INTERPRETATION

- A. A positive result in the standard assay is the formation of a distinct arrowhead of hemolysis at the intersection of the staphylococcus and test organism streaks (Fig. 3.17.8-1).
- B. A positive reverse CAMP or phospholipase D is indicated by a distinct arrow of no hemolysis at the intersection of the two hemolytic organisms (Fig. 3.17.8-1).
- C. In the disk test, a positive result is indicated by a distinct crescent- or arc-shaped zone of complete hemolysis at the intersection of the disk of beta-lysin and the isolate (Fig. 3.17.8-1).
- D. In the rapid spot test, the presence of clear enhanced hemolysis only where the diffused hemolysis overlaps is a positive result.
- E. Lack of enhanced hemolysis near the colony being tested is a negative test.

VII. REPORTING RESULTS

- A. A streptococcus which gives a positive CAMP test and is morphologically and biochemically consistent (catalase-negative, gram-positive cocci in pairs and chains) is reported as *Streptococcus agalactiae*.
- B. The following gram-positive rods are CAMP test positive: *Rhodococcus equi*, *L. monocytogenes*, *Propionibacterium avidum/granulosum*, *Actinomyces neui*, *Turicella otitidis*, *Corynebacterium glucuronolyticum*, *Corynebacterium co-lyeae*, *Corynebacterium imitans*, and some strains of *Corynebacterium striatum* and *Corynebacterium afermentans* group.
- C. *Corynebacterium pseudotuberculosis*, *Corynebacterium ulcerans*, and *A. haemolyticum* are reverse-CAMP positive.
- D. *C. perfringens* is reverse CAMP or phospholipase D positive.

VIII. LIMITATIONS

- A. Increased nonspecific hemolysis at the intersections (a "matchstick" effect) may be seen with other streptococci, but only group B streptococci will produce a definite arrowhead. Verify typical group B streptococcus, colony morphology, and hemolysis.
- B. The test has a 98% sensitivity in detecting *S. agalactiae* (9). Isolates with a negative CAMP test could still be *S. agalactiae* and require further testing.
- C. *S. pyogenes* can give a reaction that may be interpreted as positive. When there is a question, *S. pyogenes* is pyrrolidonyl-β-naphthylamide (PYR) positive but *S. agalactiae* is PYR negative.
- D. The CAMP test separates *L. monocytogenes*, the human pathogen, from most other *Listeria* species.
- E. If the agar is too thin or hemolyzed, the reaction may be very weak.

REFERENCES

1. Bernheimer, A. W., R. Linder, and L. S. Avigad. 1979. Nature and mechanism of action of the CAMP protein of group B streptococci. *Infect. Immun.* **23**:838–844.
2. Buchanan, A. 1982. Clinical laboratory evaluation of a reverse CAMP test for presumptive identification of *Clostridium perfringens*. *J. Clin. Microbiol.* **16**:761–762.
3. Christie, R., N. E. Atkins, and E. Munch-Petersen. 1944. A note on lytic phenomenon shown by group B streptococci. *Aust. J. Exp. Biol. Med. Sci.* **22**:197–200.
4. Clinical and Laboratory Standards Institute. 2004. *Quality Assurance for Commercially Prepared Microbiological Culture Media*, 3rd ed. Approved standard M22-A3. Clinical and Laboratory Standards Institute, Wayne, Pa.
5. Darling, C. L. 1975. Standardization and evaluation of the CAMP reaction for the prompt, presumptive identification of *Streptococcus agalactiae*. *J. Clin. Microbiol.* **1**:171.
6. DiPersio, J. R., J. E. Barrett, and R. L. Kaplan. 1985. Evaluation of the spot-CAMP test for the rapid presumptive identification of group B streptococci. *Am. J. Clin. Pathol.* **84**:216–219.
7. Hansen, M. V., and L. P. Elliott. 1980. New presumptive identification test for *Clostridium perfringens*: reverse CAMP test. *J. Clin. Microbiol.* **12**:617–619.
8. Hébert, G. A., C. G. Crowder, G. A. Hancock, W. R. Jarvis, and C. Thornsberry. 1988. Characteristics of coagulase-negative staphylococci that help differentiate these species and other members of the family *Micococcaceae*. *J. Clin. Microbiol.* **26**:1939–1949.
9. MacFaddin, J. F. 2000. *Biochemical Tests for the Identification of Medical Bacteria*, 3rd ed., p. 35–56. The Lippincott, Williams & Wilkins Co., Philadelphia, Pa.
10. NCCLS. 2002. *Abbreviated Identification of Bacteria and Yeast*. Approved guideline M35-A. NCCLS, Wayne, Pa.
11. Ratner, H. B., L. S. Weeks, and C. W. Stratton. 1986. Evaluation of spot CAMP test for identification of group B streptococci. *J. Clin. Microbiol.* **24**:296–297.

3.17.9

Carbohydrate Utilization Tests

I. PRINCIPLE

The ability of a microorganism to utilize carbohydrates can be key to its identification. There are a wide array of media available, using different pH indicators and nutrients. Usually the medium contains a protein source, pH indicator, and a 1% concentration of carbohydrate. The 1% concentration is optimal for most reactions and decreases the possibility of reversal reactions. Reversion occurs when the carbohydrate is depleted, thereby resulting in the masking of acid by-products by alkaline by-products of protein product utilization. It is very important that the correct base and indicator are used in testing so that the results will match the biochemical reactions expected from identification tables.

To detect fermentation with acid production of various sugars by gram-positive microorganisms, the medium used generally contains brom cresol purple (4), which turns from purple to yellow at pH 5.2. For gram-negative rods that ferment glucose, the preferred medium contains Andrade's indicator, acid fuchsin. The medium is straw colored and turns fuchsia-red when acid is produced. Andrade's indicator is very difficult to make correctly and must be aged for 6 months to produce the correct color (11). Commercial identification systems often use neutral red or phenol red, which turns from red to yellow with a drop in pH. Results using these indicators compare favorably with results with Andrade's indicator or the brom cresol purple base, and published table reactions generally apply whether media with Andrade's indicator, brom cresol purple, or phenol red or commercial systems are used for enterococcus, gram-positive rods, and gram-negative rods (reference 9, p. 426, 480, and 648, respectively). Since

staphylococcus tables were created using the brom cresol purple indicator, results with commercial systems may be slightly different from the reactions in the tables (reference 9, p. 394).

Bacteria can be divided by their ability to degrade carbohydrates anaerobically (ferment) or aerobically (oxidize). Fermentation is a metabolic process in which an organic substrate serves as the final hydrogen acceptor rather than oxygen. Agar-based media are recommended for this determination, since the oxidative reaction can be observed at the top of the tube and fermentation can be observed in the bottom of the tube. The best medium to use for the determination of whether a gram-negative rod is an oxidizer or fermenter is either triple sugar iron agar (TSI) or Kligler's iron agar (KIA) (11). Fermenters will turn the butt yellow, while oxidizers and nonutilizers produce no change or alkalization of the butt in the tube. Once the organism is determined to be a fermenting rod, either Andrade's or neutral or phenol red base broths are used to determine which other sugars are fermented. Once an organism is determined to be non-glucose-fermenting, oxidative-fermentative (OF) medium is used to establish whether an organism can oxidize glucose or other sugars or does not produce acid from sugar fermentation. This medium contains brom thymol blue, which turns yellow when acid is produced from glucose (5). King (8) developed another medium with phenol red as the indicator, but it is not commercially available. OF basal medium is used to detect utilization of other sugars by non-glucose-fermenting rods. The recommended medium to determine whether a gram-positive rod is an oxidizer or fermenting rod is reported to be semisolid

cystline Trypticase agar (CTA) with glucose, rather than KIA, TSI, or OF medium (reference 9, p. 479).

To complicate testing, some gram-negative organisms (generally those that do not grow on MAC) will not react in the KIA or TSI media, OF medium, or even Andrade's broth, because these bacteria are too fastidious for growth. In such cases rapid or conventional CTA media with phenol red indicator is the choice (6, 10) to detect the ability to utilize carbohydrates. The indicator turns from red to yellow at pH 6.8. The rapid medium does not require the growth of the organism to detect acid production but relies on preformed enzymes for the reactions. These microorganisms include *Neisseria*; *Haemophilus*, *Kingella*, *Capnocytophaga*, *Actinobacillus*; and lipid-requiring gram-positive rods (reference 9, p. 593, 609, and 479, respectively). Another alternative that is often used for demonstration of fermentation of fastidious gram-negative rods and gram-positive rods is to add a few drops of sterile rabbit serum to the Andrade's broth to allow the organism to grow. However, maltose cannot be tested since serum can split maltose (reference 9, p. 609). Carbohydrate reactions for *Neisseria* are problematic due to growth inhibition and poor oxidative metabolism; enzymatic tests are preferred (see Table 3.18.2-1 and reference 9, p. 593).

The production of gas from the fermentation of sugars can be determined by one of several methods. In agar-based tubes inoculated with gram-negative rods, disruption of the agar is evidence of gas production. If a Durham tube is inverted into broth, gas is apparent if the broth in the tube is displaced with air. When examining gram-positive bacteria, gas pro-

duction may be discerned with the use of melted paraffin overlay. The “hot loop” method is useful for fastidious gram-negative rods, such as *Haemophilus aphrophilus*, where the production is key to the identification (7). In this method, a red-hot

inoculating needle is plunged near the side of the glass tube of a 48- to 72-h culture of the organism in THIO in a biological safety cabinet (11). Gas bubbles appear along the line of insertion to indicate posi-

tive gas from glucose. Occasionally organisms ferment lactose better than glucose and will demonstrate gas from lactose in the Durham tube when no gas is seen in the glucose tube.

II. MICROORGANISMS TESTED

A. Gram-positive rods and fermentative gram-negative rods

1. Andrade’s “Enteric fermentation medium” is preferred. Add rabbit serum for fastidious gram-negative rods.
2. Heart infusion purple broth base medium can be used if the organism is not fastidious.
3. Rapid sugar fermentation media and CTA are used for fastidious rods.

B. Gram-positive cocci (*staphylococci*, *streptococci*, *enterococci*)

1. Generally these tests are present in commercial kits, and laboratories rarely stock sugar tests for identification.

2. Use heart infusion purple broth base media for conventional testing (4).

NOTE: *Leuconostoc* produces copious amounts of gas from glucose in MRS broth (procedure 3.17.32) as evidenced by displacement of a melted Vaspal, liquid paraffin or petroleum jelly plug (reference 9, p. 440). However, leucine aminopeptidase (LAP) (procedure 3.17.26) and arginine dehydrolase (procedure 3.17.15) testing can substitute for this test to separate *Leuconostoc* from *Weissella confusa* and *Pediococcus* (see Fig. 3.18.1–3).

C. Glucose-oxidizing gram-negative rods, which are generally oxidase positive and grow on MAC, as part of the identification process

1. OF basal medium is used for testing.
2. See TSI and KIA procedure (procedure 3.17.25) to determine if the organism is a glucose-fermenting organism rather than a glucose oxidizer before using the OF basal medium.

D. Identification of *Neisseria* species

1. Phenol red-based media, such as rapid sugars and CTA
2. The addition of ascitic fluid to CTA is recommended but not necessary.

III. MEDIA, REAGENTS, AND SUPPLIES

A. Media

1. Heart infusion purple broth
 - a. BHI, peptone, yeast extract, NaCl
 - b. Bromcresol purple, pH 7.4
 - c. 1% Carbohydrates, filter sterilized and added after sterilizing the broth

NOTE: This medium was developed for streptococci. A plate medium with gelatin rather than agar and another protein source was developed for staphylococci and enteric bacteria.
2. Enteric fermentation broth medium (1, 3)
 - a. Peptone, meat extract, and NaCl
 - b. Andrade’s indicator, pH 7.2
 - c. 1% Carbohydrates, filter sterilized and added after sterilizing the broth
3. CTA with or without ascitic fluid (11)
 - a. Cystine, Trypticase peptone, inorganic salts, and agar (low concentration for semisolid agar) available in dehydrated powder (BBL, BD Diagnostic Systems)
 - b. Phenol red, pH 7.3
 - c. The addition of ascitic fluid (or serum or yeast extract) is recommended for testing *Neisseria* species.
 - d. 1% Carbohydrates, filter sterilized and added after sterilizing and cooling the agar to 50°C
 - e. This medium is modified by adding 2% carbohydrates to CTA in a small-volume tube (Remel, Inc.) for rapid fermentation.

III. MEDIA, REAGENTS, AND SUPPLIES (continued)



Include QC information on reagent container and in QC records.

4. Rapid sugar broth medium
 - a. Formula for buffer-salt solution (6, 11)

K_2HPO_4 (anhydrous) ..0.04 g
 KH_2PO_4 0.01 g
 KCl 0.8 g
 1% phenol red,
 aqueous0.4 ml
 distilled water100 ml
 Adjust pH to 7.0. Filter sterilize and store at 4°C.
 - b. Prepare 20% carbohydrate solution in either distilled water or peptone broth (per liter: peptone, 10 g; meat extract, 3 g; NaCl, 5 g) and filter sterilize.
 - c. Add 1 drop (0.05 ml) of carbohydrate to 2 drops (0.1 ml) of buffer. Final concentration of sugars after adding 1 drop (0.05 ml) of culture will be 5%.
5. OF basal medium
 - a. Tryptone, inorganic salts, agar

NOTE: Hugh and Leifson's formula (5) employs a low peptone/carbohydrate ratio and a minimal amount of agar (semisolid).
 - b. Bromthymol blue, pH 7.1 (color of medium is green)
 - c. 1% Carbohydrates, filter sterilized and added after sterilizing and cooling the agar to 50°C.

B. Other products

1. Carbohydrate oxidation tablets for glucose oxidizers (Key Scientific Products)
2. Carbohydrate fermentation, Wee-Tab (Key Scientific Products) for staphylococci, streptococci, and enteric rods, but not for *Neisseria* spp.
3. Commercial kits (2, 9; procedure 3.16)
4. Ten-millimeter disks with carbohydrate are available and can be added to the phenol red broth or semisolid basal medium or OF medium without carbohydrate after inoculation (BD Diagnostic Systems).
5. Aerobic low-peptone (ALP) medium (Hardy Diagnostics) for the identification of nonfermenting, gram-negative bacilli. ALP medium can be used to detect acidification of carbohydrates or alkalization of organic salts and amides.

C. Supplies

1. Rabbit serum (catalog no. 16120-099; Invitrogen Life Technologies, Carlsbad, Calif.). Heat for 1 h at 60°C prior to use.
2. Sterile tubes
3. Wire or disposable inoculating needles or loops
4. 35°C heat block or incubator without increased CO₂

IV. QUALITY CONTROL

- A. Perform QC using a positively and negatively reacting organism on each new lot and shipment of media prior to putting them into use.
- B. Inspect agar for evidence of prior freezing, contamination, cracks, dehydration, and bubbles prior to storage and before use. Discard any tubes with bubbles in the agar. Discard tubes that are not the appropriate color.
- C. Check rabbit serum for sterility by inoculation onto CHOC.
- D. Since carbohydrates are filter sterilized, check media and carbohydrate solutions for contamination when received or prepared.
- E. Organisms

Because the number and type of organism used for QC are extensive, refer to the QC section of the manual (section 14, Table 14.2-2).

V. PROCEDURE

- A. Warm medium to room temperature.
- B. Inoculation
 1. Purple broth and enteric fermentation broth medium
 - a. Before inoculating broth with Durham tube, check for trapped air bubbles. If necessary, invert tube until air bubbles are gone.
 - b. Inoculate (carbohydrate of choice) with isolated colonies from an 18- to 24-h pure culture of the organism.
 - c. Inoculate colony directly with a loop or wire or prepare a broth or saline suspension and inoculate with 1 drop per tube if a battery of carbohydrates is being tested.

V. PROCEDURE (continued)

- d. Add 1 drop of rabbit serum for fastidious gram-negative rods or corynebacteria.
- e. Incubate for up to 7 days at 35°C in a non-CO₂ incubator with caps loose.
2. CTA
 - a. Inoculation
 - (1) Standard media: with a loop, inoculate media heavily, mixing throughout the entire top 5 to 10 mm.
 - (2) Rapid test media: fill a 3-mm loop with organisms and vigorously inoculate below the surface of the agar.
 - (3) Inoculate a control tube without carbohydrate for comparison.
 - b. *Tighten caps.*
 - c. Incubate for 2 to 4 h or up to 24 h for rapid media and up to 7 days for standard media at 35°C in a non-CO₂ incubator or heat block (preferred).
3. Rapid sugar broth medium
 - a. Prepare a very heavy suspension of organism in buffer-salt solution (equivalent to no. 2 McFarland turbidity standard).
 - b. Inoculate 1 drop (0.05 ml) to each tube containing 2 drops (0.1 ml) of buffer-salt and 1 drop (0.05 ml) of 20% carbohydrate. Alternatively, inoculate the combined carbohydrate buffer-salt solution with a loopful of the suspected organism.
 - c. Inoculate 1 drop of distilled water and 1 drop of organism suspension to 2 drops of buffered salt as a control tube.
 - d. *Tighten caps.*
 - e. Incubate for 2 h or up to 4 h at 35°C in a heat block.
4. OF basal medium
 - a. With a needle, pick up a large amount of organism from an agar plate or slant and stab into the medium four or five times to a depth of 2.5 cm.
 - b. Inoculate a control tube without carbohydrate for comparison.
 - c. *Cap the tubes loosely* and incubate for 24 h and up to 7 days at 30°C in a non-CO₂ incubator or heat block (preferred).
 NOTE: Inoculation of a tube overlaid with oil, petroleum jelly, or Vaspar (advocated for gas production of *Leuconostoc*) is not recommended to differentiate fermentation from oxidation among gram-negative rods. Rather, use KIA, TSI, or glucose broth medium with serum in conjunction with OF medium to determine if the organism is a glucose-fermenting rod (11).
5. Carbohydrate oxidation tablets (COT) for glucose oxidizers
 - a. Add each tablet to 1 ml of distilled water in a small test tube.
 - b. Heat in a boiling water bath for 10 min. Cool before using.
 - c. For multiple carbohydrate tests, inoculate with a heavy bacterial suspension made in 1 to 2 ml of sterile distilled water. Transfer 1 or 2 drops of this suspension to each tube with a sterile pipette. For a single test, inoculate the tube with a loopful of the organism being tested without making a suspension.
 - d. *Cap the tubes loosely* and incubate at 37°C for 24 h and up to 4 days.
6. Wee-Tabs
 - a. Add one tablet to a small tube and add 0.25 ml of distilled water.
 - b. Inoculate with a loopful of the organism being tested. If doing multiple tests, make a heavy suspension of the organism into 1 ml of water and add a drop of this suspension to each tube.
 - c. Add oil overlay if desired.
 - d. Incubate at 35°C for 6 to 24 h.
 NOTE: Do not use Wee-Tabs for *Neisseria* or any organism that will not grow in high salt.
- C. Examine for color change compared to control tubes without carbohydrate.

VI. INTERPRETATION**A. Purple broth**

1. Acid (positive) reaction: yellow (pH 5.2)
2. Alkaline (negative) reaction: purple
3. Gas production: bubbles in Durham tube

B. Andrade's enteric fermentation broth

1. Acid (positive) reaction: pink to red (pH 5.0)
2. Alkaline (negative) reaction: straw to tan (no change)
3. Gas production: bubbles in Durham tube

C. CTA, rapid fermentation broth or agar, COT, carbohydrate fermentation Wee-Tabs

1. Acid (positive) reaction: yellow (pH 6.8)
2. Alkaline (negative) reaction: red or no change compared to control

D. OF medium

1. Acid (positive) reaction: yellow (pH 6.0). Fermenters generally turn the entire tube yellow in 18 h, whereas oxidizers turn the top half of the tube yellow.
2. Weak acid reaction: yellow-green to green compared to the control, which is more alkaline or blue-green
3. Negative or nonoxidizer: inoculated control and carbohydrate tube are the same color.

VII. REPORTING RESULTS

A. Record each carbohydrate result separately. Indicate whether gas is produced or not. When using OF medium, also note whether the organism is an oxidizer, fermenter, or nonoxidizer.

B. *Acinetobacter* may be a nonoxidizer or an oxidizer.

C. *Neisseria*, *Pseudomonas*, *Stenotrophomonas*, and *Burkholderia* are glucose oxidizers.

D. *Enterobacteriaceae* are glucose fermenters.

E. Arabinose fermentation is important in the separation of *Enterococcus faecium* (positive) from *Enterococcus faecalis* (negative).

F. *Neisseria* species are separated by the utilization of glucose, maltose, lactose, and sucrose.

G. *Alcaligenes xylosoxidans* subsp. *xylosoxidans* oxidizes xylose but may not demonstrate oxidation of glucose.

VIII. LIMITATIONS

A. CTA basal medium without sugars is excellent for the maintenance of stock cultures at room temperature for several months (10).

B. Glucose oxidizers do not ferment other sugars.

C. If an organism appears to be a nonoxidizer in OF medium, it may be a fermenter that is not able to grow in OF medium. Repeat the test using fermentation media and rabbit serum to determine if the organism is a fermenter.

D. Inoculation of the basal medium without carbohydrate is important in the determination of true utilization of the sugar.

E. The Durham tube is usually placed in the glucose tube only, but it can be helpful in the lactose tube.

F. Even a small bubble indicates gas production.

G. Andrade's medium is not appropriate for testing *Neisseria* or streptococci.

REFERENCES

1. Andrade, E. 1906. Influence of glycerine in differentiating certain bacteria. *J. Med. Res.* **14**:551–556.
2. Evangelista, A. T., A. L. Truant, and P. Bourbeau. 2001. Rapid systems and instruments for the identification of bacteria, p. 22–49. In A. L. Truant (ed.), *Manual of Commercial Methods in Microbiology*. ASM Press, Washington, D.C.
3. Ewing, W. B., and B. R. Davis. 1970. *Media and Tests for Differentiation of Enterobacteriaceae*. Center for Disease Control, Atlanta, Ga.
4. Facklam, R. R., and J. A. Elliott. 1995. Identification, classification, and clinical relevance of catalase-negative, gram-positive cocci, excluding the streptococci and enterococci. *Clin. Microbiol. Rev.* **8**:479–495.
5. Hugh, R., and E. Leifson. 1953. The taxonomic significance of fermentative versus oxidative metabolism of carbohydrates by various gram-negative bacteria. *J. Bacteriol.* **66**:24–26.
6. Kellogg, D. S., Jr., and E. M. Turner. 1973. Rapid fermentation confirmation of *Neisseria gonorrhoeae*. *Appl. Microbiol.* **25**:550–552.
7. King, E. O., and H. W. Tatum. 1962. *Actinobacillus actinomycetemcomitans* and *Haemophilus aphrophilus*. *J. Infect. Dis.* **111**:85–94.
8. King, E. O. 1967. *Identification of Unusual Pathogenic Gram-Negative Bacteria*. Center for Disease Control, Atlanta, Ga.
9. Murray, P. R., E. J. Baron, J. H. Jorgensen, M. A. Pfaller, and R. H. Yolken (ed.). 2003. *Manual of Clinical Microbiology*, 8th ed. ASM Press, Washington, D.C.
10. Vera, H. D. 1948. A simple medium for identification and maintenance of the gonococcus and other bacteria. *J. Bacteriol.* **55**:531–536.
11. Weyant, R. S., C. W. Moss, R. E. Weaver, D. G. Hollis, J. G. Jordan, E. C. Cook, and M. I. Daneshvar. 1995. *Identification of Unusual Pathogenic Gram-Negative Aerobic and Facultatively Anaerobic Bacteria*, 2nd ed., p. 2, 7–19. Williams & Wilkins, Baltimore, Md.

I. PRINCIPLE

Bacteria that synthesize the enzyme catalase hydrolyze hydrogen peroxide into water and gaseous oxygen, which results in the liberation of gas bubbles. The test is useful in the initial characterization of most bacteria (1, 2).

II. MICROORGANISMS TESTED

- A. Young (18 h old, if possible) colonies of bacteria growing on agar media, preferably BAP or CHOC
 - B. For anaerobes, expose colonies to air for 30 min prior to testing.
 - C. See section 7 for catalase testing of mycobacteria.
-

III. REAGENTS AND SUPPLIES**A. Hydrogen peroxide reagent**

- 1. 30% For *Neisseria* (4)

Caution: 30% H_2O_2 is extremely caustic to skin. If contact occurs, wash immediately with 70% ethyl alcohol, not water.

- 2. 15% For anaerobes
- 3. 3% For other bacteria (purchase or dilute 30% 1:10 in deionized water prior to use)
- NOTE:** 30% Reagent can be used for all tests, but it is more hazardous.
- 4. Store at 2 to 8°C.

B. Supplies

- 1. Glass slide
- 2. Sterile wooden sticks or plastic or platinum loops or wires

IV. QUALITY CONTROL

- A. Perform QC on each new lot or shipment of reagent prior to putting it into use.
- B. Organisms
 - 1. *Staphylococcus aureus* ATCC 25923—catalase positive
 - 2. *Streptococcus pyogenes* ATCC 19615—catalase negative

V. PROCEDURE

- A. Touch the center of an 18- to 24-h, well-isolated colony to a clean glass slide.
 1. Be sure colony is visible to the naked eye on slide.
 2. If colony is from BAP, use care not to pick up blood.
 - B. Place 1 drop of peroxide reagent on slide and observe *immediately* for effervescence.
 1. Use a magnifying lens if necessary.
 2. Hold over dark background to enhance bubbles.
 - C. Discard slide into sharps container.
-

VI. INTERPRETATION

- A. A positive test shows immediate appearance of bubbles.
 - B. A weak reaction has one or two bubbles.
 - C. A negative test shows no bubbles or a few bubbles after 20 s.
-

VII. REPORTING RESULTS

- A. The catalase test separates staphylococci (positive) from streptococci and enterococci (negative).
 - B. *Bacillus* spp. are catalase positive, and *Clostridium* spp. are catalase negative.
 - C. The test is useful to separate among the fastidious gram-negative rods.
 - D. *Neisseria gonorrhoeae* produces an enhanced elaboration of bubbles not seen with other members of the genus due to superoxol (4).
-

VIII. LIMITATIONS

- A. RBCs contain catalase. To avoid false-positive results, do not pick up blood agar with colony. If colony does not easily pick up or grow well, repeat the test from CHOC, which does not interfere with the assay.
 - B. Do not test from Mueller-Hinton agar (2).
 - C. Selecting colonies with some metal bacteriological loop materials will yield false-positive results; platinum loops do not yield false-positive results.
 - D. Because the enzyme is present in viable cultures only, do not test colonies that are older than 24 h. Older cultures may give false-negative results.
 - E. Do not reverse the order of adding the reagent to the colony; false-negative results can occur.
 - F. Do not mix the reagent and the colony.
 - G. Some strains of *S. aureus* may appear catalase negative by this method. See the aminolevulinic acid (ALA) test (procedure 3.17.3) for further testing to identify these problematic organisms.
 - H. To confirm the lack of catalase for *Gardnerella vaginalis*, Reimer and Reller (3) recommend streaking a CHOC plate as for disk susceptibility testing and adding a dot of viridans group streptococci (*Streptococcus sanguis* ATCC 35557). A clear zone of inhibition around a dot of viridans group streptococci confirms the lack of catalase.
-

REFERENCES

1. Levin, M., and D. Q. Anderson. 1932. Two new species of bacteria causing mustiness in eggs. *J. Bacteriol.* **23**:337–347.
2. MacFaddin, J. F. 2000. *Biochemical Tests for the Identification of Medical Bacteria*, 3rd ed., p. 78–97. The Lippincott, Williams & Wilkins Co., Philadelphia, Pa.
3. Reimer, L. G., and L. B. Reller. 1985. Use of a sodium polyanetholesulfate disk for the identification of *Gardnerella vaginalis*. *J. Clin. Microbiol.* **21**:146–149.
4. Saginur, R., B. Clecner, J. Portnoy, and J. Mendelson. 1982. Superoxol (catalase) test for identification of *Neisseria gonorrhoeae*. *J. Clin. Microbiol.* **15**:475–477.

3.17.11

Cetrimide Test

I. PRINCIPLE

Cetrimide is a quaternary ammonium, cationic detergent that is toxic to most bacteria, except *Pseudomonas aeruginosa* and a few other bacteria (1, 2). When cetrimide is in contact with bacteria, nitrogen and phosphorus are released from the

bacterial cell. Organisms, other than *P. aeruginosa* and a few other pseudomonads, are unable to withstand this germicidal activity. Growth is observed on the inoculated slant of agar medium contain-

ing cetrimide for a positive result. Magnesium chloride and potassium sulfate in the medium enhance the production of pyocyanin and pyoverdin (fluorescein) by *P. aeruginosa*.

II. MICROORGANISMS TESTED

Isolated colonies of non-glucose-fermentative, gram-negative rods that are suggestive of *P. aeruginosa*

III. MEDIA, REAGENTS, AND SUPPLIES

A. Medium

- | | |
|--------------------------------------|-------------------------------------------------------------------------------|
| 1. | Agar slants containing the following ingredients per liter of deionized water |
| pancreatic digest of | |
| gelatin | 20.0 g |
| K ₂ SO ₄ | 10.0 g |
| MgCl ₂ | 1.4 g |
| cetyltrimethylammonium | |
| bromide | 0.3 g |
| agar | 13.6 g |
| glycerin | 10.0 ml |

2. Four milliliters of a 22.5% solution of cetrimide (0.9 g of hexadecyltrimethylammonium) can substitute for the cetyltrimethylammonium bromide.
3. Final pH, 7.2
4. Store at 2 to 8°C.

B. Supplies

1. Sterile inoculating loops or sticks
2. Wood's or UV light (360 nm) or short-wavelength (254-nm) UV light (preferred)

IV. QUALITY CONTROL

- A. Perform QC on each new lot or shipment of media prior to putting it into use.
- B. Inspect agar for evidence of freezing, contamination, cracks, dehydration, and bubbles prior to storage and before use.
- C. Organisms
 1. *P. aeruginosa* ATCC 27853—growth; yellow-green to blue pigment
 2. *Escherichia coli* ATCC 25922—inhibited

V. PROCEDURE

- A. Streak the slant back and forth with inoculum picked from the center of a well-isolated colony.
- B. Place cap loosely on tube.
- C. Incubate aerobically at 35 to 37°C for up to 7 days.
- D. Observe for growth and pigment.

V. PROCEDURE (continued)

- E. If no pigment is visible, examine growth under UV light for the presence of fluorescein.
- F. If negative for pigment at 24 h, incubate additional days at 25°C in the dark to enhance pigment production.

VI. INTERPRETATION

- A. Positive: growth. Optionally a yellow-green (fluorescein) to dark blue-green (pyocyanin) color may be observed.
- B. Negative: no growth

VII. REPORTING RESULTS

- A. *P. aeruginosa* is definitively identified if an oxidase-positive, gram-negative rod grows on cetrimide agar and produces a blue-green (pyocyanin) pigment.
- B. *Pseudomonas fluorescens* and *Pseudomonas putida* may also grow and may produce a fluorescent pigment on this medium (2) but are separated from *P. aeruginosa* because they do not grow at 42°C.

VIII. LIMITATIONS

- A. Growth on this medium alone is not sufficient for identification of *P. aeruginosa* to the species level, since other non-glucose-fermenting species (e.g., *Achromobacter xylosoxidans* subsp. *xylosoxidans* and *Alcaligenes faecalis*) may grow. Pigment must also be present.
- B. Lack of growth on cetrimide agar does not rule out an identification of *P. aeruginosa*.

REFERENCES

1. Lowburg, E. J. L. 1955. The use of cetrimide product in a selective medium for *Pseudomonas pyocyanea*. *J. Clin. Pathol.* **8**:47–48.
2. Weyant, R. S., C. W. Moss, R. E. Weaver, D. G. Hollis, J. G. Jordan, E. C. Cook, and M. I. Daneshvar. 1995. *Identification of Unusual Pathogenic Gram-Negative Aerobic and Facultatively Anaerobic Bacteria*, 2nd ed., p. 6. Williams & Wilkins, Baltimore, Md.

3.17.12

Citrate Utilization Test (Simmons)

[Updated March 2007]

I. PRINCIPLE

Citrate agar is used to test an organism's ability to utilize citrate as a source of energy. The medium contains citrate as the sole carbon source and inorganic ammonium salts as the sole source of nitrogen (3). Growth is indicative of utilization of citrate, an intermediate metabolite in the Krebs cycle. When the bacteria metabolize

citrate, the ammonium salts are broken down to ammonia, which increases alkalinity. The shift in pH turns the bromthymol blue indicator in the medium from green to blue above pH 7.6 (2). This medium is recommended as part of differentiating among the species of *Enterobac-*

teriaceae. With the exception of a few species, *Salmonella*, *Edwardsiella*, *Citrobacter*, *Klebsiella*, *Enterobacter*, *Serratia*, and *Providencia* usually give a positive reaction and *Escherichia*, *Shigella*, *Morganella*, and *Yersinia* give a negative reaction. *Proteus* is citrate variable.

II. MICROORGANISMS TESTED

Enterobacteriaceae, as part of the identification to the species level

III. MEDIA, REAGENTS, AND SUPPLIES

A. Medium

1. Agar slants containing citrate, ammonium salts, buffer, and bromthymol blue
2. Store at 2 to 8°C.
3. Final pH, 6.8

B. Supplies

1. Sterile inoculating loops or sticks
2. Incubator at 35°C

IV. QUALITY CONTROL

A. Perform QC on each new lot or shipment of media prior to putting it into use.

NOTE: The Clinical and Laboratory Standards Institute (formerly NCCLS) has proposed elimination of user QC for citrate agar purchased from commercial sources. Consult with current regulatory agencies prior to discontinuation of user QC (1).

B. Inspect agar for evidence of freezing, contamination, cracks, dehydration, and bubbles prior to storage and before use. Discard any tubes that are blue.

C. Organism

1. *Klebsiella pneumoniae* ATCC 13883—citrate positive (growth; blue color)
2. *Escherichia coli* ATCC 25922—citrate negative (no growth or trace of growth)

V. PROCEDURE

A. Streak the slant back and forth with a light inoculum picked from the center of a well-isolated colony.

B. Place cap loosely on tube.

C. Incubate aerobically at 35 to 37°C for up to 4 days.

D. Observe a color change from green to blue along the slant.

VI. INTERPRETATION

- A. A positive test is growth with color change from green to intense blue along the slant.
 - B. A negative test is no growth and no color change; slant remains green.
-

VII. REPORTING RESULTS

- A. *E. coli* is citrate negative.
 - B. Many other *Enterobacteriaceae* are citrate positive.
-

VIII. LIMITATIONS

- A. Luxuriant growth on the slant without an accompanying color change may indicate a positive test. However, if the agar does not turn blue on further incubation, the test should be repeated with less inoculum.
 - B. Tests with equivocal results should be repeated.
 - C. Do not stab the slant, since the test requires an aerobic environment.
 - D. Do not inoculate from broth cultures, due to carryover of media.
 - E. To avoid false-positive reactions, use a light inoculum to prevent carryover of substances from previous media.
 - F. The reactions of this medium alone are not sufficient for identification to the species level.
-

REFERENCES

1. Clinical and Laboratory Standards Institute. 2004. *Quality Assurance for Commercially Prepared Microbiological Culture Media*, 3rd ed. Approved standard M22-A3. Clinical and Laboratory Standards Institute, Wayne, Pa.
2. MacFaddin, J. F. 2000. *Biochemical Tests for the Identification of Medical Bacteria*, 3rd ed., p. 98–104. The Lippincott, Williams & Wilkins Co., Philadelphia, Pa.
3. Simmons, J. S. 1926. A culture medium for differentiating organisms of typhoid-colon-aerogenes groups and for isolation of certain fungi. *J. Infect. Dis.* **39**:209–214.

3.17.13

Coagulase Test—Protein A/ Clumping Factor Agglutination Method

I. PRINCIPLE

Staphylococcus aureus is separated from other species of staphylococci by the presence of coagulase, which is demonstrated in the 4- to 24-h tube test to detect free coagulase. Clumping factor, termed bound coagulase, can be detected rapidly in the slide test, but this test requires several colonies and lacks sensitivity. *S. aureus* produces another substance in its cell wall, protein A, which binds to the FC moiety of human immunoglobulin G (IgG) (7). If latex or erythrocyte particles are coated with IgG and with human fibrinogen, a staphylococcus will agglutinate if either clumping factor or protein A is present in the bacterial cell wall (1, 2, 3, 4, 5, 11). Other species, such as *Staphylococcus lugdunensis* and *Staphylococcus schleiferi*

subsp. *schleiferi*, produce clumping factor and can be positive in the assay. These species are important in serious infections and require that positive agglutination results be confirmed with other tests or observations. Still other staphylococci possess protein A, including *Staphylococcus saprophyticus*, so the test should be performed restrictively on urinary isolates.

More recently, it was discovered that some *S. aureus* organisms, especially methicillin-resistant *S. aureus* (MRSA), can mask the cell wall with capsular polysaccharides (6, 10) and prevent the reaction between the IgG or fibrinogen. Newer kits have added antibodies to the latex or erythrocytes, which bind to the specific

bacterial surface antigens that have been reported to interfere with the test. These new reagents are more sensitive but have reduced the specificity further (9, 12, 13). In the United States these clones of MRSA, having the specific surface antigens, are less common. Thus, most laboratories choose to use tests with higher specificity and use the tube coagulase test when it is important to detect MRSA (13).

Despite all the problems with agglutination tests, they are very useful in rapid identification of staphylococci and in increasing the sensitivity of the identification of *S. aureus*, since there are also problems of sensitivity with the tube and slide coagulase tests (procedure 3.17.14).

II. MICROORGANISMS TESTED

- A. Colonies of gram-positive cocci in clusters which are catalase positive, as part of the identification of *S. aureus*
- B. From urine cultures, use test with caution on nonhemolytic colonies that are presumptive for staphylococci, since *S. saprophyticus* may give a false-positive result.
- C. Only test colonies that are less than 24 h old and are not from mannitol salt agar.

III. REAGENTS AND SUPPLIES

A. Reagents

1. Products

- a. Murex Staphaurex and Staphaurex Plus (Wellcome Diagnostics; Remel, Inc.)
- b. Prolex Staph kit (Pro-Lab Diagnostics, Austin, Tex.)
- c. StaphTex (Hardy Diagnostics)
- d. Bacti Staph (Remel, Inc.)
- e. Staphyloslide (BD Microbiology Systems)
This is a hemagglutination test rather than a latex test.

- f. Slidex Staph kit and Slidex Staph Plus (bioMérieux Vitek, Hazelwood, Mo.)

■ NOTE: "Plus" indicates that the additional antibodies to surface antigens have been used to coat the particles.

2. Test suspension

- a. Store at 2 to 8°C in an upright position.
- b. Do not freeze. Protect reagents from bright light.

III. REAGENTS AND SUPPLIES (continued)

- c. Do not *vortex* latex reagents.
- d. Store at workbench on cold pack during working hours and return to 4°C for overnight storage.
- 3. Some kits have a negative control latex of unsensitized particles to detect autoagglutination.

B. Supplies

- 1. Loops or sterile sticks
- 2. Black coated cards (supplied by vendors for serologic agglutination tests); white cards for erythrocyte suspensions

IV. QUALITY CONTROL

- A. Inspect suspension for granularity each time the test is performed, as it is dropped onto the test card. If there is evidence of autoagglutination, do not use the suspension.
- B. At a minimum, perform QC on each new lot or shipment of reagent prior to putting it into use.
- C. Organisms
 - 1. *Staphylococcus aureus* ATCC 25923—agglutination positive
 - 2. *Staphylococcus epidermidis* ATCC 12228 or ATCC 14990—agglutination negative

V. PROCEDURE

- NOTE:** Verify any differences between this general procedure and the package insert prior to using test.
- A. Warm the reagents to room temperature.
 - B. Invert the reagent bottle several times to obtain an even suspension.
 - C. Dispense a drop of the test suspension into a circle on the reaction card for each culture to be tested.
 - D. From a plate that is <24 h old, touch two to four colonies with the flat end of a stick.
 - E. Emulsify the sample of culture in the suspension and spread over a 10-mm area of the circle.
 - F. If an autoagglutination control is included in the kit, repeat the above steps with the control reagent.
 - G. Rotate the card gently for up to, *but no longer than*, 20 s and examine for agglutination. Do not use a magnifying lens.
 - H. Dispose of the card in infectious-waste container.

VI. INTERPRETATION

- A. A positive result is indicated by the development of an agglutination pattern showing rapid strong clumping of the particles with *clearing* of the background. Most positive results are instantaneous.
- B. A negative result is indicated when the particles do not agglutinate and the appearance remains substantially unchanged throughout the test.
- C. If the control test shows clumping or the test shows clumping but the background remains unchanged, autoagglutination has likely occurred. Perform a tube coagulase test.

VII. REPORTING RESULTS

- A. Only test gram-positive cocci in clusters which are catalase positive. Report as *Staphylococcus aureus* if the test is positive and the isolate is clearly beta-hemolytic.
- B. If the test is positive and the colony is nonhemolytic and from a urine specimen or a normally sterile site, confirm with tube coagulase. In a urine specimen, this may be *S. saprophyticus*. In a blood culture, it may be *S. lugdunensis* or another coagulase-negative staphylococcus.
- C. Report negative tests from catalase-positive, gram-positive cocci in clusters as “coagulase-negative staphylococci,” but confirm with a tube test if the isolate is from a normally sterile body site, especially if it is methicillin resistant.

VIII. LIMITATIONS

- A. The advantage of agglutination assays is that they are very sensitive and rapid in separating *S. aureus* from coagulase-negative staphylococci. False-positive reactions do occur with strains of *Staphylococcus capitis*, *S. saprophyticus*, and *Staphylococcus warneri*, since these species possess protein A (2). *S. lugdunensis* and *S. schleiferi* can also give a positive result, since they possess clumping factor. Positive tests must be confirmed with the tube test for nonhemolytic strains from urine specimens or for all isolates from sites where identification to the species level is critical for clinical reasons (e.g., blood cultures).
- B. Although *S. lugdunensis* and *S. schleiferi* produce clumping factor, the reaction is less efficient in the latex test and cannot be relied upon to detect these species. Use other methods, such as pyrrolidonyl-β-naphthylamide (PYR), to detect these strains in invasive sites.
- C. A false-negative test, especially for MRSA with capsular antigens, can result. For isolates from significant sites where MRSA is important, e.g., blood and joint isolates, performing both the agglutination and the tube coagulase test will detect all strains of *S. aureus*.
- D. *Staphylococcus intermedius* and *Staphylococcus hyicus* may agglutinate the latex reagent, but it is clinically not important to separate these animal pathogens from *S. aureus*, because they are rarely found in humans (less than 0.1% in one study [8]) and they are considered as pathogenic as *S. aureus*.
- E. Do not perform testing from growth on mannitol salt agar.

REFERENCES

1. Baker, J. S., M. A. Bormann, and D. H. Boudreau. 1985. Evaluation of various rapid agglutination methods for the identification of *Staphylococcus aureus*. *J. Clin. Microbiol.* **21**:726–729.
2. Berke, A., and R. C. Tilton. 1986. Evaluation of rapid coagulase methods for the identification of *Staphylococcus aureus*. *J. Clin. Microbiol.* **23**:916–919.
3. Dickson, J. I., and R. R. Marples. 1986. Coagulase production by strains of *Staphylococcus aureus* of differing resistance characters: a comparison of two traditional methods with a latex agglutination system detecting both clumping factor and protein A. *J. Clin. Pathol.* **39**:371–375.
4. Essers, L., and K. Radebold. 1980. Rapid and reliable identification of *Staphylococcus aureus* by a latex agglutination test. *J. Clin. Microbiol.* **12**:641–643.
5. Lairscey, R., and G. E. Buck. 1987. Performance of four slide agglutination methods for identification of *Staphylococcus aureus* when testing methicillin-resistant staphylococci. *J. Clin. Microbiol.* **25**:181–182.
6. Lally, R., and B. Woolfrey. 1984. Clumping factor defective MRSA. *Eur. J. Clin. Microbiol.* **3**:151–152.
7. Langone, J. J. 1982. Protein A of *Staphylococcus aureus* and related immunoglobulin receptors produced by streptococci and pneumococci. *Adv. Immunol.* **32**:157–252.
8. Mahoudeau, I., X. Delabranche, G. Prevost, H. Monteil, and Y. Piemont. 1997. Frequency of isolation of *Staphylococcus intermedius* from humans. *J. Clin. Microbiol.* **35**:2153–2154.
9. Personne, P., M. Bes, G. Lina, F. Vandenesch, Y. Brun, and J. Etienne. 1997. Comparative performances of six agglutination kits assessed by using typical and atypical strains of *Staphylococcus aureus*. *J. Clin. Microbiol.* **35**:1138–1140.
10. Ruane, P. J., M. A. Morgan, D. M. Citron, and M. E. Mulligan. 1986. Failure of rapid agglutination methods to detect oxacillin-resistant *Staphylococcus aureus*. *J. Clin. Microbiol.* **24**:490–492.

REFERENCES (continued)

11. Smith, S. M., and C. Berezny. 1986. Comparative evaluation of identification systems for testing methicillin-resistant strains of *Staphylococcus aureus*. *J. Clin. Microbiol.* **24**:173–176.
12. Smole, S. C., E. Aronson, A. Durbin, S. M. Brecher, and R. D. Arbeit. 1998. Sensitivity and specificity of an improved rapid latex agglutination test for identification of methicillin-sensitive and -resistant *Staphylococcus aureus* isolates. *J. Clin. Microbiol.* **36**:1109–1112.
13. Wilkerson, M., S. McAllister, J. M. Miller, B. J. Heiter, and P. P. Bourbeau. 1997. Comparison of five agglutination tests for identification of *Staphylococcus aureus*. *J. Clin. Microbiol.* **35**:148–151.

3.17.14

Coagulase Test—Rabbit Plasma Method

I. PRINCIPLE

Staphylococcus aureus, the most pathogenic of the staphylococci, is separated from other species by the presence of coagulase. Coagulase is a thermostable thrombin-like substance that activates fibrinogen to form fibrin, resulting in a fibrin clot (2, 4). This is demonstrated in the test tube by the formation of a clot when plasma is inoculated with the staphylococcus. The substance is known as free coagulase, since it is liberated by the cell. In most, but not all, *S. aureus* organisms, fibrinogen binding cell surface receptor is also present in cell wall, called “bound coagulase” or “clumping factor.” Clumping factor is demonstrated by the ability of the

organism to act directly on the fibrinogen in the plasma to clump it in a slide assay. The test for clumping factor is rapid but requires several colonies, and as stated above, the factor is not present in all *S. aureus* organisms. In addition, clumping factor can be masked by cell surface capsular polysaccharides and can be present in other species, namely, *Staphylococcus lugdunensis* and *Staphylococcus schleiferi* (7). Negative slide tests must be followed with a confirmatory tube test.

Coagulase is also present in highly pathogenic species of staphylococci from animals: *Staphylococcus intermedius* from dogs, *Staphylococcus delphini* from dol-

phins, *Staphylococcus lutrae* from otters, and *Staphylococcus hyicus* from pigs. It is generally not cost-effective to separate these species in most human specimens, because infections with them either are very rare or present with morbidity similar to that of *S. aureus* (5).

In addition, the coagulase clot can be destroyed by *S. aureus* fibrinolysin or staphylokinase, a plasmid-carried enzyme which is more active at 35°C than at 25°C (2, 6, 8). Despite these caveats, algorithms can easily be implemented in the laboratory to rapidly identify *S. aureus* with a high degree of accuracy.

II. MICROORGANISMS TESTED

III. REAGENTS AND SUPPLIES



Include QC information on reagent container and in QC records.

- A. Colonies of gram-positive cocci in clusters which are catalase positive, as part of the identification of *S. aureus*
- B. Positive blood cultures containing gram-positive cocci in clusters for rapid detection of *S. aureus*

A. Plasma

NOTE: Do not use human plasma for the test, as it is less sensitive and potentially infectious with human pathogenic viruses (6, 8).

1. Dehydrated and reconstituted rabbit plasma in EDTA
 - a. Rehydrate according to manufacturer's instructions.
 - b. Dispense 0.5 ml into sterile 12- by 75-mm tubes.
 - c. Reagent expires after 1 month if stored at -20°C or 5 days if stored at 2 to 8°C.

2. Frozen rabbit plasma with EDTA (Coagulase Cryovial, Hardy Diagnostics)
3. 5% CaCl₂ (optional)

B. Supplies

1. Loops or sterile sticks
2. Glass or plastic tubes
3. Glass slides or black coated cards (supplied by vendors for serologic agglutination tests)

IV. QUALITY CONTROL

- A. Do not use plasma that has not been stored refrigerated or frozen or that appears turbid.
 - B. Perform QC of coagulase plasma on new lots prior to putting them into use.
 - C. Organisms
 - 1. *S. aureus* ATCC 25923—coagulase positive
 - 2. *Staphylococcus epidermidis* ATCC 12228 or ATCC 14990—coagulase negative
-

V. PROCEDURE**A. Slide test**

NOTE: To avoid misidentifications, only perform this test on classic-looking white to yellow, creamy, opaque, *hemolytic* colonies of gram-positive cocci in clusters that are catalase positive. Hemolysis should only be observed on fresh BAP at ≤ 18 h.

1. Add 10 μ l of deionized water to slide or black card.
2. Emulsify several colonies into the water to obtain a smooth milk-colored suspension.
 NOTE: If clumps occur and the organism does not suspend in the water, the slide test cannot be performed. The organism is inherently sticky and is said to autoagglutinate. Perform the tube test for free coagulase instead.
3. Add 1 to 3 μ l of rabbit plasma and observe for clumping immediately, not to exceed 10 s.
4. Dispose of the card in an infectious-waste container or the slide in a sharps container.

B. Tube test

1. Bring tube of plasma to 25°C.
2. Inoculate with one colony of staphylococcus growing on *noninhibitory medium* or 2 drops of blood from a positive blood culture.
3. Incubate at 35°C without CO₂ for *up to* 4 h and observe for clot formation hourly. Do not agitate the tube during observations; rather, gently tilt to observe the clot.
4. Incubate for an additional 20 h at 25°C for late clot formation (2).

NOTE: Do not leave the test at 35°C for more than 4 h, since *S. aureus* fibrinolysin can lyse the clot. If 4 h of incubation at 35°C is inconvenient for staffing, the test is most sensitive when incubated at 25°C for the entire time, but the clot may take longer to form (1). Alternatively, if the tube is left at 35°C, add a drop or two of 5% CaCl₂ at 24 h. If a clot forms, the isolate is coagulase negative; if a clot does not form, fibrinolysin has lysed a previously formed clot and this result confirms the isolate as coagulase positive (H. D. Isenberg, unpublished data).

VI. INTERPRETATION**A. Slide test**

1. A positive test is the demonstration of agglutination of the bacterial cells after the plasma is added.
2. A negative test is demonstrated by the lack of agglutination.

B. Tube test

1. A positive test meets one of the following criteria.
 - a. Complete clot formation or any degree of clot formation before 24 h
 - b. No clot formation after addition of 1 or 2 drops of 5% CaCl₂ to a tube without a clot at 24 h

VI. INTERPRETATION (continued)

2. A negative test meets one of the following criteria.
 - a. A lack of clot formation at 24 h at 25°C
 - b. No clot after 24 h at 35°C, but after addition of 1 or 2 drops of 5% CaCl₂ to the tube, a clot forms.

A flocculent or fibrous precipitate is not a true clot and is recorded as a negative test.

VII. REPORTING RESULTS

- A. Report as *Staphylococcus aureus* if the tube test is positive and the organism is catalase positive and a gram-positive coccus in clusters.
- B. For gram-positive cocci in clusters from a positive blood culture that are coagulase positive, report as “Probable *Staphylococcus aureus*; confirmation to follow” until the colony can be observed and the catalase test is performed.
- C. For a negative tube test from catalase-positive, gram-positive cocci in clusters that have creamy white colonies, report as “coagulase-negative staphylococci.”
- D. A positive slide test is reported as *S. aureus*; however, the test should be confirmed with a tube test from nonhemolytic or only slightly hemolytic colonies from sterile sites, such as blood, to separate *S. aureus* from *S. lugdunensis* and *S. schleiferi*.
- E. A negative slide test is not valid and should be followed with a tube test for confirmation.

VIII. LIMITATIONS

- A. Methicillin-resistant *S. aureus* can be deficient in bound coagulase, which results in a negative slide test (3).
- B. *S. intermedius* and *S. hyicus* may be positive in the tube test; these species are generally found only in dogs and pigs, respectively, but are as infectious as *S. aureus* when they infect humans. Both form nonhemolytic colonies on fresh (≤ 18 -h) plates and are Voges-Proskauer negative, which separates them from *S. aureus*. *S. intermedius* is also pyrrolidonyl- β -naphthylamide (PYR) positive (Table 3.18.1–1).
- C. *S. lugdunensis* and *S. schleiferi* produce slide coagulase, but the reaction is more efficient if human plasma is used rather than rabbit plasma. They can be separated from *S. aureus* by their strongly positive PYR reaction and from *S. intermedius* by a negative tube coagulase test (see Table 3.18.1–1).
- D. Do not use citrated blood, as false-positive results can occur.
- E. Coagulase testing cannot be performed from growth on mannitol salt agar.

REFERENCES

1. Baker, J. S., M. A. Bormann, and D. H. Boudreau. 1985. Evaluation of various rapid agglutination methods for the identification of *Staphylococcus aureus*. *J. Clin. Microbiol.* **21**:726–729.
2. Cowan, S. T. 1938. The classification of staphylococci by precipitation and biological reactions. *J. Pathol. Bacteriol.* **46**:31–45.
3. Lally, R., and B. Woolfrey. 1984. Clumping factor defective MRSA. *Eur. J. Clin. Microbiol.* **3**:151–152.
4. MacFaddin, J. F. 2000. *Biochemical Tests for the Identification of Medical Bacteria*, 3rd ed., p. 105–119. The Lippincott, Williams & Wilkins Co., Philadelphia, Pa.
5. Mahoudeau, I., X. Delabranche, G. Prevost, H. Monteil, and Y. Piemont. 1997. Frequency of isolation of *Staphylococcus intermedius* from humans. *J. Clin. Microbiol.* **35**:2153–2154.
6. Orth, D. S., L. R. Chung, and A. W. Anderson. 1971. Comparison of animal sera for suitability in coagulase testing. *Appl. Microbiol.* **21**:420–425.
7. Patel, R., K. E. Piper, M. S. Rouse, J. R. Uhl, F. R. Cockerill III, and J. M. Steckelberg. 2000. Frequency of isolation of *Staphylococcus lugdunensis* among staphylococcal isolates causing endocarditis: a 20-year experience. *J. Clin. Microbiol.* **38**:4262–4263.
8. Yrios, J. W. 1977. Comparison of rabbit and pig plasma in the tube coagulase test. *J. Clin. Microbiol.* **5**:221–224.

[Updated March 2007]

I. PRINCIPLE

Arginine, lysine, and ornithine decarboxylase media are used to detect an organism's ability to decarboxylate or hydrolyze an amino acid, forming an amine that produces an alkaline pH. The basal medium is usually Møller's formula (7) and contains meat peptones and beef extract, which supply nitrogenous nutrients to support bacterial growth. Glucose is the fermentable carbohydrate. The pH indicators are bromcresol purple and cresol red. Pyridoxal is an enzyme cofactor which enhances decarboxylase activity. Arginine, lysine, and ornithine are amino acids that

are singly added to basal medium to detect the production of enzymes which decarboxylate or hydrolyze these substrates. When an organism in the medium ferments glucose, acids are produced which lower the pH, resulting in a color change from purple to yellow. If decarboxylation or hydrolysis of the amino acid occurs in response to the acid pH, alkaline end products (amines) are formed which result in the medium reverting to its original color (purple). When the organism does not ferment glucose, the medium does not turn yellow; the test can still be performed, but

it is important to include a control without amino acids for comparison.

Decarboxylation of lysine yields cadaverine, decarboxylation of ornithine yields putrescine, and decarboxylation of arginine yields agmatine, which is hydrolyzed by a dihydrolase to form putrescine. In another reaction arginine dihydrolase converts arginine to citrulline, which is converted to ornithine and then to putrescine. Since decarboxylation is an anaerobic reaction, the contents of each tube must be sealed with oil or paraffin.

II. MICROORGANISMS TESTED

- A. Enteric gram-negative rods and *Vibrio*, *Plesiomonas*, and *Aeromonas* for identification to the species level
- B. Probable *Stenotrophomonas* and *Burkholderia* (lysine and arginine)
- C. Fluorescent *Pseudomonas* (arginine)
- D. Coagulase-negative staphylococci (ornithine)
- E. Viridans group streptococci (arginine)
- F. Miscellaneous non-glucose-fermenting, gram-negative rods (arginine)
- G. Spreading indole-negative *Proteus* (ornithine)

III. MEDIA, REAGENTS, AND SUPPLIES

A. Media

- 1. Peptone, beef extract, glucose, pyridoxal with bromcresol purple and cresol red indicators, and amino acids as listed below
 - a. Møller's decarboxylase base (no amino acids)
 - b. Møller's arginine decarboxylase (1% arginine)
 - c. Møller's lysine decarboxylase (1% lysine)
 - d. Møller's ornithine decarboxylase (1% ornithine)

- 2. Motility-indole-ornithine medium (MIO) (see motility tests [procedure 3.17.31] for inoculation method)
- 3. Tablets (Key Scientific Products)
Refer to <http://www.keysscientific.com> for procedure.

III. MEDIA, REAGENTS, AND SUPPLIES (continued)

4. Lysine iron agar

Used as a screen for stool pathogens to detect lysine decarboxylase-positive (purple color in butt) *Salmonella*; however, the medium is also helpful to distinguish *Salmonella* from *Proteus* and *Providencia*, which deaminate lysine and produce a red slant.

B. Reagents

1. Mineral oil
2. Vaspar, liquid paraffin, or petroleum jelly, maintained at 56°C in liquid form

C. Other supplies

1. Sterile sticks or inoculating loops
2. Incubator at 35°C

IV. QUALITY CONTROL

- A. Examine media for lack of turbidity and purple color. Discard if not purple.

- B. Test new lot or shipment of medium with a positive and negative control prior to putting it into use. Use the staphylococcus controls for validating use with staphylococci.

- C. Organisms

Test organism	Control tube	Result ^a		
		Arginine	Lysine	Ornithine
<i>Klebsiella pneumoniae</i> ATCC 13883	Yellow	—	+	—
<i>Enterobacter cloacae</i> ATCC 23355	Yellow	+	—	+
<i>Staphylococcus aureus</i> ATCC 25923	Yellow	NA	NA	—
<i>Staphylococcus lugdunensis</i> ATCC 700328	Yellow	NA	NA	+

^a —, yellow; +, purple; NA, not applicable.

V. PROCEDURE

- A. Inoculate each broth being tested with one or two colonies from an 18- to 24-h culture.

1. For non-glucose-fermenting, gram-negative rods

- a. Include the control tube

b. Inoculate the tubes *heavily* (\geq no. 8 McFarland turbidity standard [7]; see Appendix 3.16–1).

2. For *Enterobacteriaceae*, the control tube is not needed, since all strains ferment glucose.

3. For gram-positive cocci, the control tube is usually not needed.
- B. Overlay the inoculated tubes with approximately 1 ml of mineral oil or a 4-mm plug of melted petroleum jelly, Vaspar, or paraffin, being careful to cover broth layer entirely without introducing air.

- C. Tighten the caps on the tubes.

- D. Incubate aerobically at 35°C for at least 18 h and up to 7 days, and observe daily for purple color.

■ NOTE: MIO is stabbed with a wire; oil overlay is not necessary if reactions are read only at 18 h. Incubation and color interpretations are the same as for broth media. Refer to <http://www.keysscientific.com> for procedure for tablets.

VI. INTERPRETATION

- A. A positive test is turbid purple to faded-out yellow-purple color (alkaline).

- B. A negative test is bright clear yellow color (acid) or no change (nonfermenting rods).

- C. Control tube must remain its original color or turn yellow. Turbidity must be seen. An alkaline or purple color in the control invalidates the test. Compare questionable results to the control tube.

VII. REPORTING RESULTS

- A. *Staphylococcus lugdunensis* is the only staphylococcus that is pyrrolidonyl- β -naphthylamide (PYR) and ornithine positive (5).
- B. *Leuconostoc* is arginine negative, and *Weissella confusa* is arginine positive. Both are PYR-negative, vancomycin-resistant, gram-positive cocci with rod-like forms.
- C. Arginine is useful in the identification of *Enterococcus* to the species level; *Enterococcus avium* is arginine negative, but *Enterococcus faecalis* and *Enterococcus faecium* are arginine positive (2).
- D. *Stenotrophomonas maltophilia* and *Burkholderia cepacia* are among the few non-glucose-fermenting, gram-negative rods that are lysine positive.
- E. Among the polymyxin B-resistant nonfermenters, *Burkholderia mallei* and *Burkholderia pseudomallei* are arginine positive.
- F. *Streptococcus anginosus* and *Streptococcus sanguis* are the viridans group streptococci that are arginine positive (1).
- G. Reactions for *Enterobacteriaceae* are found in references 3 and 4.
- H. *Plesiomonas* is positive for lysine, arginine, and ornithine, which separates it from *Vibrio* and *Aeromonas*, whose results are variable with each species but are not positive for all three decarboxylases (see Table 3.18.2–8).
- I. Other reactions for the non-glucose-fermenting, gram-negative rods are found in the tables in Weyant et al. (8) and in the tables in procedure 3.18.2.

VIII. LIMITATIONS

- A. Mineral oil or a similar barrier to gas release must be applied to the surface of each inoculated broth medium. Oil overlay decreases the possibility of an alkaline shift occurring in the medium due to oxidation.
- B. Test interpretation should *not* be made prior to 18 to 24 h of incubation. Earlier interpretation may lead to erroneous results. Glucose fermentation occurs within the first 10 to 12 h of incubation. Fermentation produces an acidic environment which results in a yellow color development. The production of decarboxylase enzymes will not be induced until the acidic state has been established.
- C. Non-glucose-fermenting microorganisms may display weak decarboxylase activity, thereby resulting in an insufficient production of amines necessary to convert the pH indicator system. Some nonfermenters, however, will produce sufficient amines to result in a deeper purple color than in an uninoculated tube of basal medium.
- D. A gray color may indicate reduction of the indicator, rather than production of alkaline end products. To aid in reading the reaction, add additional bromcresol purple.
- E. If two layers of different colors appear, shake the tube gently before interpreting the reaction (6).
- F. Nonfermenting bacteria that are arginine positive must be lysine and ornithine negative (6).

REFERENCES

1. **Coykendall, A.** 1989. Classification and identification of the viridans streptococci. *Clin. Microbiol. Rev.* **2**:315–328.
2. **Facklam, R. R., and M. D. Collins.** 1989. Identification of *Enterococcus* species by a conventional test scheme. *J. Clin. Microbiol.* **27**:731–734.
3. **Farmer, J. J., III, B. R. Davis, F. W. Hickman-Brenner, A. McWhorter, G. P. Huntley-Carter, M. A. Asbury, C. Riddle, H. G. Wathen-Grady, C. Elias, G. R. Fanning, A. G. Steigerwalt, C. M. O'Hara, G. K. Morris, P. B. Smith, and D. J. Brenner.** 1985. Biochemical identification of new species and biogroups of *Enterobacteriaceae* isolated from clinical specimens. *J. Clin. Microbiol.* **21**:46–76.
4. **Farmer, J. J., III.** 2003. *Enterobacteriaceae*: introduction and identification, p. 636–653. In P. R. Murray, E. J. Baron, J. H. Jorgensen, M. A. Pfaller, and R. H. Yolken (ed.), *Manual of Clinical Microbiology*, 8th ed. ASM Press, Washington, D.C.
5. **Hébert, G. A.** 1990. Hemolysins and other characteristics that help differentiate and biotype *Staphylococcus lugdunensis* and *Staphylococcus schleiferi*. *J. Clin. Microbiol.* **28**:2425–2431.
6. **MacFaddin, J. F.** 2000. *Biochemical Tests for the Identification of Medical Bacteria*, 3rd ed., p. 120–135. The Lippincott, Williams & Wilkins Co., Philadelphia, Pa.
7. **Møller, V.** 1955. Simplified tests for some amino acid decarboxylases and for the arginine dihydrolase system. *Acta Pathol. Microbiol. Scand.* **36**:158–172.
8. **Weyant, R. S., C. W. Moss, R. E. Weaver, D. G. Hollis, J. G. Jordan, E. C. Cook, and M. I. Daneshvar.** 1995. *Identification of Unusual Pathogenic Gram-Negative Aerobic and Facultatively Anaerobic Bacteria*, 2nd ed., p. 7–8. Williams & Wilkins, Baltimore, Md.

3.17.16

DNase Test-Rapid Thermonuclease Test

[Updated March 2007]

I. PRINCIPLE

DNases are enzymes that hydrolyze DNA and release free nucleotides and phosphate. The DNases produced by bacteria are extracellular endonucleases that cleave DNA, yielding a high concentration of oligonucleotides. There are several media used to detect these enzymes, using no indicators (2) or various indicators (toluidine blue [5, 7] or methyl green [MG] [6]) to detect the hydrolysis of DNA. The first medium was developed by Jeffries et al.

(2), with no indicator. The hydrolysis of DNA was observed by a clearing of the agar after addition of HCl (the oligonucleotides dissolve in acid but DNA salts are insoluble). When MG indicator is added, DNA combines with the MG to produce a green color. When the DNA is hydrolyzed, the complex is released and the free MG is colorless at pH 7.5 (4). When toluidine blue O (TBO) is added, a complex is formed with the DNA, which changes

structure when DNA is hydrolyzed, resulting in a bright pink color (4). The media with dyes can inhibit some microbial growth. Using a heavy inoculum prevents this problem and makes the test more rapid, as it detects preformed enzymes. *Staphylococcus aureus* possesses a heat-stable enzyme, a thermonuclease. To detect this enzyme, first the organisms are destroyed by heat and then the free DNase reacts with the medium.

II. MICROORGANISMS TESTED

- A. Gram-negative rods that are presumptive for *Stenotrophomonas maltophilia* (positive) and are colistin or polymyxin B resistant to separate from *Burkholderia cepacia* (negative). MG agar is preferred.
- B. Gram-negative diplococci that are presumptive *Moraxella catarrhalis* (positive). TBO agar is preferred.
- C. Gram-positive cocci that are presumptive for *S. aureus* (positive) and are difficult to separate from other closely related species and have a questionable coagulase reaction. Use only TBO for staphylococcus heat-stable testing, since it is more sensitive in detection of preformed enzyme. Some staphylococci do not grow on media containing dyes.
- D. *Enterobacteriaceae* to identify *Serratia* spp. (positive) and separate them from *Klebsiella* spp. and *Enterobacter* spp. (1, 5). *Serratia fonticola* is the only *Serratia* sp. that is negative for DNase.
- E. Oxidase-positive, indole-positive, gram-negative rods to separate *Aeromonas* spp. and *Vibrio cholerae* (positive) from *Plesiomonas shigelloides* (negative) (3)

III. MEDIA, REAGENTS, AND SUPPLIES

A. Media

- 1. TBO agar
Medium is royal blue and provides better delineation of DNase activity (4).
- 2. MG agar
Medium is green and supports the growth of both gram-positive and gram-negative bacteria.
- 3. BHI for staphylococci

B. Supplies/equipment

- 1. Sterile sticks, needles or inoculating loops
- 2. Pasteur pipettes or drinking straws
- 3. Boiling heat block
- 4. Incubators at 35 and 30°C

IV. QUALITY CONTROL

- A. Inspect agar for freezing, contamination, cracks, and dehydration prior to storage and before use. Discard plates that are not blue (TBO) or green (MG).
- B. Perform QC on each new lot or shipment of media prior to putting it into use. If plates are reused after incubation, add a positive and negative control to the plate each time the test is performed.
- C. Organisms
 1. Theronuclelease test
 - a. Positive control: *S. aureus* ATCC 25923—change to pink color (TBO)
 - b. Negative control: *Staphylococcus epidermidis* ATCC 12228—no color change
 2. DNase test (choose one or more positive controls)
 - a. Positive controls
 - (1) *M. catarrhalis* ATCC 25240—change to pink color (TBO) or colorless (MG)
 - (2) *Serratia marcescens* ATCC 13880—change to pink color (TBO) or colorless (MG)
 - (3) *S. aureus* ATCC 25923—change to pink color (TBO) or colorless (MG)
 - b. Negative control: *Escherichia coli* ATCC 25922—no color change

V. PROCEDURES**A. DNase test method**

1. After touching several colonies from an 18-h culture, inoculate a segment of the agar surface with a very visible, heaping amount of organism equivalent to an entire colony. Several colonies can be placed on a single plate. Use either circular (1/4-in diameter) or line (1/2-in length) method for inoculation. Do not streak the entire plate, as it will be difficult to see the reaction.
2. Incubate without added CO₂ for 24 h and up to 72 h at the following temperatures.
 - a. 35°C for staphylococci or *M. catarrhalis* (TBO agar is more sensitive and rapid, since organism does not grow well on media with dyes)
 - b. 25°C for enteric gram-negative rods and *Vibrionaceae*
 - c. 25 or 30°C for non-glucose-fermenting, gram-negative rods (MG agar is preferred)
- NOTE:** DNases are generally most active at lower temperatures, and all testing may be performed at room temperature (e.g., 22 to 25°C) if the organism will grow at a lower temperature (4).
3. Examine for color change. Incubate a full 72 h before calling result negative.

B. Theronuclelease method

1. After touching several well-isolated staphylococcal colonies with a sterile needle, inoculate BHI.
2. Incubate at 35°C for 18 h. Growth from a positive blood culture can also be used.
3. Place broth in boiling heat block for 15 min.
4. Cool to room temperature.
5. Punch a hole in the TBO agar with the large end of a Pasteur glass pipette or drinking straw and remove the agar plug.
6. Fill the well with 2 drops of cooled broth culture.
7. Incubate at 35°C for 3 h, and observe for color change.

VI. INTERPRETATION

A. MG agar

1. Hold plates against a white background in indirect light.
2. Positive test: the development of a clear halo around the colony or the well in the agar
3. Negative test: no clear zone in the medium or around the well in the agar. Agar remains green.

B. TBO agar

1. Use transmitted light to observe changes in color.
2. Positive test: the development of a pink or red halo around the colony or the well in the agar
3. Negative test: no change in the royal blue color of the medium

VII. REPORTING RESULTS

- A. Gram-negative rods that are non-glucose-fermenting and that are lysine and DNase positive but negative or weakly positive for oxidase are *S. maltophilia*.
- B. Gram-negative diplococci that are oxidase positive and DNase positive are *M. catarrhalis*.
- C. Gram-positive cocci in clusters that have a thermostable DNase are usually *S. aureus*, but some other staphylococci can give a positive test. *S. schleiferi* is DNase and pyrrolidonyl-β-naphthylamide (PYR) positive. See Table 3.18.1–1 for other tests to separate.
- D. *Serratia* spp. produce a DNase, which separates nonpigmented strains from most other *Enterobacteriaceae* (1, 5).
- E. Oxidase-positive, indole-positive, gram-negative rods that grow on MAC and are DNase negative are likely to be *P. shigelloides*, rather than the closely related *Aeromonas* and *Vibrio* spp., which are generally DNase positive.

VIII. LIMITATIONS

- A. An inoculum that is too broad may result in complete decolorization of the media, due to the reduction of the dye. If this occurs, the test must be repeated.
- B. MG medium is better for organisms, such as gram-negative rods, that first grow on the medium and then demonstrate a positive test.
- C. For *Moraxella* and gram-positive cocci with TBO testing, a low inoculum can result in a false-negative test, since these organisms may not grow well on the medium.

REFERENCES

1. Ewing, W. H. 1986. *Edwards and Ewing's Identification of Enterobacteriaceae*, 4th ed. Elsevier Scientific Publishing Co., New York, N.Y.
2. Jeffries, C. D., F. Holtman, and G. D. Guse. 1957. Rapid method for determining the activity of microorganisms on nucleic acids. *J. Bacteriol.* **73**:590–591.
3. Krieg, N. R., and J. G. Holt (ed.). 1984. *Bergey's Manual of Systematic Bacteriology*, vol. 1, p. 484, 550. Williams & Wilkins, Baltimore, Md.
4. MacFaddin, J. F. 2000. *Biochemical Tests for the Identification of Medical Bacteria*, 3rd ed., p. 136–159. The Lippincott, Williams & Wilkins Co., Philadelphia, Pa.
5. Schreier, J. B. 1969. Modification of deoxyribonuclease test medium for rapid identification of *Serratia marcescens*. *Am. J. Clin. Pathol.* **51**:711–716.
6. Smith, P. B., G. A. Hancock, and D. L. Rhoden. 1969. Improved medium for detecting deoxyribonuclease-producing bacteria. *Appl. Microbiol.* **18**:991–993.
7. Waller, J. R., S. L. Hodel, and R. N. Nuti. 1985. Improvement of two toluidine blue O-mediated techniques for DNase detection. *J. Clin. Microbiol.* **21**:195–199.

3.17.17

Fluorescent-Pigment Agars for *Pseudomonas* Identification

I. PRINCIPLE

The kind of peptone in the basal medium markedly affects the production of pigment by bacteria (1). Two pigment-enhancing media were developed by King et al. (1). The pyocyanin or pyorubrin pigment of *Pseudomonas aeruginosa* is enhanced on P agar or "Tech" agar. The fluorescein or pyoverdin pigment of the

fluorescent pseudomonads is enhanced on F agar or "Flo" agar. When the P agar is incubated at 42°C, the ability of *P. aeruginosa*, but not *Pseudomonas fluorescens* or *Pseudomonas putida*, to grow at that temperature is also determined. The peptone choices in F agar were found to enhance the production of fluorescein and in-

hibit the production of pyocyanin. *Pseudomonas* P agar contains a peptone containing less than 0.1% phosphorus to minimize the inhibitory effect on pyocyanin production. The incorporation of magnesium chloride and potassium sulfate stimulates the production of pyocyanin and pyorubrin.

II. MICROORGANISMS TESTED

Isolated colonies of oxidase-positive, indole-negative, non-glucose-fermentative, gram-negative rods growing on MAC and suggestive of *Pseudomonas* spp.

III. MEDIA, REAGENTS, AND SUPPLIES

A. Media (3)

1. P agar slants (P agar [Remel, Inc.])

a. P agar

Bacto Peptone 20.0 g
magnesium chloride 1.4 g
potassium sulfate 10.0 g
agar 15 g
glycerol 10.0 ml
water, distilled 990 ml
pH 6.8 to 7.0

b. Cetrimide agar (procedure 3.17.11) contains all the essential ingredients to enhance both pyocyanin and fluorescein production, with 3% cetrimide to inhibit organisms other than *P. aeruginosa* (Pseudosel [BD Diagnostic Systems, Hardy Diagnostics])

2. F agar slants (F agar [Remel, Inc.])

a. F agar

Proteose Peptone
no. 3 20.0 g
magnesium sulfate
(hydrated) 1.5 g
dipotassium phosphate .. 1.5 g
agar 15 g
glycerol 10.0 ml
water, distilled 990 ml
pH 6.8 to 7.0

b. Fluorescence-denitrification (FN), fluorescence indole denitrification (FIN), and fluorescence lactose denitrification (FLN) media are similar to F agar but have added nitrate, tryptophan, and lactose, respectively, with a pH indicator to allow for additional testing (FIN and FLN; Hardy Diagnostics).

B. Supplies

1. Sterile inoculating loops or sticks
2. Wood's or long-wave (360 nm) UV light or short-wavelength (254 nm) UV light (preferred)
3. 35°C incubator or heat block
4. 42°C incubator or heat block

IV. QUALITY CONTROL

- A. Perform QC on each new lot or shipment of media prior to putting it into use.
- B. Inspect agar for freezing, contamination, cracks, dehydration, and bubbles prior to storage and before use.
- C. Organisms
 - 1. P agar
 - a. *P. aeruginosa* ATCC 27853—positive (blue-green pigment)
 - b. *P. fluorescens* ATCC 13525—negative (no blue-green pigment)
 - 2. F agar
 - a. *P. fluorescens* ATCC 13525—positive (fluorescent pigment)
 - b. *Escherichia coli* ATCC 25922—negative (no fluorescent pigment)

V. PROCEDURE

- A. Streak each slant back and forth with inoculum picked from the center of a well-isolated colony. Also stab the deep of FN, FIN, and FLN media.
- B. Place cap loosely on tube.
- C. Incubate aerobically at 35°C for up to 7 days; alternatively, when using two agar media, incubate P agar at 42°C for up to 7 days.
- D. Observe for growth and color visually and, if negative, under UV light for fluorescent pigment.
- E. If negative at 24 h, incubate additional days in the dark at 25°C.

VI. INTERPRETATION

- A. Pseudomonas F agar, cetrimide, and FN, FIN, and FLN agars
 - 1. Positive: bright yellow-green color diffusing into the agar; fluorescent zone surrounding growth
 - 2. Negative: no yellow-green pigment (or no growth on cetrimide agar)
- B. Pseudomonas P agar and cetrimide
 - 1. Positive: a blue-green color indicating pyocyanin production
 - 2. Negative: no pigment production (or no growth on cetrimide agar)
- C. Pyorubin production is indicated by a pink, red, or red-brown color on any medium.
- D. FN, FLN, and FIN agar for nitrate reduction (see nitrate test, procedure 3.17.35, for QC)
 - 1. Positive: slits in medium indicative of gas bubbles
 - 2. Negative: no slits in medium
- E. FLN for lactose acidification (see procedure 14.2 for QC of medium for acid from lactose)
 - 1. Positive: yellow color change of the slant as a result of lactose acidification
 - 2. Negative: slant remains red.

VII. REPORTING RESULTS

- A. *P. aeruginosa* is definitively identified if it is oxidase positive and does one of the following.
 - 1. Produces a blue-green pigment
 - 2. Produces a red-brown pigment
 - 3. Produces a yellow-green pigment or fluorescent pigment and grows at 42°C
☒ NOTE: *P. aeruginosa* is nitrate positive and lactose negative.
- B. *P. fluorescens* or *P. putida* is identified if there is a yellow-green pigment or fluorescent pigment and no growth at 42°C (3). See lecithinase or gelatin procedure (procedure 3.17.27 or 3.17.18, respectively) to separate.
- C. *P. putida* is nitrate negative, and *P. fluorescens* is infrequently positive.

VIII. LIMITATIONS

- A. If *P. aeruginosa* has a characteristic odor and is oxidase positive and indole negative, no further testing is indicated, except from cystic fibrosis (CF) patients (2).
- B. Not all *P. aeruginosa* isolates produce pigments, especially those from CF patients. If the isolate is from a CF patient and lacks pigments, it is identified as *P. aeruginosa* if it meets all of the following criteria.
 - 1. Is oxidase positive and indole negative
 - 2. Has characteristic mucoid colony form and has the characteristic odor
 - 3. Is polymyxin B susceptible
- C. Mueller-Hinton agar when being used for disk susceptibility testing may substitute for F agar.
- D. Not all fluorescent pseudomonads grow on cetrimide or produce pigments.

REFERENCES

- 1. King, E. O., M. K. Ward, and D. E. Raney. 1954. Two simple media for the demonstration of pyocyanin and fluorescein. *J. Lab. Clin. Med.* **44**:301–307.
- 2. NCCLS. 2001. *Abbreviated Identification of Bacteria and Yeast*. Proposed guideline M35-P. NCCLS, Wayne, Pa.
- 3. Weyant, R. S., C. W. Moss, R. E. Weaver, D. G. Hollis, J. G. Jordan, E. C. Cook, and M. I. Daneshvar. 1995. *Identification of Unusual Pathogenic Gram-Negative Aerobic and Facultatively Anaerobic Bacteria*, 2nd ed., p. 17–18. Williams & Wilkins, Baltimore, Md.

[Updated March 2007]

I. PRINCIPLE

Gelatinases are proteolytic enzymes that hydrolyze gelatin into polypeptides and individual amino acids. These enzymes destroy the structure of the gelatin, and it becomes liquid. As an alternative to a gelatin medium, X-ray film that has not been developed can be used because it is coated with gelatin. After exposure to gelatinase, the film will lose its coating and leave be-

hind a clear photographic film. Kohn (2) has reported an excellent rapid test medium for detection of small amounts of preformed gelatinase without growth of the organism. Gelatin is complexed with charcoal and formalin, and only a small amount of gelatin is needed to release the charcoal particles. This method is used in

the API 20E (bioMérieux, Inc.) and shows a reaction in 18 h rather than several days for a nutrient tube gelatin assay that requires growth of the organism and liquefaction of the medium. Gelatin testing is useful in many taxonomic identifications of both gram-negative rods (1) and gram-positive rods.

II. MICROORGANISMS TESTED

- A. Gram-negative rods that require gelatin for identification, especially for the separation of the fluorescent *Pseudomonas*: *Pseudomonas putida* (negative) from *Pseudomonas fluorescens* (positive)
- B. Gram-positive rods as needed for identification to the species level

III. MEDIA, REAGENTS, AND SUPPLIES

- A. Nutrient gelatin with heart infusion or beef extract and peptone
 1. Store at 2 to 8°C, at which it is in solid form.
 2. At room temperature, it will liquefy.
- B. Other options
 1. X-ray film
 - a. Cut X-ray film that is exposed but not developed into strips having a width and length that fit into a test tube.
 - b. Prepare sterile tubes with 1 ml of sterile distilled water or saline.
- 2. Kohn gelatin charcoal medium (required reagents are listed below, and recipe for preparation is detailed in MacFaddin [3])

nutrient gelatin	15.0 g
powdered	
charcoal	3.0 to 5.0 g
distilled water	100 ml
TSB	3 to 4 ml per tube

NOTE: Charcoal disks are soaked in 10% formalin (10 ml of 40% formaldehyde and 1 g of calcium carbonate in 90 ml of 0.85% NaCl), cut, and washed prior to sterilization. Then TSB is added to each disk.
- C. Other supplies
 1. Inoculating needle
 2. Incubator at 22, 30, or 35°C, depending on the isolate

IV. QUALITY CONTROL

- A. Inspect tubes for contamination, dehydration, and lack of liquefaction at refrigeration temperatures before storage and before use.
 - B. Inspect X-ray film for loss of gelatin coating.
 - C. Perform QC on each new lot or shipment of media prior to putting it into use. Use an uninoculated control with each use.
 - D. Organisms
 - 1. *Pseudomonas aeruginosa* ATCC 10145—liquefaction (positive)
 - 2. *Escherichia coli* ATCC 25922—no liquefaction (negative)
-

V. PROCEDURE

- A. **Incubation temperatures**
 - 1. 22°C for *Enterobacteriaceae* and fluorescent *Pseudomonas* spp.
 - 2. 30°C for nonfermenting, gram-negative rods
 - 3. 35°C for other organisms and for X-ray and Kohn methods
 - B. **Tube method**
 - 1. The medium should be solidified at room temperature. After touching several well-isolated colonies with a sterile needle, stab directly down the center of the tube to approximately 10 mm from the bottom. Repeat to inoculate heavily.
 - 2. Incubate the test and an uninoculated control tube for 48 h.
 - 3. Gently remove the inoculated and uninoculated tubes from the incubator and refrigerate for at least 30 min or until the control tube solidifies. (Gelatin is a liquid at 28°C or higher.)
 - a. Do not shake or invert the tubes prior to refrigeration.
 - b. Gently invert to detect liquefaction by the test organism after 30 min of refrigeration.
 - 4. Reincubate a negative test for up to 2 weeks if indicated by the nature of the organism, and examine at regular intervals.
 - C. **X-ray method**
 - 1. After touching several colonies from an 18-h culture, inoculate 1 ml of sterile distilled water equivalent to a no. 2 McFarland standard.
 - 2. Place a gelatin strip or X-ray strip in the water and cap tube. Do not press the strip to the side of the tube such that it is not in contact with the water.
 - 3. Prepare a tube without inoculum as a control.
 - 4. Incubate for up to 48 h at 35°C.
 - 5. Examine at regular intervals for clearing of the strip.
 - D. **Kohn method**
 - 1. Inoculate gelatin-charcoal tube heavily, equivalent to a no. 2 McFarland standard, with growth of microorganism from an 18-h culture.
 - 2. Set up an uninoculated control tube.
 - 3. Incubate for 24 h or longer at 35°C.
 - 4. Shake and observe for particles dispersed throughout medium.
-

VI. INTERPRETATION

- A. **Tube method**
 - 1. Positive test: at the end of the refrigeration period, the control tube will be resolidified and the test tube will remain liquid at least to the depth of the stab. (Some organisms only partially liquefy gelatin or liquefy just at the surface of the tube.)
 - 2. Negative test: at the end of the refrigeration period, the control tube and the test tube will be resolidified, even at the top of the tube.

VI. INTERPRETATION (continued)

B. X-ray film method

1. Positive test: green gelatin layer is removed on immersed portion of film, exposing transparent, bluish film.
2. Negative test: green gelatin layer remains undisturbed.

C. Kohn gelatin disks

1. Positive test: a visible black cloud observed after tube is shaken.
2. Negative test: no free charcoal particles in medium and tube remains as the uninoculated tube after shaking.

VII. REPORTING RESULTS

A. *Proteus* spp. are gelatinase positive.

B. *P. fluorescens* is gelatinase positive, but *P. putida* is gelatinase negative.

C. *Serratia* spp. are usually gelatinase positive.

VIII. LIMITATIONS

A. Gelatinase usually acts at the surface of the tube medium. Shaking the tube while it is warm may result in a false-negative interpretation.

B. Gelatin may vary in its gelling ability; therefore, incubate an uninoculated control with the test. The control must be refrigerated along with the test, prior to reading.

C. For the X-ray film method, the strips must be immersed in the liquid and not cling to the sides of the tube without immersion. Only part of the film must be immersed, so that a difference in reaction can be visualized.

D. Do not use photographic film.

E. False-positive results may occur with the X-ray film method in some media or saline on prolonged incubation, i.e., greater than 4 h. This can be detected in the uninoculated control tube.

REFERENCES

1. Ewing, W. H. 1986. *Edwards and Ewing's Identification of Enterobacteriaceae*, 4th ed. Elsevier Scientific Publishing Co., New York, N.Y.
2. Kohn, J. 1953. A preliminary report of a new gelatin liquefaction method. *J. Clin. Pathol.* 6:249.
3. MacFaddin, J. F. 2000. *Biochemical Tests for the Identification of Medical Bacteria*, 3rd ed., p. 170–182. The Lippincott, Williams & Wilkins Co., Philadelphia, Pa.

Glucan and Polysaccharide Production

I. PRINCIPLE

Streptococci can produce glucans (extra-cellular polysaccharides) on agar containing sucrose. The two types of glucans, dextrans and levans, yield different consistencies. Characteristic colonies and type

of glucans produced (e.g., slime, adherence) can be used to identify streptococci to the species level.

Some saprophytic *Neisseria* spp. can produce an amylosucrase enzyme that

synthesizes an iodine-reacting polysaccharide from sucrose. The polysaccharide is detected by the production of a brown to black color when iodine is added to the agar medium.

II. MICROORGANISMS TESTED

- A. Gram-positive cocci that are catalase negative
 - 1. To separate *Streptococcus bovis*, which produces copious amounts of watery slime, from other viridans group streptococci that are Voges-Proskauer positive and esculin positive
 - 2. To separate *Streptococcus mutans*, which is glucan positive, from other streptococci
- B. To detect the production of iodine-reacting polysaccharide from sucrose to separate *Neisseria meningitidis*, *Neisseria gonorrhoeae*, and *Neisseria lactamica* from saprophytic strains of *Neisseria* (4)

III. MEDIUM, REAGENT, AND SUPPLIES

A. Medium

Heart infusion agar with 5% sucrose (2) (Remel, Inc.)

B. Reagent

0.2% Iodine with 0.4% potassium iodide freshly diluted 1:5 with distilled water

C. Supplies

- 1. Sterile sticks or inoculating loops
- 2. Incubators at 35°C

IV. QUALITY CONTROL

- A. Inspect agar for freezing, contamination, cracks, and dehydration prior to storage and before use.
- B. Perform QC on new lots of media prior to putting them into use.
- C. Organisms
 - 1. *S. bovis* ATCC 33317—copious amounts of glucans (positive)
 - 2. *Enterococcus faecalis* ATCC 25922—no glucans produced (negative)
 - 3. *Neisseria polysaccharea* ATCC 43768—polysaccharides produced (positive)
 - 4. *N. gonorrhoeae* ATCC 43069—no polysaccharide production (negative)

V. PROCEDURE

- A. After touching several colonies from an 18-h culture, inoculate plate, streaking in quadrants for isolation.
- B. Incubate without added CO₂ for 24 to 48 h. The plate may be incubated with the lid up for streptococci, since glucans can fall onto the lid in the inverted position.
- C. Observe colonies for glucan production.
- D. For *Neisseria*, apply 2 drops of iodine solution and observe for dark reddish brown to black color that fades quickly. The color returns with the addition of more iodine.

VI. INTERPRETATION

- A. Positive glucan test: formation of large mucoid, runny material external to the colonies (levans). In some cases, large opaque gumdrop-like refractile colonies that are adherent are present (2). Colonies may be white-dry-adherent and form a depression in the agar.
- B. Negative glucan test: no mucoid or runny colonies and no adherent colonies
- C. Positive amylosucrase test: brown to black color with addition of iodine
- D. Negative amylosucrase test: no brown color with addition of iodine

VII. REPORTING RESULTS

- A. *S. bovis* produces great amounts of watery glucans (levans), promoting very large colonies, which run together and can drip into the cover if the plate is inverted (1).
- B. *Streptococcus salivarius* produces colonies that are opaque, gummy, and non-adherent (levans). They can be similar to those of *S. bovis* but are more gummy (1).
- C. *Streptococcus sanguis* and *Streptococcus mitis* may produce glucans, which are hard and adherent, with the texture and consistency of polyethylene. The agar can be depressed. Less than half of the strains of these species produce glucans (1).
- D. *S. mutans* produces puddles of glucans that even produce a droplet on the top of the colony (1).
- E. A positive test for amylosucrase indicates that an oxidase-positive, gram-negative diplococcus is not *N. gonorrhoeae* or *N. meningitidis*.

VIII. LIMITATIONS

- A. The identification of *S. bovis* is difficult, and the organism can be confused with *S. mutans* and *S. salivarius* (3). Glucan agar is helpful in the identification.
- B. The API Rapid Strep and RapID 32 (bioMérieux, Inc.) do an excellent job of separating *S. bovis* from other streptococci.

REFERENCES

1. Coykendall, A. 1989. Classification and identification of the viridans streptococci. *Clin. Microbiol. Rev.* **2**:315–328.
2. Facklam, R., and J. A. Elliott. 1995. Identification, classification, and clinical relevance of catalase-negative, gram-positive cocci, excluding the streptococci and enterococci. *Clin. Microbiol. Rev.* **8**:479–495.
3. Ruoff, K., S. I. Miller, C. V. Garner, M. J. Ferraro, and S. B. Calderwood. 1989. Bacteremia with *Streptococcus bovis* and *Streptococcus salivarius*: clinical correlates of more accurate identification of isolates. *J. Clin. Microbiol.* **27**:305–308.
4. Weyant, R. S., C. W. Moss, R. E. Weaver, D. G. Hollis, J. G. Jordan, E. C. Cook, and M. I. Daneshvar. 1995. *Identification of Unusual Pathogenic Gram-Negative Aerobic and Facultatively Anaerobic Bacteria*, 2nd ed., p. 5. Williams & Wilkins, Baltimore, Md.

I. PRINCIPLE

In the Gram stain, some gram-positive rods appear gram variable or gram negative, especially members of the genera *Bacillus*, *Erysipelothrix*, *Lactobacillus*, and *Listeria*, which may result in misclassification. Alternatively, some organisms that are gram negative may, at times, appear to be gram positive, since they have been known to resist the alcohol-acetone decolorization step in the Gram stain. Among these problem organisms are members of the *Neisseriaceae* family, which include

Moraxella catarrhalis, *Neisseria*, *Kinella*, and *Acinetobacter* spp. Erroneous Gram stain reactions represent one of the most frequent causes of misidentification, which can result in a delay of appropriate therapy.

In 1976, Cerny reported using L-alanine-4-nitroanilide to detect the presence of cell wall aminopeptidase, which correlated with gram negativity (2). In their comparison study with the 3% KOH method, Carbone et al. yielded more cor-

rect Gram stain determinations with L-alanine-4-nitroanilide than with the 3% KOH method (1). Manafi and Kneifel in 1990 (3) evaluated various chromogenic and fluorogenic substrates for their ability to distinguish gram-positive from gram-negative bacteria. They preferred the fluorogenic compound L-alanine-7-amido-4-methylcoumarin for a rapid test due to the pronounced blue fluorescence of a positive reaction and increased test sensitivity.

II. MICROORGANISMS TESTED

Use a fresh colony, less than 48 h old, of an aerobic or facultative microorganism growing on any laboratory medium.

III. REAGENTS AND SUPPLIES

A. Disks

1. Impregnated with L-alanine-7-amido-4-methylcoumarin (Gram-Sure; Remel, Inc.), L-alanine-4-nitroanilide (LanaGram; Hardy Diagnostics), and L-alanine-p-nitroanilide (APNA; Key Scientific)
2. Store in original container at 2 to 8°C until used.
3. Minimize exposure to light.

4. Protect disks from moisture by removing only those disks necessary for testing from the vial. Promptly replace the cap and return the vial to 2 to 8°C.

B. Supplies

1. Inoculating loop, swab, or stick
2. Long-wave UV light

IV. QUALITY CONTROL

A. Do not use product if any of the following is true.

1. The color of the disk has changed (refer to manufacturer's package insert).
2. The color of the desiccant has changed to pink.
3. There are other signs of deterioration.

B. Perform QC on each new lot or shipment of reagent prior to putting it into use, with the following QC organisms.

1. *Escherichia coli* ATCC 25922—positive
2. *Staphylococcus aureus* ATCC 25923—negative

V. PROCEDURE

- A. Add 0.25 ml of purified water to a 10- by 75-mm test tube.
 - B. Inoculate heavily with an isolated bacterial colony to produce a milky suspension.
 - C. Add one disk to the inoculated tube.
 - D. Incubate at room temperature (25°C) for 5 to 20 min.
 - E. Observe under long-wave UV light for the appearance of blue fluorescence (methylcoumarin substrate), or observe visually for the development of a pale yellow to bright yellow color (nitroanilide substrate).
-

VI. INTERPRETATION

- A. A positive test (gram negative) shows blue fluorescence (methylcoumarin substrate) or pale to bright yellow color (nitroanilide substrate).
 - B. A negative test (gram positive) shows no fluorescence (methylcoumarin substrate) or no change in color (nitroanilide substrate).
-

VII. REPORTING RESULTS

- A. Aerobic, gram-negative rods and coccobacilli demonstrate a bright blue fluorescence (methylcoumarin substrate) or a pale to bright yellow color change (nitroanilide substrate).
 - B. Gram-positive rods and coccobacilli demonstrate no fluorescence (methylcoumarin substrate) or no change in color (nitroanilide substrate).
-

VIII. LIMITATIONS

- A. This test is designed to serve as an adjunct to (not a replacement for) the traditional Gram stain method.
 - B. Obligate anaerobic and gram-positive cocci may fail to give the expected results and should not be tested with this product. Such genera as *Campylobacter*, *Bacteroides*, and *Streptococcus* are likely to produce false results.
 - C. Do not use the nitroanilide substrate to test yellow colonies or colonies from media with dye.
 - D. Nitroanilide substrate requires up to 20 min. Negative test reactions (no color change) should be held for the full 20 min.
-

REFERENCES

1. Carbone, G. M., M. J. Valadez, and M. J. Pickett. 1982. Methods for distinguishing gram-positive from gram-negative bacteria. *J. Clin. Microbiol.* **16**:1157–1159.
2. Cerny, G. 1976. Method for the distinction of gram-negative from gram-positive bacteria. *Eur. J. Appl. Microbiol.* **33**:223–225.
3. Manafi, M., and W. Kneifel. 1990. Rapid methods for differentiating gram-positive from gram-negative aerobic and facultative anaerobic bacteria. *J. Appl. Bacteriol.* **69**:822–827.

I. PRINCIPLE

The hippurate test is used in the identification of *Campylobacter jejuni*, *Listeria monocytogenes*, *Gardnerella vaginalis*, and *Streptococcus agalactiae*, by detecting the ability of the organism to hydrolyze sodium hippurate to benzoic acid and glycine by the action of the enzyme hippuricase.

The ability of bacterial species to hydrolyze hippurate was classically tested using ferric chloride indicator to detect benzoic acid. However, a 2-h rapid method, as opposed to the 48-h classical method, for detecting hippurate hydrolysis has since been developed (3). The rapid test employs ninhydrin as the indicator,

which reacts with any protein or amino acid and, in this case, detects glycine. The rapid hippurate hydrolysis test has been shown to be as specific and as sensitive as the classical method, which detects the benzoic acid by-product.

II. MICROORGANISMS TESTED

- A. Presumptive *L. monocytogenes*: tiny gram-positive rods that are catalase positive, motile at 25°C, and beta-hemolytic
- B. Presumptive *S. agalactiae*: gram-positive cocci that are catalase negative, displaying a translucent colony with a characteristic narrow zone of beta-hemolysis
- C. Presumptive *C. jejuni*: curved gram-negative rods that are oxidase and catalase positive and do not grow aerobically at 35°C (2, 4)
- D. Presumptive *G. vaginalis*: catalase-negative, tiny gram-variable rods

III. MEDIA, REAGENTS, AND SUPPLIES



Include QC information on reagent container and in QC records.

A. Rapid hippurate tubes

1. Commercial hippurate reagents
 - a. Tube containing 20 g of sodium hippurate per liter when rehydrated (Hardy Diagnostics)
 - b. Disks or tablets to be reconstituted in a tube (BD Diagnostic Systems; Key Scientific; Oxoid, Inc.; Remel, Inc.)
2. Prepare 1.0% hippurate.
 - a. Add 1 g of sodium hippurate (Sigma Chemical Co.) to 100 ml of distilled water.
 - b. Dissolve completely by mixing well.
 - c. Dispense in capped tubes in 0.2- to 0.4-ml amounts (e.g., Durham tubes work well).

- d. Freeze at -20°C. Shelf life is very long. Discard when QC fails.

■ **NOTE:** Piot et al. (6) found that a pH of 6.4 was optimal to detect positive reactions for *G. vaginalis*.

B. Ninhydrin

1. If reagent is lyophilized, reconstitute by adding distilled water in the amount recommended by the manufacturer.
2. Ninhydrin preparation
 - a. Mix 50 ml of acetone and 50 ml of 1-butanol thoroughly in a dark glass bottle.
 - b. Add 3.5 g of ninhydrin; mix.

III. MEDIA, REAGENTS, AND SUPPLIES (continued)

- c. Store at room temperature for up to 6 months.

■ NOTE: Care must be taken when handling this solution not to spill reagent onto skin or clothing, as a blue color will develop that does not wash off but will wear off the skin in 24 to 48 h.

C. Supplies

1. Sterile wooden sticks or inoculating loops
2. Incubator at 35°C
3. Test tubes
4. Distilled water

IV. QUALITY CONTROL

- A. Test each new lot and shipment of reagent with known positive and negative controls, and retest at least monthly thereafter. Discard all reagents and prepare new ones if the reagents do not pass QC.

B. Organisms

1. *S. agalactiae* ATCC 12386—hippurate positive
2. *Streptococcus pyogenes* ATCC 19615—hippurate negative

V. PROCEDURE

A. Prepare hippurate tube.

1. Add 0.2 ml (3 or 4 drops) of distilled water at a pH of 6.8 to 7.2 to reconstitute lyophilized tube test reagent.
 2. Add 2 drops of distilled water to empty tube for disk or tablet tests.
 3. Defrost one 0.4-ml tube per test for prepared reagent.
- B. In the tube, make a heavy suspension (equivalent to no. 3 McFarland standard) from an 18- to 24-h culture. Use care not to pick up agar, which contains protein.
- C. For disk or tablet tests, add reagent after inoculation of the tube with the culture.
- D. Incubate the tube for 2 h at 35 to 37°C.
- E. After the 2-h incubation period, add 2 drops of the ninhydrin solution to the hippurate reagent-organism mixture. Add an additional 2 drops if test has 0.4 ml of hippurate.
- F. Reincubate at 35 to 37°C for 30 min. Observe the tubes at 10-min intervals for the appearance of a deep blue color, which is a positive test. The color change will usually appear within 10 to 15 min after the ninhydrin indicator solution has been added.

VI. INTERPRETATION

- A. A positive reaction is indicated by the appearance of a deep blue color (about the color of crystal violet) within 30 min.
- B. A negative reaction is indicated by a faint purple color or no color change.

VII. REPORTING RESULTS

- A. *L. monocytogenes* organisms are tiny gram-positive rods that are catalase positive, motile at 25°C, beta-hemolytic, CAMP positive, and hippurate positive.
- B. *S. agalactiae* organisms are catalase-negative, gram-positive cocci that are identified by having a characteristic narrow zone of beta-hemolysis and are hippurate positive.
- C. *C. jejuni* organisms are curved, gram-negative rods that are identified by having a positive oxidase and catalase reaction, having no growth aerobically at 35°C, and being hippurate positive.
- D. *G. vaginalis* organisms are catalase-negative, gram-variable rods that are hemolytic on human blood agar and are hippurate positive.

VII. REPORTING RESULTS (continued)

- E.** *Arcanobacterium haemolyticum* is hippurate negative and reverse-CAMP positive, while *Arcanobacterium pyogenes* is hippurate positive and reverse-CAMP negative. Both are hemolytic.

VIII. LIMITATIONS

- A.** Not all *S. agalactiae* organisms are beta-hemolytic. Viridans group streptococci can be hippurate positive; another test must be done on nonhemolytic colonies to confirm the identification.
- B.** A small number of enterococci are beta-hemolytic and may hydrolyze hippurate, but they are pyrrolidonyl- β -naphthylamide (PYR) positive.
- C.** A small percentage of *C. jejuni* organisms are hippurate negative and must be identified by other methods (5).
- D.** A negative test does not rule out the identification of *G. vaginalis*, since the biotypes that cause bacterial vaginosis can be hippurate negative (1).
- E.** False-positive results can occur if incubation with ninhydrin exceeds 30 min.

REFERENCES

1. Aroutcheva, A. A., J. A. Simoes, K. Behbakht, and S. Faro. 2001. *Gardnerella vaginalis* isolated from patients with bacterial vaginosis and from patients with healthy vaginal ecosystems. *Clin. Infect. Dis.* **33**:1022–1027.
2. Harvy, S. M. 1980. Hippurate hydrolysis by *Campylobacter fetus*. *J. Clin. Microbiol.* **11**:435–437.
3. Hwang, M. N., and G. M. Ederer. 1975. Rapid hippurate hydrolysis method for presumptive identification of group B streptococci. *J. Clin. Microbiol.* **1**:114–115.
4. Morris, G. K., M. R. el Sherbeeny, C. M. Patton, H. Kodaka, G. L. Lombard, P. Edmonds, D. G. Hollis, and D. J. Brenner. 1985. Comparison of four hippurate hydrolysis methods for identification of thermophilic *Campylobacter* species. *J. Clin. Microbiol.* **22**:714–718.
5. Nicholson, M. A., and C. M. Patton. 1995. Evaluation of disk method for hippurate hydrolysis by *Campylobacter* species. *J. Clin. Microbiol.* **33**:1341–1343.
6. Piot, P., E. Van Dyck, P. A. Totten, and K. K. Holmes. 1982. Identification of *Gardnerella (Haemophilus) vaginalis*. *J. Clin. Microbiol.* **15**:19–24.

[Updated March 2007]

I. PRINCIPLE

Certain organisms are capable of enzymatically liberating sulfur from inorganic sulfur as hydrogen sulfide (H_2S) compounds or from sulfur-containing amino acids through proteolysis of proteins. Detection of H_2S from inorganic sulfur compounds is a two-step process where the bacterium reacts with sodium thiosulfate in the medium to yield a sulfite and H_2S . The colorless H_2S gas released reacts with ferric ions or lead acetate to yield ferrous sulfide or lead sulfide, which are insoluble black precipitates. The most common sul-

fide indicators are ferrous sulfate, ferric citrate, ferric ammonium sulfate or citrate, peptonized iron, and lead acetate. The sensitivity of each indicator varies, and H_2S detected by one indicator may not be detected by another (3).

Lead acetate is the most sensitive reagent and should be used for organisms that produce trace amounts of H_2S . Since all members of the family *Enterobacteriaceae* are capable of producing various amounts of H_2S , the least sensitive approach (Kligler's iron agar [KIA] or triple

sugar iron agar [TSI]) is best for routine identification of these organisms. Several plate media, such as xylose-lysine-desoxycholate (XLD), Hektoen enteric agar, and salmonella-shigella agar (SS), are also capable of detecting H_2S production. While these media are used primarily as screening tests for fecal pathogens, the tube tests are also useful biochemical tests for H_2S production by *Erysipelothrix* and fastidious gram-negative rods, such as *Campylobacter*.

II. MICROORGANISMS TESTED

A. Organisms

1. Gram-negative rods, to determine their ability to produce hydrogen sulfide
2. Catalase-negative gram-positive rods, to detect H_2S production to separate lactobacilli from *Erysipelothrix*
3. *Campylobacter*, to detect H_2S production (For use in testing campylobacters, media should be less than 1 week old or boiled and reslanted prior to use.)

B. Use as part of the battery to distinguish enteric pathogens (procedure 3.8.1).

III. MEDIA, REAGENTS, AND SUPPLIES

A. Media

1. KIA (refer to procedure 3.17.25 for details on preparation)
2. TSI (refer to procedure 3.17.25 for details on preparation)
3. Sulfide-indole-motility agar (SIM)
4. XLD, Hektoen, or other primary culture media to select for fecal pathogens

B. Supplies

1. Wire or disposable inoculating needles for tubed media and inoculating loops for plate media
2. 35°C heat block for tubes or incubator without increased CO_2
3. Optional: lead acetate paper

IV. QUALITY CONTROL

A. Perform QC on each new lot or shipment of tubed media prior to putting it into use.

1. Refer to motility procedure (procedure 3.17.31) for QC of SIM and the KIA and TSI procedure (procedure 3.17.25) for QC of KIA and TSI.
2. It is not necessary for users to QC Hektoen, XLD, and SS agar if it has been done by the manufacturer. Refer to Clinical and Laboratory Standards Institute (formerly NCCLS) document M22-A3 for more information (2).

IV. QUALITY CONTROL
(continued)

- B.** Inspect agar for freezing, contamination, cracks, dehydration, and bubbles prior to storage and before use. Discard any tubes with bubbles in the agar. Discard TSI or KIA tubes that are not red or that lack a deep butt. The slant and butt should be equal in length.
-

V. PROCEDURE**A. Tube media**

1. Warm medium to room temperature and examine for cracks. Do not use if cracks appear.
2. Using a sterile inoculating needle, touch the center of a well-isolated colony.
3. Stab to within 3 to 5 mm from the bottom of the tube.
4. Withdraw the needle.
5. For KIA or TSI, streak the entire surface of the agar slant.
6. Optional for fastidious organisms: add a strip of lead acetate paper to top of tube and hold in place with the cap of the tube so that it extends 1 in. into the tube.
7. *Place cap loosely on tube.* Do not tighten the cap to allow for release of gas in the tube.
8. Incubate aerobically at 35 to 37°C for 18 to 24 h.
9. Observe for black precipitate indicating hydrogen sulfide production.
10. If desired, extend incubation only to detect H₂S production. Campylobacters may take 3 days for production of H₂S.

B. Plate media

1. Streak plate so as to obtain isolated colonies.
 2. Incubate aerobically at 35 to 37°C for 18 to 24 h.
 3. Observe for blackened colonies.
-

VI. INTERPRETATION**A. Positive reactions**

1. H₂S production in tube media: black color throughout the medium, a black ring at the junction of the butt and slant, or any black precipitate in the butt. Blackening usually begins at the line of inoculation.
2. H₂S production in plate media: black colonies surrounded by a brownish black zone or metallic sheen
3. Lead acetate paper: brownish black coloration of the paper strip

B. Negative reactions

1. H₂S production in tube media: no blackening in tube
 2. H₂S production in plate media: no blackening and no metallic-sheen colonies
 3. Lead acetate paper: no change in color of strip
-

VII. REPORTING RESULTS

- A. If a catalase-negative, vancomycin-resistant, gram-positive rod is H₂S positive, report as "*Erysipelothrix rhusiopathiae*."
- B. If a campylobacter-like organism is H₂S positive, report as "Probable *Campylobacter hyoilestinalis*." See Table 3.8.2–4 for other tests to confirm the identification.
- C. For evaluation of enteric pathogens, see procedure 3.8.1.
- D. *Shewanella putrefaciens/algae* is a foul-smelling, oxidase-positive, gram-negative rod that is H₂S positive and does not produce acid on TSI or KIA (4).

VIII. LIMITATIONS

- A. H₂S production may be inhibited on TSI for organisms that utilize sucrose and suppress the enzyme mechanism that results in production of H₂S (1).
 - B. SIM is more sensitive in the detection of H₂S than either TSI or KIA, because of its semisolid nature, its lack of interfering carbohydrates, and the use of peptonized iron as an indicator (3).
 - C. Lead acetate paper is 10 times more sensitive than other media (5).
 - D. Lead acetate is toxic to bacteria and may inhibit the growth of some bacteria. Do not allow the media to touch the strip.
-

REFERENCES

1. Bulmash, J. M., and M. Fulton. 1964. Discrepant tests for hydrogen sulfide. *J. Bacteriol.* **88**:1813.
2. Clinical and Laboratory Standards Institute. 2004. *Quality Assurance for Commercially Prepared Microbiological Culture Media*, 3rd ed. Approved standard M22-A3. Clinical and Laboratory Standards Institute, Wayne, Pa.
3. MacFaddin, J. F. 2000. *Biochemical Tests for the Identification of Medical Bacteria*, 3rd ed., p. 205–220. The Lippincott, Williams & Wilkins Co., Philadelphia, Pa.
4. Weyant, R. S., C. W. Moss, R. E. Weaver, D. G. Hollis, J. G. Jordan, E. C. Cook, and M. I. Daneshvar. 1995. *Identification of Unusual Pathogenic Gram-Negative Aerobic and Facultatively Anaerobic Bacteria*, 2nd ed. Williams & Wilkins, Baltimore, Md.
5. ZoBell, C. E., and C. B. Reltham. 1934. A comparison of lead, bismuth, and iron as detectors of hydrogen sulphide produced by bacteria. *J. Bacteriol.* **28**:169–176.

I. PRINCIPLE

The ability of an organism to split indole from the amino acid tryptophan is due to the presence of tryptophanase. Indole, if present, combines with the aldehyde in the reagent to produce a pink to red-violet quinoidal compound (benzaldehyde reagent) or a blue to green color (cinnamaldehyde reagent). In the rapid spot test, indole is detected directly from a colony growing on a medium rich in tryptophan.

II. MICROORGANISMS TESTED

- A. Fresh growth of a gram-negative rod on medium that does not contain dyes and contains tryptophan, e.g., BAP or CHOC
- B. Anaerobic gram-positive rods
- C. Anaerobic gram-negative rods

III. MEDIA, REAGENTS, AND SUPPLIES**A. Reagents**

Obtain indole reagents commercially, or prepare in-house.

- 1. Rapid spot indole: see Appendix 3.17.23–1.
 - a. Prepare either 5% *p*-dimethylaminobenzaldehyde or 1% paradimethylaminocinnamaldehyde in 10% (vol/vol) concentrated HCl (1, 6)
 - b. Do not use the spot benzaldehyde reagent for anaerobes.
 - 2. Tube indole
 - a. Kovács' method for aerobically growing organisms (3)
 - (1) Reagent: see Appendix 3.17.23–1.
 - (2) Medium: broth that contains tryptophan (containing peptone, tryptone, or casein). Semisolid agar, such as motility-indole-ornithine agar or sulfide-indole-motility agar, can be used.
- b. Ehrlich's method for anaerobes and weak indole producers (2)
 - (1) Reagent: see Appendix 3.17.23–1.
 - (2) Medium: heart infusion or anaerobic medium with tryptophan
 - (3) Xylene
 - 3. Clearly label indole reagents, indicating preparation and expiration dates.
 - a. Record the expiration date in a work record (in-house preparation) or on a receipt record (commercial record).
 - b. Store indole reagents in a dark bottle at 4°C.
- B. Supplies**
- 1. Sterile loop, swab, or stick for harvesting
 - 2. Filter paper (optional)

IV. QUALITY CONTROL

- A. Do not use benzaldehyde reagents (including Ehrlich's and Kovács') if color is not pale yellow.
- B. Perform QC
 - 1. On each new lot or shipment of reagent prior to putting it into use
 - 2. Although not required, QC in-house-prepared reagents weekly, as they can deteriorate, especially if not stored at 4°C. Discard if reactions become weak (4).
- C. Organisms
 - 1. *Escherichia coli* ATCC 25922—indole positive
 - 2. *Pseudomonas aeruginosa* ATCC 27853—indole negative
 - 3. For Ehrlich's reagent for use with anaerobic microorganisms
 - a. *Porphyromonas asaccharolytica* ATCC 25260—indole positive
 - b. *Bacteroides fragilis* ATCC 25285—indole negative

V. PROCEDURE**A. Rapid spot indole**

Use one of the methods below.

- 1. Moisten filter paper with reagent. Using a wooden stick, rub portion of colony onto paper.
- 2. Sweep the colony onto a swab. Add drop of indole reagent to the colony swab.
- 3. Add reagent directly to the colony growing on the agar surface.

B. Tube test

- 1. Inoculate liquid tube medium or stab agar medium with colony.
- 2. Incubate for 18 to 24 h. If broth is used for indole production, decant a portion of the medium to a second tube before testing.
- 3. For Ehrlich's method
 - a. Add 0.5 ml of xylene to tube and invert to mix. Let settle.
 - b. Add 6 drops of Ehrlich's indole reagent down the side of the tube and observe color below the xylene layer.
- 4. For Kovács' method, add 3 drops of Kovács' reagent down the side of the tube and observe color change at meniscus.
- 5. If test is negative, repeat test after an additional 24 h of incubation, if desired.

VI. INTERPRETATION

- A. The development of a brown-red to purple-red color (benzaldehyde reagents) or blue color (cinnamaldehyde reagent) within 20 s indicates the presence of indole.
- B. A negative test is colorless or slightly yellow.

VII. REPORTING RESULTS

- A. *E. coli* is indole positive, as are many other *Enterobacteriaceae*, *Vibrio*, *Aeromonas*, *Plesiomonas*, and *Pasteurella*.
- B. Several fastidious gram-negative rods are indole positive, such as *Cardiobacterium hominis* and *Pasteurella bettyae*. See Table 3.18.2–3 for others.
- C. *Propionibacterium acnes* is indole positive.
- D. *Delftia (Comamonas) acidovorans* produces a pumpkin orange reaction from the production of anthranilic acid, rather than indole, from tryptophan with Kovács' reagent (5).

VIII. LIMITATIONS

- A. Detectable indole will diffuse to colonies within 5 mm of a 2- to 3-mm colony, giving false-positive results.
- B. Do not use media that contain dyes (e.g., EMB, MAC).
- C. Growth medium must contain an adequate amount of tryptophan. Do not use Mueller-Hinton agar for test, because tryptophan is destroyed during the acid hydrolysis of casein.
- D. Only the cinnamaldehyde reagent can be used for spot testing of anaerobic microorganisms. It is the more sensitive reagent, but it is less stable.
- E. Do not use a plate with a nitrate disk to perform the indole test, as nitrate can interfere with the spot indole test by inducing false-negative results.
- F. If the rapid indole test is negative, the isolate could be positive in the more sensitive tube test. Extraction with xylene is the most sensitive test. Xylene substitutes are less sensitive.
- G. For fastidious gram-negative rods, such as *C. hominis*, a heavy inoculum and extraction are necessary.

REFERENCES

1. Bale, M. J., S. M. McLaws, and J. Matsen. 1984. The spot indole test for identification of swarming *Proteus*. *Am. J. Clin. Pathol.* **83**:87–90.
2. Böhme, A. 1905. Die Anwendung der Ehrlichischen Indolreaktion für bakteriologische Zwecke. *Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. I Abt. Orig.* **40**:129–133.
3. Kovács, N. 1928. Eine vereinfachte Methode zum Nachweis der Indolbildung durch Bakterien. *S. Immunitätsforsch.* **55**:311–315.
4. MacFaddin, J. F. 2000. *Biochemical Tests for the Identification of Medical Bacteria*, 3rd ed., p. 221–232. The Lippincott, Williams & Wilkins Co., Philadelphia, Pa.
5. Marraro, R. V., J. L. Mitchell, and C. R. Payet. 1977. A chromogenic characteristic of an aerobic pseudomonad species in 2% tryptone (indole) broth. *Am. Med. Technol.* **39**:13–19.
6. Vracko, R., and J. C. Sherris. 1963. Indole-spot test in bacteriology. *Am. J. Clin. Pathol.* **39**:429–432.

APPENDIX 3.17.23-1



Include QC information on reagent container and in QC records.

Reagent Preparation

Caution: HCl is toxic and burns. Make indole reagents in a fume hood. Add acid to water; do not add water to acid.

A. Ehrlich's reagent

<i>p</i> -dimethylaminobenzaldehyde	1 g
ethyl alcohol, 95%	95 ml
hydrochloric acid, concentrated	20 ml

1. Dissolve the aldehyde in the alcohol (this may require gentle heating).
2. Working under a fume hood, slowly add the acid (never add alcohol to acid). Mix constantly.
3. The reagent should be pale yellow and stable for 1 year.

B. Kovács' reagent

<i>p</i> -dimethylaminobenzaldehyde	10 g
isobutyl or isoamyl alcohol (absolute)	150 ml
hydrochloric acid, concentrated	50 ml

1. Dissolve the aldehyde in the alcohol (this may require gentle heating).
2. Working under a fume hood, slowly add the acid (never add alcohol to acid). Mix constantly.
3. The reagent should be pale yellow and stable for 1 year.

APPENDIX 3.17.23-1 (continued)**C. 5% *p*-Dimethylaminobenzaldehyde**

p-dimethylaminobenzaldehyde 5 g
10% (vol/vol) hydrochloric acid 100 ml

1. Add 10 ml of concentrated HCl to 90 ml of water.
2. Dissolve the aldehyde in the acid (use a fume hood).
3. Shelf life is 4 months.

D. 1% *p*-Dimethylaminocinnamaldehyde

p-dimethylaminocinnamaldehyde 1 g
10% (vol/vol) hydrochloric acid 100 ml

1. Add 10 ml of HCl to 90 ml of water.
2. Dissolve the aldehyde in the acid (use a fume hood).
3. Shelf life is 2 months (1).

Reference

1. MacFaddin, J. F. 2000. *Biochemical Tests for the Identification of Medical Bacteria*, 3rd ed., P. 221–232. The Lippincott, Williams & Wilkins Co., Philadelphia, Pa.

I. PRINCIPLE

Indoxyl is a tryptophan product of putrefactive decomposition in the intestines of humans by a bacterial esterase (3). The presence of this enzyme can be confirmed *in vitro* by the bacterial hydrolysis of indoxyl acetate to release indoxyl. Indoxyl then combines with oxygen to spontane-

ously form indigo. In 1987, Mills and Gherna (2) found this test to be useful to distinguish *Campylobacter jejuni* and *Campylobacter coli* (positive) from *Campylobacter lari* and *Campylobacter fetus* (negative).

II. MICROORGANISMS TESTED

- A. Oxidase-positive, motile, curved, gram-negative rods suggestive of *Campylobacter* spp. and related organisms (*Helicobacter* and *Arcobacter*)
- B. Gram-negative, oxidase-positive diplococci growing on BAP as white colonies that remain together when lifted with a loop or wire (4)

III. REAGENT AND SUPPLIES

- A. Disks
 - 1. Purchase (Hardy Diagnostics, Rемel, Inc.) or
 - 2. Prepare in-house.
 - a. 10% Indoxyl acetate (Sigma Chemical Co., St. Louis, Mo.) in acetone
 - b. Add 50 µl (25 µl at a time) to a 6-mm blank paper disk (BD Diagnostic Systems, Hardy Diagnostics).
- c. Dry the disks at room temperature.
- 3. Store at 4°C in a brown bottle to protect from light; add desiccant to keep dry.
- B. Supplies
 - 1. Sterile wooden sticks
 - 2. Petri dish
 - 3. Sterile distilled water or saline

IV. QUALITY CONTROL

- A. Do not use if disks are discolored and no longer white.
- B. Perform QC on each lot or shipment of disks using a positive and negative control prior to putting it into use.
- C. Organisms
 - 1. *C. jejuni* ATCC 33560 or *Moraxella catarrhalis* ATCC 25240—positive (blue color)
 - 2. *Campylobacter hyoilei* ATCC 35217 or *C. fetus* ATCC 27374—negative (no color change)

V. PROCEDURE

- A. Place indoxyl acetate disk in a plastic petri dish and transfer a large loopful of 24- to 48-h growth from plate onto the disk.
- B. Add a drop of sterile distilled water to the disk for *Campylobacter* (3). For *Moraxella*, premoisten the disk with saline prior to inoculation.
- C. Incubate at room temperature for up to 30 min for *Campylobacter* (3) and up to 3 min for *Moraxella* (4).

VI. INTERPRETATION

- A. A positive test shows dark blue color development on disk in 3 min for *Moraxella* and 5 to 10 min for *Campylobacter*.
- B. A negative test shows no color change.
- C. Weakly positive reactions are pale blue in 10 to 30 min.

VII. REPORTING RESULTS

- A. *C. jejuni*, *C. coli*, *Campylobacter upsaliensis*, *Arcobacter cryaerophilus*, and *Helicobacter fennelliae* are indoxyl acetate positive (1, 3).
- B. *C. lari*, *Helicobacter pylori*, *Helicobacter cinaedi*, and *Helicobacter pullorum* are negative for indoxyl acetate (1, 3).
- C. Report as *Moraxella catarrhalis* if oxidase-positive, gram-negative diplococci grow on BAP as colonies that remain together when sampled and are strongly indoxyl acetate positive in 3 min (4).

VIII. LIMITATIONS

- A. The test is reliable regardless of the media used for growth (2).
- B. The disk test is more rapid and reliable than the tube method (3).

REFERENCES

1. Hodge, D. S., A. Borczyk, and L. L. Wat. 1990. Evaluation of the indoxyl acetate hydrolysis test for the differentiation of campylobacters. *J. Clin. Microbiol.* **28**:1482–1483.
2. Mills, C. K., and R. L. Gherne. 1987. Hydrolysis of indoxyl acetate by *Campylobacter*. *J. Clin. Microbiol.* **25**:1560–1561.
3. Popovic-Uroic, T., C. M. Patton, M. A. Nicholson, and J. A. Kiehlbauch. 1990. Evaluation of the indoxyl acetate hydrolysis test for rapid differentiation of *Campylobacter*, *Helicobacter*, and *Wolinella* species. *J. Clin. Microbiol.* **28**:2335–2339.
4. Speeleveld, E., J. M. Fossépré, B. Gordts, and H. W. Van Landuyt. 1994. Comparison of three rapid methods, tributyrine, 4-methylumbelliferyl butyrate, and indoxyl acetate, for rapid identification of *Moraxella catarrhalis*. *J. Clin. Microbiol.* **32**:1362–1363.

3.17.25

Kligler's Iron Agar Test and Triple Sugar Iron Agar Test

[Updated March 2007]

I. PRINCIPLE

Kligler's iron agar (KIA) contains casein and meat peptones, phenol red as the pH indicator, 0.1% glucose, and 1% lactose for fermentation (4, 5). Ferric or ferrous ions and sodium thiosulfate are present to detect hydrogen sulfide production. Triple sugar iron agar (TSI) is similar in formulation; however, TSI also contains 1% sucrose, which is not included in KIA (3, 7).

Organisms that are non-lactose fermenting initially produce a yellow slant due to the production of acid from the glucose. The small amount of glucose is rapidly depleted. Oxidative metabolism continues in the slant after the low concentration of glucose has been depleted, producing an alkaline pH from the aerobic

breakdown of peptone; the slant turns red. There is no oxygen penetration into the butt and no oxidative metabolism; the butt remains acid and yellow. Thus, a non-lactose fermenting organism yields an alkaline (K) slant over an acid (A) butt (K/A; red slant; yellow butt). Lactose-fermenting (and/or sucrose-fermenting in the case of TSI) bacteria continue to produce a large amount of acid in the slant and in the butt so the reaction in both remains acid (A/A; yellow slant; yellow butt). If the slant and the butt remain neutral, the organism is not capable of fermenting glucose or other sugars (K/K; red slant; red butt). Gas production from sugar fermentation is indicated by bubbles, fracturing

of the medium, or displacement of the medium.

Hydrogen sulfide is produced by the action of the bacteria with sodium thiosulfate. This is detected by the reduction of ferric ions to produce a black precipitate. While these media are used primarily as screening tests for fecal pathogens, they are useful biochemical tests for H₂S production by *Erysipelothrix* and other organisms, such as *Campylobacter*. In addition, these media are very useful for the determination of the ability of fastidious organisms to ferment glucose, since many organisms will not react in OF medium and appear to be non-glucose fermenting (8).

II. MICROORGANISMS TESTED

A. Organisms

1. Gram-negative rods, especially fastidious organisms, to determine their ability to ferment glucose and lactose (or sucrose, for TSI) and to produce hydrogen sulfide
 2. Oxidase-negative, catalase-positive, nonmotile, gram-negative coccobacilli that grow on MAC, to identify *Acinetobacter* spp.
 3. Catalase-negative, gram-positive rods to detect H₂S production, to separate lactobacilli from *Erysipelothrix*
 4. *Campylobacter*, to detect H₂S production
- B. Use as part of the battery to screen for enteric pathogens (procedure 3.8.1).
- C. Use to determine whether a gram-negative rod is glucose fermenting, in deciding whether to use an oxidative or fermentative system for the identification of the organism.

III. MEDIA, REAGENTS, AND SUPPLIES

A. Media

1. Characteristics
 - a. Agar slants with deep butts
 - b. Purchase commercially or prepare from dehydrated powder (6).
- (1) Rehydrate commercial TSI or KIA according to manufacturer's instructions.
- (2) Dispense 5- to 7-ml aliquots into 16- by 125-mm screw-cap tubes.

III. MEDIA, REAGENTS, AND SUPPLIES (continued)

- (3) Autoclave at 121°C for 15 min. Do not overheat.
- (4) Cool as slants with deep butts.
- (5) Store at 2 to 4°C. Some prefer to store at 25°C so that cracks will not form when medium is warmed prior to inoculation.

2. Options
 - a. KIA
 - b. TSI

NOTE: For best results with campylobacters, agar must be freshly prepared and used in 1 week. One manufacturer recommends that KIA be prepared fresh or melted and resolidified just before use (6).

B. Supplies

1. Wire or disposable inoculating needles
2. 35°C heat block or incubator without increased CO₂
3. Optional: lead acetate paper

IV. QUALITY CONTROL

- A. Perform QC on each new lot and shipment of media prior to putting it into use.
 NOTE: The Clinical and Laboratory Standards Institute (formerly NCCLS) has proposed elimination of user QC for TSI purchased from commercial sources. Consult with current regulatory agencies prior to discontinuation of user QC (2).
- B. Inspect agar for freezing, contamination, cracks, dehydration, and bubbles prior to storage and before use. Discard any tubes with bubbles in the agar. Discard tubes that are not red or that lack a deep butt. The slant and butt should be equal in length.
- C. Organisms

Organism	ATCC no.	TSI	KIA	H ₂ S
<i>Escherichia coli</i>	25922	A/A, gas	A/A, gas	—
<i>Salmonella enterica</i> serovar Typhimurium	14028	K/A, ± ^a gas	K/A, ± ^a gas	+
<i>Pseudomonas aeruginosa</i>	27853	K/K, no gas	K/K, no gas	—
Optional for special species: <i>Campylobacter hyoilealis</i>	35217	K/K, no gas	K/K, no gas	+

^a ±, with or without.

V. PROCEDURE

- A. Warm medium to room temperature and examine for cracks. Do not use if cracks appear.
- B. Using a sterile inoculating needle, touch the center of a well-isolated colony.
- C. Stab to within 3 to 5 mm from the bottom of the tube.
- D. Withdraw the needle, and streak the entire surface of the agar slant.
- E. Optional for fastidious organisms: add a strip of lead acetate paper to top of tube and hold in place with the cap of the tube.
- F. Place cap loosely on tube. Do not tighten the cap.
- G. Incubate aerobically at 35 to 37°C for 18 to 24 h.
- H. Examine the reaction in the slant and the butt. Observe for gas and hydrogen sulfide production.
- I. Do not attempt to interpret sugar fermentation reactions after 24 h. Refrigerate tubes if readings will be delayed.
- J. If desired, extend incubation only to detect H₂S production. Campylobacters may take 3 days for production of H₂S.

VI. INTERPRETATION

A. Observations

1. Acid reaction: yellow
2. Alkaline reaction: red

VI. INTERPRETATION (continued)

3. H₂S production: black color throughout the medium, a black ring at the junction of the butt and slant, or a black precipitate in the butt. Low levels of H₂S production present as no blackening of the agar but blackening of the lead acetate paper.
 4. Gas production: bubbles, cracks, or displacement of media
- B. Interpretation of carbohydrate observations**
1. A/A: glucose and lactose (and/or sucrose for TSI) fermented
 2. K/A: only glucose fermented (non-lactose fermenter or glucose fermenter).
 3. K/K: no carbohydrates fermented (non-glucose fermenter).

VII. REPORTING RESULTS

- A. If a catalase-negative, gram-positive rod is vancomycin resistant and H₂S positive, report as “probable *Erysipelothrix rhusiopathiae*.” See Table 3.18.1–5.
- B. If a campylobacter-like organism is H₂S positive, report as “probable *Campylobacter hyoilealis*.” See Table 3.8.2–4 for other tests to confirm identification.
- C. For evaluation of the identification of *Salmonella*, see the screening flowchart in Fig. 3.8.1–2.
- D. For other organisms, refer to procedure 3.18.2.

VIII. LIMITATIONS

- A. Do not read the test before 18 h, since false readings of acid in the slant may result.
- B. Copious amounts of H₂S may mask the glucose reaction. If this exists, glucose has been fermented and should be recorded as such. Check for gas production.
- C. When using KIA for screening fecal cultures, it is not able to separate some uncommon non-lactose fermenters (e.g., *Providencia*) that ferment sucrose from fecal pathogens which are lactose and sucrose negative.
- D. If TSI is used to screen fecal cultures, the sugar reactions of acid/acid may be obtained with *Yersinia enterocolitica* and *Edwardsiella tarda*, since both can ferment sucrose but not lactose (6).
- E. Neither KIA nor TSI, without the use of pyrrolidonyl-β-naphthylamide (PYR), will separate lactose-positive *Salmonella* (lysogenized) from *Citrobacter*.
- F. Gas production is better in TSI than in KIA. However, H₂S production may be inhibited on TSI for organisms that utilize sucrose and suppress the enzyme mechanism that results in production of H₂S (1).
- G. Sulfide-indole-motility (SIM) agar is more sensitive in the detection of H₂S than either TSI or KIA.

REFERENCES

1. Bulmash, J. M., and M. Fulton. 1964. Discrepant tests for hydrogen sulfide. *J. Bacteriol.* **88**:1813.
2. Clinical and Laboratory Standards Institute. 2004. *Quality Assurance for Commercially Prepared Microbiological Culture Media*, 3rd ed. Approved standard M22-A3. Clinical and Laboratory Standards Institute, Wayne, Pa.
3. Hajna, A. A. 1945. Triple-sugar iron agar medium for identification of the intestinal group of bacteria. *J. Bacteriol.* **49**:516–517.
4. Kligler, I. J. 1917. A simple medium for the differentiation of members of the typhoid-paratyphoid group. *Am. J. Public Health* **7**:1042–1044.
5. Kligler, I. J. 1918. Modifications of culture media used in the isolation and differentiation of typhoid, dysentery and allied bacilli. *J. Exp. Med.* **28**:319–322.
6. MacFaddin, J. F. 2000. *Biochemical Tests for the Identification of Medical Bacteria*, 3rd ed., p. 239–253. The Lippincott, Williams & Wilkins Co., Philadelphia, Pa.
7. Sulkin, S. E., and J. C. Willett. 1940. A triple sugar-ferrous sulfate medium for use in identification of enteric organisms. *J. Lab. Clin. Med.* **25**:649–653.
8. Weyant, R. S., C. W. Moss, R. E. Weaver, D. G. Hollis, J. G. Jordan, E. C. Cook, and M. I. Daneshvar. 1995. *Identification of Unusual Pathogenic Gram-Negative Aerobic and Facultatively Anaerobic Bacteria*, 2nd ed., p. 2–3, 22. Williams & Wilkins, Baltimore, Md.

3.17.26

LAP (Leucine Aminopeptidase) Test

[Updated March 2007]

I. PRINCIPLE

Detection of the enzyme leucine aminopeptidase (LAP) is one of the tests in the definitive identification of catalase-negative, gram-positive cocci. Specifically, it differentiates *Aerococcus* and *Leuconos-*

toc from *Streptococcus*, *Enterococcus*, *Lactococcus*, and *Pediococcus* (1, 2). The former are LAP negative, and the latter are positive. Hydrolysis of the leucine-*p*-naphthylamide substrate on a paper disk

inoculated with the bacteria releases leucine and free β -naphthylamide. The β -naphthylamide combines with cinnamaldehyde reagent to form a pink to cherry-red pigment.

II. MICROORGANISMS TESTED

Fresh growth less than 24 h old of catalase-negative, gram-positive cocci. If growth is poor, a culture that has been incubated for up to 72 h may be used.

III. MEDIA, REAGENTS, AND SUPPLIES

A. Reagents

1. Disks (Key Scientific Products Company; Hardy Diagnostics; Remel, Inc.; Oxoid, Inc.)
NOTE: BactiCard Strep (Remel, Inc.) incorporates both LAP and pyrrolidonyl- β -naphthylamide (PYR) on one card. StrepQuick (Hardy Diagnostics) incorporates LAP, PYR, and esculin on one card.
2. 0.01% *p*-Dimethylaminocinnamaldehyde (the same reagent as used in PYR test)

B. Supplies

1. Sterile loop or stick for harvesting
2. Distilled water (neutral pH)
3. Petri dish

IV. QUALITY CONTROL

- A. Perform QC on each new lot or shipment of disks and color reagent prior to putting it into use. Since the test is performed infrequently and most gram-positive cocci are LAP positive, verify potency by testing the positive control each time a negative test is obtained.
- B. Organisms
 1. *Enterococcus faecalis* ATCC 29212—LAP positive
 2. *Aerococcus viridans* ATCC 11563—LAP negative

V. PROCEDURE

- A. Place disk onto slide or petri dish. Moisten (do not saturate the disk) with a loopful of distilled water.
- B. Using a sterile stick or loop, smear with the suspected isolate.
- C. Incubate at room temperature for 5 min.
- D. Add 1 drop of cinnamaldehyde developer reagent and wait 2 min to observe color.

VI. INTERPRETATION

- A. A positive test after adding reagent shows a deep red to reddish purple color.
 - B. Weak reactions are pink.
 - C. A negative test is colorless or yellow.
-

VII. REPORTING RESULTS

- A. *Leuconostoc* is LAP negative, whereas other organisms in the group (*Streptococcus*, *Enterococcus*, *Lactococcus*, and *Pediococcus*) are almost always positive. *Aerococcus* is LAP variable.
 - B. See Table 3.18.1–4 for further testing (2).
-

VIII. LIMITATIONS

- A. This test is only one of a battery of useful tests for identifying catalase-negative, gram-positive cocci and is most helpful when kit tests yield uncommon identifications or Gram stain results do not show chaining cocci.
 - B. False negatives may result from insufficient inoculum.
-

REFERENCES

1. Facklam, R., and J. A. Elliott. 1995. Identification, classification, and clinical relevance of catalase-negative, gram-positive cocci, excluding the streptococci and enterococci. *Clin. Microbiol. Rev.* **8**:479–495.
2. LaClaire, L. L., and R. R. Facklam. 2000. Comparison of three commercial rapid identification systems for the unusual gram-positive cocci *Dolosigranulum pigrum*, *Ignavigranum ruoffiae*, and *Facklamia* species. *J. Clin. Microbiol.* **38**:2037–2042.

[Updated March 2007]

I. PRINCIPLE

Lecithinases or phospholipases are enzymes released by the bacteria that destroy animal tissues. Lecithin is a normal component of egg yolks. In egg yolk agar (EYA) the lipoprotein component, lecithovitellin, can be split by lecithinase into phosphorylcholine and an insoluble diglyceride, which forms a precipitate in the

medium (2). This precipitate appears as a white opaque halo surrounding the colony that produces the lecithinase. CCFA agar (procedure 3.8.3), used to identify *Clostridium difficile*, also contains egg yolks.

Bacterial lipases hydrolyze the breakdown of triglycerides into glycerol and

free fatty acids. Fatty acids are mostly insoluble and cause opacity on EYA, producing an iridescent sheen on the colonies and the surface of EYA. Unlike lecithinase, lipase is not diffusible, and the reaction occurs only on the surface of the agar in the immediate vicinity of the colony.

II. MICROORGANISMS TESTED

- A. Spore-forming gram-positive rods: lecithinase will separate *Bacillus cereus* group (positive) and *Bacillus anthracis* (positive) from other *Bacillus* species (negative) and *Clostridium perfringens* (positive) from some other *Clostridium* spp.
- B. Catalase-negative, hemolytic, gram-positive rods: lecithinase separates *Aracobacterium haemolyticum* from *Actinomyces pyogenes*.
- C. Spore-forming, catalase-negative, gram-positive rods: *Clostridium sporogenes* is lipase positive. *C. difficile* is both lipase and lecithinase negative.
- D. Non-glucose-fermenting, gram-negative rods of the fluorescent group: lecithinase will separate *Pseudomonas putida* (negative) from *P. fluorescens* (positive).
- E. Catalase-negative, gram-variable rods identified as presumptive *Gardnerella vaginalis*, which is often lipase positive

III. MEDIA, REAGENTS, AND SUPPLIES

A. Media: EYA

- 1. Media available from most medium vendors, or see Appendix 3.17.27–1 for preparation.
- 2. Store in moist container at 2 to 8°C until expiration date (usually 2 to 4 weeks).

B. Supplies

- 1. Sterile sticks or inoculating loops
- 2. Incubators at 42, 35, and 30°C

IV. QUALITY CONTROL

- A. Inspect agar for freezing, contamination, cracks, and dehydration prior to storage and before use. Discard plates that are opaque.
 - B. Perform QC on each new lot or shipment of media prior to putting it into use.
 - C. Organisms
 - 1. *B. cereus* ATCC 14579—opaque halo around colony (lecithinase positive)
 - 2. *Staphylococcus aureus* ATCC 12600—iridescent sheen on and around colony (lipase positive)
 - 3. *Escherichia coli* ATCC 25922—no change in medium (negative)
-

V. PROCEDURE

- A. After touching several colonies from an 18- to 72-h culture, inoculate a segment of the agar surface with a very *visible circular* amount of organism about the size of a dime. Then, streak an area to obtain isolated colonies.
 - B. For accurate results, incubate as follows without added CO₂ for 24 to 48 h or anaerobically for up to 72 h for anaerobes.
 - 1. 35°C for *Bacillus*, other gram-positive rods, and anaerobes
 - 2. 25°C for fluorescent non-glucose-fermenting, gram-negative rods, except *Pseudomonas aeruginosa*
 - 3. 30°C for other non-glucose-fermenting, gram-negative rodsExamine for halo and iridescence.
-

VI. INTERPRETATION

- A. **Lecithin**
Use transmitted light to observe halo.
 - 1. Positive test: development of a milky white opaque halo around the colony
 - 2. Negative test: no halo in the medium or around the colony
 - B. **Lipase**
Hold the plate on an angle with good lighting.
 - 1. Positive test: development of an iridescent sheen (mother-of-pearl) on the surface of the colony and the surrounding agar
 - 2. Negative test: no change in the medium
-

VII. REPORTING RESULTS

- A. Catalase-positive, spore-forming, gram-positive rods that are lecithinase positive, with large zones of opacity, belong to the *B. cereus* group. Lack of motility separates *B. anthracis* from the other members of the group.
- B. *G. vaginalis* organisms are catalase-negative, gram-variable coccobacilli that are hemolytic on human blood and are lipase positive. A negative test does not rule out the identification, since the biotypes that cause bacterial vaginosis are often lipase negative (1).
- C. A gram-positive rod that is catalase negative, hemolytic, and lecithinase positive is *A. haemolyticum*.
- D. Lecithinase and lipase are useful as part of identification of *Clostridium* to the species level.
 - 1. *C. perfringens* is lipase negative and lecithinase positive, which can be neutralized by adding anti-α toxin prior to inoculation of the agar (the Nagler reaction).
 - 2. *C. sporogenes* is lipase positive.
 - 3. *C. difficile* is both lipase and lecithinase negative.
 - 4. See section 4 for further tests for identification of *Clostridium* spp.
- E. Among the fluorescent group of non-glucose-fermenting, gram-negative rods, *P. putida* is lecithinase negative and most *P. fluorescens* isolates are lecithinase positive. This test can substitute for gelatin hydrolysis.
- F. *Burkholderia* organisms are often lecithinase positive.

VIII. LIMITATIONS

- A. Ignore a slight clearing around the inoculum.
 - B. Zones of opacity are small with non-glucose-fermenting rods.
 - C. A negative lecithinase test should be compared to an uninoculated control plate, as lecithinase can diffuse throughout the entire agar plate and make interpretation difficult.
 - D. Some microorganisms may require up to 1 week to produce a positive lipase reaction.
 - E. Not all *P. fluorescens* isolates are lecithinase positive.
-

REFERENCES

1. Aroutcheva, A. A., J. A. Simoes, K. Behbakht, and S. Faro. 2001. *Gardnerella vaginalis* isolated from patients with bacterial vaginosis and from patients with healthy vaginal ecosystems. *Clin. Infect. Dis.* 33:1022–1027.
 2. MacFaddin, J. F. 2000. *Biochemical Tests for the Identification of Medical Bacteria*, 3rd ed., p. 273–281, 286–293. The Lippincott, Williams & Wilkins Co., Philadelphia, Pa.
-

APPENDIX 3.17.27-1

Preparation of Egg Yolk Agar Medium (1)

McClung and Toabe agar, modified for lecithinase and lipase tests

Proteose no. 2 peptone or Polypeptone (BBL)	40.0 g
disodium phosphate	5.0 g
monopotassium phosphate	1.0 g
sodium chloride	2.0 g
magnesium sulfate	0.1 g
glucose	2.0 g
hemin solution, 5 mg/ml	1.0 ml
agar	20.0 g
water	1.0 liter

- A. Suspend ingredients, and adjust the pH to 7.6.
- B. Mix, and boil to dissolve.
- C. Dispense 20 ml per tube, and autoclave at 118°C for 15 min.
- D. Cool to 50°C, and to each tube, add 2 ml of commercial egg yolk emulsion or 1 ml of egg yolk emulsion prepared as follows.
 1. Scrub, and then soak, an antimicrobial agent-free hen egg in 95% ethanol for 1 h.
 2. Aseptically aspirate or separate the egg yolk.
 3. Add equal volumes of egg yolk to sterile saline and stir to make smooth suspension.
- E. Mix, and pour into plates.

Reference

1. Phillips, E., and P. Nash. 1985. Culture media, p. 1064–1065. In E. H. Lennette, A. Balows, W. J. Hausler, Jr., and H. J. Shadomy (ed.), *Manual of Clinical Microbiology*, 4th ed. American Society for Microbiology, Washington, D.C.

3.17.28

Lipophilism Test for *Corynebacterium*

I. PRINCIPLE

Some strains of *Corynebacterium* grow better in the presence of added lipid. Such strains are called lipophilic or rabbit serum stimulated. They may also require lipid for growth (3). By adding a drop of serum to a plain Mueller-Hinton agar (MH) plate on which the isolate has been inoculated, the

requirement or enhancement of growth with added lipid can be determined. In the tube test, growth is evaluated compared to growth in a broth culture that has not been enriched with rabbit serum. 1.0% Tween 80 can be used as a substitute for serum.

II. MICROORGANISMS TESTED

Catalase-positive, gram-positive rods as part of the identification of *Corynebacterium* to the species level

III. MEDIA, REAGENTS, AND SUPPLIES

A. Media and reagents

1. MH
2. Andrade's glucose broth or BHI
3. Filter-sterilized rabbit serum (no. 16120-099; Invitrogen Life Technologies, Carlsbad, Calif.)
 - a. Store at -20°C in small aliquots.
 - b. Heat for 1 h at 60°C prior to use.
4. Sterile 0.85% NaCl

B. Other supplies

1. Sterile sticks and swabs
2. Incubator at 35°C with 5% CO₂
3. 0.5 McFarland standard

IV. QUALITY CONTROL

- A. Test each lot of rabbit serum for sterility by inoculating 1 drop into BHI or onto MH. No increase in turbidity should be seen, and Gram stain of broth should show no organisms. No colonies should be present on MH.

- B. Test each lot of rabbit plasma to verify that it will enhance the growth of *Corynebacterium*.

C. Organism

Corynebacterium jeikeium ATCC 43734—growth in broth or on MH with rabbit serum but not without it

V. PROCEDURE

A. Plate method

1. Emulsify the test organism in sterile saline to match a no. 0.5 McFarland standard.
2. Dip swab into saline and inoculate MH for confluent growth as for disk susceptibility testing (procedure 5.1). Allow to dry.
3. Place 1 drop of rabbit serum on plate. Allow to dry.
4. Incubate for 24 h at 35°C in 5% CO₂.
5. Observe for growth.

V. PROCEDURE (continued)**B. Broth method**

1. Warm two tubes of either Andrade's broth with glucose or BHI broth to room temperature.
2. Add 1 drop of rabbit serum to one tube; label as "rabbit serum added."
3. Inoculate both tubes from several colonies of a 24- to 48-h culture.
4. Incubate for 24 h at 35°C in 5% CO₂.
5. Observe for growth.

VI. INTERPRETATION**A. Positive test**

1. Plate test: enhanced growth on top of and immediately around the drop size area where the serum was inoculated compared to the areas where no serum was present
2. Tube test: enhanced growth (and red color in Andrade's tube if glucose positive) in tube with serum but no growth or poor growth and no color change in tube without serum

B. Negative test

1. Plate test: no enhancement of growth around the serum drop. Growth will be uniform on MH.
2. Tube test: growth (and red color in Andrade's tube if glucose positive) in both tubes.

VII. REPORTING RESULTS

The following species of *Corynebacterium* are lipophilic (3): *C. afermentans* subsp. *lipophilum*, *C. accolens*, *C. bovis*, *C. diphtheriae/intermedius*, *C. jeikeium*, CDC group G, *C. lipophiloflavum*, *C. macginleyi*, CDC group F-1, and *C. urealyticum*.

VIII. LIMITATIONS

- A. This test is an excellent adjunct to kit identification of *Corynebacterium*; results with the kit should be consistent with the results of this test (1, 2, 4).
- B. This test cannot be performed using CHOC, since most species of *Corynebacterium* will grow on this medium.
- C. Tween 80 (polysorbate 80) can be used as an alternative to rabbit serum.

REFERENCES

1. Funke, G., K. Peters, and M. Aravena-Roman. 1998. Evaluation of the RapID CB Plus system for identification of coryneform bacteria and *Listeria* spp. *J. Clin. Microbiol.* **36**:2439–2442.
2. Funke, G., F. N. R. Renaud, J. Freney, and P. Riegel. 1997. Multicenter evaluation of the updated and extended API (RAPID) Coryne database 2.0. *J. Clin. Microbiol.* **35**:3122–3126.
3. Funke, G., A. von Graevenitz, J. E. Claridge III, and K. A. Bernard. 1997. Clinical microbiology of coryneform bacteria. *Clin. Microbiol. Rev.* **10**:125–159.
4. Hudspeth, M. K., S. Hunt Gerardo, D. M. Citron, and E. J. C. Goldstein. 1998. Evaluation of the RapID CB Plus system for identification of *Corynebacterium* species and other gram-positive rods. *J. Clin. Microbiol.* **36**:543–547.

Malonate Test

[Updated March 2007]

I. PRINCIPLE

The medium for the malonate test contains sodium malonate (1). Malonate is an enzyme inhibitor and inhibits utilization of succinic acid by bacteria, shutting down the Krebs and glyoxylic cycles. Growth is indicative of malonate utilization as a carbon source; a small amount of glucose is also present to stimulate growth of some

organisms. The medium also contains inorganic ammonium salts as the sole source of nitrogen. When the bacterium ferments sodium malonate, sodium hydroxide and sodium bicarbonate are formed, which increases alkalinity of the medium. The shift in pH turns the bromthymol blue indicator

in the medium from green to blue (2). Malonate-negative organisms that ferment glucose cause the indicator to turn yellow or acid. This medium is recommended as part of differentiating among the *Enterobacteriaceae*, especially species of *Klebsiella* and *Salmonella*.

II. MICROORGANISMS TESTED

Enterobacteriaceae as part of the identification to the species level

III. MEDIA, REAGENTS, AND SUPPLIES

A. Media

1. Ingredients per liter of deionized water

yeast extract	1.0 g
ammonium sulfate	2.0 g
dipotassium phosphate	0.6 g
monopotassium phosphate	0.4 g
sodium chloride	2.0 g
sodium malonate	3.0 g
glucose	0.25 g
bromthymol blue	0.025 g

- a. Final pH, 6.7
- b. Store at 2 to 8°C.

2. Malonate tablets are also available (Key Scientific). Refer to <http://www.keysientific.com> for procedure.

B. Supplies

1. Sterile inoculating loops or sticks
2. Incubator at 35 to 37°C

IV. QUALITY CONTROL

- A. Perform QC on each new lot or shipment of media prior to putting it into use.
- B. Inspect broth for contamination prior to storage and before use. Discard any tubes that are blue.

C. Organisms

1. *Klebsiella pneumoniae* ATCC 13883—malonate positive (blue color)
2. *Escherichia coli* ATCC 25922—malonate negative (green color)

V. PROCEDURE

- A. Using a loop or stick, inoculate tube with a light inoculum picked from the center of a well-isolated colony (turbidity should be less than a no. 0.5 McFarland standard, i.e., no visible turbidity).
- B. Incubate aerobically at 35 to 37°C for up to 48 h.
- C. Observe a color change from green to blue along the slant.

VI. INTERPRETATION

- A. A positive test is growth and change to light blue or deep Prussian blue color throughout the medium.
 - B. A negative test is no color change or change from green to yellow due to fermentation of glucose.
-

VII. REPORTING RESULTS

- A. *Klebsiella ozaenae* is malonate negative; *K. pneumoniae* is malonate positive.
 - B. *Citrobacter amalonaticus* is malonate negative; *Citrobacter koseri* is malonate positive.
 - C. Use as part of the identification of other *Enterobacteriaceae*.
-

VIII. LIMITATIONS

- A. Some reactions are slight. Compare to an uninoculated tube when reading.
 - B. Do not read reactions before 48 h.
 - C. Yeast extract and glucose are needed to stimulate growth of some *Salmonella* organisms but are not generally necessary for other species.
-

REFERENCES

1. Leifson, E. 1933. Fermentation of sodium malonate as a means of differentiating *Aerobacter* and *Escherichia*. *J. Bacteriol.* **26**:329–330.
2. MacFaddin, J. F. 2000. *Biochemical Tests for the Identification of Medical Bacteria*, 3rd ed., p. 310–315. The Lippincott, Williams & Wilkins Co., Philadelphia, Pa.

3.17.30

MGP (Methyl Glucopyranoside) Test

[Updated March 2007]

I. PRINCIPLE

MGP broth is used to differentiate enterococci based on the ability to acidify the carbohydrate methyl- α -D-glucopyranoside (MGP). Vancomycin-resistant *Enterococcus faecium* (VREF) is a serious nosocomial problem. *Enterococcus gallinarum* and *Enterococcus casseliflavus*, while not important for purposes of control of nosocomial infections, show intrinsic resistance to vancomycin due to the *vanC* gene (6). *E. gallinarum* and *E. casseliflavus* are difficult to differentiate from

VREF by conventional biochemical tests (5), since they are phenotypically closely related.

Typically, *E. gallinarum* and *E. casseliflavus* can be differentiated from *E. faecium* based on motility: *E. gallinarum* and *E. casseliflavus* are motile, while *E. faecium* is not. However, nonmotile strains of *E. gallinarum* have been detected using standard motility agar (2, 8), although this has not been reported when

the rapid (2-h) motility test is performed (9). Correct identification of these strains is critical due to their difference in pathogenicity. *E. gallinarum* and *E. casseliflavus* acidify MGP, but *Enterococcus faecalis* and *E. faecium* do not (1, 2, 4, 7, 8). This makes the MGP test useful in preventing the misidentification of vancomycin-resistant *E. gallinarum* as VREF for laboratories that cannot perform the rapid tube motility test.

II. MICROORGANISMS TESTED

A. As an alternative to the motility test

1. Test any gram-positive coccus that is catalase negative and pyrrolidonyl- β -naphthylamide (PYR) positive and grows on plates with 6 μ g of vancomycin per ml or demonstrates resistance to vancomycin by antimicrobial susceptibility testing but shows susceptibility to ampicillin.
2. Test any isolate that is identified as *E. faecium* by a commercial kit system but is ampicillin susceptible.
- B. Test any nonmotile enterococcus that demonstrates resistance to vancomycin with an MIC less than 32 μ g/ml but is ampicillin susceptible.
- C. Test can be performed from any BAP or any plate medium with enterococcal growth, including media with bile-esculin or azide.

III. MEDIA AND SUPPLIES

A. Media (Hardy Diagnostics)

1. Standard medium per liter of deionized water

pancreatic digest of

casein	10.0 g
sodium chloride	5.0 g
MGP	10.0 g
phenol red	18.0 mg

2. Rapid medium per liter of deionized water

basal medium	15.0 g
indicator	50.0 mg
MGP	50.0 g

3. Store at 2 to 8°C, away from direct light, for up to 6 months.

B. Supplies

1. Incubator

2. Sterile sticks, loops, or inoculating needle

IV. QUALITY CONTROL

- A. Examine media for red-orange color (standard tube) or blue color (rapid tube) and lack of turbidity.
 - B. Test each lot of medium with a positive and negative control prior to putting it into use.
 - C. Organisms
 - 1. *E. gallinarum* ATCC 49573—medium turns yellow (positive).
 - 2. *E. faecalis* ATCC 29212—medium remains red or orange (negative).
-

V. PROCEDURE

- A. **Standard medium**
 - 1. Inoculate the MGP broth by lightly touching a single, isolated colony. Do not use a heavy inoculum.
 - 2. Incubate aerobically at 35°C for 24 h.
 - 3. Observe for yellow color change in the media.
 - B. **Rapid medium**
 - 1. Using a “sweep” of colonies from an 18 to 24-h pure culture of the organism to be tested, stab the rapid MGP medium with an inoculating needle. There should be a cell paste visible on the needle as the medium is being inoculated.
 - 2. Incubate aerobically at 35°C for 5 h.
 - 3. Observe for the development of a yellow color along the stab line; this is indicative of a positive test.
 - 4. Replace weak or inconclusive reactions in the incubator for a total of 24 h for development of a yellow color.
 - 5. Optional: run controls with each test run to easily distinguish between a negative and positive reaction.
-

VI. INTERPRETATION

- A. A yellow color change is a positive test for acidification of MGP.
 - B. Red or orange color is a negative test in standard media. Blue color is a negative test in the rapid medium.
-

VII. REPORTING RESULTS

- A. Report as either *Enterococcus gallinarum* or *Enterococcus casseliflavus* if the reaction is positive and the vancomycin susceptibility test shows an intermediate or resistant result for vancomycin. The yellow pigment of *E. casseliflavus* separates these two species. Report the vancomycin MIC result for treatment purposes but do not label or comment that either of these two species, which carry the *vanC* genotype, are vancomycin-resistant enterococci (VRE).
▣ **NOTE:** *E. faecium* and *E. faecalis* carry the *vanA* and *vanB* genotypes, which confer high-level vancomycin resistance and may be transferred within the hospital environment. Patients colonized or infected with true VRE are placed on contact isolation. Patients colonized or infected with either *E. casseliflavus* or *E. gallinarum*, which carry the *vanC* genotype, which confers low-level intrinsic resistance to vancomycin, do not require the same infection control measures to prevent nosocomial spread (3).
- B. Report as *Enterococcus faecium* if result is negative and the organism keys out as *E. faecium* by a kit identification method. Also alter infection control to VRE if isolate is vancomycin resistant.
- C. Other enterococci are positive for MGP but are not resistant or intermediate in susceptibility to vancomycin. See Table 3.18.1–3.

VIII. LIMITATIONS

- A. A heavy inoculum may give false-positive results in the standard test.
- B. Extended incubation times may result in false-positive reactions.
- C. Do not test organisms identified as *E. faecalis*, since false-positive results have been reported (1).
- D. Rapid MGP has a sensitivity of 100% and a specificity of 96.5%. The standard test has reported sensitivities of 100% (2) and 98% (6) and a specificity of 95% (2), but two of the three misidentified *E. faecium* isolates were not VRE.
- E. *Vagococcus* gives a positive result (1).
- F. Use of an inoculating needle is recommended for the rapid test, as inocula from a loop can overwhelm the media.
- G. Negative rapid MGP tests may change from their original color, becoming fainter shades of blue. This color change is easily distinguished if positive and negative control tubes are used in parallel with each test being performed.

REFERENCES

1. Carvalho, M. G., L. M. Teixeira, and R. R. Facklam. 1998. Use of tests for acidification of methyl- α -D-glucopyranoside and susceptibility to efrotomycin for differentiation of strains of *Enterococcus* and some related genera. *J. Clin. Microbiol.* **36**:1584–1587.
2. Chen, D. K., L. Pearce, A. McGeer, D. E. Low, and B. M. Willey. 2000. Evaluation of D-xylose and 1% methyl- α -D-glucopyranoside fermentation tests for distinguishing *Enterococcus gallinarum* from *Enterococcus faecium*. *J. Clin. Microbiol.* **38**:3652–3655.
3. Clinical and Laboratory Standards Institute. 2006. *Performance Standards for Antimicrobial Susceptibility Testing*. Sixteenth informational supplement. Approved standard M100-S16. Clinical and Laboratory Standards Institute, Wayne, Pa.
4. Devriese, L. A., B. Pot, K. Kersters, S. Lauwers, and F. Haesebrouck. 1996. Acidification of methyl- α -D-glucopyranoside: a useful test to differentiate *Enterococcus casseliflavus* and *Enterococcus gallinarum* from *Enterococcus faecium* species group and from *Enterococcus faecalis*. *J. Clin. Microbiol.* **34**:2607–2608.
5. Facklam, R. R., and M. D. Collins. 1989. Identification of *Enterococcus* species isolated from human infections by a conventional test scheme. *J. Clin. Microbiol.* **27**:731–734.
6. Gin, A. S., and G. G. Zhanel. 1996. Vancomycin-resistant enterococci. *Ann. Pharmacother.* **30**:615–624.
7. Hanson, K. L., and C. P. Cartwright. 1999. Comparison of simple and rapid methods for identifying enterococci intrinsically resistant to vancomycin. *J. Clin. Microbiol.* **37**:815–817.
8. Turenne, C. Y., D. J. Hoban, J. A. Karlowsky, G. G. Zhanel, and A. M. Kabani. 1998. Screening of stool samples for identification of vancomycin-resistant *Enterococcus* isolates should include the methyl- α -D-glucopyranoside test to differentiate nonmotile *Enterococcus gallinarum* from *E. faecium*. *J. Clin. Microbiol.* **36**:2333–2335.
9. Van Horn, K. G., and K. M. Rodney. 1998. Colonization and microbiology of the motile enterococci in a patient population. *Diagn. Microbiol. Infect. Dis.* **31**:525–530.

I. PRINCIPLE

The motility test is used to detect the presence of flagella by bacteria, allowing them to travel in and out of the microscopic field or beyond their initial inoculation in agar (3). For the wet preparation, a drop of organism in broth is suspended on a clean glass slide, a coverslip is added, and the culture is observed microscopically for

motility. Occasionally the organism is incubated in the broth prior to examination.

In the tube test, semisolid motility medium is inoculated in a straight line down through the center of a tube (4). Motile organisms will migrate out from the line of inoculation, causing visible turbidity

throughout the tube. Nonmotile organisms will grow only along the line of inoculation. Other substrates which allow simultaneous testing of other biochemical reactions that aid in the identification of microorganisms may be added to the medium.

II. MICROORGANISMS TESTED

Campylobacter; Legionella; enterococci; Enterobacteriaceae; Listeria; Bacillus; other gram-positive rods; non-glucose-fermenting, gram-negative rods; and any other organism where motility is useful for identification

III. MEDIA, REAGENTS, AND SUPPLIES

A. Media and reagents

1. Broth medium for wet preparation
 - a. TSB, nitrate, or BHI broth
 - b. Voges-Proskauer (VP) cupule of the API strip
 - c. Any agar slants with TSB or BHI broth added (*see item VIII*)
 - d. Saline or distilled water
■ NOTE: Cleary (1) reported that some motile organisms can become immobile in distilled water.
2. Motility tube media
 - a. Combination of sulfide for H₂S detection, indole, and motility test medium (SIM)
 - b. Motility test medium with or without TTC (triphenyltetrazolium chloride, a colorless vital dye incorporated into the medium, which turns red as it is reduced when incorporated into bacteria; the dye allows visualization of the bacteria)

c. Motility nitrate medium

- d. Combination of motility test medium, indole, and lysine decarboxylase
- e. Combination of motility test medium, indole, and ornithine decarboxylase (MIO)

■ NOTE: Motility medium contains 5 g of agar per liter and can be prepared in-house in agar plates that are incubated agar side down. Motile organisms move from the point of inoculum to the edges of the plate.

B. Supplies

1. 22- by 22-mm coverslips and microscope slides
2. Phase-contrast or bright-field microscope
3. Sterile inoculating needle or sticks

IV. QUALITY CONTROL

- A.** Test each new lot or shipment of tube media prior to use with a positively and negatively reacting organism and for sterility.
B. Organisms

Organism	ATCC no.	Media	Motility	Indole	H ₂ S	Lysine	Ornithine
<i>Escherichia coli</i>	25922	All	+	+	-	-	+
<i>Klebsiella pneumoniae</i>	13883, 27736	All	-	-	-	+	-
<i>Proteus vulgaris</i>	33420	SIM	+	+	+		

- C.** Competence of technologists in the hanging-drop test is validated by testing known motile enterococci or *Listeria* in the broth assay.

V. PROCEDURE**A. Wet mount preparation**

1. Inoculum: use *fresh* growth from an agar plate and suspend isolated colonies in broth. Use a light inoculum (not visibly turbid).
 - a. It is acceptable to suspend the organism in a small amount of medium for an initial wet mount, but follow with incubation of a larger amount of broth media if the result is negative.
 - b. Choosing the medium
 - (1) Use any broth which does not contain carbohydrate and will support the growth of the organisms (BHI, nitrate broth).
 - (2) Always use a broth for *Bacillus* spp. (1).
 - (3) Use 0.5 ml of BHI or TSB for enterococci.
 - (4) Saline can be used for gram-negative rods.
 - (5) Use warm sterile tap water for *Legionella*.
 - (6) A drop from the VP microtube of the incubated API 20E strip may be used before the reagents are added. However, a negative test must be repeated in a non-carbohydrate-containing broth.
 - (7) For problem organisms, streak an agar slant and then carefully add a few drops of BHI or nutrient broth to the base of the slant, covering just a bit of the streaked slant. After overnight incubation, use a drop of the BHI broth for the wet mount (this works very well for non-fermenting gram-negative rods).

2. Examination

- a. While wearing gloves, place a small drop of fresh liquid on the center of a microscope slide; add coverslip. Allow organisms to "settle" for a minute.
- b. Observe under high power (40×).
 - (1) For a light microscope, decrease the light by closing the diaphragm.
 - (2) Preferably, use a phase-contrast microscope.
3. For all organisms negative for motility by initial wet mount, repeat the wet mount after incubation in broth, or test by tube method below.
 - a. Incubate at 30°C for nonfermenting, gram-negative rods (24 h).
 - b. Incubate enterococci and *Listeria* at 30°C for 2 h (5, 8).
 - c. Other organisms may be incubated at temperatures optimal for their growth, usually 35°C.

B. Tube media for *Enterobacteriaceae*; nonfermenting, gram-negative rods; and *Listeria*

1. With a sterile inoculating wire, pick an isolated colony and stab the medium straight down through the center to a depth of 1/2 in. for small tubes and 1 in. for larger tubes.

V. PROCEDURE (continued)

2. Incubate as follows.
 - a. At 35°C for *Enterobacteriaceae* for 24 h.
 - b. At 30°C for nonfermenting, gram-negative rods and enterococci for 24 h.
 - c. If there is a question regarding a negative result, incubate at 25°C.
 - d. For *Listeria* and *Yersinia*, incubate two tubes, one at 35°C and one at 25°C.

VI. INTERPRETATION**A. Wet mount preparation**

1. Directional purposeful motility is a positive test. Motile organisms change position with respect to one another. Brownian movement (random jiggling or shaking due to molecular bombardment), where the organisms remain in the same relative position with respect to each other, should not be mistaken for true motility (9).
2. *Campylobacter* organisms display a very active motility which appears as tiny dots darting in and out of the field.

B. Tube media

1. Diffuse growth outward away from stab line or turbidity of the media is a positive test.
2. A clear tube (the same as the uninoculated media) with growth only along the line of inoculation indicates that the organism is nonmotile.
3. In media with TTC, the red color forms in the area of bacterial growth. Motile organisms produce a pink color that diffuses from the stab line. Organisms that are nonmotile produce a pinkish red pigment that is confined to the stab line.

VII. REPORTING RESULTS

- A. *Bacillus* spp. should be motile. Lack of motility could indicate *Bacillus anthracis*.
- B. *Enterococcus casseliflavus* and *Enterococcus gallinarum* are motile.
- C. *Listeria* organisms are motile at 25°C but not at 35°C, with a characteristic umbrella-shaped growth at the top of the tube. On wet mount they exhibit tumbling motility.
- D. *Yersinia enterocolitica* is motile at 25°C but not at 35°C.
- E. *Acinetobacter* organisms are nonmotile.
- F. Nonfermenting, gram-negative rods and *Enterobacteriaceae* vary in their motility.

VIII. LIMITATIONS

- A. Studies by Edmondson and Sanford (2) showed that nonmotile, mucoid *Klebsiella* strains may give a false-positive motile reaction in tube media; this is due to mucoid strains spilling between medium and the tube, giving a cloudy appearance which is often confused with motility. False-positive results can be avoided by use of media with adequate tube depth and careful reading with attention to the density of growth in the central stab.
- B. *Bacillus* species are best tested directly from a fresh plate. If a fresh plate is not available, inoculate a plate and incubate for 4 h. Then perform wet mount.
- C. A large number of *E. casseliflavus* and *E. gallinarum* organisms have been reported as nonmotile using some tube motility agar (6, 7). If a vancomycin MIC is between 4 to 16 µg/ml and the isolate is ampicillin susceptible, but the enterococcus is nonmotile, confirm results with the 2-h broth method or perform the MGP test (procedure 3.17.30).

VIII. LIMITATIONS (continued)

- D. Motility results for enterococcus with MIO have been reported to have poor sensitivity (7).
- E. Excessive heat on a microscope slide can affect the results.
- F. False-negative reactions may occur if bacterial flagella are damaged due to heating, shaking, or other trauma. Such environmental shock will render the organism nonmotile.
- G. Some microorganisms do not produce flagellar proteins at 35 to 37°C but do so at 22°C.
- H. TTC may be inhibitory to certain fastidious bacteria.

REFERENCES

1. Cleary, T. 2002. Evaluation of wet-prep motility test for presumptive identification of *Bacillus* species. *J. Clin. Microbiol.* **40**:730. (Letter to the editor.)
2. Edmondson, E. B., and J. P. Sanford. 1967. The Klebsiella-Enterobacter (Aerobacter), Serratia group. *Medicine* **46**:323–340.
3. Leifson, E. 1960. *Bacterial Flagellation*. Academic Press, New York, N.Y.
4. Tittler, R. P., and L. A. Sandholzer. 1936. The use of semi-solid agar for the detection of bacterial motility. *J. Bacteriol.* **31**:575–580.
5. Toth, C., and K. Van Horn. 1999. Evaluation of motility media for detection of motility in enterococci, abstr. C-442, p. 196. *Abstr. 99th Gen. Meet. Am. Soc. Microbiol.* American Society for Microbiology, Washington, D.C.
6. Turenne, C. Y., D. J. Hoban, J. A. Karlowsky, G. G. Zhanel, and A. M. Kabani. 1998. Screening of stool samples for identification of vancomycin-resistant *Enterococcus* isolates should include the methyl- α -D-glucopyranoside test to differentiate nonmotile *Enterococcus gallinarum* from *E. faecium*. *J. Clin. Microbiol.* **36**:2333–2335.
7. Van Horn, K., C. Tóth, R. Kariyama, R. Mitsuhata, and H. Kumon. 2002. Evaluation of 15 motility media and a direct microscopic method for detection of motility in enterococci. *J. Clin. Microbiol.* **40**:2476–2479.
8. Van Horn, K. G., and K. M. Rodney. 1998. Colonization and microbiology of the motile enterococci in a patient population. *Diagn. Microbiol. Infect. Dis.* **31**:525–530.
9. Weyant, R. S., C. W. Moss, R. E. Weaver, D. G. Hollis, J. G. Jordan, E. C. Cook, and M. I. Daneshvar. 1995. *Identification of Unusual Pathogenic Gram-Negative Aerobic and Facultatively Anaerobic Bacteria*, 2nd ed., p. 13–14. Williams & Wilkins, Baltimore, Md.

I. PRINCIPLE

Gas production by gram-positive rods is tested in glucose-containing medium called lactobacillus MRS broth, named for the authors of the publication (1). MRS broth is helpful to differentiate *Leuconostoc* spp. and *Weissella confusa* (previously called *Lactobacillus confusus*) from

Pediococcus, which is gas negative (2, 4, 5). The gas is detected by a rise in a petroleum jelly plug or displacement of broth with air in the Durham tube. Sufficient gas is not produced in ordinary sugar fermentation tubes to detect these organisms.

II. MICROORGANISMS TESTED

Vancomycin-resistant, catalase-negative, gram-positive coccobacilli that are pyrrolidonyl- β -naphthylamide (PYR) negative and grow aerobically

III. MEDIUM, REAGENTS, AND SUPPLIES**A. MRS broth**

- Store at 4 to 8°C.
1. Purchase from most vendors.
2. Prepare from dehydrated powder (Difco, BBL, BD Diagnostic Systems, Hardy Diagnostics), dispense into tubes with Durham tube (optional), and autoclave.

B. Gas detection options

1. Vaspar, liquid paraffin, or petroleum jelly, maintained at 56°C in liquid form
2. Durham tube

C. Other supplies

1. Sterile sticks
2. Incubator at 35°C

IV. QUALITY CONTROL

- A. Examine media for lack of turbidity. Invert if there is a Durham tube and it contains a bubble.
B. Test each new lot or shipment of medium with a positive and negative control prior to putting it into use.
C. Organisms
1. *Leuconostoc mesenteroides* ATCC 10830—Gas produced (positive)
2. *Enterococcus faecalis* ATCC 29212—no gas (negative)

V. PROCEDURE

- A. Inoculate MRS broth lightly with one or two colonies from an 18- to 24-h BAP.
- B. If a Durham tube is not used, overlay the inoculated MRS broth with a plug of melted Vaspar or petroleum jelly, being careful to cover broth layer entirely without introducing air.
- C. Incubate aerobically at 35°C for up to 7 days, and observe daily for gas trapped in the Durham tube or solid plug.

VI. INTERPRETATION

- A. A positive test shows a visible lifting of the Vaspar plug resulting in complete separation from the broth surface or trapped gas in the Durham tube. Positive reactions usually raise wax plugs within 48 h.
- B. A negative test shows no lifting of the wax plug or no gas bubble in the Durham tube.

VII. REPORTING RESULTS

- A. Streptococci, lactobacilli, and *Pediococcus* do not produce gas from glucose.
- B. *Leuconostoc* and *W. confusa* are positive for gas.
 1. Prepare a Gram stain from the MRS broth.
 2. *Leuconostoc* is the only genus that produces coccoid forms as well as coccobacilli and rods. It is arginine deaminase negative and does not grow at 42°C (3).
 3. *W. confusa* is a gas producer but does not have coccoid forms, is generally arginine positive, and grows at 42°C (3).

VIII. LIMITATIONS

- A. The Durham tube is a safer alternative to petroleum jelly, since strains of *Leuconostoc* can produce copious amounts of gas. However, the Durham tube method does not work as well.
- B. The difficulty in separating *Weissella* and *Leuconostoc* may result in a misidentification, if the Gram stain is equivocal and other biochemical tests are not performed.

REFERENCES

1. De Mann, J. D., M. Rogosa, and M. E. Sharpe. 1960. A medium for the cultivation of lactobacilli. *J. Appl. Bacteriol.* **23**:130–135.
2. Facklam, R., and J. A. Elliott. 1995. Identification, classification, and clinical relevance of catalase-negative, gram-positive cocci, excluding the streptococci and enterococci. *Clin. Microbiol. Rev.* **8**:479–495.
3. Facklam, R., D. Hollis, and M. D. Collins. 1989. Identification of gram-positive coccal and coccobacillary vancomycin-resistant bacteria. *J. Clin. Microbiol.* **27**:724–730.
4. Isenberg, H. D., E. M. Vellozzi, J. Shapiro, and L. G. Rubin. 1988. Clinical laboratory challenges in the recognition of *Leuconostoc* spp. *J. Clin. Microbiol.* **26**:479–483.
5. Olano, A., J. Chua, S. Schroeder, A. Minari, M. La Salvia, and G. Hall. 2001. *Weissella confusa* bacteremia: a case report. *J. Clin. Microbiol.* **39**:1604–1607.

3.17.33

MR-VP (Methyl Red–Voges-Proskauer) Tests

[Updated March 2007]

I. PRINCIPLE

The methyl red (MR) test is used to determine if an organism is able to produce stable acid end products from glucose fermentation (2). Methyl red indicator (red color below pH 4.4; yellow color at pH 5.8) is used to determine the pH after an enteric gram-negative rod has fermented glucose to completion. All members of the *Enterobacteriaceae* give a positive methyl red reaction when tested up to 24 h due to conversion of glucose to pyruvic acid by the Embden-Meyerhof pathway. After further incubation (2 to 5 days) those organisms that are MR positive continue to me-

tabolize pyruvic acid to lactic, acetic, and formic acids by the mixed acid pathway and are able to maintain the acid pH (<4.4). Organisms utilizing the butylene glycol pathway produce acetyl methylcarbinol (acetoin) and butanediol, neutral end products that raise the pH towards neutrality (pH > 6) and result in a high final pH. Most *Enterobacteriaceae* demonstrate one or the other metabolic pathway but rarely both.

The Voges-Proskauer (VP) test (9) is used to determine if an organism produces

acetyl methylcarbinol from glucose fermentation. If present, acetyl methylcarbinol is converted to diacetyl in the presence of α-naphthol, strong alkali (40% KOH), and atmospheric oxygen. The α-naphthol was not part of the original procedure but was found to act as a color intensifier by Barratt (1) and must be added first. The diacetyl and quanidine-containing compounds found in the peptones of the broth then condense to form a pinkish red polymer (8).

II. MICROORGANISMS TESTED

- A. MR test for enteric gram-negative rods, as part of identification to species level
- B. VP test for identification to the species level of the following groups of organisms
 - 1. Enteric gram-negative rods, *Aeromonas*, and *Vibrio*
 - 2. Viridans group streptococci (4)
 - 3. Staphylococci

III. MEDIA, REAGENTS, AND SUPPLIES

- A. Media
 - Store broths at 4 to 8°C.
 - 1. Andrade's 1% glucose broth (*to be used for VP testing only, not for MR testing*)
 - 2. MRVP broth (pH 6.9)
Ingredients per liter of deionized water:
 - buffered peptone 7.0 g
 - glucose 5.0 g
 - dipotassium phosphate 5.0 g
 - 3. Volumes
 - a. Generally dispense approximately 5 ml per tube.
 - b. Use enough broth to cover an inverted Durham tube, if it is used.
- c. Dispense 2 ml of MRVP broth for rapid VP testing and 0.5 ml for rapid MR testing.
- 4. MRVP tablets with creatine (Key Scientific) for rapid test. Refer to <http://www.keysientific.com> for procedures.
- B. Reagents
 - 1. 40% Potassium hydroxide
Caution: KOH is hygroscopic and becomes caustic when moist. Weigh quickly in tared beaker. Store away from acids. Avoid exposure to skin.
 - a. Dissolve 40 g of potassium hydroxide pellets in 100 ml of distilled water in a polyethylene bottle. Keep bottle in cool water bath during preparation.

III. MEDIA, REAGENTS, AND SUPPLIES (continued)



Include QC information on reagent container and in QC records.

- b. Optional: add 0.3 g of creatine (*N*-methyl-*N*-guanylglycine) for more rapid and sensitive reaction (3). Creatine should *not* be added if reagent is used for the API 20E or the tablets from Key Scientific, since it is in the media.
- c. Store at 4 to 8°C.
- d. Shelf life: 2 to 3 weeks (8)
- 2. 5% α-Naphthol (5 g/100 ml) in 95% ethyl alcohol (3)
 - a. Store at 4 to 8°C in the dark.
 - b. Shelf life: 2 to 3 weeks
 - c. Reagent should be almost colorless.
- 3. Methyl red solution, 0.02%
 - a. Dissolve 0.1 g of methyl red in 300 ml of ethyl alcohol, 95%.
 - b. Add sufficient distilled water to make 500 ml.
 - c. Store at 4 to 8°C in a brown bottle.
 - d. Solution is stable for 1 year.
- 4. Reagents with extended shelf life are commercially available from most vendors.

C. Supplies

- 1. Sterile wooden sticks or inoculating loops
- 2. Test tubes

IV. QUALITY CONTROL

- A. Examine broth for signs of contamination, dehydration, and deterioration.
- B. Perform QC on each new lot or shipment of media and reagent prior to use with one organism known to demonstrate a positive reaction and one organism known to give a negative reaction.
- C. Organisms
 - 1. *Klebsiella pneumoniae* ATCC 13883—MR negative (yellow), VP positive (red)
 - 2. *Escherichia coli* ATCC 25922—MR positive (red), VP negative (no change)

V. PROCEDURE

A. VP test

1. Inoculate a tube of 1% glucose broth (Andrade's base) or MRVP broth with colony of the organism to be tested and incubate at 35°C for 18 to 24 h. *Do not tighten caps.*
 - NOTE:** Some organisms may produce acetyl methyl carbinol at room temperature and not 35°C (e.g., *Hafnia alvei*, *Yersinia*, *Listeria*). In this case, inoculate another broth and incubate at room temperature.
2. If a 5-ml broth culture is used, aliquot 2.0 ml of broth into a nonsterile 13-by 100-mm test tube. Hold the remainder for possible reincubation.
 - NOTE:** If the glucose broth contains a Durham tube, be careful not to introduce air bubbles into it when the 2.0 ml of broth is poured into the nonsterile tube for testing.
3. Add 6 drops of 5% α-naphthol, and mix well to aerate.
4. Add 2 drops of 40% potassium hydroxide, and mix well to aerate.
5. Observe for a pink-red color at the surface within 30 min. Shake the tube vigorously during the 30-min period.
 - NOTE:** If the result is negative, the glucose or MRVP broth can be incubated for up to 48 h and the test repeated.

B. MR test

1. Inoculate tube as for VP test and incubate at 35°C for at least 48 h.
 - NOTE:** If the center of one colony is inoculated to a 0.5-ml volume of MRVP broth, the test can be read at 18 to 24 h (8).
2. Remove approximately 1 ml of the 48-h broth to a nonsterile 13-by 100-mm tube. (The remainder should be reserved for testing at 3 to 5 days if necessary.)
3. Add 3 to 6 drops (or 1 drop to 0.5 ml) of methyl red indicator to aliquot.
4. Observe for red color immediately.

VI. INTERPRETATION

A. VP test

1. A pink-red color at the surface is a positive reaction.
2. A lack of a pink-red color is a negative reaction.
3. A copper color should be considered negative. A rust color is a weak positive reaction.

B. MR test

1. A positive reaction is a distinct red color.
2. A negative reaction is a yellow color.
3. A weak positive is red-orange.
4. If an orange color is seen, incubate the remainder of the broth for up to 4 days and repeat the test after further incubation. In this case it may also be helpful to set up a duplicate broth at 25°C.

VII. REPORTING RESULTS

- A. Organisms in the *E. coli* group are MR positive, and those in the *Enterobacter-Klebsiella* group are MR negative (5, 7).
- B. Most members of the family *Enterobacteriaceae* give opposite MR and VP reactions; however, certain organisms, like *H. alvei* and *Proteus mirabilis*, may give both a positive MR reaction and a positive VP reaction (often delayed) (5, 7).
- C. *Streptococcus mitis* group organisms are VP negative, whereas the other viridans group streptococci are VP positive, except *Streptococcus vestibularis*, which is VP variable (4).
- D. Refer to procedure 3.8.1 for VP reactions of *Vibrio*, *Plesiomonas*, and *Aeromonas*.
- E. *Listeria* organisms are beta-hemolytic, gram-positive rods that are VP positive at 25°C, but this test is not a key test in the identification.

VIII. LIMITATIONS

- A. Increased exposure of the organism to atmospheric oxygen in the microtechnique decreases the incubation period.
- B. Avoid overinoculation. Bacterial growth is inhibited when the inoculum exceeds approximately 10⁹ viable cells per ml (8).
- C. If not enough incubation time is allowed before the methyl red indicator is added, a false-positive result may be obtained.
- D. With prolonged incubation (>3 days), some VP-positive organisms can produce an acid condition in the medium, yielding weak positive reactions or false-negative VP reactions.
- E. Shaking the tubes enhances the VP reaction.
- F. Do not add more than 2 drops of KOH per 2 ml of medium. Excess amounts of KOH can give a weak positive reaction, which may be masked by the formation of a copperlike color because of the reaction of KOH with α-naphthol alone.
- G. Do not read the test more than 1 h after adding the VP reagents. A copperlike color may develop, resulting in a potential false-positive interpretation.
- H. Reagents must be added in the specified order. A reversal of order may result in a weak positive or false-negative VP result.
- I. When performing the VP test for the API 20E, add 1 drop of KOH followed by 1 drop of α-naphthol. This is opposite to the order for adding reagents in the standard test. Creatine is present in the cupule and is not needed in the KOH reagent used for the test (API 20E package insert).

VIII. LIMITATIONS (continued)

J. The original study by Barritt (1) using α -naphthol indicated that it should be prepared in absolute (100%) ethanol, but the Coblenz method (3) using 95% ethanol and creatine in the KOH is more rapid and sensitive (6, 8, 10). For the Coblenz method, use a very heavy inoculum in 2 ml of MRVP broth and creatine in the KOH, and read the test as soon as 6 h (6, 10).

REFERENCES

1. Barritt, M. M. 1936. The intensification of the Voges-Proskauer reaction by the addition of α -naphthol. *J. Pathol. Bacteriol.* **42**:441–454.
2. Clark, W. M., and H. A. Lubs. 1915. The differentiation of bacteria of the colon-aerogenes family by the use of indicators. *J. Infect. Dis.* **17**:161–173.
3. Coblenz, L. M. 1943. Rapid detection of the production of acetyl-methyl-carbinol. *Am. J. Public Health* **33**:815–817.
4. Coykendall, A. 1989. Classification and identification of the viridans streptococci. *Clin. Microbiol. Rev.* **2**:315–328.
5. Ewing, W. H. 1986. *Edwards and Ewing's Identification of Enterobacteriaceae*, 4th ed. Elsevier Scientific Publishing Co., New York, N.Y.
6. Facklam, R., and J. A. Elliott. 1995. Identification, classification, and clinical relevance of catalase-negative, gram-positive cocci, excluding the streptococci and enterococci. *Clin. Microbiol. Rev.* **8**:479–495.
7. Farmer, J. J., III, B. R. Davis, F. W. Hickman-Brenner, A. McWhorter, G. P. Huntley-Carter, M. A. Asbury, C. Riddle, H. G. Wathen-Grady, C. Elias, G. R. Fanning, A. G. Steigerwalt, C. M. O'Hara, G. K. Morris, P. B. Smith, and D. J. Brenner. 1985. Biochemical identification of new species and biogroups of *Enterobacteriaceae* isolated from clinical specimens. *J. Clin. Microbiol.* **21**:46–76.
8. MacFaddin, J. F. 2000. *Biochemical Tests for the Identification of Medical Bacteria*, 3rd ed., p. 321–326, 439–450. The Lippincott, Williams & Wilkins Co., Philadelphia, Pa.
9. Voges, O., and B. Proskauer. 1898. Beitrag zur Ernährungsphysiologie und zur Differentialdiagnose der Bakterien der hämorrhagischen Septicemia. *Z. Hyg. Infektkr.* **28**:20–37.
10. Weyant, R. S., C. W. Moss, R. E. Weaver, D. G. Hollis, J. G. Jordan, E. C. Cook, and M. I. Daneshvar. 1995. *Identification of Unusual Pathogenic Gram-Negative Aerobic and Facultatively Anaerobic Bacteria*, 2nd ed., p. 22–23. Williams & Wilkins, Baltimore, Md.

3.17.34

MUG (4-Methylumbelliferyl- β -D-Glucuronide) Test

[Updated March 2007]

I. PRINCIPLE

Escherichia coli and rare other *Enterobacteriaceae* (*Salmonella*, *Yersinia*, and *Shigella*) produce the enzyme β -glucuronidase. This enzyme hydrolyzes the substrate 4-methylumbelliferyl- β -D-glucuronide (MUG), releasing 4-methylumbelliferone, which fluoresces blue under

long-wave UV light (4). Since 97% of *Escherichia coli* strains possess the β -glucuronidase enzyme, the MUG test can be used for rapid identification of *E. coli*, the most common gram-negative rod seen in clinical specimens (1, 2, 4, 5, 6, 8, 10). Since verotoxin-producing *E. coli* strains

are among the few *E. coli* strains that do not produce MUG, this test can also be used to detect the absence of the enzyme in a fecal isolate of *E. coli* to alert the microbiologist to the possible presence of a verotoxin-producing strain (7, 9).

II. MICROORGANISMS TESTED

- A. Fresh colonies on BAP of possible *E. coli* organisms that are indole-positive, oxidase-negative, gram-negative rods, whether they are lactose positive or negative.
- NOTE:** For the tube test, colonies from EMB, but not MAC, can be used.
- B. Do not use this test as part of an algorithm to rapidly identify *E. coli* in abdominal sources, since occasional isolates of both *Salmonella* and *Shigella* can be MUG positive. However, the test can be used to separate potential verotoxin-producing *E. coli* (MUG negative) from other *E. coli* strains (usually MUG positive) in gastrointestinal specimens, once the isolate has been identified as *E. coli*.

III. MEDIA, REAGENTS, AND SUPPLIES



Include QC information on reagent container and in QC records.

A. Reagents

- 1. Purchase MUG as disks from commercial vendors.
 - a. MUG disks: Hardy Diagnostics, Inc.; Key Scientific; Oxoid, Inc.; Remel, Inc.
 - b. BactiCard *E. coli*: MUG and indole on a filter card (Remel, Inc.)
- 2. Prepare MUG from powder (Sigma Chemical Co.).
 - a. M/15 Sorensen's phosphate buffer, pH 7.5
 - (1) M/15 Na₂PO₄ (solution A)
 - (a) 4.730 g of Na₂HPO₄
 - (b) q.s. to 500 ml with sterile distilled water.
- (2) M/15 KH₂PO₄ (solution B)
 - (a) 4.535 g of KH₂PO₄
 - (b) q.s. to 500 ml with sterile distilled water.
- (3) Combine 85 ml of solution A and 15 ml of solution B.
 - NOTE:** Check final pH; adjust to pH 7.5 with either solution if necessary (solution A to make more alkaline; solution B to acidify).
- (4) Store at room temperature.

III. MEDIA, REAGENTS, AND SUPPLIES (continued)

- b.** MUG liquid reagent or stock for disks
 - (1) Dissolve 50 mg of MUG in 100 ml of Sorenson's buffer.
 - (2) This is a 500- μ g/ml stock solution.
 - (3) Label, aliquot, and store at -20°C in convenient amounts.
 - (4) Shelf life: 1 year at -20°C or 1 week at 4°C in the dark (cover tubes in refrigerator with foil to keep from light).
- c.** Disk preparation
 - (1) Add 0.2 ml of stock MUG solution to 3.2 ml of M/15 Sorenson's phosphate buffer to prepare a 1:16 dilution.
 - (2) Add 1.25 ml to a vial of 50 sterile 6-mm-diameter paper disks (BD Diagnostic Systems; Hardy, Inc.). Allow to thoroughly saturate disks so that no fluid hangs on the sides of vial (0.78 $\mu\text{g}/\text{disk}$).
 - (3) Spread the saturated disks onto a metal screen and place in a dry environment until the disks are completely dry. Protect from light.
 - (4) Store disks at -20°C , away from the light, for up to 1 year or at 4°C for up to 1 month.

B. Supplies

- 1. Long-wave UV light (366 nm; e.g., Wood's lamp)
- 2. Wooden sticks or inoculating loops
- 3. 35 to 37°C incubator
- 4. Empty petri dish or Durham tubes

IV. QUALITY CONTROL

- A. Perform QC on each new lot or shipment of disks or liquid reagent prior to putting it into use.
- B. Organisms
 - 1. *E. coli* ATCC 25922—blue fluorescence (positive)
 - 2. *Klebsiella pneumoniae* ATCC 13883 or ATCC 27736—no fluorescence (negative)

V. PROCEDURE

- A. MUG tube test
 - 1. Place 2 drops of MUG reagent in Durham tube.
 - 2. Remove one colony from BAP or EMB with sterile stick and inoculate tube.
 - NOTE: For commercial products, place 0.25 ml of deionized water in a glass (not soda lime glass) tube and add the MUG disk to the tube after inoculation with the colony.
 - 3. Incubate for at least 2 h at 35°C without added CO_2 .
 - NOTE: MUG reactions occasionally take 4 h to turn positive.
 - 4. Observe fluorescence using long-wave UV light in a darkroom.
- B. MUG disk method
 - 1. Place disk in sterile empty petri dish and wet with 1 drop of water.
 - NOTE: If excess water is used, test may be falsely negative.
 - 2. Using a wooden stick or bacteriological loop, roll colony from a BAP onto the disk.
 - NOTE: Alternatively, place the disk directly over the colony and incubate for 30 min. Observe for fluorescence and reincubate disk for up to 2 h.
 - 3. Incubate at 35°C for a minimum of 2 h (test can be read up to 24 h, but 2 h is usually sufficient).
 - 4. Observe the disk using long-wave UV light in a darkened room.

VI. INTERPRETATION

- A. A positive test is bright blue-white fluorescence.
 - B. A negative test is lack of blue fluorescence.
- NOTE:** It may be helpful, when interpreting the tube test, to observe an uninoculated tube when evaluating the fluorescence. When interpreting weak reactions with the disk test, after incubation, place the disk in 2 ml of sterile saline. Observe as for tube test after 10 min.

VII. REPORTING RESULTS

- A. *E. coli* is definitively identified if a gram-negative rod is indole positive, oxidase negative, and MUG positive (11).
- B. *E. coli* O157:H7 is indole positive and MUG negative.

VIII. LIMITATIONS

- A. Not all *E. coli* organisms are MUG positive. A negative test does not mean that the organism is not *E. coli*.
- B. Do not use media that contain dyes (e.g., EMB, MAC) for the disk test, although the dyes do not interfere with the tube test.
- C. Of *Shigella* species that are indole positive, approximately 8% are also MUG positive. Rare isolates of *Salmonella* and *Yersinia* are also MUG positive. However, they are rarely indole positive. Thus, to avoid a misidentification, lactose-negative organisms from abdominal sources or from blood should not be tested using this method.
- D. Some fluorescing organisms, such as *Pseudomonas aeruginosa*, may resemble a positive MUG result. Therefore, the test should not be performed on oxidase-positive organisms.
- E. Some organisms fluoresce orange, which is not considered a positive reaction.
- F. Rare MUG-positive *E. coli* O157 strains have been reported (3).

REFERENCES

1. Edberg, S. C., and C. M. Kontnick. 1986. Comparison of β -glucuronidase-based substrate systems for identification of *Escherichia coli*. *J. Clin. Microbiol.* **24**:368–371.
2. Feng, P. C. S., and P. A. Hartman. 1982. Fluorogenic assays for immediate confirmation of *Escherichia coli*. *Appl. Environ. Microbiol.* **43**:1320–1329.
3. Hayes, P. S., K. Blom, P. Feng, J. Lewis, N. A. Strockbine, and B. Swaminathan. 1995. Isolation and characterization of a beta-D-glucuronidase-producing strain of *Escherichia coli* serotype O157:H7 in the United States. *J. Clin. Microbiol.* **33**:3347–3348.
4. Iritani, B., and T. J. Inzana. 1988. Evaluation of a rapid tube assay for presumptive identification of *Escherichia coli* from veterinary specimens. *J. Clin. Microbiol.* **26**:564–566.
5. Kilian, M., and P. Bülow. 1976. Rapid diagnosis of Enterobacteriaceae. I. Detection of bacterial glucosidases. *Acta Pathol. Microbiol. Scand. Sect. B* **84**:245–251.
6. Perez, J. L., C. I. Berrocal, and L. Berrocal. 1986. Evaluation of a commercial β -glucuronidase test for rapid and economical identification of *Escherichia coli*. *J. Appl. Bacteriol.* **61**:541–545.
7. Ratnam, S., S. B. March, R. Ahmed, G. S. Bezanson, and S. Kasatiya. 1988. Characterization of *Escherichia coli* serotype O157:H7. *J. Clin. Microbiol.* **26**:2006–2012.
8. Thaller, M. C., F. Berluttì, B. Dainelli, and R. Pezzi. 1988. New plate medium for screening and presumptive identification of gram-negative urinary tract pathogens. *J. Clin. Microbiol.* **26**:791–793.
9. Thompson, J. S., D. S. Hodge, and A. A. Borczyk. 1990. Rapid biochemical test to identify verocytotoxin-positive strains of *Escherichia coli* serotype O157. *J. Clin. Microbiol.* **28**:2165–2168.
10. Trepeta, R. W., and S. C. Edberg. 1984. Methylumbelliferyl- β -D-glucuronide-based medium for rapid isolation and identification of *Escherichia coli*. *J. Clin. Microbiol.* **19**:172–174.
11. York, M. K., E. J. Baron, M. Weinstein, R. Thomson, and J. E. Clarridge. 2000. A multilaboratory validation of rapid spot tests for identification of *Escherichia coli*. *J. Clin. Microbiol.* **38**:3394–3398.

I. PRINCIPLE

Nitrate broth and nitrite broth are used to determine if an organism can reduce nitrate (NO_3^-) to nitrites (NO_2^-) and reduce nitrites further. The reduction of nitrate is associated with anaerobic respiration in which the organism derives its oxygen from nitrate. The end products of nitrate reduction include nitrite, ammonia, molecular nitrogen (N_2), hydroxylamine, and other related compounds.

The presence of nitrites resulting from the reduction of nitrate is detected by the formation of a red diazonium compound when sulfanilic acid, α -naphthylamine, and nitrite react. The nitrite combines with

acetic acid to form nitrous acid which will diazotize sulfanilic acid to a diazonium salt. The dimethyl-naphthylamine couples with the diazo compound to form a red dye. Nitrogen gas is detected as gas trapped in a Durham vial inverted in the nitrate or nitrite broth (3).

The reduced nitrogen compounds are not detected directly but are inferred if there is no gas or nitrite but nitrate has been reduced. This is tested by adding zinc dust to the reaction tube to detect unreduced nitrate after the sulfanilic acid and α -naphthylamine have been added but did not produce the red color. The zinc dust

will reduce the remaining nitrate to nitrite and the red color develops. If nitrite is not present, because it has been previously reduced to nitrogen compounds, no red color develops after addition of zinc and the test is positive for both nitrate and nitrite reduction.

The test is useful in the evaluation of non-glucose-fermenting and fastidious gram-negative rods and in differentiating *Moraxella catarrhalis* from *Neisseria* (2). The test also confirms membership in the family *Enterobacteriaceae*. The disk test is used for anaerobic nitrate testing (see section 4).

II. MICROORGANISMS TESTED

- A. Gram-negative rods and gram-negative cocci (*Neisseria* spp.) as part of their identification (2, 4)
- B. Gram-positive, non-spore-forming bacilli that grow aerobically
- C. Anaerobic organisms

III. MEDIA, REAGENTS, AND SUPPLIES



Include QC information on reagent container and in QC records.

A. Media

1. Nitrate broth: store at 2 to 8°C.

peptone 20 g or
heart infusion broth for
fastidious organisms 25 g
potassium nitrate 2 g
distilled water 1,000 ml

Dispense 4-ml aliquots in 16- by 125-mm screw-cap tubes with Durham tube. Autoclave at 121°C for 15 min.

2. Nitrite broth: store at 2 to 8°C.

heart infusion broth 25 g
potassium nitrite 0.1 to 1 g
distilled water 1,000 ml

Dispense 4-ml aliquots in 16- by 125-mm screw-cap tubes with Dur-

ham tube. Autoclave at 121°C for 15 min.

3. Glucose cupule of the API 20E strip
4. Nitrate disk (available commercially) for anaerobic organisms
5. Nitrate tablets (Key Scientific) for both media and reagents

B. Reagents

1. 0.8% Sulfanilic acid (reagent A)

sulfanilic acid 0.8 g
distilled water 70 ml
glacial acetic acid 30 ml

- a. Mix sulfanilic acid with water; heat to dissolve.
- b. Cool, and then add acetic acid.

III. MEDIA, REAGENTS, AND SUPPLIES (continued)

- c. Store at 2 to 8°C.
 - d. Shelf life is 3 months.
 - 2. 0.5% *N,N*-Dimethyl- α -naphthylamine (reagent B)
glacial acetic acid 30 ml
distilled water 70 ml
N,N-dimethyl- α -naphthylamine 0.5 g
 - a. Combine acetic acid and water.
Add α -naphthylamine.
 - b. Store at 2 to 8°C.
 - c. Shelf life is 3 months.
- NOTE:** The purchase of this reagent is preferred to preparation in-house, since special precautions must be taken in handling concentrated *N,N*-dimethyl- α -naphthylamine, a potential carcinogen.
- 3. Zinc metal dust

IV. QUALITY CONTROL

- A. Examine tubes for contamination. Invert to remove bubbles in the Durham tube prior to storage of product.
- B. Test each new lot or shipment of media and reagents with a positively and negatively reacting organism prior to putting it into use.
NOTE: New lots of reagent can be checked without inoculation either with nitrate broth by adding zinc after the reagents are added or with nitrite broth directly. If the reagents give a red color, they are acceptable and have detected nitrite.
- C. Organisms
 - 1. *Escherichia coli* ATCC 25922—nitrate positive, gas negative
 - 2. *Pseudomonas aeruginosa* ATCC 27853—nitrate positive, gas positive
 - 3. *Acinetobacter baumannii* ATCC 19606—nitrate negative
 - 4. Alternative positive control: *Campylobacter jejuni* ATCC 33560—nitrate positive

V. PROCEDURE

- A. **Tube method**
 - 1. Before inoculating the broth, check the Durham tube for trapped air bubbles. If necessary, invert the tube until the air bubbles are gone.
 - 2. Inoculate the tube of nitrate broth from an isolated colony; a pure subculture or, preferably, 1 to 2 drops of an overnight broth culture of the organism. Subsequently, if nitrate has *not* been reduced, determine reduction of nitrite by inoculation of nitrite broth in the same manner.
 - 3. Incubation
 - a. Nonfermenting, gram-negative rods, 25 to 30°C
 - b. Other organisms, 35°C
 - c. Two to five days of incubation may be necessary for some organisms.
 - d. Incubate *Campylobacter* at 35°C in a microaerobic atmosphere for 72 h.
 - 4. Look for and record any gas in the Durham vial and growth in the tube. *Do not add reagents if there is no visible growth in the tube.*
 - 5. *If gas is present* and the organism does not ferment glucose in Kligler's iron agar or triple sugar iron agar, do not add reagents because the test is considered positive for nitrate reduction, nitrite reduction, and gas.
 - 6. If no gas is present *or* the organism is a glucose-fermenting, gram-negative rod (i.e., *Enterobacteriaceae*), remove approximately 0.5 ml of broth into a nonsterile 13- by 100-mm tube.
 - a. Add 2 or 3 drops of reagent A. Mix well by tapping or shaking tube.
 - b. Then, add 2 or 3 drops of reagent B. Mix again.
 - c. Look for a red color within 1 to 2 min.

V. PROCEDURE (continued)

7. If no red color is observed, add a small amount of zinc dust to the nitrate tube. *Do not add zinc dust to nitrite.*
 - a. The amount of zinc dust should not exceed that which adheres to the end of an applicator stick.
 - b. Examine for red color within 10 min.
 8. If nitrate or nitrite has not been reduced or there is no gas production, reincubate the remaining broth and retest at 48 h. Test nitrite broth additionally at 5 days.
- B. Disk method (used only for anaerobes)**
1. Make a fresh subculture of the organism to be tested onto a supportive agar and place a nitrate disk in the heavy inoculum area. Incubate anaerobically for 24 to 48 h.
 2. Remove the disk from the surface of the plate and place it in a clean petri dish or on a slide.
 3. Add 1 drop each of reagents A and B. If no color develops in a few minutes, drop a small amount of zinc dust onto the surface of the disk and observe for up to 5 min.

C. Rapid method

1. Prepare 0.5 ml of broth or use tablet (Key Scientific). Refer to <http://www.keysscientific.com> for instructions on tablet preparation and testing.
2. Inoculate *heavily* with organism.
3. Incubate for 2 h at 35°C.
4. Add reagents A and B and observe for red color.
5. Interpret as for standard test.

VI. INTERPRETATION

- NOTE:** If nitrate has been reduced, inoculation of nitrite broth is unnecessary.
- A. Gas bubbles (even a single gas bubble) in the Durham vial, except for glucose-fermenting, gram-negative rods (no reagent added)
 1. In nitrate tube: nitrate reduction positive, nitrite reduction positive, gas positive
 2. In nitrite tube: nitrite reduction positive, gas positive
 - B. No gas bubbles: gas negative; add reagents for evaluation of reduction.
 - C. Organisms that ferment glucose and non-glucose-fermenting, gram-negative rods that are gas negative
 1. In nitrate tube
 - a. Red color after the addition of reagents: nitrate reduction positive, nitrite reduction negative
 - b. No red color after the addition of reagents *plus* no red color after the addition of zinc to nitrate broth: nitrate reduction positive, nitrite reduction positive
 2. In nitrite tube—no color development after addition of reagents: nitrite reduction positive
 - D. Negative results (final reading at ≥ 48 h)
 1. In nitrate broth—no color development after adding reagents and red color development after adding zinc (zinc catalyzes the change from nitrate to nitrite): nitrate reduction negative
 2. In nitrite broth—a red color after addition of reagents: nitrite reduction negative

VII. REPORTING RESULTS

- A. Report as nitrate reduction positive or negative and nitrite reduction positive or negative.
 - B. Report the production of gas for non-glucose-fermenting rods.
 - C. Use the results in the determination of the species, using charts in procedures 3.18.1 and 3.18.2.
 - D. EF-4 is among the few unique microorganisms that reduce nitrate and nitrite, but no gas is produced (4).
 - E. Among the gram-negative diplococci that infect humans, only *Neisseria mucosa* and *M. catarrhalis* are able to reduce nitrate (2).
-

VIII. LIMITATIONS

- A. Gas production in *Enterobacteriaceae* is not due to nitrate reduction but is hydrogen gas.
 - B. Failure to recognize that the organism did not grow in the medium will result in possible false-negative test(s).
 - C. Interpretation of color reactions should be made immediately, as color reactions with a positive test may fade rapidly.
 - D. Failure to remove air bubbles prior to inoculation may result in reading the result as a false-positive reaction for gas reduction.
 - E. A faint pink color may be produced following addition of the nitrate reagents. This is not a positive result.
 - F. A negative zinc reduction (no color change) test, in combination with a negative nitrite reaction, is presumptive indication that the nitrate was reduced beyond the nitrite stage. Although a very common end product of nitrite reduction is nitrogen gas, other end products may be formed.
 - G. Excess zinc dust has been reported to cause false-positive nitrite reduction reactions due to complete reduction of previously unreduced nitrate to ammonia.
 - H. Most positive reactions are detected in 12 to 24 h; however, rare isolates may take up to 5 days (1).
-

REFERENCES

1. Cowan, S. T. 1974. *Cowan & Steels Manual for the Identification of Medical Bacteria*, 2nd ed., p. 38–39, 167. Cambridge University Press, Cambridge, United Kingdom.
2. Janda, W. M., and J. S. Knapp. 2003. *Neisseria* and *Moraxella catarrhalis*, p. 585–608. In P. R. Murray, E. J. Baron, J. H. Jorgensen, M. A. Pfaffer, and R. H. Yolken (ed.), *Manual of Clinical Microbiology*, 8th ed. ASM Press, Washington, D.C.
3. MacFaddin, J. F. 2000. *Biochemical Tests for the Identification of Medical Bacteria*, 3rd ed., p. 348–358. The Lippincott, Williams & Wilkins Co., Philadelphia, Pa.
4. Weyant, R. S., C. W. Moss, R. E. Weaver, D. G. Hollis, J. G. Jordan, E. C. Cook, and M. I. Daneshvar. 1995. *Identification of Unusual Pathogenic Gram-Negative Aerobic and Facultatively Anaerobic Bacteria*, 2nd ed., p. 14–15. Williams & Wilkins, Baltimore, Md.

3.17.36

O/129 Disk Susceptibility Testing for *Vibrio* and *Aeromonas* spp.

I. PRINCIPLE

Disk containing 10 and 150 µg of O/129, the vibriostatic agent 2,4-diamino-6,7-diisopropylpteridine phosphate, are used in a method resembling disk susceptibility testing to differentiate *Vibrio* spp. from *Aeromonas* spp. (3, 4, 5). *Aeromonas* species are resistant, with no zone of inhibi-

tion at 24 h, with both disks. *Vibrio* and *Plesiomonas* species will show susceptibility, with a distinct zone of inhibition with the 150-µg disk; results for the 10-µg disk will vary among the *Vibrio* and *Plesiomonas* species.

Some *Vibrio* spp. require salt for growth. Therefore, the test is run in duplicate on Mueller-Hinton media (MH) with low salt (approximately 0.5%) and with added NaCl (4%) to ensure growth on at least one of the plates.

II. MICROORGANISMS TESTED

- A. Gram-negative rods which are oxidase positive, indole positive, and nonpigmented and grow on MAC
- B. Microorganisms identified by kits as either *Vibrio* or *Aeromonas* species
- C. *Corynebacterium*, to separate *Corynebacterium amycolatum* from *Corynebacterium xerosis*

III. MEDIA, REAGENTS, AND SUPPLIES



Include QC information on reagent container and in QC records.

- A. **O/129 disks**
 - 1. Purchase (Oxoid, Inc.; Hardy Diagnostics) or
 - 2. Prepare in-house.
 - a. Using sterile technique, dissolve 30 mg of O/129 (2,4-diamino-6,7-diisopropylpteridine phosphate, Sigma Chemical Company, St. Louis, Mo.) in a small amount of sterile deionized water.
 - b. Add sufficient water to make a total of 4.0 ml at 7.5 mg/ml. This is solution A.
 - c. Dilute this 1:15 (add 0.5 ml of stock solution to 7.0 ml of H₂O) to make solution B of 0.5 mg/ml.
 - d. Lay out blank 1/4-in. paper disks (6 mm) (BD Diagnostic Systems, Hardy Diagnostics) in an empty petri dish.
 - e. Pipette 20 µl of solution A onto each disks to produce 150-µg disks.
 - f. Pipette 20 µl of solution B onto each disk to produce 10-µg disks.
 - g. Place petri dishes with lids slightly ajar at 35°C until disks are dry, 10 to 30 min.
 - h. Return disks to blank vials and close tops tightly.
 - i. Label each vial with type of content (10-µg O/129 disks or 150-µg O/129 disks).
 - j. Expiration date is set at 1 year from date of preparation but can be extended if the zone sizes of controls remain within a 5-mm range over time.
 - k. Place each vial in a separate container half-filled with desiccant.
 - l. Label this container similarly.
 - m. Store at -20°C.
 - B. **Media**
 - 1. MH
 - 2. MH with 4% NaCl (sold by most vendors for oxacillin screening)
 - C. **Supplies**
 - 1. Swabs
 - 2. Broth for inoculum

IV. QUALITY CONTROL

- A.** Perform QC on each new lot of disks and with each use with both an organism that is susceptible and one that is resistant to O/129.
B. Organisms

Test organism	MH		MH with 4% salt	
	10 µg	150 µg	10 µg	150 µg
<i>Aeromonas hydrophila</i> ATCC 7966	6 mm	6 mm	NA	NA
<i>Vibrio fluvialis</i> ATCC 33809	NA ^a	NA	7–15 mm	≥20 mm

^a NA, not applicable because the organism will not grow on the medium.

V. PROCEDURE

- A.** Remove disks from freezer and equilibrate to room temperature.
- B.** Allow test media (MH and MH with 4% NaCl) to equilibrate to room temperature. If the plates have excess moisture, dry them for 10 to 30 min at 35°C with lids slightly ajar.
- C.** Suspend an overnight growth of the organism and each of the controls in TSB to a turbidity of a no. 0.5 McFarland standard.
- D.** Divide each plate in half with a marker.
- E.** Using a swab dipped into the broth, inoculate the surface of one-half of each plate with organism suspension in three planes as for antimicrobial susceptibility disk testing.
- F.** In the same manner, inoculate the other half of the MH plate with 4% salt with the *Vibrio* control and the MH plate without salt with the *Aeromonas* control.
- G.** Allow plates to dry for 3 to 5 min but no longer than 15 min.
- H.** Place a 10-µg O/129 disk and a 150-µg O/129 disk on each half of the two plates in well-separated locations. Add antimicrobial susceptibility disks, if desired.
- I.** Using sterile forceps, press each disk gently onto the agar surface to ensure good contact.
- J.** Incubate for 18 to 24 h at 35°C in a non-CO₂ incubator.
- K.** Read zone of inhibition on plate with best growth and most clear-cut zone(s).

VI. INTERPRETATION

- A.** The organism is O/129 resistant if there is no zone of inhibition around the respective disk.
- B.** The organism is O/129 susceptible if there is any zone around the respective disk.
- C.** The organism is generally salt requiring if it does not grow on MH.
- D.** The organism is salt tolerant or enhanced if it grows on MH with 4% salt.
- E.** The organism is inhibited by salt if it does not grow on MH with 4% salt.

VII. REPORTING RESULTS

- A.** Gram-negative rods that are oxidase positive, grow on MAC, and ferment glucose (most are indole positive) are reported as follows.
 - 1.** *Aeromonas*, if resistant to 150 µg of O/129, grows without salt, and is arginine positive. Strains are also usually DNase positive.
 - a.** *Aeromonas veronii* bv. Veronii is arginine negative.
 - b.** *Vibrio cholerae* is arginine negative, and some strains are resistant to O/129 and may not grow on media with 4% salt (1). Perform further testing on strains with these characteristics, or submit to reference laboratory to separate *V. cholerae* from *A. veronii* bv. Veronii.
 - c.** *Vibrio fluvialis* is arginine positive and may be resistant to 150 µg of O/129, but it usually grows only with 4% salt.

VII. REPORTING RESULTS

(continued)

2. *Plesiomonas shigelloides*, if susceptible to 150 µg of O/129 and positive for indole, arginine dihydrolase, and ornithine and lysine decarboxylases. *P. shigelloides* is also inhibited on 4% salt agar and is DNase negative.
 3. *Vibrio* spp., if susceptible to 150 µg of O/129 even if they do *not* grow on MH with 4% salt but are either negative for arginine dihydrolase or negative for ornithine and lysine decarboxylases.
- NOTE:** Some vibrios may be resistant to 150 µg of O/129, but they will be arginine negative.
- B. Identify *Vibrio* to the species level with commercial kit using saline diluent.
 1. If isolate is resistant to 10 µg of O/129, it is likely to be *V. fluvialis*, *Vibrio alginolyticus*, or *Vibrio parahaemolyticus*, the most common isolates.
 2. If isolate is susceptible to 10 µg of O/129, it is likely to be *Vibrio vulnificus*.
 - C. Unless the organism identification from the kit agrees with the results of the O/129 and salt tolerance tests, submit to a reference laboratory for further identification. See Fig. 3.8.1–1 in procedure 3.8.1 for flowchart.
 - D. Submit *V. cholerae* and possibly *V. vulnificus* to the local health department, in accordance with local health department policies, or to a reference laboratory for confirmation of toxicity and identification.
 - E. *C. amycolatum* is usually resistant to 150 µg of O/129, with no zone of inhibition, while *C. xerosis*, *Corynebacterium minutissimum*, and *Corynebacterium striatum* are susceptible.

VIII. LIMITATIONS

- A. Kits can misidentify *V. vulnificus* as *V. parahaemolyticus*, *Aeromonas* as *V. fluvialis*, and *Vibrio damselae* as *Vibrio cholerae* (2). Use both O/129 disks and growth with salt testing to prevent initial misidentifications and potential public health consequences.
- B. *V. cholerae* strains that are resistant to O/129 have been reported, which is probably related to antimicrobial therapy (1).
- C. The 10-µg disk is not as important in the identification as the 150-µg disk and may be omitted.
- D. Some vibrios do not grow on MH with 4% salt, and some grow without added salt. All *Aeromonas* organisms grow without salt but may grow on medium with salt.

REFERENCES

1. Abbott, S. L., W. K. Cheung, B. A. Portoni, and J. M. Janda. 1992. Isolation of vibrio-static agent O/129-resistant *Vibrio cholerae* non-O1 from a patient with gastroenteritis. *J. Clin. Microbiol.* **30**:1598–1599.
2. Abbott, S. L., L. S. Seli, M. Catino, Jr., M. A. Hartley, and J. M. Janda. 1998. Misidentification of unusual *Aeromonas* species as members of the genus *Vibrio*: a continuing problem. *J. Clin. Microbiol.* **36**:1103–1104.
3. Furniss, A. L., J. V. Lee, and T. J. Donovan. 1978. *The Vibrios*. Public Health Laboratory Service monograph ser. no. 11. Her Majesty's Stationery Office, London, United Kingdom.
4. Janda, J. M., C. Powers, R. G. Bryant, and S. L. Abbott. 1988. Current perspectives on the epidemiology and pathogenesis of clinically significant *Vibrio* spp. *Clin. Microbiol. Rev.* **1**:245–267.
5. Janda, J. M. 1991. Recent advances in the study of the taxonomy, pathogenicity, and infectious syndromes associated with the genus *Aeromonas*. *Clin. Microbiol. Rev.* **4**:397–410.

ONPG (*o*-Nitrophenyl- β -D-Galactopyranoside) Test

[Updated March 2007]

I. PRINCIPLE

o-Nitrophenyl- β -D-galactopyranoside (ONPG) is used in differentiating members of the *Enterobacteriaceae* and *Neisseria* based on β -galactosidase activity. The ability of a bacterium to ferment lactose depends on two enzymes, permease and β -galactosidase. Permease allows lactose to enter the bacterial cell wall, where it is then broken down into glucose and galactose by β -galactosidase. The glucose and galactose can then be metabolized by the bacteria. The enzymes are inducible

and are only present when lactose, rather than glucose, is available to the organism for metabolism. Some organisms lack permease and appear as late lactose fermenters or non-lactose fermenters. The ONPG test will detect true lactose fermenters that have the β -galactosidase enzyme, even if they lack the permease enzyme. A lactose fermentation test will not detect organisms lacking the permease.

ONPG is a colorless substrate, similar in structure to lactose, used in this test as

the substrate for β -galactosidase. If the organism possesses β -galactosidase, the enzyme will split the β -galactoside bond, releasing galactose and *o*-nitrophenol, which is a yellow compound. The activity of the galactosidase enzyme is increased in the presence of sodium ions (1). Other substrates can be used to detect β -galactosidase and are found in the commercial identification kits, especially those used to identify *Neisseria* species.

II. MICROORGANISMS TESTED

- A. Gram-negative rods growing aerobically
- B. Gram-negative diplococci growing aerobically

III. MATERIALS, REAGENTS, AND SUPPLIES

- | |
|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| <ul style="list-style-type: none">A. Reagents<ul style="list-style-type: none">1. Because of the instability of ONPG, the reagent should not be prepared but purchased.2. Purchase ONPG as broth, disks, or tablets.<ul style="list-style-type: none">a. Store as directed by the manufacturer. Usually store broth at less than -10°C and disks and tablets at 4°C.b. Store away from direct light; ONPG is <i>light sensitive</i>.B. Supplies/equipment<ul style="list-style-type: none">1. Plastic or glass tubes for disk test2. Wooden sticks or inoculating loops3. Sterile saline4. Incubator at 35 to 37°C |
|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|

IV. QUALITY CONTROL

- | |
|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| <ul style="list-style-type: none">A. Do not use if broth media or disk, after reconstitution, is not light amber and clear, with no precipitates.B. Perform QC on each new lot or shipment prior to putting it into use.C. Organisms<ul style="list-style-type: none">1. <i>Escherichia coli</i> ATCC 25922—ONPG positive2. <i>Proteus mirabilis</i> ATCC 12453—ONPG negative or3. <i>Neisseria lactamica</i> ATCC 23971—ONPG positive4. <i>Neisseria gonorrhoeae</i> ATCC 43069—ONPG negative |
|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|

V. PROCEDURE**A. Tube medium**

1. Bring test medium to room temperature.
2. Using a heavy inoculum (no. 2 McFarland) from a pure 18- to 24-h culture, inoculate the test medium.
3. Incubate aerobically at 35°C.
4. Examine for yellow color development at 1 h.
5. If the tube has not changed color after 1 h of incubation or if the colony was not taken from lactose-containing medium, continue incubation up to 24 h.

B. Disk test

1. Inoculate colonies into 0.5 ml of saline to produce a heavy suspension (no. 2 McFarland).
2. Add disk to tube.
3. Incubate aerobically at 35°C for up to 6 h.
4. Observe for yellow color change.

VI. INTERPRETATION

A. A yellow color change is a positive test for ONPG.

B. A lack of color change is a negative test.

VII. REPORTING RESULTS

A. An organism that produces a yellow color is considered ONPG positive and generally a lactose fermenter.

B. An organism that does not produce a yellow color is considered ONPG negative and non-lactose fermenting.

C. Among the *Neisseria* spp., *N. lactamica* is identified by its ONPG-positive reaction (2).

VIII. LIMITATIONS

A. For the rapid test, colonies should be from a lactose-containing medium (e.g., triple sugar iron agar or MAC).

B. Cultures that naturally produce a yellow pigment cannot be tested with this medium.

C. If medium is not properly buffered, results may be inaccurate.

D. Do not use if medium is yellow.

E. Since glucose inhibits lactose fermentation by bacteria, organisms growing on glucose-containing medium show less activity than they would in the presence of lactose.

REFERENCES

1. Negut, M., and G. Hermann. 1975. A comparison of two methods for detecting β-D-galactosidase. *Public Health Lab.* **33**:190–193.
2. Weyant, R. S., C. W. Moss, R. E. Weaver, D. G. Hollis, J. G. Jordan, E. C. Cook, and M. I. Daneshvar. 1995. *Identification of Unusual Pathogenic Gram-Negative Aerobic and Facultatively Anaerobic Bacteria*, 2nd ed., p. 16. Williams & Wilkins, Baltimore, Md.

Optochin Susceptibility Test

[Updated March 2007]

I. PRINCIPLE

Streptococcus pneumoniae is found commonly in the human respiratory tract, as are other streptococci, and has a hemolytic pattern indistinguishable from that of other alpha-hemolytic streptococci and lactobacilli. Optochin susceptibility is used to differentiate *S. pneumoniae* from other alpha-hemolytic streptococci, either as isolated colonies or as a test to recog-

nize *S. pneumoniae* colonies directly on cultures of respiratory specimens.

Sensitivity to optochin (ethylhydrocupreine hydrochloride) has been well-established for *S. pneumoniae* since the early 20th century (5). In 1955 Bowen and Jeffries impregnated disks with the reagent to demonstrate the susceptibility of the pneumococcus for identification purposes (2).

A positive identification in an organism with Gram stain and colony morphology consistent with *S. pneumoniae* is made when a well-defined zone of inhibition forms around the impregnated disk. Other alpha-hemolytic streptococci do not display this clear zone of inhibition in the presence of optochin.

II. MICROORGANISMS TESTED

- A. Test any fresh culture of alpha-hemolytic, catalase-negative, gram-positive cocci in pairs, growing on BAP or Columbia colistin-nalidixic acid agar (CNA) and having the characteristic central depression (flattened center) or mucoid colony morphology suggestive of *S. pneumoniae*.
- B. As a rapid detection and identification method, place the disks on plates inoculated with either respiratory specimens or subcultures from blood cultures. However, results for disks placed on plates directly inoculated with respiratory specimens can be very misleading unless a Gram-stained smear indicates an almost singular presence of lanceolate gram-positive diplococci.
- C. As a test of purity of culture, add the disk for any known *S. pneumoniae* isolate that is being tested for antimicrobial resistance.

III. REAGENTS AND SUPPLIES

A. Reagents

- 1. Optochin disks—purchase from most vendors.
 - a. Each disk is impregnated with 5 µg of optochin.
 - b. Store stock at 2 to 8°C. Protect from light, excessive heat, and moisture.
 - c. The expiration date is at least 9 months (2) but may be considerably longer.

- 2. BAP—use only 5% sheep blood agar for identification test.

B. Supplies/equipment

- 1. Standard microbiological loops
- 2. CO₂ incubator at 35 to 37°C
- 3. Sterile forceps

IV. QUALITY CONTROL

- A. Perform QC with known positive and negative controls prior to use of new lots or shipments and, optionally, at weekly intervals.
 - B. Organisms and zone sizes
 - 1. *S. pneumoniae* ATCC 49619, ≥ 14 mm
 - 2. *Enterococcus faecalis* ATCC 29212, no zone
- NOTE:** For a 10-mm disk, use ≥ 16 mm as the breakpoint for susceptibility. Compare weekly zone size results and if the size is decreasing, open a new vial, as the current supply in use may be deteriorating even before the expiration date.

V. PROCEDURE

- A. Using an inoculating loop, select a well-isolated colony of the alpha-hemolytic organism to be tested. Alternatively, a broth culture with a turbidity corresponding to a 0.5 McFarland standard can be used (3).
- B. Using a loop from a colony, or swab from a broth culture, streak the isolate onto BAP in at least two directions so as to obtain confluent growth. Several isolates may be placed on one plate by dividing the plate into quadrants and streaking one isolate per quadrant.
 - NOTE:** Use of media other than 5% sheep blood agar is not recommended, as smaller zone sizes can result in lack of definitive identification (3). However, when performing a susceptibility test for purposes of detection of contamination of a culture of known *S. pneumoniae*, the Mueller-Hinton blood agar used for the susceptibility testing can be used without strict measurement of the zone size.
- C. Using sterile forceps, place an optochin disk onto the inoculated surface of the agar.
- D. Press disk gently with the sterile forceps or loop so that the disk adheres firmly to the agar surface.
- E. Incubate the plate at 35 to 37°C for 18 to 24 h in 5 to 10% CO₂.
- F. If zone of inhibition is present, measure the diameter with a millimeter ruler or caliper.

VI. INTERPRETATION

- A. A zone of inhibition of ≥ 14 mm (15 to 30 mm) around the disk is considered a susceptible result.
- B. Organisms with zone sizes of < 14 mm around the disk should be considered intermediate in susceptibility to optochin.
- C. If there is no zone around the disk, the organism is considered resistant to optochin.
- D. Colonies *within* the zone may or may not be *S. pneumoniae*, since this phenomenon can occur with *S. pneumoniae* (7).

VII. REPORTING RESULTS

- A. Report an identification of *S. pneumoniae*, if the alpha-hemolytic colony from gram-positive cocci in pairs is catalase negative and susceptible to optochin.
- B. For any alpha-hemolytic colonies from gram-positive cocci in pairs that are catalase negative but produce zone sizes with *intermediate* results, perform spot bile solubility test for confirmation of identification. Report as *Streptococcus pneumoniae* if positive.
- C. If the organism is optochin resistant and is a catalase-negative, alpha-hemolytic colony from gram-positive cocci in pairs, report as a viridans group streptococcus.
- D. For purposes of determination of contamination of a susceptibility test, any zone of inhibition of ≥ 10 mm indicates lack of contamination.

VIII. LIMITATIONS

- A. *S. pneumoniae* isolates should be incubated in a CO₂-enriched environment, as some isolates will grow poorly or not at all without increased CO₂ (3, 8).
- B. If the organism is optochin resistant, it is likely to be a nonpneumococcal alpha-hemolytic streptococcus; however, rare exceptions have been reported (1, 7).
- C. Optochin susceptibility is an excellent test to identify *S. pneumoniae*, with a 99% sensitivity for *encapsulated* strains and 98 to 99% specificity (4, 6). However, Mundy et al. (6) reported that only 10 of 33 nonencapsulated strains positive by DNA probe were detected as susceptible by optochin; the remainder had intermediate zone diameters with optochin.
- D. If the tube bile test is performed to confirm strains with zone of inhibition between 7 and 14 mm, the sensitivity in detection of nonencapsulated strains is increased; however, bile-resistant strains with intermediate zone sizes may still be *S. pneumoniae*. Further testing with a DNA probe (GenProbe, Inc.) is needed for definitive identification (6). Since most (85%) will *not* be *S. pneumoniae* and most nonencapsulated strains are from noninvasive sites, this approach may not be cost-effective (3).
- E. If there are colonies present within the zone of inhibition, these colonies may or may not be pneumococci. Pikis et al. (7) reported that resistant strains may exhibit this phenomenon; however, it is rare. True optochin-resistant strains have only been reported for three cases (7). The resistance was shown to be a point mutation in strains from patients that had been treated with antimicrobial agents (7). Subculture such colonies and confirm with Gram stain, catalase test, and bile solubility to determine if they are contaminants or pneumococci prior to reporting susceptibility results.

REFERENCES

1. Borek, A. P., D. C. Dressel, J. Hussong, and L. R. Peterson. 1997. Evolving clinical problems with *Streptococcus pneumoniae*: increasing resistance to antimicrobial agents, and failure of traditional optochin identification in Chicago, Illinois, between 1993 and 1996. *Diagn. Microbiol. Infect. Dis.* **29**:209–214.
2. Bowen, E. F., and L. R. Jeffries. 1955. Optochin in the identification of *Str. pneumoniae*. *J. Clin. Pathol.* **8**:58–60.
3. Gardam, M. A., and M. A. Miller. 1998. Optochin revisited: defining the optimal type of blood agar for presumptive identification of *Streptococcus pneumoniae*. *J. Clin. Microbiol.* **36**:833–834.
4. Kellogg, J. A., D. A. Bankert, C. J. Elder, J. L. Gibbs, and M. C. Smith. 2001. Identification of *Streptococcus pneumoniae* revisited. *J. Clin. Microbiol.* **39**:3373–3375.
5. Moore, H. F. 1915. The action of ethylhydrocupreine (optochin) on type strains of pneumococci *in vitro* and *in vivo*, and on some other microorganisms *in vitro*. *J. Exp. Med.* **22**:269–285.
6. Mundy, L. S., E. N. Janoff, K. E. Schwebke, C. J. Shanholtzer, and K. E. Willard. 1998. Ambiguity in the identification of *Streptococcus pneumoniae*. Optochin, bile solubility, quellung, and the AccuProbe DNA probe tests. *Am. J. Clin. Pathol.* **109**:55–61.
7. Pikis, A., J. M. Campos, W. J. Rodriguez, and J. M. Keith. 2001. Optochin resistance in *Streptococcus pneumoniae*: mechanism, significance, and clinical implications. *J. Infect. Dis.* **184**:582–590.
8. Ragsdale, A. R., and J. P. Sanford. 1971. Interfering effect of incubation in carbon dioxide on the identification of pneumococci by optochin discs. *Appl. Microbiol.* **22**:854–855.

Oxidase Test

[Updated March 2007]

I. PRINCIPLE

In the presence of atmospheric oxygen, a bacterium's intracellular cytochrome oxidase enzymes oxidize the phenylenediamine reagent (an electron acceptor) to form a deep purple compound, indol phenol (3). The test is useful in the initial characterization of gram-negative bacteria (2).

II. MICROORGANISMS TESTED

- A. Aerobic, facultatively anaerobic, or microaerobic gram-negative rods and cocci from BAP, Mueller-Hinton agar, brucella agar, BHI, or CHOC
- B. Restrictions
 - 1. Do not use media with dyes, such as EMB or MAC.
 - 2. Do not test strictly anaerobic organisms.
 - 3. Do not test organisms growing on media that contain glucose.

III. REAGENTS AND SUPPLIES

- A. Reagents
 - 1. Kovács' reagent
 - NOTE: Other formulas exist, but Kovács' is the most sensitive reagent.
 - a. For 0.5 to 1% solution, dissolve 0.1 g (size of a pea) *N,N,N,N*-tetramethyl-*p*-phenylenediamine dihydrochloride in 10 ml of sterile distilled water.
 - b. Mix well and allow to sit for 15 min.
 - c. Make fresh daily (preferred method), or store aliquots of the reagent in foil-wrapped test tubes at -20°C. Remove from the freezer and thaw before use.
 - d. Discard unused portion daily.
 - 2. Dried filter paper disks or strips impregnated with reagent
 - 3. Disposable glass ampoules (available from most vendors)
 - B. Supplies
 - 1. Filter paper or swab
 - 2. Disposable petri dish
 - 3. Sterile wooden sticks or plastic or platinum loops or wires
 - 4. Deionized water

IV. QUALITY CONTROL

- A. Do not use once the pale purple color of the reagent or the filter paper begins to darken.
 - B. Perform QC on each new lot of powdered reagent prior to putting it into use.
 - C. Organisms
 - 1. *Pseudomonas aeruginosa* ATCC 27853—oxidase positive
 - 2. *Escherichia coli* ATCC 25922—oxidase negative
-

V. PROCEDURE**A. Filter paper method**

- 1. Optional preparations
 - a. Place a small square of Whatman no. 1 filter paper in a petri dish and moisten with 1 or 2 drops of prepared Kovács' oxidase reagent *or*
 - b. Place impregnated dry disk or strip in petri dish and moisten with deionized water.
 - **NOTE:** Some manufacturers may not require that strip be moistened.
 - 2. Optional methods of colony testing
 - a. Pick an isolated colony with a stick and smear onto Kovács' reagent-dampened filter paper. Observe paper for purple color.
 - b. For fastidious bacteria, swipe colony onto white cotton swab and rub onto the dampened filter paper. Observe *swab* for purple color.
 - c. Touch moistened filter paper to colony and observe paper for purple color.
-

B. Plate method

- 1. Drop a few drops of reagent directly on top of a few suspected colonies. Do not flood entire plate because bacteria covered by the reagent generally are not viable for subculture.
 - 2. Expose the colonies to air by tilting the culture after flooding with oxidase reagent to allow oxygen to reach the colonies.
 - 3. Observe colony for purple color. Ignore any discoloration of surrounding medium.
 - **NOTE:** Colonies tested by this method are quickly nonviable. Subculture immediately.
-

VI. INTERPRETATION**A. Positive test**

- 1. Development of a deep blue to purple color in 10 to 30 s is a positive reaction.
- 2. Development of the color in 30 to 60 s is a weak positive reaction, characteristic of many *Pasteurella* spp.
- 3. Do not read after 60 s.

B. Negative test is no color change in 60 s.

VII. REPORTING RESULTS

- A. To avoid misidentifications, perform oxidase test on *all* gram-negative rods, except those that swarm.
 ■ **NOTE:** This test is especially important in separating *Aeromonas* from *Enterobacteriaceae*. Most commercial kits do not include an oxidase test as part of their biochemical reactions but require the user to record the result as part of their identification scheme. If the test is omitted, errors in identification can be made. In addition, oxidase testing can aid in rapid identifications, avoiding the need for costlier kit identifications (4).
- B. Gram-negative diplococci should give a positive reaction, since all members of the genus *Neisseria* are oxidase positive. *Moraxella* spp. which are either gram-negative diplococci or coccobacilli are also oxidase positive.

VII. REPORTING RESULTS (continued)

- C. Gram-negative rods that are oxidase positive do not belong to the *Enterobacteriaceae*, with the exception of *Plesiomonas shigelloides*, which is both oxidase and indole positive (see Table 3.18.2–8).
- D. *Campylobacter* spp. are oxidase positive.
- E. Use this test as a major characteristic for identification of gram-negative rods that are not in the *Enterobacteriaceae* family.

VIII. LIMITATIONS

- A. To avoid false-positive results
 - 1. Do not use a Nichrome wire to pick colony.
 - 2. Do not test organisms growing on media that contain glucose or dyes (e.g., MAC or EMB).
 - 3. Do not use if reagent or filter paper is purple.
- B. Mixed cultures of *Neisseria* and pseudomonads can give false-negative results, since the pseudomonads can elaborate an inhibitory substance that interferes with the production of oxidase by *Neisseria*.
- C. Timing is critical to accurate testing.
- D. Dry filter paper may be rewet with reagent for further use until it is purple in color.
- E. Some organisms may require subculture to produce a positive reaction.
- F. The modified oxidase test (Microdase disks; Remel, Inc.) to detect cytochrome *c* is one of several tests that can be used to separate *Micrococcus* (positive) from staphylococci (negative). The test is performed in the same manner as the oxidase test, except that the reagent is 6% tetramethylphenylenediamine hydrochloride in dimethyl sulfoxide (1).

REFERENCES

1. Faller, A., and K. H. Schleifer. 1981. Modified oxidase and benzidine tests for separation of staphylococci from micrococci. *J. Clin. Microbiol.* **13**:1031–1035.
2. Kovács, N. 1956. Identification of *Pseudomonas pyocyanea* by the oxidase reaction. *Nature* **178**:703.
3. MacFaddin, J. F. 2000. *Biochemical Tests for the Identification of Medical Bacteria*, 3rd ed., p. 368–378. The Lippincott, Williams & Wilkins Co., Philadelphia, Pa.
4. NCCLS. 2002. *Abbreviated Identification of Bacteria and Yeast*. Approved guideline M35-A. NCCLS, Wayne, Pa.

3.17.40

Phenylalanine Deaminase Test

[Updated March 2007]

I. PRINCIPLE

The phenylalanine deaminase (PDA) test is used to differentiate among the urea-positive gram-negative bacilli based on the ability of the microorganisms to produce phenylpyruvic acid by oxidative deamination. Phenylalanine is an amino acid that, upon deamination by oxidase enzymes, results in the formation of phenylpyruvic acid. The deamination of phenylalanine to phenylpyruvic acid is detected by the addition of a ferric chloride solution that acts as a chelating agent with the α -keto acid by-product to pro-

duce a light to deep green cyclic compound.

Hendriksen, in 1950, demonstrated that *Proteus* spp. were able to convert phenylalanine to phenylpyruvic acid (3). This observation was incorporated into a medium by Ewing et al. (2) and into a disk along with urea by Ederer et al. (1). Of the *Enterobacteriaceae* that are urea positive, only members of the *Proteus*, *Providencia*, and *Morganella* group are capable of deaminating phenylalanine (2). The test

can also be used to identify other *Enterobacteriaceae*, *Buttiauxella*, *Rahnella*, and *Tatumella*, which are PDA positive but are urea negative.

Tryptophan can be substituted for phenylalanine; tryptophan deamination releases indole-pyruvic acid, which results in a purple to black color with the addition of ferric chloride. Either tryptophan or phenylalanine can be used to differentiate among the *Proteus* group of gram-negative rods.

II. MICROORGANISMS TESTED

- A. Gram-negative rods that grow well on MAC, are oxidase negative, and are usually urea positive
- B. This test can be used as part of screening for fecal pathogens to rule out *Proteus*, *Providencia*, and *Morganella*.
- C. Oxidase-positive, gram-negative coccobacilli and rods that are urea positive, to separate *Oligella* spp. and *Psychrobacter phenylpyruvicus* and others from PDA-negative strains

III. MEDIA, REAGENT, AND SUPPLIES



Include QC information on reagent container and in QC records.

A. Media

- 1. Phenylalanine agar slants (available from most medium vendors)
 - a. Contain L-phenylalanine and yeast extract in a buffered agar
 - b. Store at 15 to 30°C. Shelf life, 1 year
- 2. Rapid tests
 - a. Urea-PDA disks (Hardy Diagnostics; Remel, Inc.; or prepare in-house [see reference 1])
 - b. Phenylalanine tablets (Key Scientific)
 - c. Indole-IPA tablets containing tryptophan (Key Scientific)

- d. ONGP-PDA broth (Hardy Diagnostics)—store frozen at less than -10°C.

■ NOTE: Commercial multitest systems often include a substrate to detect either PDA or tryptophan deaminase (TDA).

B. Reagent

- 10% Ferric chloride, acidified
- 1. Dissolve 12 g of ferric chloride in 97.5 ml of water.
- 2. Slowly add 2.5 ml of concentrated HCl in a fume hood.
- 3. Store in brown bottle at 4°C.

III. MEDIA, REAGENT, AND SUPPLIES (continued)

4. For in-house-prepared reagent, test monthly and discard when reaction is weak.

NOTE: Acidified ferric chloride is recommended, but 10% aqueous ferric chloride (10 g in 100 ml of deionized water) can be used (4).

C. Supplies/equipment

1. Sterile wooden sticks or inoculating loops
2. Saline or water in small plastic tube for disk test
3. Incubator at 35 to 37°C

IV. QUALITY CONTROL

- A. Examine phenylalanine agar to be sure that it is slightly opalescent and light amber. Check for signs of prior freezing, contamination, cracks, dehydration, and deterioration.

- B. Perform QC on each new lot or shipment of medium, disks, and ferric chloride prior to use with one organism known to demonstrate a positive reaction and one organism known to give a negative reaction.

C. Organisms

1. *Proteus mirabilis* ATCC 12453—turns green after the addition of 4 or 5 drops of ferric chloride with agitation; may take 1 to 5 min (positive)
2. *Escherichia coli* ATCC 25922—remains yellow after addition of ferric chloride (negative)

V. PROCEDURE

A. Agar test

1. Prior to inoculation, allow the medium to equilibrate to room temperature.
2. Using a heavy inoculum from an 18- to 24-h pure culture, streak the slant surface using a fishtail motion.
3. Incubate the inoculated slant aerobically at 35°C for 18 to 24 h. If a heavy inoculum is used, incubate for 4 to 6 h.
4. Following incubation, apply 4 or 5 drops of ferric chloride directly to the slant.
5. Gently roll the reagent over the slant to dislodge surface colonies, and observe for the development of a green color within 1 to 5 min.

B. Rapid tests

Check the package insert for differences from procedure below.

1. Prepare a small test tube with 0.25 ml (5 drops) of saline or water.
- NOTE:** Preferably use plastic tubes for disk tests.
2. Make a heavy suspension of the actively growing organism.
3. Add disk (tablets are already in tube).
4. Incubate aerobically at 37°C for 1 h or up to 2 h for the urea-PDA test.
5. If urea is present in disk, observe for pink color indicative of a positive urea reaction. Add 2 drops of 1 N HCl to acidify the alkaline urea reaction (optional).
6. Add 2 drops of 10% ferric chloride, shake, and observe for green color.

VI. INTERPRETATION

- A. A positive phenylalanine deamination reaction is indicated by the development of a light to dark green color (PDA) or purple to black color (TDA) within 1 to 5 min after applying ferric chloride reagent.
- B. A negative test is indicated by the absence of a green color reaction. Negative results will take on a yellow color due to the color of the ferric chloride.

VII. REPORTING RESULTS

- A. A positive or negative PDA test is only one test in the identification of urea-positive *Proteus*, *Providencia*, and *Morganella*.
 - B. If the PDA test is used as a screening test, PDA-positive *Enterobacteriaceae* can be eliminated as fecal pathogens.
 - C. A positive PDA test can eliminate the identification of *Brucella* from oxidase-positive, urea-positive, gram-negative coccobacilli.
-

VIII. LIMITATIONS

- A. The green color reaction of a positive test fades rapidly. Test results must be interpreted within 5 min following the application of ferric chloride or false-negative results may occur.
 - B. Slight agitation of the tube containing ferric chloride will produce a faster, more pronounced color reaction.
 - C. TDA can be detected instead of PDA with the same interpretation of results.
-

REFERENCES

1. Ederer, G. M., J. H. Chu, and D. J. Blazevic. 1971. Rapid test for urease and phenylalanine deaminase production. *Appl. Microbiol.* **21**:545.
2. Ewing, E. H., B. R. Davis, and R. W. Reaves. 1957. Phenylalanine and malonate media and their use in enteric bacteriology. *Public Health Lab.* **15**:153–160.
3. Hendriksen, S. D. 1950. A comparison of the phenylalanine acid reaction and urease test in the differentiation of *Proteus* from other enteric organisms. *J. Bacteriol.* **60**:225–231.
4. MacFaddin, J. F. 2000. *Biochemical Tests for the Identification of Medical Bacteria*, 3rd ed., p. 388–393. The Lippincott, Williams & Wilkins Co., Philadelphia, Pa.

3.17.41

PYR (L-Pyrrolidonyl- β -Naphthylamide) Test

[Updated March 2007]

I. PRINCIPLE

L-Pyrrolidonyl- β -naphthylamide (PYR) serves as a substrate for the detection of pyrrolidonyl peptidase. Following hydrolysis of the substrate by the peptidase, the resulting β -naphthylamide produces a red color upon the addition of 0.01% cinnamaldehyde reagent. When a visible inoculum of microorganism is rubbed onto a small area of a disk impregnated with the

substrate, the hydrolysis occurs within 2 min, at which time the cinnamaldehyde reagent is added to detect the reaction by a color change to purple.

Godey et al. (5) first reported that the hydrolysis of this substrate serves as a useful tool in the identification of *Streptococcus pyogenes* (beta-hemolytic group A) and enterococci. Others soon tested the

method and demonstrated its accuracy (1, 3, 4). York et al. (7) and Chagla et al. (2) also found a high degree of accuracy using PYR for identification of *Escherichia coli*, separating it from other indole-positive, lactose-positive, gram-negative rods. Hébert et al. (6) demonstrated the utility of using PYR to differentiate among the coagulase-negative staphylococci.

II. MICROORGANISMS TESTED

- A. Catalase-negative, beta-hemolytic, gram-positive cocci with typical group A streptococcal morphology
- B. Catalase-negative, gamma- or alpha-hemolytic, gram-positive cocci with typical enterococcal morphology
- C. Oxidase-negative, indole-positive, gram-negative rods that are lactose positive on MAC, to identify *E. coli*
- D. Coagulase-negative staphylococci, to screen for *Staphylococcus lugdunensis* and identify other staphylococci to the species level (6)

III. MATERIALS, REAGENTS, AND SUPPLIES

A. Reagents

- 1. Disks impregnated with PYR
 - a. Store at 2 to 8°C in the dark.
 - b. Vendors: BD Diagnostic Systems; EY Laboratories; Hardy Diagnostics; Key Scientific; Oxoid, Inc.; PML; Remel, Inc.
- Caution:** PYR powder or liquid is a potential carcinogen; making the reagent in the clinical laboratory is discouraged.

c. A number of vendors combine several spot tests for identification of streptococci into a card with PYR. PYR in some kits is a liquid reagent, which is more suitable for staphylococci (6).

- 2. 0.01% *p*-Dimethylaminocinnamaldehyde

B. Other supplies

- 1. Sterile water
- 2. Sterile sticks
- 3. Petri dish and forceps

IV. QUALITY CONTROL

- A. Perform QC on each new lot or shipment of disks and color reagent prior to putting it into use.
- B. Organisms
 - 1. *Enterococcus faecalis* ATCC 29212—PYR positive
 - 2. *E. coli* ATCC 25923—PYR negative

V. PROCEDURE

- A. Using forceps, place PYR disk in petri dish.
- B. Moisten, but do not saturate, disk with sterile water.
- C. Using a sterile stick, remove one or two loopfuls of culture from a blood agar plate that is 24 to 48 h old. Use several loopfuls for organisms that take 48 h or more to grow.
- D. Rub onto PYR disk.
- E. Allow to react for 2 min (extend time to 10 min for poorly growing organisms).
- F. After incubation period, add 1 drop of cinnamaldehyde reagent and observe for red color.

VI. INTERPRETATION

- A. A positive test is indicated by the appearance of a bright pink or cherry-red color usually within 1 min.
- B. A negative test is indicated by no color change or a blue color due to a positive indole reaction.
- C. A pale pink reaction (weak) is considered negative.

VII. REPORTING RESULTS

- A. An identification of *S. pyogenes* may be made for PYR-positive, catalase-negative, beta-hemolytic, gram-positive cocci with typical group A streptococcal morphology from throat cultures.
 - 1. Confirm negative PYR tests on beta-hemolytic streptococci with further testing or a fresh subculture if inoculum is low or results are questionable.
 - 2. Confirm positive tests from nonrespiratory sites with either negative bile-esculin, negative esculin, or serologic typing for the group A antigen.
- B. An identification of *Enterococcus* may be made on PYR-positive, catalase-negative, nonhemolytic, gram-positive cocci with typical enterococcal morphology, short chains, and diplococci, *not* tetrads or clusters.
- C. Gram-negative rods are identified as *E. coli* if they are oxidase-negative, indole-positive, lactose-positive colonies that are PYR negative.
- D. Slide coagulase-positive staphylococci that are PYR positive could be *Staphylococcus lugdunensis*. Use a positive ornithine or other tests to confirm (see Table 3.18.1–1).
- E. *Citrobacter* is PYR positive and can be differentiated from H₂S-positive *Salmonella*, which is PYR negative.

VIII. LIMITATIONS

- A. Odd gram-positive cocci will be positive in this test but are not enterococci. Gram stain is most helpful with this issue. They are generally in tetrads or clusters in the smear, are tiny colonies, or are not significant pathogens.
- B. A false-negative test can result if the disk is too moist.
- C. Weak, pale results occur with the disk test for *Staphylococcus aureus*; positive results may need to be confirmed with other tests or with the tube PYR test, which is available in rapid identification kits, such as API Rapid Strep (6).
- D. False-negative tests result if selective media or tube biochemical agars are used to provide inocula.

REFERENCES

1. **Bosley, G. S., R. R. Facklam, and D. Grossman.** 1983. Rapid identification of enterococci. *J. Clin Microbiol.* **18**:1275–1277.
2. **Chagla, A. H., A. A. Borczyk, J. E. Aldom, S. Dalla Rosa, and D. D. Cole.** 1993. Evaluation of the L-pyrrolidonyl β -naphthylamide hydrolysis test for the differentiation of members of the families *Enterobacteriaceae* and *Vibrionaceae*. *J. Clin. Microbiol.* **31**:1946–1948.
3. **Ellner, P. D., D. A. Williams, M. E. Hosmer, and M. A. Cohenford.** 1985. Preliminary evaluation of a rapid colorimetric method for the presumptive identification of group A streptococci and enterococci. *J. Clin. Microbiol.* **22**:880–881.
4. **Facklam, R. R., L. G. Thacker, B. Fox, and L. Eriquez.** 1982. Presumptive identification of streptococci with a new test system. *J. Clin. Microbiol.* **15**:987–990.
5. **Godsey, J., R. Schulman, and L. A. Eriquez.** 1981. The hydrolysis of L-pyrrolidonyl- β -naphthylamide as an aid in the rapid identification of *Streptococcus pyogenes*, *S. avium*, and group D enterococci, abstr. C84, p. 276. *Abstr. 81st Annu. Meet. Am. Soc. Microbiol.* 1981. American Society for Microbiology, Washington, D.C.
6. **Hébert, G. A., C. G. Crowder, G. A. Hancock, W. R. Jarvis, and C. Thornsberry.** 1988. Characteristics of coagulase-negative staphylococci that help differentiate these species and other members of the family *Micococcaceae*. *J. Clin. Microbiol.* **26**:1939–1949.
7. **York, M. K., E. J. Baron, M. Weinstein, R. Thomson, and J. E. Clarridge.** 2000. A multilaboratory validation of rapid spot tests for identification of *Escherichia coli*. *J. Clin. Microbiol.* **38**:3394–3398.

[Updated March 2007]

I. PRINCIPLE

As a rapid method to definitively identify *Streptococcus pneumoniae* directly in sputum, pleural fluid, CSF, blood, or other body fluids, capsular swelling of the pneumococcus in the presence of specific capsular antibody is observed (3, 5). This procedure can also be used to identify difficult

isolates of presumptive pneumococci that are bile insoluble or optochin negative or to type these organisms after they have already been isolated and identified, using specific capsular typing sera, for evaluation of vaccine efficacy. Antigen-antibody

reaction between antiserum and the capsule causes the capsule to appear to swell, although the mechanism is probably due to a change in the refractive index of the capsule that enhances its visibility (Neufeld reaction [1]).

II. SPECIMEN OR MICROORGANISM TESTED

- A. Positive blood cultures containing lancet-shaped, gram-positive cocci in pairs suggestive of pneumococci
- B. Respiratory specimens or CSF with direct Gram stains positive for lancet-shaped, gram-positive cocci in pairs
- C. Colonies with characteristic pneumococcal morphology that are bile resistant

III. REAGENTS AND SUPPLIES

- A. Reagents
 - 1. Polyclonal *S. pneumoniae* antisera from Omniserum (Erna Lund, Pneumococcus Department, Statens Serum Institut, Copenhagen, Denmark; distributed by DAKO, Carpinteria, Calif.).
 - a. Contents: antisera to 84 pneumococcal types in a small amount of methylene blue
 - b. Store at 4°C.
 - 2. Methylene blue solution (store at room temperature)
 - a. 0.3 g of methylene blue
 - b. 100 ml of H₂O
 - 3. Rabbit serum (stored at -20°C)
- B. Supplies
 - 1. Loops
 - 2. Slides and coverslips
 - 3. Pasteur pipettes

IV. QUALITY CONTROL

- A. Prior to use and every 6 months thereafter, test a fresh isolate of a bile-soluble colony (preferably from a blood culture) and a bile-insoluble colony to obtain the expected positive Quellung reaction (bile-soluble colony) and negative Quellung reaction (bile-insoluble colony).
- B. QC strains are not suitable for controls, as they easily lose their capsule.

V. PROCEDURE

- A. Divide a slide into two sections with a wax pencil. Label one section as the test and the other as the control. (Two slides may be used instead.)
- B. Specimen
 1. Dilute growth from blood culture bottles containing pneumococci 1:10 in broth or saline before the slide is made, depending on the number of organisms seen in smear. Four organisms per field is ideal.
 2. Prepare a small saline or broth suspension of a colony; it should be barely visibly turbid.
 3. Dilute other direct specimens if needed in saline, especially if they are viscous.
- C. Using a sterile 10-µl loop (1:100 ml), place a drop of specimen suspension onto each of two sections of a slide; spread out and allow to air dry.
- D. Using a sterile 10-µl loop (1:100 ml), place a small drop of antiserum on the first section of the slide and spread out over the specimen.
NOTE: Antiserum is very expensive, and a small amount will give the desired reaction.
- E. Using a 10-µl loop, place a similar-size drop of rabbit serum on the second section of the slide.
- F. Put a small drop of methylene blue solution on each of two coverslips. Invert the coverslips onto each section of the slide.
NOTE: Adding additional methylene blue solution to the pneumococcal omeniserum will give a more readable preparation, as there is only a small amount of methylene blue in the reagent.
- G. After 10 min, read the test and control slides.
 1. Adjust the microscope to obtain oblique light by adjusting the iris diaphragm so that only one-third of the light passes through the condenser at low power (p. 163 in reference 2).
 2. Switch to oil immersion lens to view capsules.
- H. If the test is negative
 1. Repeat if inoculum is too heavy. Approximately four organisms per field is ideal.
 2. Hold for 30 min in a wet chamber before discarding as negative. (A petri dish with damp gauze upon which to rest slide(s) works well.)

VI. INTERPRETATION

- A. A positive test shows marked swelling of the capsules in comparison to the control, giving a sharply demarcated halo. Capsules may be visible in the control but do not produce a clear demarcation and glassy appearance.
- B. A negative test is marked by no difference between the test and control cells.

VII. REPORTING RESULTS

- A. Report a positive test as “*Streptococcus pneumoniae* by Quellung test.”
- B. Report a negative test as “Negative for *Streptococcus pneumoniae* by Quellung test.”

VIII. LIMITATIONS

- A. A negative test does not indicate that the organism is not *S. pneumoniae*, since the organism could have lost the ability to express a capsule (6).
- B. The test can be falsely negative if there are too many organisms on the slide (antigen excess); if this occurs, the test should be repeated with fewer organisms.

VIII. LIMITATIONS (continued)

C. The Phadebact pneumococcus test (Boule Diagnostics, Huddinge, Sweden) is a coagglutination test using antibodies directed against the capsular antigens of *S. pneumoniae*. In the package insert, the manufacturer reports an 8% false-positive rate with other viridans group streptococci, but it had a 100% sensitivity and 98% specificity in one published study (4). The Directogen and Pneumo-slide (BD Diagnostic Systems) had an 85 to 87% specificity in the same study (4).

REFERENCES

1. Austrian, R. 1976. The quellung reaction, a neglected microbiologic technique. *Mt. Sinai J. Med.* **43**:699–709.
2. Facklam, R. R., and R. B. Carey. 1985. Streptococci and aerococci, p. 154–175. In E. H. Lennette, A. Ballows, W. J. Hausler, Jr., and H. J. Shadomy (ed.), *Manual of Clinical Microbiology*, 4th ed. American Society for Microbiology, Washington, D.C.
3. Heineman, H. S. 1973. Quellung test for pneumonia. *N. Engl. J. Med.* **288**:1027.
4. Kellogg, J. A., D. A. Bankert, C. J. Elder, J. L. Gibbs, and M. C. Smith. 2001. Identification of *Streptococcus pneumoniae* revisited. *J. Clin. Microbiol.* **39**:3373–3375.
5. Merrill, C. W., J. M. Gwaltney, Jr., J. W. Hendley, and M. A. Sande. 1973. Rapid identification of pneumococci. Gram stain vs. the quellung reaction. *N. Engl. J. Med.* **288**:510–512.
6. Mundy, L. S., E. N. Janoff, K. E. Schwebke, C. J. Shanholtzer, and K. E. Willard. 1998. Ambiguity in the identification of *Streptococcus pneumoniae*. Optochin, bile solubility, quellung, and the AccuProbe DNA probe tests. *Am. J. Clin. Pathol.* **109**:55–61.

6.5% Salt and Temperature Tolerance Test

[Updated March 2007]

I. PRINCIPLE

As part of the differentiation of catalase-negative, gram-positive cocci, growth in 6.5% salt and growth at low (10°C) and high (42 to 45°C) temperatures are used (1). Salt tolerance broth, containing heart infusion (HI), glucose, and bromcresol purple indicator with 6.5% salt, was initially designed by Qadri et al. (4) to aid in the differentiation of *Enterococcus* spp. from streptococci. Enterococci can grow in the presence of 6.5% NaCl, but streptococci do not. The salt concentration, acting as a selective agent, interferes with membrane permeability and osmotic equi-

librium for most bacteria. Salt-tolerant organisms produce heavy growth in the medium within 48 h. Organisms that are capable of growing in the high salt concentration will also ferment glucose. Glucose fermentation produces an acid reaction which results in the bromcresol purple indicator turning yellow. Appearance of a yellow color change in broth with indicator is indicative of a positive salt tolerance test, although turbidity of the media is also considered a positive reaction. The broth media, HI with dextrose and indicator

(without additional salt), can be used as a growth control medium for the organism, for comparison of its growth in the same formula with the added salt. The control medium can also be used to demonstrate growth of the microorganism at various temperatures.

The pyrrolidonyl-β-naphthylamide (PYR) test has replaced the salt agar test to differentiate enterococci, but salt agar continues to be useful in the separation of the more recently described genera of catalase-negative cocci (1, 2, 3).

II. MICROORGANISMS TESTED

- A. Catalase-negative, aerobic, gram-positive cocci, to aid in the identification of organisms that do not key out by multitest kits as streptococci and enterococci
- B. Catalase-negative, aerobic, gram-positive cocci in tetrads and clusters, as part of the identification, including *Aerococcus* and *Alloiococcus*
- C. For some gram-negative rods, growth at various temperatures is used in their identification (reference 5 and procedure 3.8.2).

III. MEDIA, REAGENTS, AND SUPPLIES

A. Media

Store at 4 to 8°C.

1. HI (Difco, BD Diagnostic Systems; Remel, Inc.)

Approximate formula per liter of purified water

beef HI	500.0 g
tryptose	10.0 g
sodium chloride	5.0 g

2. Add 0.1 to 1% glucose and bromcresol purple indicator (1 ml of 1.6-g/100 ml 95% ethanol to a liter of medium) for temperature studies.
3. Add 0.5 to 1% glucose and bromcresol purple indicator plus 6% NaCl (HI contains 0.5% NaCl).

NOTE: Agar may be added to solidify the medium. Slant tube while cooling. Color reactions are easier to observe when agar, rather than broth,

medium is used since reaction (yellow color change) on the slant is visible before entire broth tube changes pH to produce yellow color.

4. Commercial HI broth with 6.5% salt is available from Remel, Inc. BHI with 6.5% salt is available from BD Diagnostic Systems and Hardy Diagnostics. SF broth (BD Diagnostic Systems) is similar but contains 5% salt and sodium azide to inhibit organisms other than *Enterococcus* spp.

B. Other supplies/equipment

1. Sterile sticks or inoculation loop
2. Beakers of water
3. Incubator at 35°C
4. Refrigerator at 10°C
5. Incubator or heat block at 42 to 45°C

IV. QUALITY CONTROL

- A. Examine medium for purple color and lack of turbidity.
 - B. Test each new lot or shipment of medium with a positive and negative control prior to putting it into use.
 - C. Organism
 - 1. *Enterococcus faecalis* ATCC 29212—growth at all temperatures and in salt (positive)
 - 2. *Streptococcus bovis* ATCC 33317—no growth at 10°C or in salt broth (negative)
-

V. PROCEDURE

- A. Add 1 or 2 drops from an overnight broth culture or one or two colonies from a fresh plate to the broth.
 - B. Tighten tube caps.
 - C. Incubate aerobically for up to 7 days and observe for growth (turbidity) and color change.
 - 1. To hold the temperatures, place the tube in a beaker of water at 10°C and at 42 to 45°C if an incubator, rather than a heat block, is used.
 - 2. Incubation temperatures
 - a. HI at 35°C
 - b. HI with 6.5% salt at 35°C
 - c. HI at 10°C
 - d. HI at 42 to 45°C
-

VI. INTERPRETATION

- A. A positive test shows visible turbidity and/or change in color from purple to yellow under the above-listed conditions; this occurs usually within 24 h, but tests can be incubated for up to 14 days.
 - B. A negative test shows no turbidity or yellow color.
-

VII. REPORTING RESULTS

- A. Viridans group streptococci (including *S. bovis*), *Granulicatella*, *Dolosicoccus*, and *Gemella* do not grow in 6.5% salt.
 - B. *Streptococcus agalactiae* may grow in salt broth.
 - C. *Enterococcus*, *Lactococcus*, *Globicatella*, and *Vagococcus* will grow at 10°C, but other catalase-negative, gram-positive cocci generally will not (3).
 - D. *S. bovis* and *Enterococcus* will grow at 45°C (1).
 - E. *Pediococcus* and *Leuconostoc* have variable reactions but are easily separated by their vancomycin resistance and PYR-negative reactions.
 - F. *Weissella confusa* usually grows at 42°C (2).
-

VIII. LIMITATIONS

- A. Adding agar to 6.5% salt broth and slanting the tube will provide a medium that will easily allow visualization of weak reactions that start on the top of the slant.
- B. Use of BHI, instead of HI, has not been tested with the unusual gram-positive cocci that are confused with streptococci and enterococci.

REFERENCES

1. Facklam, R., and J. A. Elliott. 1995. Identification, classification, and clinical relevance of catalase-negative, gram-positive cocci, excluding the streptococci and enterococci. *Clin. Microbiol. Rev.* **8**:479–495.
2. Facklam, R., D. Hollis, and M. D. Collins. 1989. Identification of gram-positive coccal and coccobacillary vancomycin-resistant bacteria. *J. Clin. Microbiol.* **27**:724–730.
3. LaClaire, L., and R. Facklam. 2000. Comparison of three commercial rapid identification systems for the unusual gram-positive cocci *Dulosigranulum pigrum*, *Ignavigranum ruoffiae*, and *Facklamia* species. *J. Clin. Microbiol.* **38**:2037–2042.
4. Qadri, S. M., C. W. Nichols, and S. G. Qadri. 1978. Rapid sodium chloride tolerance test for presumptive identification of enterococci. *J. Clin. Microbiol.* **7**:238.
5. Weyant, R. S., C. W. Moss, R. E. Weaver, D. G. Hollis, J. G. Jordan, E. C. Cook, and M. I. Daneshvar. 1995. *Identification of Unusual Pathogenic Gram-Negative Aerobic and Facultatively Anaerobic Bacteria*, 2nd ed. Williams & Wilkins, Baltimore, Md.

[Updated March 2007]

I. PRINCIPLE

Haemophilus influenzae requires two factors, hemin and NAD, for growth. NAD is also referred to as V factor. BAP supplies hemin but not NAD. Although *H. influenzae* requires both hemin and NAD, other *Haemophilus* species require only NAD for growth. Species that require NAD will not grow on BAP unless they are hemolytic and can release the NAD from the RBCs. However, they will grow well on BAP near colonies of staphylococci,

which are capable of providing NAD. The NAD diffuses into the surrounding medium and stimulates growth of *Haemophilus* in the vicinity of the staphylococcus colony. This is known as satellitism. For *Haemophilus* spp., the satellite test substitutes for the V factor test and does not require the purchase or QC of any reagents or strips (3, 4).

Similarly, nutritionally variant streptococci (NVS; include *Abiotrophia defectiva*

and *Granulicatella adiacens*, *Granulicatella elegans*, and *Granulicatella balaenopterae*, previously in the genus *Abiotrophia*) require either L-cysteine or pyridoxal to grow in vitro (2). These organisms do not grow on BAP, since it lacks the nutritional requirements, but will grow in the medium surrounding staphylococci, which supplies the needed nutrients.

II. MICROORGANISM TESTED

- A. Any organism growing only on CHOC and not on BAP that meets the following criteria
 - 1. Is suggestive of *Haemophilus* by Gram stain (gram-negative coccobacilli or short rods)
 - 2. Is a possible NVS by Gram stain (e.g., cocci, filaments, and bulbous forms in smear)
- B. Perform test only in conjunction with Gram stain. If bizarre forms are seen on Gram stain, repeat the smear after subculture to fresh CHOC (1).
- C. Use for detection of *Haemophilus* from lower respiratory specimens by dotting directly on the primary BAP.

III. ORGANISMS, MEDIUM, AND SUPPLIES

A. Microorganisms

- 1. *Staphylococcus aureus* ATCC 25923 or
- 2. *Staphylococcus epidermidis* strain that is nonhemolytic and penicillinase negative

▣ **NOTE:** Most any staphylococcus will work for the test. However, if using on primary BAP, it might be useful to choose coagulase-negative, beta-lactamase-negative, nonhemolytic staphylococci to be able to easily detect contamination of primary media and see hemolysis of colonies that grow from the specimen.

B. BAP

C. Supplies/equipment

- 1. Sterile sticks or inoculating loop
- 2. Incubator at 35°C with 5 to 10% CO₂ or a CO₂-generating system

IV. QUALITY CONTROL

- A. Demonstrate satelliting on *each lot* of BAP using the staphylococcus and *H. influenzae* ATCC 43065. Record lot numbers of BAP and results in QC log.
 - B. Verify satellitism with an NVS (e.g., *A. defectiva* ATCC 49176) when choosing a staphylococcus for the test.
 - C. Periodically verify that the strain has not become contaminated, especially if it is being used on the direct specimen plates for sputum cultures. Alternatively, get a new stock of staphylococci from the freezer monthly.
■ **NOTE:** It is convenient to do the QC test when new lots of CHOC are received and must be quality controlled. The fresh growth of *Haemophilus* on the CHOC provides a stock for use in QC of the satellite test.
-

V. PROCEDURE

- A. Gram stain suspected colony.
 - B. Subculture colony to BAP and CHOC.
 - 1. Use a sterile stick to dot the staphylococcus on the inoculated BAP.
 - 2. Incubate plates for 24 h at 35 to 37°C in 5% CO₂.
 - 3. Examine for the presence of colonies that satellite around staphylococcus dots on BAP.
 - 4. Use colonies from CHOC to perform further tests for identification to the species level or for susceptibility testing.
-

VI. INTERPRETATION

- A. Growth of tiny colonies surrounding the staphylococcus dots is a positive test.
 - B. Growth on BAP without the staphylococcus dots is a negative test. Colonies appear on media with no concentration around staphylococcus dots or there is no growth on BAP.
-

VII. REPORTING RESULTS

- A. A positive result for a tiny gram-negative rod or coccobacillus indicates that the organism is in the genus *Haemophilus*.
 - B. A positive result for a gram-positive coccus indicates a *Granulicatella* or *Abiotrophia* sp. For practical purposes, report as “Nutritionally variant streptococci.”
 - C. Some microorganisms grow only on CHOC and will not grow on BAP even with a staphylococcus dot. These include *Francisella tularensis*, *Haemophilus ducreyi*, and some *Methylobacterium* spp.
-

VIII. LIMITATIONS

- A. *Haemophilus ducreyi* does not require V factor (NAD) but due to its fastidious nature does not grow on BAP even with the staphylococcus dot.
- B. *Haemophilus haemolyticus* and *Haemophilus parahaemolyticus* may grow without the staphylococcus and thus may not demonstrate the satellite phenomenon even though they require V factor (NAD). Since they are hemolytic, they can release NAD into the medium.
- C. *Brucella* can be confused with *Haemophilus*; this test separates these genera, since *Brucella* spp. grow on BAP without staphylococci to supply NAD.
- D. A positive satellite test for NVS does not definitely demonstrate that the organism requires pyridoxal to grow. Disks for this purpose are available commercially (Hardy Diagnostics; Remel, Inc.).

REFERENCES

1. Bottone, E. J., C. A. Thomas, D. Lindquist, and J. M. Janda. 1995. Difficulties encountered in identification of a nutritionally deficient streptococcus on the basis of its failure to revert to streptococcal morphology. *J. Clin. Microbiol.* **33**:1022–1024.
2. Collins, M. D., and P. A. Lawson. 2000. The genus *Abiotrophia* (Kawamura et al.) is not monophyletic: proposal of *Granulicatella* gen. nov., *Granulicatella adiacens* comb. nov., *Granulicatella elegans* comb. nov., and *Granulicatella balaenopterae* comb. nov. *Int. J. Syst. Evol. Microbiol.* **50**:365–369.
3. Kilian, M. 1974. A rapid method for the differentiation of *Haemophilus* strains—the porphyrin test. *Acta Pathol. Microbiol. Scand. Sect. B* **82**:835–842.
4. Kilian, M. 2003. *Haemophilus*, p. 623–635. In P. R. Murray, E. J. Baron, J. H. Jorgensen, M. A. Pfaffer, and R. H. Yolken (ed.), *Manual of Clinical Microbiology*, 8th ed. ASM Press, Washington, D.C.

SPS (Sodium Polyanetholesulfonate) Disk Test

[Updated March 2007]

I. PRINCIPLE

Gardnerella vaginalis is believed to be a contributing cause of bacterial vaginitis (BV). It has also occasionally been reported to cause bacteremia in postpartum women and in men after transurethral resection of the prostate. Differentiation of *G. vaginalis* from other catalase-negative, gram-variable coccobacilli is achieved by

demonstration of the susceptibility of *G. vaginalis* to sodium polyanetholesulfonate (SPS), a common anticoagulant. Using a standardized inoculum, susceptibility to SPS is easily determined by measuring a zone of inhibition around a disk containing SPS (3).

Other microorganisms are susceptible to SPS, including *Haemophilus ducreyi*, which can be demonstrated by this test. Unfortunately, the use of SPS as an anti-coagulant in blood cultures may interfere with the ability to isolate SPS-susceptible organisms from bacteremic patients.

II. MICROORGANISMS TESTED

- A. Catalase-negative, gram-variable coccobacilli presumed to be *G. vaginalis*
- B. Catalase-negative, gram-negative rods, to separate *H. ducreyi* from other *Haemophilus* spp.
- C. See section 4 for use of this test to identify anaerobic cocci.

III. MEDIA AND SUPPLIES

- A. Medium and reagent
 - 1. Brucella or other agar on which the organism will grow
 - 2. SPS disks—1 mg
- B. Other supplies/equipment
 - 1. Sterile sticks, swabs, or inoculation loops
 - 2. Sterile saline
 - 3. Incubator at 35°C with 5 to 10% CO₂ or CO₂-generating system
 - 4. 0.5 McFarland standard

IV. QUALITY CONTROL

- A. Test each new lot and/or shipment of disks with a positive and negative control prior to putting it into use and monthly or with use if test is performed less often.
- B. Organisms
 - 1. *G. vaginalis* ATCC 14108, ≥12 mm
 - 2. *Streptococcus sanguis* ATCC 35557, <12 mm

V. PROCEDURE

- A. Using a 24- to 48-h culture, prepare a suspension of the test organism in sterile saline to match a no. 0.5 McFarland standard.
 - B. Dip a sterile swab into the saline suspension and inoculate brucella or other agar in three directions for confluent growth, as for disk susceptibility testing.
▣ **NOTE:** Reimer and Reller (3) recommend use of brucella agar for *G. vaginalis*. Other media may work well and may be preferred for other organisms.
 - C. Place SPS disk on plate.
 - D. Incubate for 24 to 48 h at 35°C in 5% CO₂.
 - E. Observe zone of inhibition.
-

VI. INTERPRETATION

- A. Susceptible is a zone of inhibition of ≥ 12 mm.
 - B. Resistant is a zone of inhibition of ≤ 11 mm.
-

VII. REPORTING RESULTS

- A. The definitive identification of *G. vaginalis* consists of the following (1).
 1. Colonies appear pinpoint and transparent, with no greening of the agar on BAP, Columbia colistin-nalidixic acid agar (CNA), or CHOC.
 2. Gram-variable to gram-negative small, pleomorphic coccobacilli that do not elongate into filaments or chains
 3. Catalase negative
 4. SPS sensitive
 5. Beta-hemolytic on human blood agar
 - B. The identification of *G. vaginalis* may include positive hippurate and lipase reactions, but negative results for these tests do not rule out the identification.
 - C. *H. ducreyi* is susceptible, with a zone of ≥ 12 mm; no other *Haemophilus* species are susceptible. The addition of 0.002% Tween 80 may aid in dispersion of the cells (4). *Capnocytophaga* is reported to be SPS susceptible, but it is aminolevulinic acid (ALA) positive (4).
-

VIII. LIMITATIONS

- A. A critical factor in performance of the test is the inoculum size, which must be 10^8 CFU/ml (3).
 - B. It is not necessary to confirm the identification of *G. vaginalis* with tests other than colony morphology, catalase, and typical smear, if the direct Gram stain of a vaginal specimen is consistent with diagnosis of BV (2).
-

REFERENCES

1. Aroutcheva, A. A., J. A. Simoes, K. Behbakht, and S. Faro. 2001. *Gardnerella vaginalis* isolated from patients with bacterial vaginosis and from patients with healthy vaginal ecosystems. *Clin. Infect. Dis.* **33**:1022–1027.
2. Catlin, B. W. 1992. *Gardnerella vaginalis*: characteristics, clinical considerations, and controversies. *Clin. Microbiol. Rev.* **5**:213–237.
3. Reimer, L. G., and L. B. Reller. 1985. Use of a sodium polyanetholesulfonate disk for the identification of *Gardnerella vaginalis*. *J. Clin. Microbiol.* **21**:146–149.
4. Shawar, R., J. Sepulveda, and J. E. Claridge. 1990. Use of the RapID-ANA system and sodium polyanetholesulfonate disk susceptibility testing in identifying *Haemophilus ducreyi*. *J. Clin. Microbiol.* **28**:108–111.

3.17.46

SS (Salmonella-Shigella) Agar Test for Growth

[Updated March 2007]

I. PRINCIPLE

Salmonella-shigella (SS) agar is commonly used for the selective isolation and differentiation of *Salmonella* and *Shigella* in both clinical and nonclinical samples. However, SS agar is also useful as part of an overall identification scheme for other, more unusual gram-negative organisms

based on their ability to grow on the medium (3). SS agar is inhibitory to most gram-positive and many gram-negative organisms, because it contains bile salts and brilliant green and neutral red dyes. SS agar is similar to MAC and can differentiate among the lactose fermenting and

non-lactose-fermenting organisms, but it is more inhibitory. It is a modification of a formula originally described by Leifson (2). Sodium thiosulfate and ferric citrate enable the detection of hydrogen sulfide production as evidenced by colonies with black centers.

II. MICROORGANISMS TESTED

- A. Gram-negative rods that are able to grow on MAC, if needed, as part of the identification (3)
- B. Gram-negative rods identified as either *Ralstonia paucula* (CDC group IVc-2) or *Bordetella bronchiseptica*

III. MEDIUM AND SUPPLIES

- A. SS agar
 - 1. Store at 2 to 8°C.
 - 2. If purchased or prepared in tubes, the shelf life is extended beyond 6 months.
 - 3. Prepare from dehydrated medium (Difco, BBL; BD Diagnostic Systems; Hardy Diagnostics).
 - a. Weigh desired amount per manufacturer's instructions.
 - b. Boil for 2 to 3 min to dissolve. Cool to 50 to 60°C.
 - c. Dispense in tubes, slant, and tighten caps. Do not autoclave medium.
- B. Other supplies/equipment**
- 1. Sterile sticks or inoculating loop
 - 2. Incubator at 35°C

IV. QUALITY CONTROL

- A. Check plates for signs of contamination, cracks, dehydration, and deterioration.
- B. Test each lot or shipment of medium with a positive and negative control prior to putting it into use. End-user QC testing of commercially prepared SS agar is not required, if the manufacturer certifies that each lot has been verified to meet or exceed Clinical and Laboratory Standards Institute (formerly NCCLS) guidelines (1).
- C. Organisms
 - 1. *Shigella flexneri* ATCC 12022—growth and colorless colonies (positive)
 - 2. *Escherichia coli* ATCC 25922—partial or complete inhibition of growth (negative); colonies are pink to rose.

V. PROCEDURE

- A. Inoculate 1 or 2 drops from an overnight broth culture or one or two colonies from a fresh plate to the agar slant or plate. Streak slant in a zigzag direction across the slant and streak plate in quadrants for isolation of colonies.
 - B. Incubate aerobically for up to 7 days at 35°C, and observe for visible growth.
-

VI. INTERPRETATION

- A. A positive test shows visible growth, usually within 24 h, but tests can be incubated for up to 7 days (3).
 - B. A negative test shows no growth on the agar slant.
-

VII. REPORTING RESULTS

- A. For oxidase-positive, urea-positive, gram-negative, non-glucose-oxidizing rods that are nonpigmented and grow on MAC, separation of species is accomplished with SS agar.
 - 1. *R. paucula* (CDC IVc-2) does not grow on SS agar (negative).
 - 2. *B. bronchiseptica* grows on SS agar (positive).
 - B. Refer to Weyant et al. (3) for other gram-negative rods for which SS agar is useful in identification (e.g., *Alcaligenes faecalis*, *Achromobacter xylosoxidans*, *Bordetella avium/hinzii*, *Pseudomonas mendocina* [Vb-2], and *Ralstonia eutropha*).
-

VIII. LIMITATIONS

- A. *E. coli* may grow, but will grow poorly, on SS agar. *Enterococcus faecalis* will not grow.
 - B. Organisms that do not grow on MAC will not grow on SS agar.
 - C. Due to the dyes and bile salts, growth of some *Shigella* strains is inhibited.
-

REFERENCES

1. Clinical and Laboratory Standards Institute. 2004. *Quality Assurance for Commercially Prepared Microbiological Culture Media*, 3rd ed. Approved standard M22-A3. Clinical and Laboratory Standards Institute, Wayne, Pa.
2. Leifson, E. 1935. New culture media based on sodium desoxycholate for the isolation of intestinal pathogens and for the enumeration of colon bacilli in milk and water. *J. Pathol. Bacteriol.* **40**:581–599.
3. Weyant, R. S., C. W. Moss, R. E. Weaver, D. G. Hollis, J. G. Jordan, E. C. Cook, and M. I. Daneshvar. 1995. *Identification of Unusual Pathogenic Gram-Negative Aerobic and Facultatively Anaerobic Bacteria*, 2nd ed., p. 20. Williams & Wilkins, Baltimore, Md.

3.17.47

Starch Hydrolysis Test

[Updated March 2007]

I. PRINCIPLE

Bacteria can excrete an amylase, endoamylase, which hydrolyzes amylose to maltose and glucose. If there is no enzyme present, and therefore no hydrolysis, the starch agar will turn blue in the presence of iodine, due to the action of iodine and the helical structure of amylose. When hy-

drolisis occurs and thus no amylose is present, there is no color development in the medium immediately surrounding the colony. To observe this hydrolysis, an organism is grown on medium containing amylose. Since amylose and iodine react together to form a blue color, the plates

can be flooded with iodine to visually detect the absence or presence of amylose, one of the components of starch. Mueller-Hinton agar (MH) contains amylose and can be used to test for starch hydrolysis, avoiding the purchase of additional media.

II. MICROORGANISMS TESTED

- A. To separate *Streptococcus bovis* (positive) from other viridans group streptococci that are bile-esculin positive, 6.5% NaCl negative, and pyrrolidonyl- β -naphthylamide (PYR) negative
- B. To separate *Chryseobacterium meningosepticum* (negative) from *Chryseobacterium indologenes* (positive)

III. MEDIA, REAGENT, AND SUPPLIES

- A. Media
 - 1. MH
 - 2. Heart infusion agar with 2% starch
- B. Reagent
 - Gram's iodine (Appendix 3.2.1–1)
- C. Supplies/equipment
 - 1. Sterile sticks or inoculating loops
 - 2. Incubator at 35°C

IV. QUALITY CONTROL

- A. Inspect agar for freezing, contamination, cracks, and dehydration prior to storage and before use.
- B. Perform QC on each new lot or shipment of starch agar prior to putting it into use. MH agar can be tested with use for starch hydrolysis.
- C. Organisms
 - 1. *S. bovis* ATCC 33317—clear halo around colony with addition of iodine (starch positive)
 - 2. *Enterococcus faecalis* ATCC 25922—blue color with addition of iodine (starch negative)

V. PROCEDURE

- A. After touching several colonies from an 18-h culture, inoculate several segments of one-half of the agar surface with a very *visible circular* amount of organism about the size of a dime on each spot. *Be sure plate has no surface moisture.*
- B. Inoculate a positive and negative control on the other half of the plate in the same manner.

V. PROCEDURE (continued)

- C. Incubate without increased CO₂ for at least 48 h.
- D. To detect starch hydrolysis, transfer a piece of agar from one of the growth dots of the test or control organism to an empty sterile petri dish. Flood dropwise with Gram's iodine and observe for halos around colony.
- E. If negative for starch hydrolysis, reincubate and retest additional pieces of agar at 72 h or later.

VI. INTERPRETATION

- A. A positive test is development of a clear halo without color around the colony and a blue to blue-purple color in the surrounding medium after the addition of Gram's iodine.
- B. A negative test shows no clear halo around the colony. The medium around the colony turns blue to blue-purple after the addition of Gram's iodine.

VII. REPORTING RESULTS

- A. Catalase-negative, PYR-negative, gram-positive cocci that are bile-esculin positive, do not grow on 6.5% salt, and hydrolyze starch are *S. bovis*. If they are positive for group D antigen, they are definitively identified, without starch or salt testing.
- B. Catalase-negative, PYR-negative, gram-positive cocci that are bile-esculin positive, do not grow on 6.5% salt, and do not hydrolyze starch may be *S. bovis* variants, but other tests need to be done to confirm the identification.
- C. Among the yellow-pigmented indole-, catalase-, esculin-, and oxidase-positive gram-negative rods, *Chryseobacterium gleum/indologenes* is starch positive.

VIII. LIMITATIONS

- A. Avoid using glucose starch medium, since the metabolism of glucose may interfere with the assay (3).
- B. Read plates immediately after addition of iodine, as the blue color fades.
- C. Once the iodine is added, the organisms are nonviable.
- D. A red-violet color is due to partial hydrolysis, and the test should be repeated after further incubation.
- E. The identification of *S. bovis* is difficult, and the organism can be confused with *Streptococcus mutans* (starch negative) and *Streptococcus salivarius* (1, 4).
- F. *S. bovis* strains from humans are said to be biotype I if they hydrolyze starch. *S. bovis* biotype II strains do not hydrolyze starch (2).
- G. *Gardnerella vaginalis* organisms are starch positive but this test is not a reliable method to identify them, since they do not grow on MH and require special starch media to grow.

REFERENCES

1. Coykendall, A. 1989. Classification and identification of the viridans streptococci. *Clin. Microbiol. Rev.* **2**:315–328.
2. Facklam, R. R. 1972. Recognition of group D streptococcal species of human origin by biochemical and physiological tests. *Appl. Microbiol.* **23**:1131–1139.
3. MacFaddin, J. F. 2000. *Biochemical Tests for the Identification of Medical Bacteria*, 3rd ed., p. 412–423. The Lippincott, Williams & Wilkins Co., Philadelphia, Pa.
4. Ruoff, K., S. I. Miller, C. V. Garner, M. J. Ferraro, and S. B. Calderwood. 1989. Bacteremia with *Streptococcus bovis* and *Streptococcus salivarius*: clinical correlates of more accurate identification of isolates. *J. Clin. Microbiol.* **27**:305–308.

3.17.48

Urea Test

[Updated March 2007]

I. PRINCIPLE

Urea medium, whether a broth or agar, contains urea and the pH indicator phenol red. Many organisms, especially those that infect the urinary tract, have a urease enzyme, which is able to split urea in the presence of water to release two molecules of ammonia and carbon dioxide. The ammonia combines with the carbon dioxide and water to form ammonium carbonate, which turns the medium alkaline (6), turning the indicator from its original orange-yellow color to bright pink (2).

This test can be used as part of the identification of several genera and species of *Enterobacteriaceae*, including *Proteus*, *Klebsiella*, and some *Yersinia* and *Citrobacter* species, as well as some *Corynebacterium* species. It is also useful to identify *Cryptococcus* spp. (see section 8), *Brucella*, *Helicobacter pylori*, and many other bacteria that produce the urease enzyme.

Disks are available that combine urea and phenylalanine deaminase (PDA), allowing a one-disk test to identify *Proteus*, *Providencia*, and *Morganella* and separate them from *Klebsiella* and *Yersinia enterocolitica* (4). The disk reactions are rapid and sensitive and allow for the rapid detection of agents of serious infections, e.g., *Brucella* and *Cryptococcus*.

II. MICROORGANISMS TESTED

- A. The urea test is part of the battery of tests to identify the following.
 - 1. Gram-negative enteric pathogens, including *Yersinia* spp.
 - 2. Fastidious gram-negative rods—*Brucella*, *H. pylori*, and *Pasteurella*
 - 3. Gram-positive rods—*Corynebacterium* and *Rhodococcus* spp.
 - 4. Yeasts—*Cryptococcus* spp.
- B. Directly, this test is performed on gastric biopsy samples to detect the presence of *H. pylori* (see procedure 3.8.4 for details).

III. MEDIA, REAGENTS, AND SUPPLIES

A. Media and reagents

Use one of the following.

- 1. Christensen's urea agar slants containing urea, peptone, dextrose, and agar in buffered phenol red solution
NOTE: Urea cannot be autoclaved but must be filter sterilized and added to agar. Christensen's urea agar base 10× concentrate (BD Diagnostic Systems) can be added to autoclaved and cooled agar to prepare medium.
- 2. Christensen's urea broth
 - a. Dilute Christensen's urea agar base 10× concentrate 1:10 with sterile distilled water.
 - b. Dispense in 0.5-ml amounts into 13- by 100-mm tubes.
- 3. Rapid urea medium (Remel, Inc.; Hardy Diagnostics) containing agar

and urea in buffered phenol red solution. This medium can also be used for gastric biopsy specimens (1, 5).

- 4. Rapid urea broth (Remel, Inc.)
- 5. RSU broth containing sucrose and urea for rapid identification of *Corynebacterium* (Hardy Diagnostics)
- 6. Urea disks or tablets—stored at 2 to 8°C
 - a. Supplied by Remel, Inc.; Key Scientific; or Hardy Diagnostics
 - b. Supplied as urea-PDA disks (Remel, Inc.; Hardy Diagnostics)

B. Supplies/equipment

- 1. Sterile wooden sticks or loops
- 2. Saline or water in small plastic tube for disk test
- 3. Incubator at 35 and 30°C

IV. QUALITY CONTROL

- A. Inspect agar for evidence of prior freezing, contamination, cracks, and dehydration prior to storage and before use.
- B. Each time the test is performed for direct gastric specimens, test negative reactions for the ability of the medium to split urea and turn the medium red.
 1. Insert a urease tablet (Kimberly Clark, Draper, Utah) into the negative patient test, as you would a biopsy sample.
 2. After 5 min, inspect for a positive red color change.
- C. Test each new lot or shipment of medium or reagent with a positive and negative control prior to putting it into use.
 - NOTE:** The Clinical and Laboratory Standards Institute (formerly NCCLS) has proposed elimination of user QC for urease agar purchased from commercial sources. Consult with current regulatory agencies prior to discontinuation of user QC (3).
- D. Organisms
 - 1. *Proteus mirabilis* ATCC 12453—positive (red color)
 - 2. *Escherichia coli* ATCC 25922—negative (no color change)
 - NOTE:** Alternate controls should be used if test is being done for detection of *H. pylori*. The reaction time of *H. pylori* is much shorter than that of *P. mirabilis*.
 - H. pylori* ATCC 43504—positive (red color)
 - Campylobacter jejuni* ATCC 33560—negative (no color change)

V. PROCEDURE

- A. **Christensen's urea agar**
 1. Using a sterile stick or loop, inoculate the agar slant surface from a well-isolated colony. Do not stab the butt.
 2. Incubate, with cap loosened, aerobically at 35 to 37°C.
 3. For nonfermenters, incubate at 30°C.
 4. Examine for the development of a pink color for as long as 7 days.
 - NOTE:** If the organism does not grow on the slant, inoculate heavily with growth from the plate to detect preformed urease.
- B. **Urea agar deeps or rapid urea broth**
 1. Pick up colonies with either a stick or needle.
 2. Stab the agar or rotate in the broth with the inoculated stick or needle.
 3. For biopsy samples, refer to procedure 3.8.2.
- C. **Urea disks or tablets**

Check the package insert for differences from procedure below.

 1. Prepare a small test tube with 0.25 ml (5 drops) of saline or water.
 - NOTE:** Preferably use plastic tubes for disk tests.
 2. Make a heavy suspension of the actively growing organism.
 3. Add urea disk. (Tablets are already in tube.)
 4. Incubate aerobically at 35°C for 1 to 24 h.
- D. **Gastric specimens**

See procedure 3.8.4.

VI. INTERPRETATION

- A. A positive test is development of an intense magenta to bright pink color in 15 min to 24 h.
- B. A negative test shows no color change.

VII. REPORTING RESULTS



It is imperative that these cultures be handled in a biosafety hood.

- A. This test will help differentiate among gram-negative rods that grow well on MAC and are likely members of the *Enterobacteriaceae*.
- B. Urea-positive, oxidase-positive, gram-negative coccobacilli that do not grow on MAC in 24 h are presumptively identified as *Brucella*, unless they are isolated from urine. *Immediately transfer cultures to a biosafety cabinet*.
- C. Urea-positive, oxidase-positive, gram-negative coccobacilli that are isolated from the urinary tract may be *Oligella ureolytica*.
- D. Identify further any significant numbers of urea-positive corynebacteria from respiratory or urine specimens. See procedure 3.18.1.
- E. Urea-positive, oxidase-positive, curved rods from gastric specimens are identified as *H. pylori* (7).
- F. Use urea reaction as part of the identification of other microorganisms, following charts in procedure 3.18.2.

VIII. LIMITATIONS

- A. Some organisms rapidly split urea (*Brucella* and *H. pylori*), while others react slowly.
- B. When performing overnight tests from medium that contains peptone, the alkaline reaction may be due not to urease but to hydrolysis of peptone.
- C. Urea is light sensitive and can undergo autohydrolysis. Store at 2 to 8°C in the dark.
- D. The test is less sensitive if the medium is not buffered.

REFERENCES

1. Abdalla, S., F. Marco, R. M. Perez, J. M. Pique, J. M. Bordas, M. T. Jimenez de Anta, and J. Teres. 1989. Rapid detection of gastric *Campylobacter pylori* colonization by a simple biochemical test. *J. Clin. Microbiol.* **27**:2604–2605.
2. Christensen, W. B. 1946. Urea decomposition as a means of differentiating *Proteus* and paracolon cultures from each other and from *Salmonella* and *Shigella* types. *J. Bacteriol.* **52**:461–466.
3. Clinical and Laboratory Standards Institute. 2004. *Quality Assurance for Commercially Prepared Microbiological Culture Media*, 3rd ed. Approved standard M22-A3. Clinical and Laboratory Standards Institute, Wayne, Pa.
4. Ederer, G. M., J. H. Chu, and D. J. Blazevic. 1971. Rapid test for urease and phenylalanine deaminase production. *Appl. Microbiol.* **21**:545.
5. Goldie, J., S. J. O. Veldhuyzen van Zanten, S. Jalali, J. Hollingsworth, R. H. Riddell, H. Richardson, and R. H. Hunt. 1989. Optimization of medium for rapid urease test for detection of *Campylobacter pylori* in gastric antral biopsies. *J. Clin. Microbiol.* **27**:2080–2082.
6. Mobley, H. L., M. D. Island, and R. P. Hau-singer. 1995. Molecular biology of microbial ureases. *Microbiol. Rev.* **59**:451–480.
7. Owen, R. J., S. R. Martin, and P. Boman. 1985. Rapid urea hydrolysis by gastric campylobacters. *Lancet* **i**:111.

3.18.1

Identification of Gram-Positive Bacteria

[Updated March 2007]

I. PRINCIPLE

Unlike gram-negative rods, it can be very difficult to sort out the identification of gram-positive cocci and rods. Many kits for staphylococcal identification have proved to be less sensitive than desired, and new DNA studies indicate that we have misidentified many streptococci. Gram-positive rods have been difficult to identify because there are hundreds of named species and thousands of genotypes or biochemical variants found in the environment and the normal microbiota of the human body, including skin, mucosal membranes, oropharynx, and genitourinary and gastrointestinal tracts. Thus, it is not within the scope of this handbook to identify all isolates, but to detect and iden-

tify the known pathogenic microorganisms in the human biosphere and to limit other identifications to those bacteria that are involved in disease from invasively collected specimens. The figures and tables that follow are designed to rapidly determine the agents of infection and to provide guidance for when to perform a kit identification or pursue other microorganisms.

The charts may not include all the options at each step, since the observance of a colony morphology may lead to performing a test but the lack of that morphology may suggest doing a different test. The figures are designed to do the minimum to arrive at an excellent identi-

fication, without doing further tests unless the morphology warrants them. For species identification of viridans group streptococci, enterococci, and corynebacteria, commercial kits are easier to perform than standard tube biochemical tests (see Tables 3.16–1, 3.16–3, and 3.16–4) but are still limited in their accuracy. No system available can do as good a job as cell wall analysis and DNA studies, both of which are beyond the scope of most laboratories and beyond reasonable cost. For identification of other aerobic gram-positive rods in the actinomycete group, refer to section 6 of this handbook; for further information on *Actinomyces* spp., refer to section 4 and reference 5.

II. MICROORGANISMS TESTED

Gram-positive rods or cocci as determined by Gram stain.

■ NOTE: Because of the lack of a unique colony morphology of many gram-positive microorganisms, the Gram stain *must* be performed on all isolates. Colonies of *Streptococcus* and *Aerococcus* may appear similar, but these organisms can be differentiated by the arrangement of the cells in the Gram stain. Group B streptococci and *Listeria* colonies may also have a similar appearance. Gram-variable microorganisms are considered to be gram positive. When in doubt, the Gram reaction enzymatic test (procedure 3.17.20) may be helpful.

III. MEDIA, REAGENTS, AND SUPPLIES

■ NOTE: See individual tests in procedure 3.17 for methods for use of tests. To the extent that tests are available in kits, it is not necessary to stock the separate tests.

A. Media

1. Bile-esculin (procedure 3.17.5)
2. Glucans (optional) (procedure 3.17.19)
3. H₂S production medium (procedure 3.17.22)
4. Human blood agar for *Gardnerella*

5. Lipophilism test (procedure 3.17.28)

6. Ornithine and arginine decarboxylase (procedure 3.17.15)

7. 6.5% NaCl broth (procedure 3.17.43)

8. Broth for motility (procedure 3.17.31)

9. Lecithinase agar (procedure 3.17.27)

B. Reagents and supplies

1. Catalase (3% H₂O₂) (procedure 3.17.10)

III. MEDIA, REAGENTS, AND SUPPLIES (continued)

2. Polymyxin B (300 U), novobiocin (5 g), bacitracin (0.4 U), and vancomycin disks (procedure 3.17.4)
3. CAMP test (procedure 3.17.8)
4. Coagulase by rabbit plasma and (optionally) agglutination (procedures 3.17.13 and 3.17.14)
5. Spot bile reagent (procedure 3.17.6)
6. Hippurate (optional) (procedure 3.17.21)
7. Leucine aminopeptidase (LAP) (procedure 3.17.26)
8. Optochin disks (procedure 3.17.38)
9. Streptococcal grouping antisera (section 11)
10. Pyrrolidonyl-β-naphthylamide (PYR) (procedure 3.17.41)
11. Urea test (procedure 3.17.48)
12. Kits for identification of enterococci and viridans group streptococcus gram-positive and gram-positive rod identifications (*corynebacterium* and anaerobe kits) (Tables 3.16–1, 3.16–3, and 3.16–4)

IV. PROCEDURE

- A. Observe colony morphology on BAP and CHOC if growth is lacking on BAP.
- B. Perform catalase and Gram stain from BAP or CHOC.
 - NOTE:** To avoid misidentifications, do not skip this step.
- C. Use Fig. 3.18.1–1 if the organism is a catalase-positive, gram-positive coccus.
- D. Use Fig. 3.18.1–2 if organism is a beta-hemolytic, catalase-negative, gram-positive coccus.
- E. Use Fig. 3.18.1–3 if organism is a catalase-negative, gram-positive coccus that is not hemolytic (except those with characteristic group B streptococcus morphology) and either is PYR negative or does not grow on BAP, except around staphylococci.
- F. Proceed to Fig. 3.18.1–4 if the catalase-negative, gram-positive coccus is PYR positive and not identified from the other figures.
- G. For gram-positive rods, proceed to Fig. 3.18.1–5.
 1. If isolate is catalase positive
 - a. Perform CAMP test (and test for lipophilism, if microorganism demonstrates small colonies or poor growth at 24 h).
 - b. Check for motility by wet mount from young growth of either broth or agar cultures.
 2. If isolate is catalase negative, follow Fig. 3.18.1–5 based on the anatomical site of isolation.

V. REPORTING AND INTERPRETATION OF RESULTS

- A. Follow tables and kit identifications to report genus and species as appropriate without delay.
- B. Use commercial kits for identification of *Enterococcus faecalis* and *Enterococcus faecium*, but perform motility testing on vancomycin-intermediate or -resistant *E. faecium*.

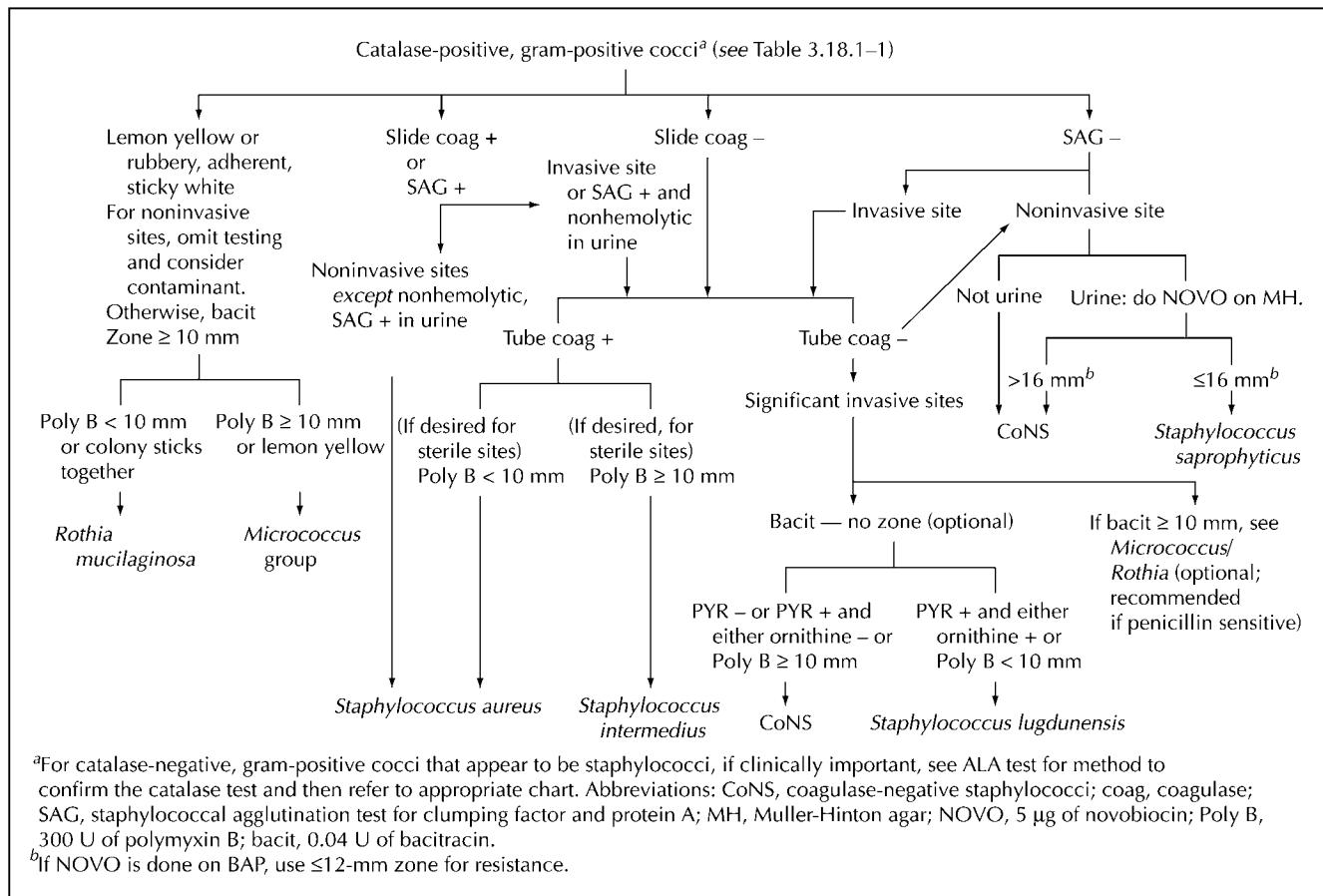


Figure 3.18.1–1 Flowchart for identification of catalase-positive, gram-positive cocci.

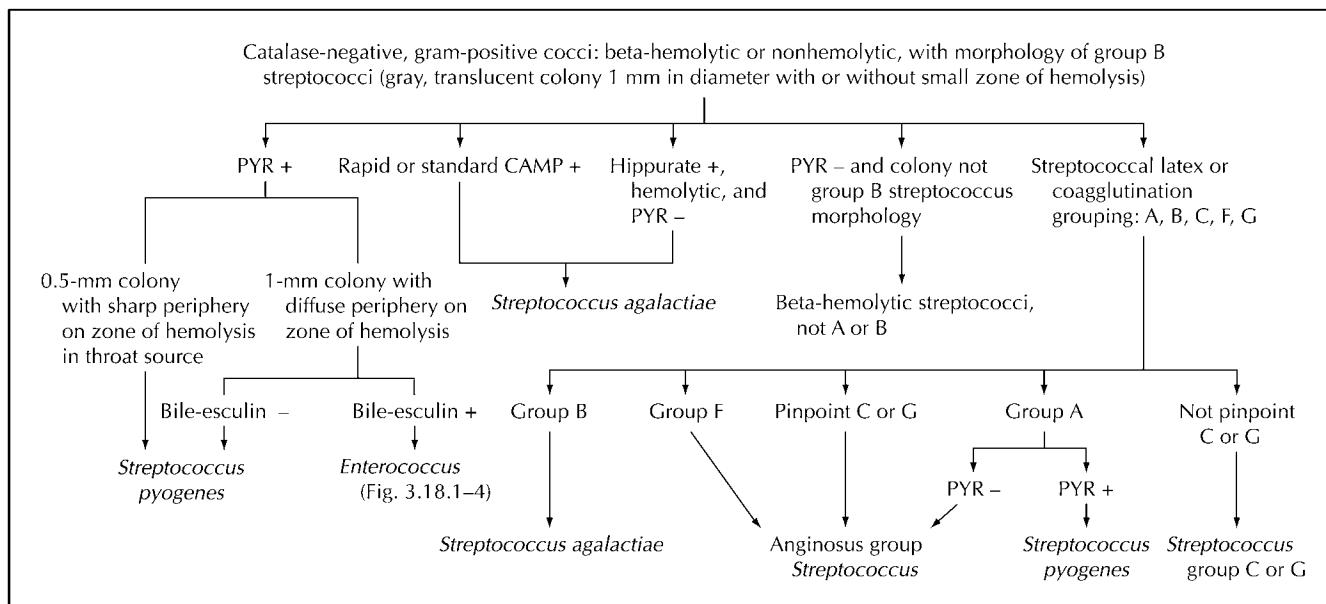


Figure 3.18.1–2 Flowchart for identification of catalase-negative, gram-positive cocci, either beta-hemolytic or nonhemolytic, with morphology of group B streptococci.

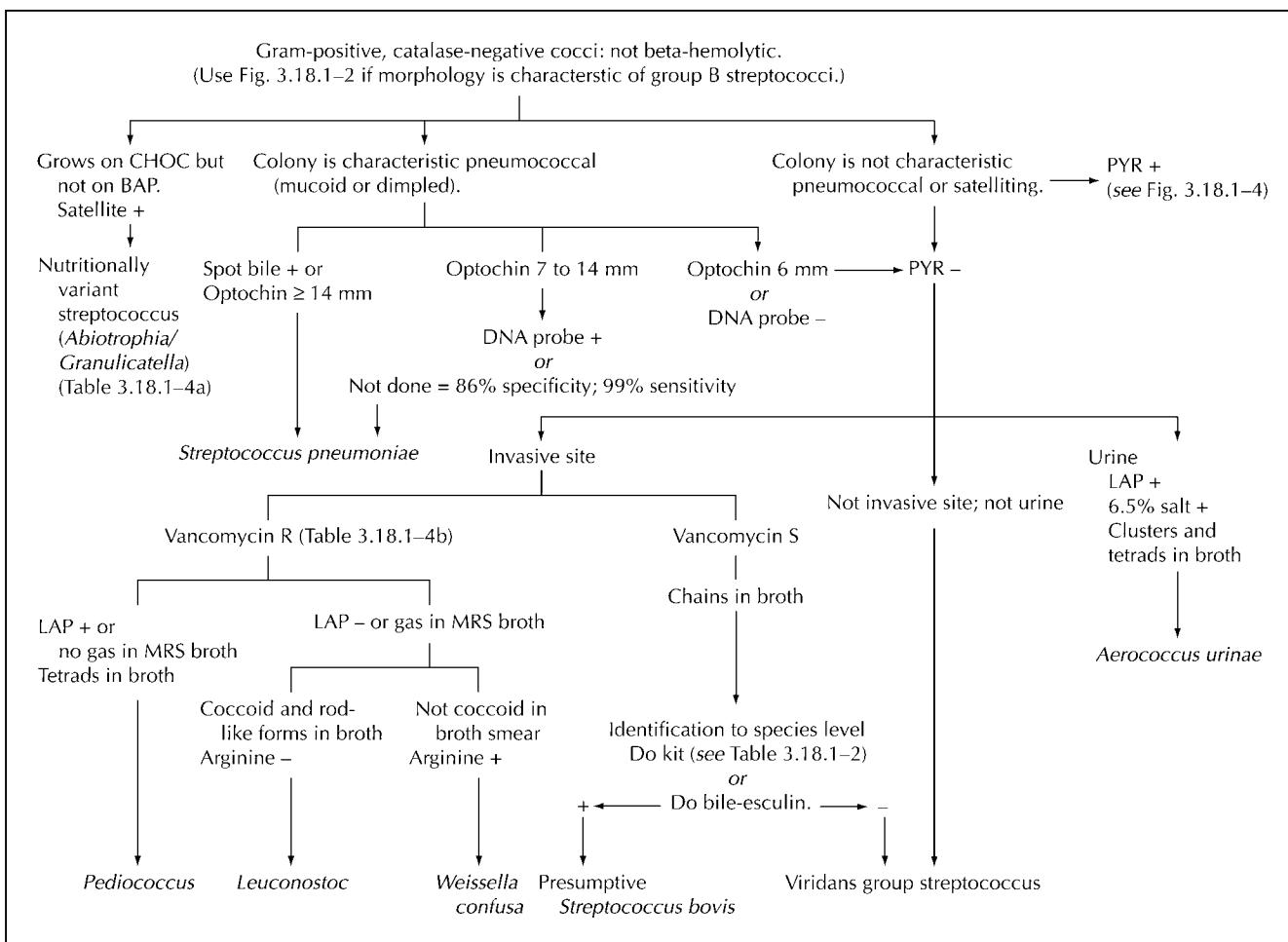


Figure 3.18.1–3 Flowchart for identification of gram-positive, catalase-negative, cocci that are not beta-hemolytic. R, resistant; S, susceptible.

VI. LIMITATIONS

- A. A number of gram-positive cocci are either coagulase or agglutination positive but are not *Staphylococcus aureus*, making identification problematic.
- B. Some staphylococci are catalase-negative. See the aminolevulinic acid (ALA) test for options.
- C. Streptococci are increasingly difficult to identify to the species level, even with commercial kits. The LAP test is important to at least confirm the genus of streptococci or enterococci.
- D. Gram-positive rods are most difficult to identify. Every laboratorian should be able to recognize *Listeria monocytogenes*, *Erysipelothrix rhusiopathiae*, *Bacillus cereus*, *Arcanobacterium haemolyticum*, and *Gardnerella vaginalis* and be able to presumptively recognize *Bacillus anthracis* and *Corynebacterium diphtheriae*. Some other *Corynebacterium* species are identified using a commercial kit. For other gram-positive rods of significance, a reference laboratory is usually needed.

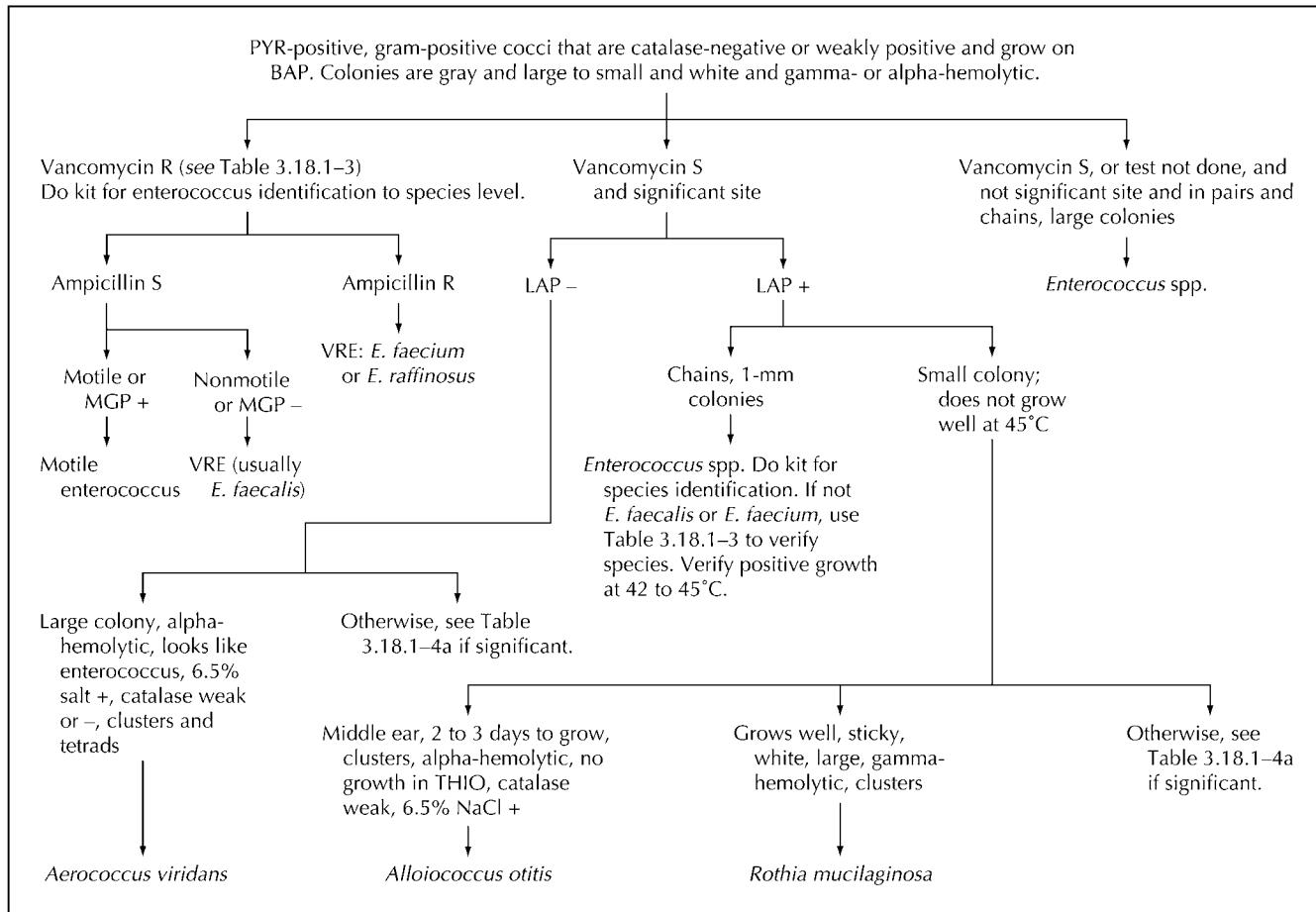


Figure 3.18.1-4 Flowchart for identification of PYR-positive, catalase-negative, gram-positive cocci. R, resistant; S, susceptible; VRE, vancomycin-resistant enterococci; MGP, methyl- α -D-glucopyranoside.

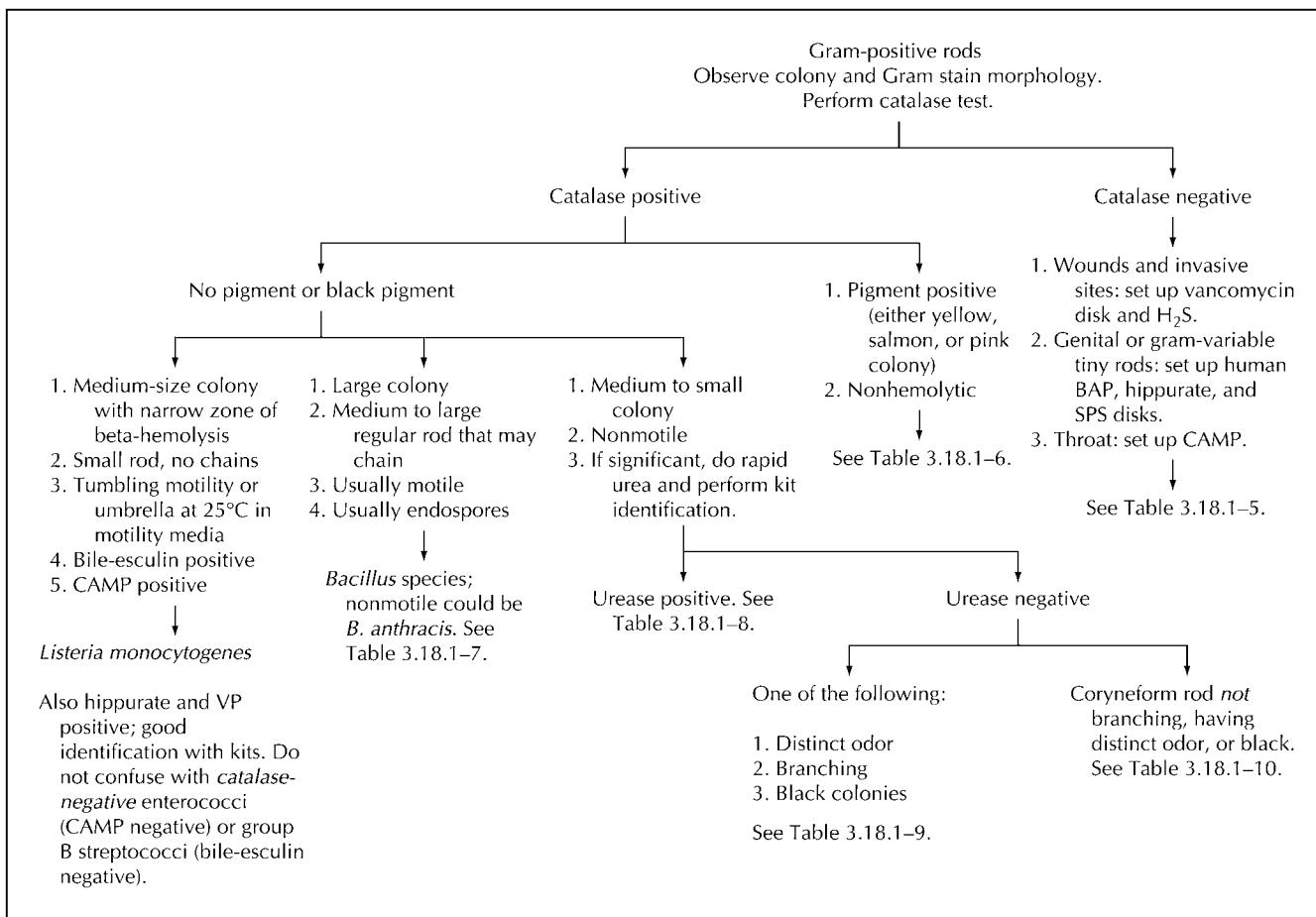


Figure 3.18.1–5 Guide to distinguish genera and significant species of gram-positive rods. VP, Voges-Proskauer. (Refer to section 6 for aerobic actinomycetes.)

Table 3.18.1-1 Key biochemical reactions of the common and/or significant gram-positive cocci that are catalase positive with large white to yellow colonies^a

Organism(s)	Selected characteristic(s)	Slide coag	SAG	Tube coag	Bacit (0.04 U)	Poly B (300 U)	PYR ^b
<i>Rothia mucilaginosa</i>	Adherent, sticky				S	R	V
<i>Micrococcus</i> group ^c	Often yellow				S	S	V
<i>S. aureus</i>	VP +	V	+	+	R	R	—
<i>S. intermedius</i> (dogs)	VP —	V	V	+	R	S	+
<i>Staphylococcus delphini</i> (dolphins)	VP —	—	NA	+	R	NA	NA
<i>Staphylococcus hyicus</i> (pigs)	VP —	—	NA	V	R	R	—
<i>S. lugdunensis</i>	Ornithine +	V	V	—	R	R	+
<i>S. schleiferi</i>	Ornithine —	+	V	— ⁺	R	S	+
<i>S. saprophyticus</i>	Urine; novo R; nonhemolytic	—	V	—	R	S	—
<i>Staphylococcus epidermidis</i>	Nonhemolytic	—	—	—	R	R	—
<i>S. haemolyticus</i>	Urease —, VP +, DNase —	—	—	—	R	S	+
<i>Staphylococcus caprae</i>	Urease +, DNase +	—	—	—	R	S	+
Other coagulase-negative staphylococci	Novo V, urease V; nonhemolytic or delayed hemolysis	—	V	—	R	S	V

^a Symbols and abbreviations: +, greater than 90% of strains positive in 48 h; —, greater than 90% of strains negative; V, results are between 90 and 10% positive; —+, most strains are negative but rare positive strains exist; NA, not applicable or available; R, resistant; S, susceptible; Bacit, bacitracin; Poly B, polymyxin B; coag, coagulase; SAG, staphylococcal protein A or clumping factor agglutination; VP, Voges-Proskauer; Novo, novobiocin. Data are from references 1, 6, 12, 15, 16, 19, 23, and 30. Catalase can be weak for *Rothia*.

^b PYR data are for broth test. Weak positive results with *S. aureus* ATCC 29213 and ATCC 25923 occur with the disk test, suggesting that this test is unreliable to separate *S. aureus* from *S. intermedius* (M. York, personal communication).

^c Includes related taxa. The genus *Micrococcus* has been divided into additional genera, including *Kytococcus* and *Kocuria*.

Table 3.18.1–2 Separation of the common groups of viridans group streptococci (PYR-negative, LAP-positive, 6.5% NaCl-negative cocci in chains)^a

Group	Species	VP	ARG	MAN	SOR	Esculin	Glucans	Hemolysis and comments
Mutans	<i>S. mutans</i> , <i>S. sobrinus</i> , <i>S. ratti</i>	+	–	+	+	V	+ , puddles and droplets	α, β. Sometimes dry and adherent. <i>S. sobrinus</i> can be sorbitol negative; <i>S. ratti</i> is arginine positive.
Salivarius	<i>S. salivarius</i> , <i>S. vestibularis</i>	+	–	–	–	+	+ , mucoid, firm	α, γ. <i>S. vestibularis</i> is α, VP and esculin variable, and glucan negative
Bovis	I	+	–	+	–	+	+ , watery, spreads	γ. Bile-esculin positive (3). Taxonomic revisions suggest that human <i>S. bovis</i> I isolates be renamed as <i>S. gallolyticus</i> , and biotypes II/2 and II/1 have proposed species names <i>S. pasteurianus</i> and <i>S. infantarius</i> , respectively (3, 4, 10, 27)
	II/1	+	–	–	–	+	+ , watery, spreads	
	II/2	+	–	–	–	+	–	
Anginosus ("S. milleri")	<i>S. anginosus</i> , <i>S. constellatus</i> , <i>S. intermedius</i>	+	+	– +	–	+ –	–	α, β, γ. <i>S. constellatus</i> is divided into subspecies <i>constellatus</i> and <i>pharyngis</i> . All species are esculin positive, except that <i>S. constellatus</i> subsp. <i>constellatus</i> is esculin variable (28).
Mitis	<i>S. sanguis</i> , <i>S. parasanguis</i> , <i>S. gordoni</i> , <i>S. cristatus</i>	–	+	–	V	+ –	V, hard, adheres to agar	α. <i>S. cristatus</i> may be arginine and esculin negative. <i>S. parasanguis</i> may be esculin negative. <i>S. sanguis</i> may be sorbitol positive.
	<i>S. mitis</i> , <i>S. oralis</i>	–	–	–	–	– +	V	α. Can be penicillin resistant. <i>S. oralis</i> can be esculin positive.

^a Abbreviations: ARG, hydrolysis of arginine; MAN, acid production from mannitol; SOR, acid production from sorbitol; + –, most strains positive but rare negative strains exist. See Table 3.18.1–1, footnote a for other abbreviations and symbols. Strains do not always produce glucans; test is only useful if positive. Commercial kits for identification of streptococci are helpful to resolve variable reactions. Data are extrapolated from reference 35. Also see references 7, 10, 25, and 28.

Table 3.18.1-3 Common species of enterococci and related PYR-positive cocci in chains^a

Organism	Motility ^b	Pigment ^c	MGP	Arginine dihydrolase	Arabinose	Mannitol	Lactose	Raffinose	Ribose	Sorbitol	45°C growth	Vancomycin
<i>E. avium</i>	—	—	V	—	+	+	—	—	+	+	+	S
<i>E. raffinosus</i>	—	—	V	—	+	+	+	+	+	+	+	V
<i>Vagococcus fluvialis</i>	+	—	+	—	—	+	—	—	+	+	—	S
<i>E. faecium</i>	—	—	—	+	—	+	—	+	—	+	+	V
<i>E. gallinarum</i>	+ ^d	—	+	+ ^d	+	+	+	+	+	+	D	R
<i>E. casseliflavus</i>	+ ^d	+ ^d	+	+ ^d	+	+	+	+	D	V	+	R
<i>E. mundtii</i>	—	+	—	+	+	+	+	+	+	+	+	S
<i>E. faecalis^d</i>	—	—	—	+ ^d	—	+ ^d	—	—	+	+	+	V
<i>Lactococcus garvieae</i>	—	—	—	+	—	+	—	—	+	+	—	S
<i>E. durans</i>	—	—	—	+	—	—	+	—	+	—	+	S
<i>E. hirae</i>	—	—	—	+	—	—	+	—	+	—	+	S
<i>E. dispar</i>	—	—	—	+	—	—	+	—	+	—	—	S

^a All species grow well on BAP and in 6.5% NaCl and are PYR, bile-esculin, and LAP positive. PYR-negative species *E. cecorum*, *E. columbae*, and *E. saccharolyticus* are not included and have not been isolated from humans. PYR-positive strains *E. malodorans*, *E. pseudalatum*, *E. asini*, and *E. sulfureus* (H_2S^+) also are not listed since they have not been isolated from humans. *E. gilvus* and *E. pallens* have been described to occur in humans but are extremely rare. Abbreviations: MGP, methyl- α -D-glucopyranoside; D, different reactions in references. See footnote ^a to Tables 3.18.1-1 and 3.18.1-2 for other abbreviations and symbols. Table adapted from references 11, 26, and 33. Also see references 20, 21, and 32.

^b Motility is done in 0.5 ml of BHI or TSB incubated at 30°C for 2 h.

^c Pigment (yellow) is observed by swabbing a blood agar plate incubated at 35°C in 5% CO₂ for 24 to 48 h and observing swab for bright yellow color (+).

^d Lactose-negative asaccharolytic *E. faecalis* exists.

Table 3.18.1–4a Biochemical reactions of PYR-positive, catalase-negative or weakly positive, gram-positive cocci (excluding *Streptococcus pyogenes*)

Genus or species	Gram stain	Phenotypic characteristics ^a							
		CAT	LAP	NaCl	10°C	45°C	Colony on BAP	Hemolysis	Bile-esculin
<i>Enterococcus</i> (some motile)	CH	—	+	+	+	+	Large	α, γ, β	+
<i>Lactococcus</i>	CH	—	+	V	+	—	Large	α, γ	+
<i>Vagococcus</i> (motile)	CH	—	+	+	+	—	Large	α, γ	+
<i>Abiotrophia/Granulicatella</i>	CH	—	+	—	—	—	Satellite	α, γ	—
<i>Globicatella</i>	CH	—	—	+	—	—	Small	α	V
<i>Dulosicoccus</i>	CH	—	—	—	—	—	Small	α	NA
<i>Aerococcus viridans</i>	CL/T	—, W	—	+	—	—	Large	α	V
<i>Helcococcus kunzii</i>	CL/T	—	—	V	—	—	Tiny	γ	—
<i>Gemella</i>	CL/T/CH	—	V	—	—	—	Tiny, 48 h to grow	α, γ	—
<i>Facklamia</i> (hippurate +)	CL/CH	—	+	+	—	—	Small	γ	—
<i>Alloiococcus otitis</i>	CL/T	W, +	+	+	—	—	Tiny, 72 h to grow	α	NA
<i>Ignavigranum</i> (hippurate —)	CL/CH	—	+	+	—	—	Satellite (V) or small	γ	—
<i>Rothia mucilaginosa</i>	CL	—, W, +	+	—	NA	NA	Sticky	γ	V
<i>Dulosigranulum</i>	CL/T	—	+	+	—	—	Small	γ	NA

^a CAT, catalase production; NaCl, growth in broth containing 6.5% NaCl; 10°C and 45°C, growth at 10 and 45°C, respectively (for the latter, use campylobacter incubator if heat block not available). Abbreviations for cell arrangement in Gram stain: CL, clusters; T, tetrads; CH, chains; W, weak. Large colonies are approximately 1 mm; small colonies are about the size of viridans group streptococci. See Table 3.18.1–1, footnote *a*, for other abbreviations and symbols. Tables adapted from references 9, 17, 22, and 25. Also see reference 6.

Table 3.18.1–4b Biochemical reactions of PYR-negative, catalase-negative gram-positive cocci

Genus or species	Gram stain	Phenotypic characteristics ^a						
		LAP	NaCl	Van	Arginine	45°C	MRS	
<i>Leuconostoc</i>	CH, rods	—	V	R	—	—	—	+
<i>Weissella confusa</i>	CH, rods	—	V	R	+	+	+	+
<i>Pediococcus</i>	CL/T	+	V	R		V	—	
<i>Streptococcus</i>	CH	+	—	S		V	—	
<i>Aerococcus urinae</i>	CL/T	+	+	S		—	—	

^a Van, vancomycin; MRS, gas production in MRS broth. See footnote *a* to Table 3.18.1–4a and Table 3.18.1 for other abbreviations.

Table 3.18.1–5 Catalase-negative, gram-positive rods that can grow aerobically^a

Organism(s)	H ₂ S	Vancomycin	Hemolysis	Hippurate	Motility	Nitrate	Esulin	Gram stain morphology
<i>Weissella</i> spp.	—	R	Alpha	—	—	NA	+	Small, short GPR; gas in MRS broth (22)
<i>Erysipelothrix rhusiopathiae</i>	+	R	Alpha	—	—	—	—	Has two cell forms; the long-chainning form can be confused with <i>Lactobacillus</i> , and the short form can be confused with <i>Actinomyces</i> or even <i>Enterococcus</i> (PYR positive)
<i>Lactobacillus</i> spp.	—	R	Alpha	—	—	V	V	Most lactobacilli are long regular-chainning rods. Some are C shaped.
<i>Arcanobacterium haemolyticum</i>	—	S	Beta	—	—	—	—	Branching, which can be rudimentary; reverse-CAMP positive, lecithinase positive, gelatin negative
<i>Arcanobacterium pyogenes</i>	—	S	Beta	+	—	—	—	Branching, which can be rudimentary; reverse-CAMP negative, lecithinase negative, gelatin positive
<i>Arcanobacterium bernardiae</i>	—	S	V	V	—	—	—	Reverse-CAMP negative, gelatin negative; does not branch; not beta-hemolytic on human blood. Kits can misidentify as <i>Gardnerella</i> .
<i>Gardnerella vaginalis</i>	—	S	—	+ —	—	—	NA	SPS sensitive; beta-hemolytic on human blood
<i>Bifidobacterium</i> spp.	—	S	—	+	—	—	+	Some are aerotolerant; can look like <i>Actinomyces</i> or <i>Gardnerella</i> ; not beta-hemolytic on human blood
<i>Actinomyces israelii</i>	—	S	—	NA	—	+	+	Branching, which can be rudimentary; anaerobic kits will identify; urease negative
<i>Actinomyces</i> spp.	—	S	—	V	—	V	V	Not all species show branching; <i>A. naeslundii</i> and others are urease positive. Some colonies of <i>A. meyerii-odontolyticus</i> group turn red after 1 wk.
Aerotolerant <i>Clostridium</i>	—	S	—	NA	V	+ —	+	Forms spores; medium to large regular rod; grows slowly compared to <i>Bacillus</i> ; anaerobic kits will identify

^a Data from references 2, 5, 8, and 24. Once *G. vaginalis*, *Arcanobacterium*, *Weissella*, and *E. rhusiopathiae* are ruled out, either call “Anaerobic gram-positive rod” or do anaerobic identification kit. GPR, gram-positive rods; SPS, sodium polyanethol sulfonate. See footnote *a* to Tables 3.18.1–1 and 3.18.1–2 for other abbreviations and symbols. See Table 3.18.1–9 for catalase-positive *Actinomyces*.

Table 3.18.1–6 Catalase-positive, usually yellow- or pink-pigmented gram-positive rods^a

Organism(s)	Motility	Fermentation	Nitrate	Urease	Esculin	Gelatin	Glucose	Comment
<i>Cellulosimicrobium/Cellulomonas (Oerskovia) spp.</i>	+	+	+/V	-	+	+	+	Subsurface or surface hyphae/pseudohyphae may be present.
<i>Microbacterium (Aureobacterium) spp.</i>	+ -	V	V	V	+ -	V	+	
<i>Exiguobacterium acetylicum</i>	+	+	V	-	+	V	+	Golden yellow to orange
<i>Leifsonia aquatica</i>	V	-	V	-	V	V	+	Previously called "Corynebacterium aquaticum"
<i>Corynebacterium falsenii</i>	-	W	V	W	V	NA	W	Yellow after 72 h
<i>Corynebacterium lipophiloflavum</i>	-	+	-	W	-	-	-	Lipophilic, rarely isolated
<i>Corynebacterium mucificiens</i>	-	-	-	-	-	-	+	Slightly to deep yellow mucoid colonies, CAMP negative
<i>Rhodococcus equi</i>	-	-	V	+	-	-	-	Mucoid in 48 h, usually pink after 4–7 days, can be yellowish, CAMP positive. Can be acid fast. Important pathogen.

^a If motile and yellow or positive for esculin and/or gelatin, report as "Motile coryneform, not *Corynebacterium* spp." If of clinical significance, use kit (e.g., Coryne API or RapID Coryne) or send to reference laboratory. Other yellow *Corynebacterium* spp. (*C. aurimucosum*, *C. falsenii*, and *C. sanguinis*) are rarely isolated or associated with human disease. Data are from references 13, 14, 29, and 31; also see section 6. See footnote ^a to Tables 3.18.1–1, 3.18.1–2, and 3.18.1–3 for abbreviations and symbols.

Table 3.18.1–7 Large, regular catalase-positive, gram-positive rods that usually produce spores and are usually motile^a

Organism(s)	Diam of cell usually above 1 µm	Motility ^b	Beta-hemolysis	Lecithinase	Penicillin	Large colony at 24 h	Sticky, tenacious colony at 24 h
<i>B. anthracis</i>	+	-	-	+	S ^b	+	+
<i>B. cereus</i>	+	+	+	+	R	+	-
<i>Bacillus thuringiensis</i>	+	+ , V	+	+	R	+	-
<i>Bacillus mycoides</i>	+	-	W	+	R	+	-
<i>Bacillus megaterium</i>	+	+ , V	V	-	S	+	-
Other <i>Bacillus</i> spp. and related groups ^c	-	+ , V	- +	-	V	V	V

^a *B. cereus*, *B. thuringiensis* (insect pathogen used in horticulture), *B. mycoides* (rhizoids or hairy projections in agar), and *B. anthracis* are included in the *B. cereus* group. If organism is motile with spores, penicillin resistant, hemolytic, with cells greater than 1 µm in diameter, and/or lecithinase positive, report as "*Bacillus cereus* group, not *B. anthracis*." Otherwise, if organism is motile, with spores, but nonhemolytic and/or lecithinase negative, report as "*Bacillus*, not *B. anthracis* or *B. cereus*." Data are from references 18 and 34. Spores can be induced by growing on urea, bile-esculin agar, or an agar plate with vancomycin disk or at 45°C. Spores can be proved by heating broth culture to 80°C for 10 min and subculturing to BAP. Viable colonies indicate that spores survived the heating. See Table 3.18.1–1, footnote ^a, for other abbreviations and symbols.

^b Submit any nonmotile, spore-forming strain to designated higher reference laboratory to rule out *B. anthracis*, regardless of the penicillin susceptibility.

^c *Kurthia* (diameter, 0.8 to 1.2 µm) organisms are motile and nonhemolytic and do not produce spores (13).

Table 3.18.1–8 Urease-positive *Corynebacterium* spp. of clinical importance^a

Organism	Nitrate	Urease	Pyrazinamidase	Glucose	Sucrose	Lipophilism	CAMP reaction
<i>C. glucuronolyticum</i>	V	V	+	+	+	-	+
<i>C. pseudotuberculosis</i> ^b	V	+	-	+	V	-	Reverse +
<i>C. ulcerans</i> ^b	-	+	-	+	-	-	Reverse +
<i>C. pseudodiphtheriticum</i> ^c	-	+	+	-	-	-	-
<i>C. riegelii</i> ^d	-	+	V	-	-	-	-
<i>C. urealyticum</i> ^e	-	+	+	-	-	+	-
<i>C. amycolatum</i>	V	V	+	+	V	-	-
CDC group F1	V	+	+	+	+	+	-

^a Data are from references 13 and 14. Other urease-positive or -variable species of less clinical significance include *C. durum*, *C. falsenii*, *C. singulare*, *C. sundsvallense*, and *C. thomssenii*, which are CAMP and reverse-CAMP negative, are not lipophilic, and ferment glucose. See Table 3.18.1–1, footnote *a*, for abbreviations and symbols.

^b Submit to reference laboratory for diphtheria toxin testing. *C. pseudotuberculosis* is associated with sheep handlers.

^c Respiratory pathogen; not able to acidify maltose, ribose, or trehalose.

^d *C. riegelii* is rarely isolated but has been found in urine and other body sites (13). It is able to acidify maltose.

^e Urinary pathogen; multiresistant to antimicrobials.

Table 3.18.1–9 Catalase-positive, urease-negative, gram-positive rods, excluding *Corynebacterium* spp. and yellow- or pink-pigmented rods^a

Organism(s)	Fermentation	Nitrate	Esculin	Gelatin	Glucose	CAMP	Gram stain or colony appearance
<i>Actinomyces neuii</i>	+	V	-	-	+	+	Nonhemolytic, slight branching
<i>Actinomyces viscosus</i>	+	+	-	NA	+	-	
<i>Propionibacterium avidum/granulosum</i>	+	-	V	V	+	+	Beta-hemolytic, branching
<i>Turicella otitidis</i>	-	-	-	-	-	+	Large rod, branching; ear pathogen CAMP-positive <i>C. auris</i> and <i>C. af fermentans</i> have similar reactions
<i>Brevibacterium</i> spp.	-	V	-	+	-	-	Some yellowish, distinct odor
<i>Dermabacter hominis</i>	+	-	+	+	+	-	Coccoid rods, distinct odor Lysine +, arginine -, ornithine +
<i>Rothia</i> spp.	+	+	+	V	+	-	Some branching, some black pigmented; if sticky, refer to Tables 3.18.1–1 and 3.18.1–4a for <i>R. mucilaginosa</i> (previously <i>Stomatococcus mucilaginosus</i>) (6).

^a Usually irregular rods. Identify only if clinically significant, but all can be pathogens (13, 14). The important tests are fermentation (use Andrade's base or cysteine Trypticase agar), CAMP, and Gram stain, with careful reading of Gram stain morphology. Coryneform identification kits can be helpful. For abbreviations and symbols, see Table 3.18.1–1, footnote *a*.

Table 3.18.1–10 Urease-negative *Corynebacterium* spp. of clinical importance^a

Organism(s)	Nitrate	Urease	Pyrazinamidase	Alkaline phosphatase	Glucose	Maltose	Sucrose	Lipophilism	CAMP	Comment(s)
<i>C. accolens</i>	+	–	V	–	+	–	V	+	–	
CDC group G ^b	V	–	+	+	+	V	V	+	–	Fructose positive
<i>C. jeikeium</i> ^b	–	–	+	+	+	V	–	+	–	Fructose negative
<i>C. afermentans</i>	–	–	V	+	–	–	–	V	V	One subspecies is lipophilic.
<i>C. macginleyi</i>	+	–	–	+	+	–	+	+	–	Found in eye specimens.
<i>C. diphtheriae</i> ^c	+	–	–	–	+	+	–	–	–	<i>C. diphtheriae/belfanti</i> is nitrate negative; <i>C. diphtheriae/internmedius</i> is lipophilic.
<i>C. propinquum</i>	+	–	V	V	–	–	–	–	–	
<i>C. amycolatum</i> ^b	V	V	+	+	+	V	V	–	–	Dry colony, O/129 R, a common species of human resident microbiota; can be misidentified as <i>C. xerosis</i> .
<i>C. minutissimum</i>	–	–	+	+	+	+	V	–	–	O/129 S, DNase positive, PYR positive
<i>C. striatum</i>	+	–	+	+	+	–	V	–	V	O/129 S
<i>C. xerosis</i>	V	–	+	+	+	+	+	–	–	Creamy colony, O/129 S, LAP positive

^a Other species that are rare are not listed (see references 13 and 14), including some CAMP test-positive species. Some *Corynebacterium* organisms have black-pigmented colonies. For identification of species in this table, the combination of CAMP test, lipophilism, O/129 disk, and commercial kits for corynebacteria should be used if identification is clinically important. For abbreviations and symbols, see footnote ^a to Tables 3.18.1–1 and 3.18.1–9.

^b Multiresistant to antimicrobials.

^c Submit to reference laboratory for diphtheria toxin testing.

REFERENCES

- Bannerman, T. 2003. *Staphylococcus, Micrococcus*, and other catalase-positive cocci that grow aerobically, p. 384–404. In P. R. Murray, E. J. Baron, J. H. Jorgensen, M. A. Pfaller, and R. H. Yolken (ed.), *Manual of Clinical Microbiology*, 8th ed. ASM Press, Washington, D.C.
- Bille, J., J. Rocourt, and B. Swaminathan. 2003. *Listeria* and *Erysipelothrix*, p. 461–471. In P. R. Murray, E. J. Baron, J. H. Jorgensen, M. A. Pfaller, and R. H. Yolken (ed.), *Manual of Clinical Microbiology*, 8th ed. ASM Press, Washington, D.C.
- Chuard, C., and L. B. Reller. 1998. Bile-esculin test for presumptive identification of enterococci and streptococci: effects of bile concentration, inoculation technique, and incubation time. *J. Clin. Microbiol.* **36**:1135–1136.
- Clarridge, J. E., III, J. S. M. Attorri, Q. Zhang, and J. Bartell. 2001. 16S ribosomal DNA sequence analysis distinguishes biotypes of *Streptococcus bovis*: *Streptococcus bovis* biotype II/2 is a separate genospecies and the predominant clinical isolate in adult males. *J. Clin. Microbiol.* **39**:1549–1552.
- Clarridge, J. E., III, and Q. Zhang. 2002. Genotypic diversity of clinical *Actinomyces* species: phenotype, source, and disease correlation among genospecies. *J. Clin. Microbiol.* **40**:3442–3448.
- Collins, M. D., R. A. Hutson, V. Baverud, and E. Falsen. 2000. Characterization of a *Rothia*-like organism from a mouse: description of *Rothia nasimurium* sp. nov. and reclassification of *Stomatococcus mucilaginosus* as *Rothia mucilaginosa* comb. nov. *Int. J. Syst. Evol. Microbiol.* **50**:1247–1251.
- Coykendall, A. L. 1989. Classification and identification of the viridans streptococci. *Clin. Microbiol. Rev.* **2**:315–328.
- Dunbar, S. A., and J. E. Clarridge III. 2000. Potential errors in the recognition of *Erysipelothrix rhusiopathiae*. *J. Clin. Microbiol.* **38**:1302–1304.
- Facklam, R., and J. A. Elliott. 1995. Identification, classification, and clinical relevance of catalase-negative, gram-positive cocci, excluding the streptococci and enterococci. *Clin. Microbiol. Rev.* **8**:479–495.

REFERENCES (continued)

10. Facklam, R. 2002. What happened to the streptococci: overview of taxonomic and nomenclature changes. *Clin. Microbiol. Rev.* **15**:613–630.
11. Facklam, R. R., and M. D. Collins. 1989. Identification of *Enterococcus* species isolated from human infections by a conventional test scheme. *J. Clin. Microbiol.* **27**:731–734.
12. Falk, D., and S. J. Guering. 1983. Differentiation of *Staphylococcus* and *Micrococcus* spp. with the Taxo A bacitracin disk. *J. Clin. Microbiol.* **18**:719–721.
13. Funke, G., and K. A. Bernard. 2003. Coryneform gram-positive rods, p. 472–501. In P. R. Murray, E. J. Baron, J. H. Jorgensen, M. A. Pfaller, and R. H. Yolken (ed.), *Manual of Clinical Microbiology*, 8th ed. ASM Press, Washington, D.C.
14. Funke, G., A. von Graevenitz, J. E. Clarridge III, and K. Bernard. 1997. Clinical microbiology of coryneform bacteria. *Clin. Microbiol. Rev.* **10**:125–159.
15. Hébert, G. A. 1990. Hemolysins and other characteristics that help differentiate and biotype *Staphylococcus lugdunensis* and *Staphylococcus schleiferi*. *J. Clin. Microbiol.* **28**:2425–2431.
16. Hébert, G. A., C. G. Crowder, G. A. Hancock, W. R. Jarvis, and C. Thornsberry. 1988. Characteristics of coagulase-negative staphylococci that help differentiate these species and other members of the family *Micrococcaceae*. *J. Clin. Microbiol.* **26**:1939–1949.
17. LaClaire, L. L., and R. R. Facklam. 2000. Comparison of three commercial rapid identification systems for the unusual gram-positive cocci *Dolosigranulum pigrum*, *Ignavigranum ruoffiae*, and *Facklamia* species. *J. Clin. Microbiol.* **38**:2037–2042.
18. Logan, N. A., and P. C. B. Turnbull. 2003. *Bacillus* and other aerobic endospore-forming bacteria, p. 445–460. In P. R. Murray, E. J. Baron, J. H. Jorgensen, M. A. Pfaller, and R. H. Yolken (ed.), *Manual of Clinical Microbiology*, 8th ed. ASM Press, Washington, D.C.
19. Mahoudeau, I., X. Delabranche, G. Prevost, H. Monteil, and Y. Piemont. 1997. Frequency of isolation of *Staphylococcus intermedius* from humans. *J. Clin. Microbiol.* **35**:2153–2154.
20. Manero, A., and A. R. Blanch. 1999. Identification of *Enterococcus* spp. with a biochemical key. *Appl. Environ. Microbiol.* **65**:4425–4430.
21. Murray, B. E. 1990. The life and times of the *Enterococcus*. *Clin. Microbiol. Rev.* **3**:46–65.
22. Olano, A., J. Chua, S. Schroeder, A. Minari, M. La Salvia, and G. Hall. 2001. *Weissella confusa* (basonym: *Lactobacillus confusus*) bacteremia: a case report. *J. Clin. Microbiol.* **39**:1604–1607.
23. Patel, R., K. E. Piper, M. S. Rouse, J. R. Uhl, F. R. Cockerill III, and J. M. Steckelberg. 2000. Frequency of isolation of *Staphylococcus lugdunensis* among staphylococcal isolates causing endocarditis: a 20-year experience. *J. Clin. Microbiol.* **38**:4262–4263.
24. Reimer, L. G., and L. B. Reller. 1985. Use of a sodium polyanetholesulfate disk for the identification of *Gardnerella vaginalis*. *J. Clin. Microbiol.* **21**:146–149.
25. Ruoff, K. L. 2002. Miscellaneous catalase-negative, gram-positive cocci: emerging opportunists. *J. Clin. Microbiol.* **40**:1129–1133.
26. Ruoff, K. L., L. de la Maza, M. J. Murtagh, J. D. Spargo, and M. J. Ferraro. 1990. Species identities of enterococci isolated from clinical specimens. *J. Clin. Microbiol.* **28**:435–437.
27. Ruoff, K. L., S. I. Miller, C. V. Garner, M. J. Ferraro, and S. B. Calderwood. 1989. Bacteremia with *Streptococcus bovis* and *Streptococcus salivarius*: clinical correlates of more accurate identification of isolates. *J. Clin. Microbiol.* **27**:305–308.
28. Ruoff, K. L., R. A. Whiley, and D. Beighton. 2003. *Streptococcus*, p. 405–421. In P. R. Murray, E. J. Baron, J. H. Jorgensen, M. A. Pfaller, and R. H. Yolken (ed.), *Manual of Clinical Microbiology*, 8th ed. ASM Press, Washington, D.C.
29. Schumann, P., N. Weiss, and E. Stackebrandt. 2001. Reclassification of *Cellulomonas cellulans* (Stackebrandt and Keddie 1986) as *Cellulosimicrobium cellulans* gen. nov., comb. nov. *Int. J. Syst. Evol. Microbiol.* **51**:1007–1010.
30. Shuttleworth, R., R. J. Behme, A. McNabb, and W. D. Colby. 1997. Human isolates of *Staphylococcus caprae*: association with bone and joint infections. *J. Clin. Microbiol.* **35**:2537–2541.
31. Takeuchi, M., and K. Hatano. 1998. Union of the genera *Microbacterium* Orla-Jensen and *Aureobacterium* Collins et al. in a redefined genus *Microbacterium*. *Int. J. Syst. Bacteriol.* **48**:739–747.
32. Teixeira, L. M., M. G. Carvalho, V. L. Merquior, A. G. Steigerwalt, D. J. Brenner, and R. R. Facklam. 1997. Phenotypic and genotypic characterization of *Vagococcus fluvialis*, including strains isolated from human sources. *J. Clin. Microbiol.* **35**:2778–2781.
33. Teixeira, L. M., and R. R. Facklam. 2003. *Enterococcus*, p. 422–433. In P. R. Murray, E. J. Baron, J. H. Jorgensen, M. A. Pfaller, and R. H. Yolken (ed.), *Manual of Clinical Microbiology*, 8th ed. ASM Press, Washington, D.C.
34. Turnbull, P. C. B., and J. M. Kramer. 1991. *Bacillus*, p. 296–303. In A. Balows, W. J. Hausler, Jr., K. L. Herrmann, H. D. Isenberg, and H. J. Shadomy (ed.), *Manual of Clinical Microbiology*, 5th ed. American Society for Microbiology, Washington, D.C.
35. Whiley, R. A., and D. Beighton. 1998. Current classification of the oral streptococci. *Oral Microbiol. Immunol.* **13**:195–216.

3.18.2

Identification of Gram-Negative Bacteria

[Updated March 2007]

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

The gram-negative bacteria are a heterogeneous group of organisms consisting of numerous families, genera, and species, many of which cause serious disease and endotoxic shock. The accurate, rapid identification of gram-negative rods and diplococci provides timely, meaningful treatment and diagnosis of disease. Lack of identification or recognition of a serious pathogen can have long-term effects on the cure and spread of disease. The figures and tables that follow are designed to rapidly determine the agent of infection and to provide guidance in order to avoid misidentification of serious pathogens.

The algorithms presented include a combination of rapid tests for identification, some of which are based on Clinical and Laboratory Standards Institute (formerly NCCLS) guidelines for rapid identification (12) and others of which are based on established references on identification of gram-negative rods (6, 8, 11, 13, 14; G. L. Gilardi, unpublished identification tables). The use of commercial automated and manual multitest systems (kits) is encouraged when colony morphology and a few rapid tests do not provide a definitive identification. Commercial kit systems do an excellent job of identifying most pathogens with minimal work, and they should be a part of any laboratory's identification system. Deciding when to use a commercial system and which system to use for identification is key to success in providing the identification in a timely manner. In addition, knowing the limitations of the databases of the kit systems can be helpful in decid-

ing when to pursue the identification further using the tests listed in the tables that follow. Commercial kit systems are presented in Tables 3.16–1 and 3.16–2 and are also reviewed elsewhere (5, 11). These references should be consulted when deciding which kits to purchase and what are the limitations of the databases. In the algorithms presented here, specific manufacturers' kits are not presented.

The great majority of gram-negative strains found in clinical specimens are either *Escherichia coli* or *Pseudomonas aeruginosa*. The identification schemes that follow include rapid identification of these two pathogens, when they produce typical colony morphologies and reactions. The figures also show the identification of many of the gram-negative rod agents of bioterrorism in less than 2 h (refer to section 16). The figures and tables are designed to prevent misidentifications of agents of bioterrorism, as well as many other gram-negative rods which cause life-threatening infections.

The figures that follow include rapid tests (oxidase, indole, and catalase) that lead the user to the appropriate table or definitive identification, when combined with colony morphology. Most are performed in 2 min. The figures are designed to do the minimum amount of work to arrive at a good identification or lead to the appropriate table or kit, without doing tests that are not helpful. For that reason, the figures may not include all of the options at each step, because the observance of one colony morphology may lead to performing a certain test, while the lack of

that morphology may suggest a different test. The figures send the reader to tables which include many organisms that may not be clinically significant. These organisms are listed to avoid misidentifications and to provide biochemical reactions for identification, should they be isolated from a significant site. Finally, the reader is referred to other texts (11, 14) for a more extensive list of tests when the minimal tests indicate what microorganism is suspected but other testing might be helpful to confirm the identification.

An example of using the figures may be helpful. If a nonpigmented respiratory isolate did not grow on MAC but grew at 48 h on BAP, Fig. 3.18.2–3 indicates performing Gram stain, indole, catalase, and oxidase. If the catalase is positive and the oxidase and indole are negative, the figure indicates testing with a polymyxin B disk and consulting Table 3.18.2–5. In Table 3.18.2–5 there are only five microorganisms listed that are oxidase negative or variable, and only one of those is a respiratory coccobacillus. In our case, the Gram stain showed the coccobacillary morphology. A positive rapid (2-h) urea test will complete a preliminary identification of *Bordetella parapertussis*. Inoculation to Mueller-Hinton (MH) agar with a polymyxin B disk for observation of the brown pigment and susceptibility to polymyxin B would be confirmatory and rule out the rare possibility of *Burkholderia mallei*, the agent of glanders (potential bioterrorist agent). However, the report would have already been communicated to

the caregiver for appropriate treatment and isolation techniques. Thus, *B. parapertussis* was identified to the species level in less than 2 h with three <1-min tests, a Gram stain, and one 2-h biochemical test.

To have performed a commercial identification system on this isolate would have delayed the final identification and resulted in needless cost and erroneous results, since this pathogen is not in the databases of commonly used identification systems. The user is encouraged to follow the charts to arrive at appropriate testing and rapid reporting, often without the need for using more expensive commercial kits.

II. MICROORGANISMS

III. REAGENTS AND SUPPLIES

- A. Gram-negative rods or cocci as determined by Gram stain
- B. Assumed gram-negative rods because of colony morphology or growth on MAC or EMB

NOTE: Procedures for methods for biochemical tests are listed in procedures 3.17.1 through 3.17.48. To the extent that tests are available in kits, it is not necessary to stock the separate tests.

A. Minimum useful for most laboratories

1. Catalase (3 and 30%) (procedure 3.17.10)
2. Indole (spot, Kovács', and Ehrlich's) (procedure 3.17.23)
3. Oxidase test reagent (procedure 3.17.39)
4. Polymyxin B (300-U) (or colistin [10-µg]) and penicillin (10-U) disks (procedure 3.17.4)
5. MH agar
6. Butyrate disks (procedure 3.17.7)
7. δ-Aminolevulinic acid (ALA) test (procedure 3.17.3)
8. Pyrrolidonyl-β-naphthylamide (PYR) (procedure 3.17.41)
9. Triple sugar iron agar (TSI) or Kligler's iron agar (KIA) (procedure 3.17.25)
10. Urea—rapid method preferred (procedure 3.17.48)
11. Motility medium—wet mount may suffice (procedure 3.17.31)
12. Kit for *Neisseria* identification (see procedure 3.9.3 for options)
13. Kit(s) for identification of enteric and nonfermenting, gram-negative rods and fastidious other gram-negative rods (Tables 3.16–1 and 3.16–2)

B. Desired for large-volume and reference laboratories and for those laboratories that provide service to cystic fibrosis patients

1. 4-Methylumbelliferyl-β-D-glucuronide (MUG) (procedure 3.17.34)
2. Andrade's glucose broth with rabbit serum (procedure 3.17.9)
3. MH agar with 4% salt (procedure 3.17.43)
4. O/129 150-µg disks (procedure 3.17.36)
5. Acetamide or F and P agar for *Pseudomonas* (procedures 3.17.1 and 3.17.17)
6. Gelatin (procedure 3.17.18)
7. Lysine, ornithine, arginine decarboxylase-dihydrolase broth (Møller's) (procedure 3.17.15)
8. OF medium (procedure 3.17.9)
9. Nitrate medium (procedure 3.17.35)
10. DNase (procedure 3.17.16)
11. Esculin (procedure 3.17.5)
12. Phenylalanine deaminase (PDA) (procedure 3.17.40)
13. Sugar fermentation media or commercial kit with sugar fermentation tests for fastidious microorganisms (procedure 3.17.9)

ANALYTICAL CONSIDERATIONS

IV. PROCEDURE

- A. Observe colony morphology on BAP, CHOC, and MAC or EMB.
- B. Perform oxidase from BAP or CHOC.

NOTE: To avoid misidentifications, do not skip this step. Omit only for obvious spreading *Proteus* or satelliting colonies.

IV. PROCEDURE (continued)

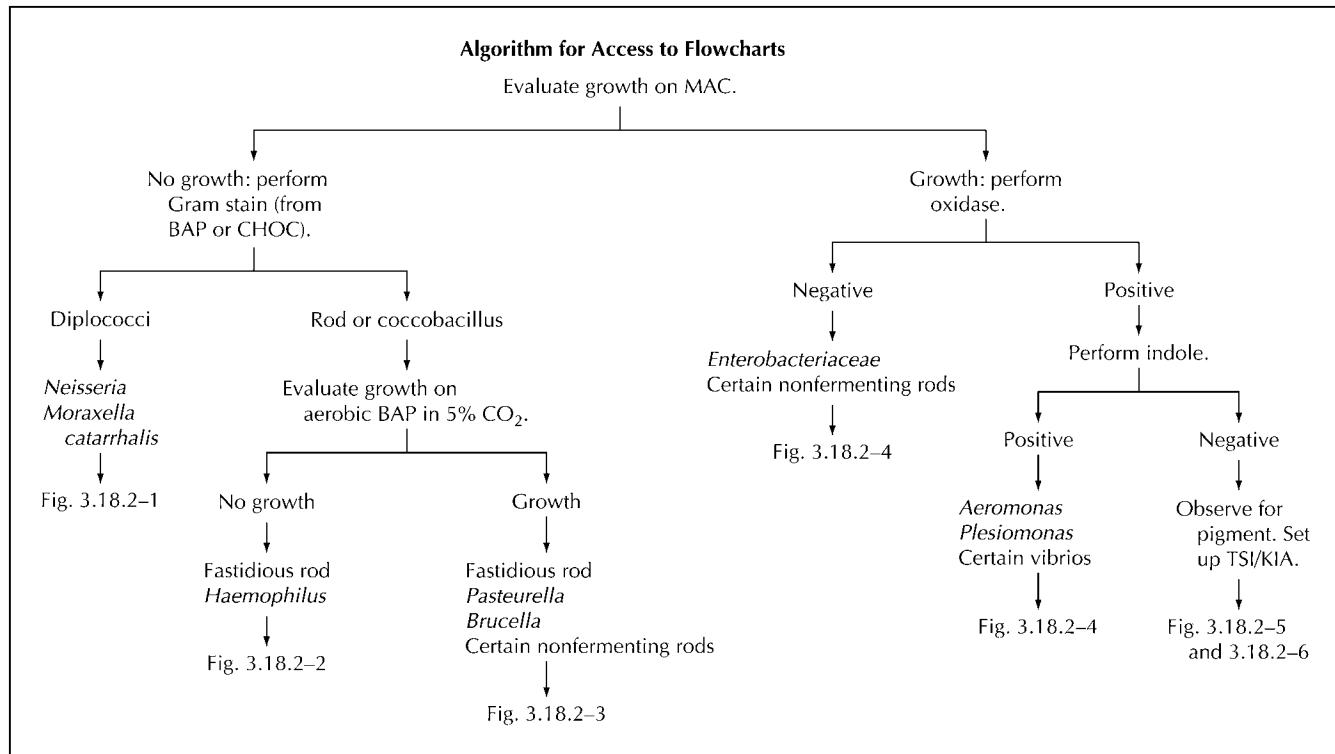
- C. If microorganism is not growing on MAC, perform Gram stain and catalase test.
■ **NOTE:** With rare exceptions, organisms that grow on MAC are catalase-positive, gram-negative rods.
- D. Follow Fig. 3.18.2–1 if the organism is a diplococcus.
- E. Proceed to Fig. 3.18.2–2 if the organism is a gram-negative rod that does not grow on BAP.
- F. Perform spot indole test on gram-negative rods that are growing on BAP. For organisms that grow poorly on MAC, performing the indole test with Kovács' reagent may be necessary. For organisms that are yellow, perform the tube indole test with Ehrlich's method.
■ **NOTE:** To avoid misidentifications, do not skip this step.
- G. Proceed to Fig. 3.18.2–3 for microorganisms that grow on BAP but do not grow or grow poorly on MAC.
 1. In order to determine if a gram-negative rod ferments glucose, inoculate TSI or KIA.
■ **NOTE:** Many organisms that do not grow on MAC will not show fermentation in OF media, which leads to erroneous identifications. Users may skip this step if they have the experience to recognize that a specific colony morphology represents a suspected nonfermenting rod. For example, many technologists can recognize colonies of *P. aeruginosa* or *Stenotrophomonas maltophilia* and go directly to Fig. 3.18.2–6.
 2. To see if a nonfermenting rod is an oxidizer, inoculate OF medium or use a commercial test system designed to identify nonfermenting rods.
- H. Follow Fig. 3.18.2–4 to identify organisms that grow on MAC and are either oxidase negative or oxidase positive and indole positive.
- I. Go to Fig. 3.18.2–5 and 3.18.2–6 sequentially to identify oxidase-positive, indole-negative rods or oxidase-negative, nonfermenting rods that grow on MAC.
- J. Observe plates at 48 h for colonies that were not present the first day of incubation. This step is particularly important for respiratory specimens.

POSTANALYTICAL CONSIDERATIONS**V. REPORTING AND INTERPRETATION OF RESULTS**

- A. Follow tables and kit identifications to report genus and species as appropriate without delay.
- B. Significant organisms are noted in the flowcharts to alert the user. Less significant microorganisms can be reported with minimal testing (e.g., yellow pseudomonad).

VI. LIMITATIONS

Because many tests are not 100% positive or negative for a particular strain, it is desirable that more than one positive reaction be used in the confirmation of an identification.



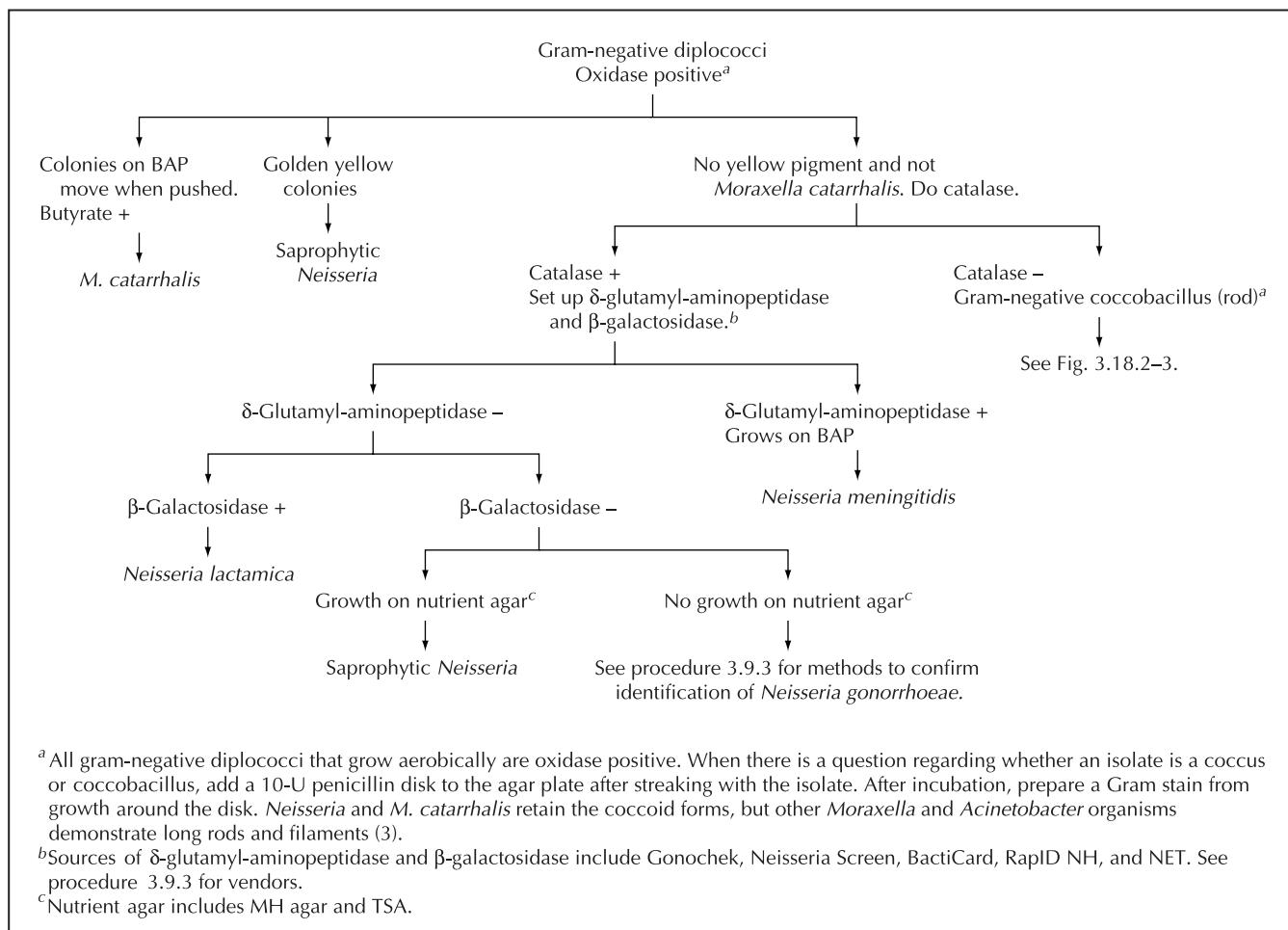


Figure 3.18.2-1 Identification scheme for gram-negative diplococci; also see Table 3.18.2-1.

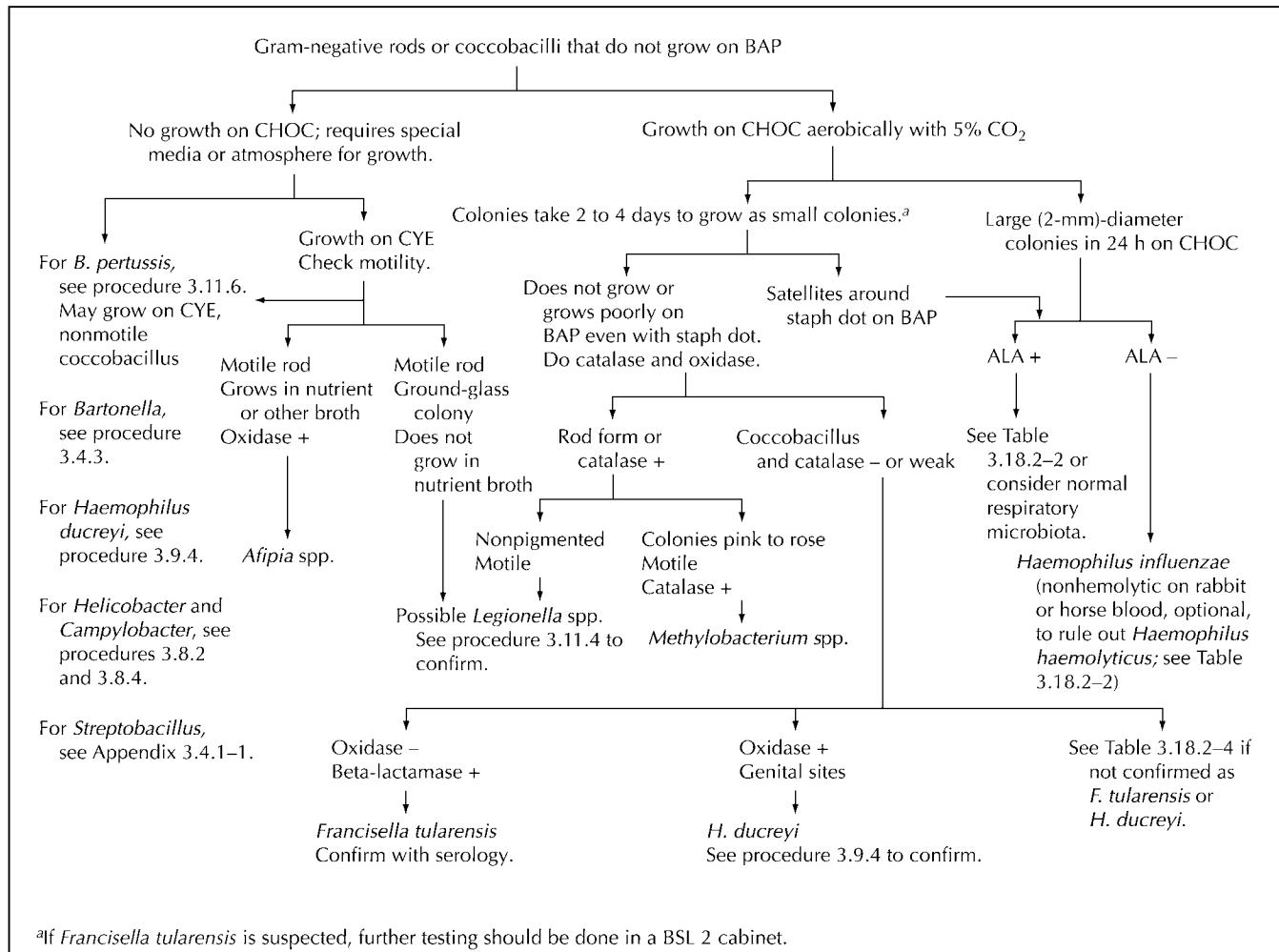


Figure 3.18.2–2 Identification scheme for gram-negative rods that do not grow on BAP aerobically in 5% CO₂. CYE, charcoal-yeast extract agar.

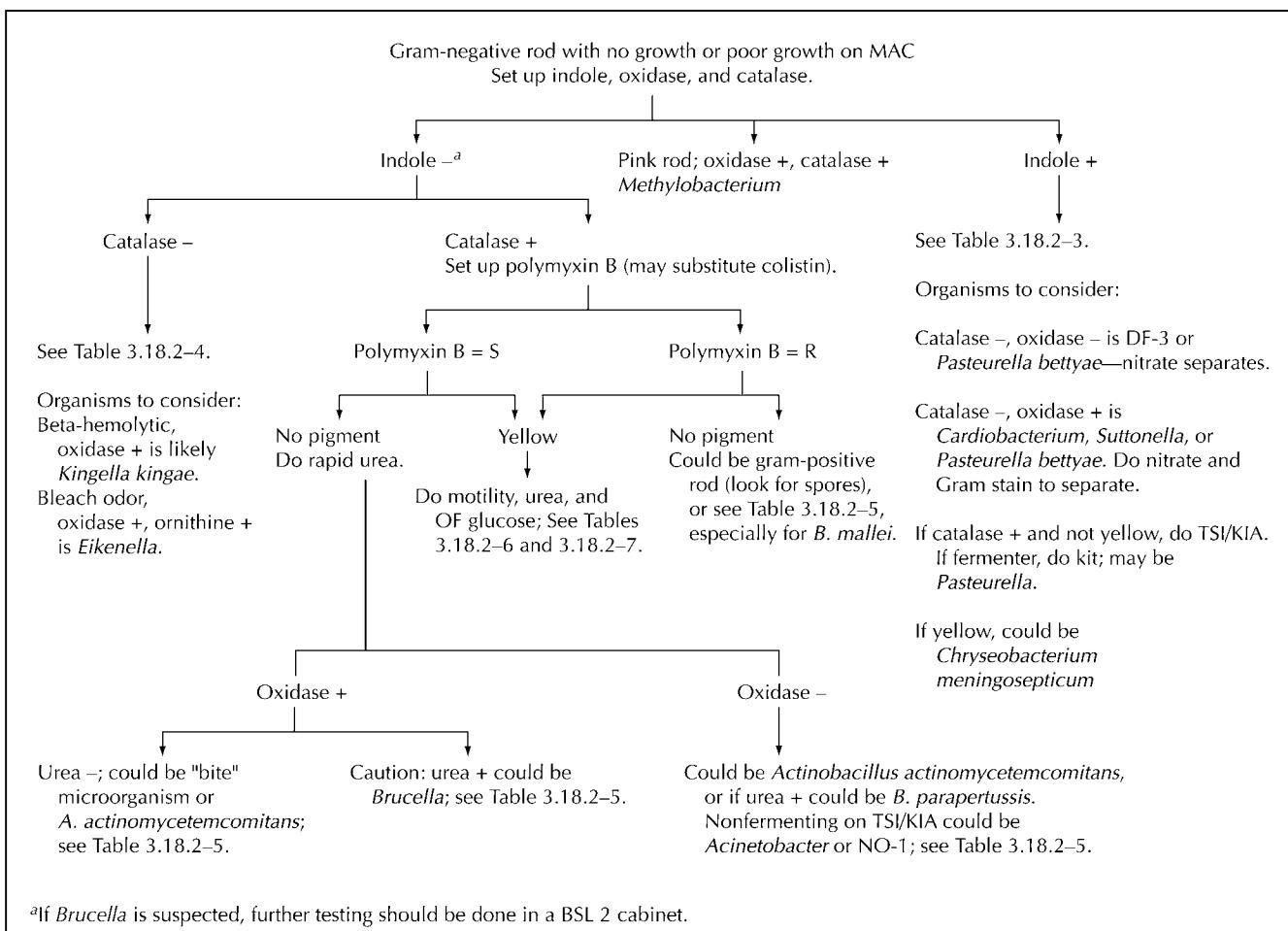


Figure 3.18.2–3 Identification scheme for gram-negative rods that grow on BAP with 5% CO₂ but do not grow well on MAC in 48 h.

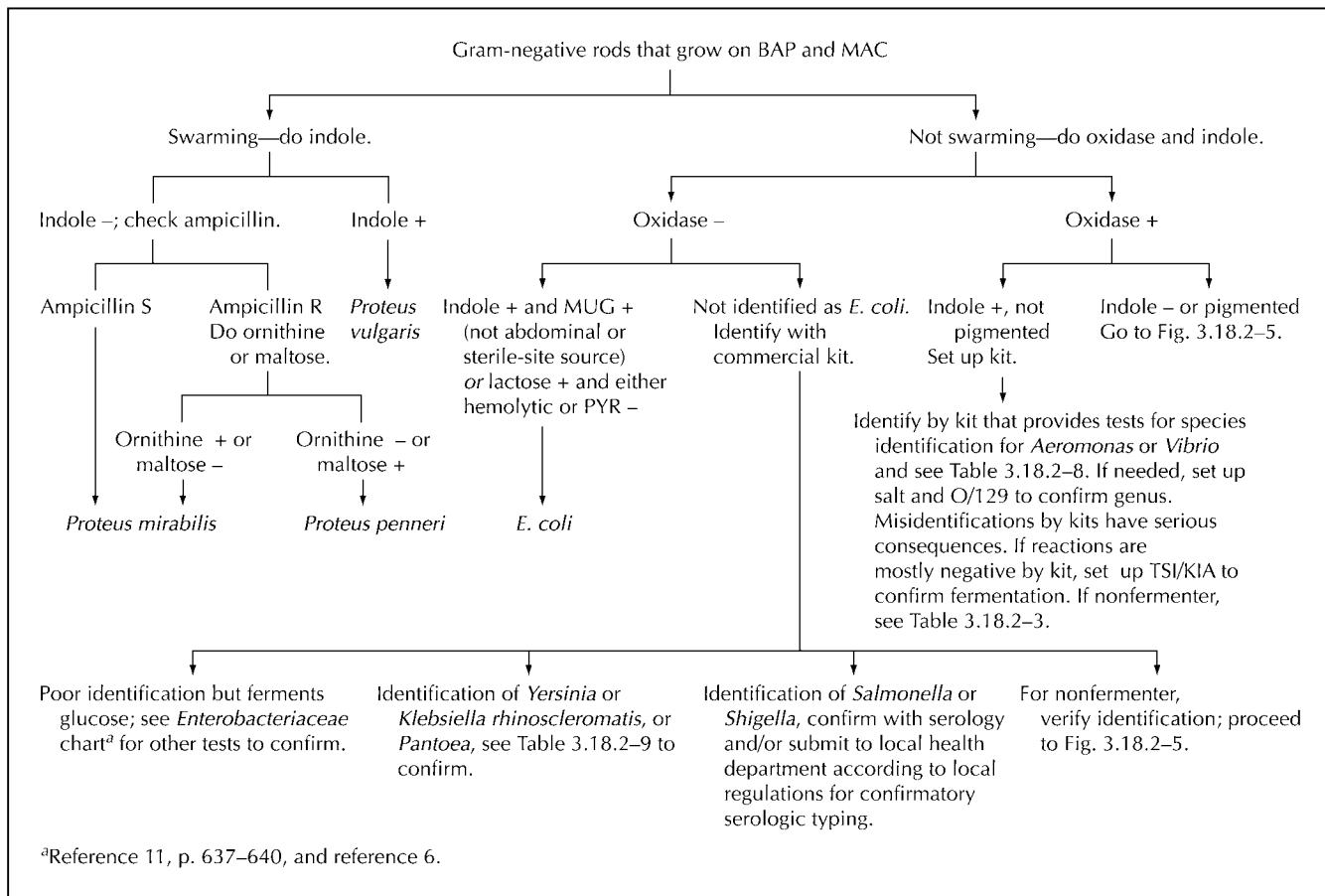


Figure 3.18.2-4 Identification scheme for gram-negative rods that grow on BAP and MAC.

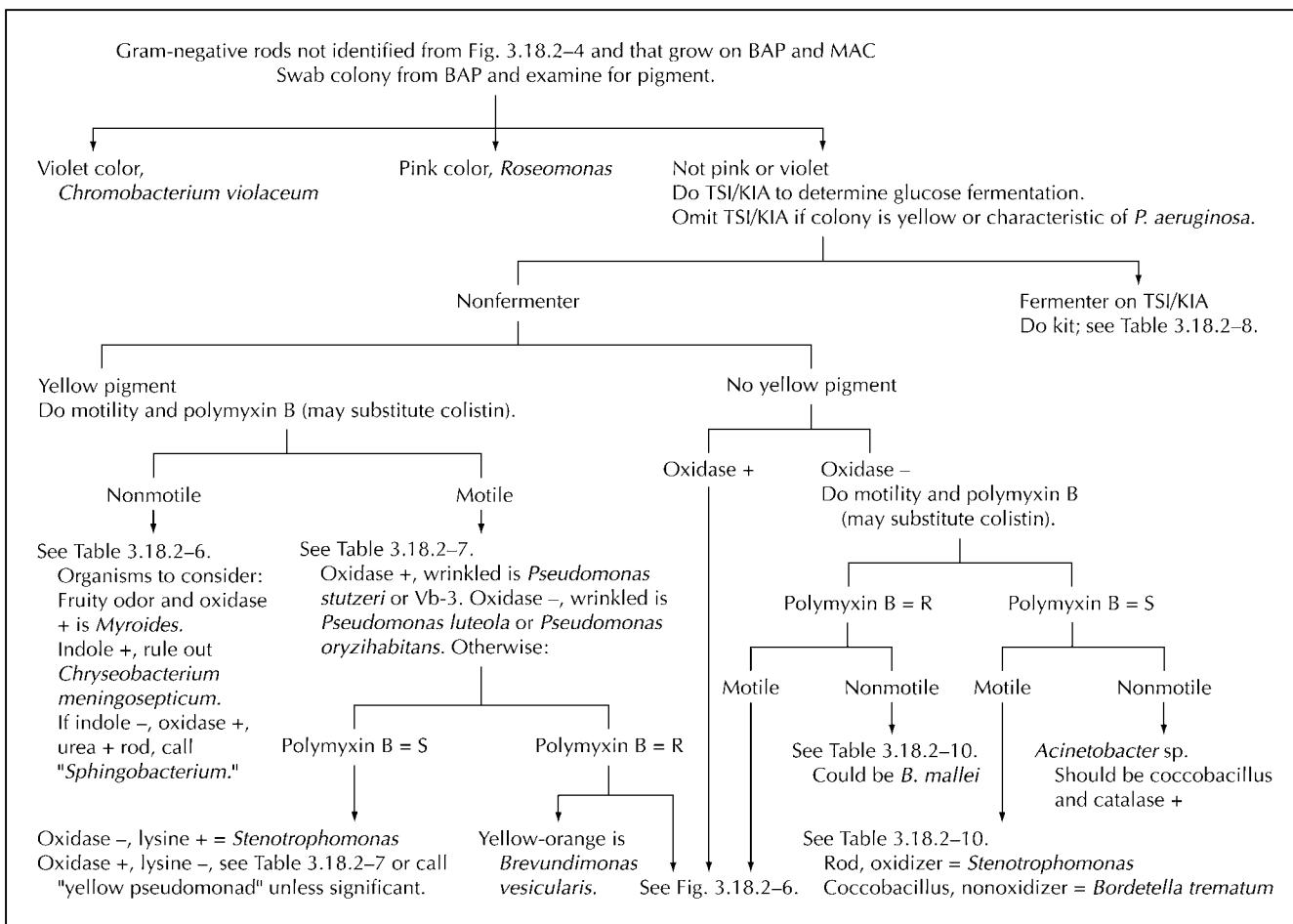


Figure 3.18.2-5 Identification scheme for gram-negative rods that grow well on BAP and MAC and are not identified from Fig. 3.18.2-4.

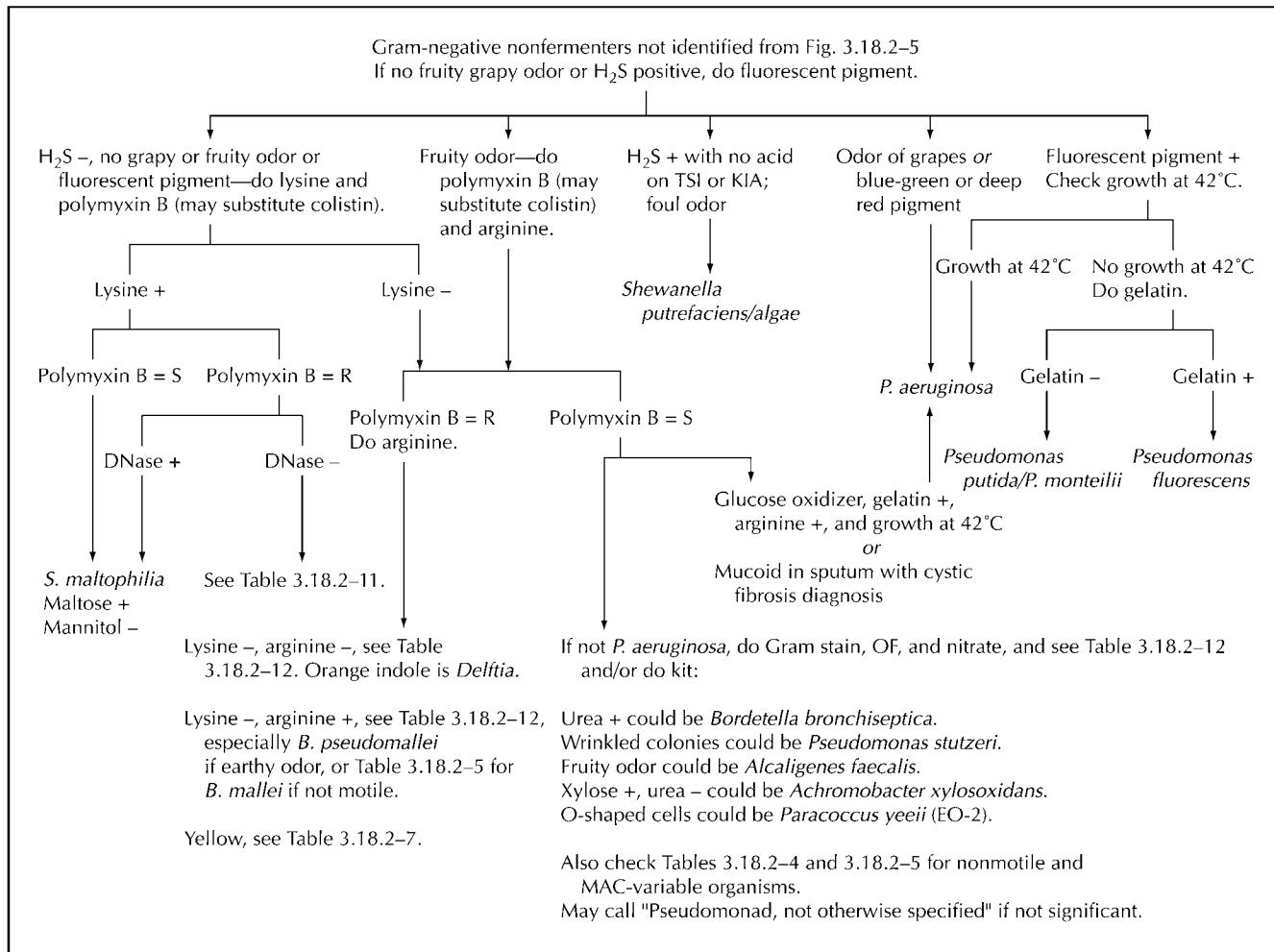


Figure 3.18.2-6 Identification scheme for gram-negative rods that grow on BAP and MAC and are not identified by Fig. 3.18.2-4 and 3.18.2-5.

Table 3.18.2–1 Biochemical reactions of *Neisseria* and related oxidase-positive diplococcus and rods that may grow on Thayer-Martin or similar selective agar^{a,b}

Organism(s)	Superoxol, 30% H ₂ O ₂	Growth on basic agar media at 35°C ^d	Colistin 10-µg disk	Glucose	Maltose	Lactose, ONPG, or BGAL	Sucrose	PRO	GLUT	Butyrate ^e
<i>Neisseria gonorrhoeae</i>	4+	—	R	+ −	—	—	—	+	—	—
<i>Neisseria meningitidis</i>	2–4+	V	R	+	+ −	—	—	V	+	—
<i>Neisseria lactamica</i>	2+	+	R	+	+	+	—	+	—	—
<i>Neisseria cinerea</i> ^c	2+	+	V	V	—	—	—	+	—	—
<i>Neisseria flavescens</i> ^c	2+	+	S	—	—	—	—	+	—	—
<i>Neisseria elongata</i> ^c	V	+	S	—	—	—	—	V	—	—
Other nonpathogenic <i>neisseriae</i> ^c	2+	+	V	+	+	—	V	V	V ^d	—
<i>Moraxella catarrhalis</i>	2–4+	+	V	—	—	—	—	V	—	+
<i>Kingella denitrificans</i>	—	+	R	+	—	—	—	+	—	—

^a Abbreviations for tests: PRO, prolyl iminopeptidase; ONPG, *o*-nitrophenyl-β-D-galactopyranoside; BGAL, β-galactosidase; GLUT, D-glutamyl-aminopeptidase. Reactions are from package inserts, the website <http://www.CDC.gov/ncidod/dastlr/gcdir/neident/index.html>, and references 4, 9 and 15. Polymyxin B can be substituted for colistin; alternatively, resistance to these agents can be determined by growth or lack of growth on Thayer-Martin or other selective agar with colistin or polymyxin B. Results for sugars are in cysteine Trypticase agar.

^b Abbreviations and symbols for results: +, greater than 90% of strains positive in 48 h; —, greater than 90% of strains negative; V, results are between 90 and 10% positive; + −, most strains are positive but a few are known to be negative, resulting in critical misidentifications if other tests are not also performed; R, 90% of strains resistant or no zone around disk; S, 90% of strains susceptible or zone around disk.

^c Does not usually grow on selective media for *N. gonorrhoeae*. *N. subflava* and *N. flavescens* colonies are yellow; *N. subflava* is the only species other than *N. meningitidis* to be GLUT positive.

^d Nutrient or MH agar or TSA without blood at 35°C.

^e See procedure 3.17.7. Do not read after time period in package insert, as this delay may result in false-positive reactions. Many *Moraxella* spp. and *Acinetobacter* spp. are butyrate positive. Isolate must be a diplococcus for identification of *M. catarrhalis* to be accurate.

Table 3.18.2–2 Biochemical reactions of *Haemophilus* species that satellite on BAP^a

Organism	Satellite (V factor)	ALA	Lactose ^c	Urea	Indole	Ornithine	Hemolysis ^b	Catalase
<i>H. influenzae</i>	+	—	—	V	V	V	—	+
<i>H. haemolyticus</i>	+ ^b	—	—	+	V	—	+	+
<i>H. parahaemolyticus</i>	+ ^b	+	—	+	—	—	+	+
<i>H. parainfluenzae</i>	+	+	—	V	V	V	—	V
<i>H. paraphrophilus</i>	+	+	+	—	—	—	—	—

^a Data for table from reference 14, p. 218–219. See footnote ^b of Table 3.18.2–1 for abbreviations and symbols.

^b Hemolysis demonstrated on horse, sheep, or rabbit blood agar. Note that hemolytic strains may grow on BAP without staphylococcal dot even though they require V factor.

^c Test is performed in 1% lactose in phenol red broth base (BD Diagnostic Systems) supplemented with hemin and NAD (10 mg of each per liter; Sigma Chemical Co.) (reference 11, p. 628).

Table 3.18.2-3 Differential biochemical reactions for indole-positive, gram-negative rods that grow poorly on MAC in 48 h^a

Organism	MAC	Oxidase	Catalase	Nitrate	Glucose	Mannitol	Maltose	Urea	Other differential trait(s)
<i>Dysgonomonas capnocytophagoïdes</i> (DF-3)	—	—	—	—	F ^s	—	+	—	Small rod, acid on slant and in butt of TSI, fecal pathogen
<i>Pasteurella bettyae</i>	V	V	—	+	F, gas	—	—	—	Rod, genital sites, acid on slant and in butt of TSI
<i>Cardiobacterium hominis</i>	—	+	—	—	F ^s	+ or (+)	+ or (+)	—	Pleomorphic rod; sorbitol +, alkaline phosphatase —
<i>Suttonella indologenes</i>	—	+	V	—	F	—	+ or (+)	—	Plump rod; sorbitol —, alkaline phosphatase +; rare
<i>Pasteurella dagmatis</i>	—	+	+	+	F, gas	—	+	+	Decarboxylase — ^d
Bisgaard's taxon 16	V	V	+	+	F	—	+	—	Decarboxylase —
<i>Pasteurella stomatis</i>	—	+	+	+	F	—	—	—	Decarboxylase —
<i>Pasteurella pneumotropica</i>	V	+	+	+	F	—	+	+	Ornithine +
<i>Pasteurella multocida</i>	—	+	+	+	F	+	—	—	Ornithine +; from cat bites
<i>Pasteurella canis</i>	—	+	+	+	F	—	—	—	Ornithine +
<i>Bergeyella zoohelcum</i>	—	+	+	—	n-o	—	—	+	Arginine +; rod from animal bites; polymyxin B = R
<i>Weeksella virosa</i>	—	+	+	—	n-o	—	—	—	Light yellow, decarboxylase —, polymyxin B = S
CDC group II-g	+	+	+	—	n-o	—	—	—	Reduces nitrite
CDC groups II-c, II-e, II-h, II-i	—	+	+	—	O	—	V	—	Rarely isolated
<i>Balneatrix alpica</i> ^b	—	+	+	+	O	(+)	+	—	Yellow, esculin —, motile, polymyxin B = S
<i>Chryseobacterium gleum</i> / <i>Chryseobacterium indologenes</i> ^c	V	+	+	V	O	—	+	V	Deep yellow, esculin +, polymyxin B = R
<i>Chryseobacterium meningosepticum</i> ^c	V	+	+	—	O	+	+	—	Yellow V, esculin +, polymyxin B = R

^a Indole is done with Kovács at 48 h directly on plate. For yellow colonies, Ehrlich's method may be needed. CDC group II-g grows on MAC but is a non-glucose oxidizer. See Table 3.18.2-8 for indole-positive, fermenting rods that grow on MAC. Data are from reference 14. (+), greater than 90% of strains positive in 3 to 7 days; F, glucose fermenting in TSI or KIA or Andrade's glucose broth or other sugar fermentation medium; n-o, nonoxidizer in glucose OF medium and no reaction in fermentation medium; O, oxidizer in glucose OF medium; F^s, addition of rabbit serum to Andrade's or other glucose fermentation medium may be required to demonstrate fermentation; gas, gas from either glucose or nitrate, depending on the test. Results for carbohydrates are in OF medium for oxidizers and Andrade's or rapid sugar agar for fermenters. See footnote b to Table 3.18.2-1 for other abbreviations and symbols.

^b All strains except *Balneatrix alpica* are nonmotile.

^c See Table 3.18.2-6 to separate from *Empedobacter brevis*, which is yellow and indole positive and grows on MAC.

^d Decarboxylase — indicates negative reaction for lysine, arginine, and ornithine.

Table 3.18.2-4 Gram-negative rods that grow on BAP but are catalase negative or weak, with poor growth on MAC in 48 h^a

Organism	MAC	Catalase	Oxidase	Nitrate	Indole	Glucose	Xylose	Mannitol	Sucrose	Maltose	Urea	Other differential trait(s)
<i>Leptotrichia</i>	—	—	—	—	—	F	—	—	+	+	—	Long filamentous rods, grows poorly
<i>Streptobacillus moniliformis</i>	—	—	—	—	—	F	—	—	—	+	—	Needs 20% ascitic fluid, blood, or serum to grow; pleomorphic forms; arginine +
<i>Capnocytophaga ochracea</i> (DF-1) and related species	—	—	V	—	F	—	—	+	V	—	—	Gliding motility; esculin V, tapered ends, yellow
<i>Dysgonomonas capnocytophagoïdes</i> (DF-3)	—	—	—	V	F ^s	V	—	V	V	—	—	Coccobacilli, acid on slant and in butt of TSI/KIA; esculin V
<i>Bordetella holmesii</i>	V	V	—	—	n-o	—	—	—	—	—	—	Coccoid rod, brown pigment on MH agar, nonhemolytic
<i>Haemophilus aphrophilus</i>	—	V	+	—	F, gas	—	—	+	+	—	—	Tiny rod, acid on slant and in butt of TSI/KIA; esculin —
<i>Pasteurella bettyae</i>	V	—	V	+	F, gas	—	—	—	—	—	—	Rod, genital sites, acid on slant and in butt of TSI
<i>Kingella kingae</i>	—	+	—	—	F	—	—	—	—	+	—	Beta-hemolytic, nitrite V
<i>Moraxella bovis</i>	—	V	+	—	n-o	—	—	—	—	—	—	Beta-hemolytic
<i>Brevundimonas vesicularis</i>	V	V	+	—	O	V	—	—	—	+	—	Half the strains are yellow, motile, polymyxin B = R
<i>Simonsiella muelleri</i>	—	V	+	V	—	F	—	—	—	+	—	“Roll of coins” smear, beta-hemolytic, rarely isolated
<i>Neisseria elongata</i>	—	V	+	V	—	n-o	—	—	—	—	—	Often nitrite +; polymyxin B = S
<i>Eikenella corrodens</i>	—	—	+	+	—	n-o	—	—	—	—	—	Ornithine + is definitive; odor of bleach, can be yellow
<i>Paracoccus yeui</i> (EO-2), <i>Psychrobacter</i> spp.	V	V	+	+	—	O	+	—	—	V	V	Coccoid but large cells with vacuoles and mucoid colony is <i>Paracoccus yeui</i>
<i>Kingella denitrificans</i>	—	—	+	, gas	—	F	—	—	—	—	—	Grows on Thayer-Martin; polymyxin B = R
<i>Cardiobacterium hominis</i>	—	—	+	—	+	F ^s	—	(+)	V	V	—	Slender rod; sorbitol +; alkaline phosphatase —
<i>Sutonella indologenes</i>	—	V	+	—	+	F	—	—	+	V	—	Plump rod; sorbitol —, alkaline phosphatase +; rarely isolated

^a All are nonmotile except *B. vesicularis*. Data are from reference 14. See footnote b to Table 3.18.2-1 and footnote a to Table 3.18.2-3 for abbreviations and symbols.

Table 3.18.2–5 Biochemical differentiation of non-yellow-pigmented, gram-negative rods that are catalase-positive and indole-negative but do not grow well on MAC^a

Organism(s)	MAC	Oxidase	Urea ^b	Nitrate	Glucose	Arginine	Other differential trait(s)
<i>Bordetella parapertussis</i>	—	—	+	—	n-o	NA	Coccobacilli, brown pigment on MH agar, beta-hemolytic
CDC group EO-5	—	—	W	—	O	—	Coccobacilli; 20% are yellow
CDC group NO-1	V	—	—, W	+	n-o	—	Rod; from dog and cat bites. <i>Acinetobacter</i> organisms that fail to grow on MAC are coccoid and nitrate —.
<i>Actinobacillus actinomycetemcomitans</i>	—	V	—	+	F, gas V	—	Tiny rod; esculin —; acid slant and butt in TSI; sucrose fermentation —.
<i>Burkholderia mallei</i>	V	V	V	+	O	+	Coccobacilli, hazardous; resistant to polymyxin B; citrate —
<i>Pasteurella gallinarum</i>	V	+	—	+	F	—	Rod, acid slant and butt in TSI; sucrose +
CDC group EF-4b	V	+	—	+	O	—	Reduces nitrite with no gas; no reaction in TSI; from dog and cat bites
CDC group EF-4a	V	+	—	+ , gas	F	V	May reduce nitrite with no gas; 73% acid in butt of TSI; from dog and cat bites
<i>Capnocytophaga canimorsus/cynodegmi</i> (DF-2)	—	+	—	—	F ^s	+	Rod; gliding motility, ONPG + ; from dog and cat bites
<i>Pasteurella haemolytica</i>	V	+	—	+	F	—	Acid slant and butt in TSI; beta-hemolytic
<i>Methylobacterium</i> spp.	V	+	V	V	n-o, O	NA	Vacuolated rod pink in 72 h; grows faster on CHOC; motile
<i>Actinobacillus</i> spp. (animal)	V	+	+	+	F	—	Acid slant and butt in TSI
<i>Brucella</i> ^b	V	+	+	+	O	NA	Coccoid tiny cells; work in safety cabinet
<i>Paracoccus yeeii</i> (EO-2)	V	+	V	+	O	NA	Coccoid but large cells with vacuoles; mucoid colony
<i>Psychrobacter immobilis</i>	V	+	V	V	n-o, O	NA	Coccoid; may have rose-like odor
<i>Psychrobacter phenylpyruvicus</i>	V	+	+	V	n-o	NA	Coccoid, PDA + , nitrite — , 6.5% salt +
<i>Oligella ureolytica</i>	V	+	+	+ , gas V	n-o	NA	Coccoid, PDA + , motile
<i>Oligella urethralis</i>	V	+	—	—	n-o	NA	Coccoid, PDA + , nitrite +
<i>Moraxella</i> spp.	V	+	—	V	n-o	NA	Coccoid, thick cells
<i>Neisseria weaveri</i>	V	+	—	—	n-o	—	Rod; PDA V, nitrite + ; Gilardi rod group 1 similar, except nitrite — and PDA +

^a For indole-positive strains, see Table 3.18.2–3. All strains nonmotile, except as noted, but even with those, motility is difficult to demonstrate. See Table 3.18.2–4 for catalase-variable rods. Data are from references 11, 13, and 14 and from G. L. Gilardi, unpublished identification tables. W, weak reaction; NA, not applicable or available. See footnote ^b to Table 3.18.2–1 and footnote ^a to Table 3.18.2–3 for other abbreviations and symbols.

^b Urea-positive, oxidase-positive coccobacilli are presumed to be *Brucella* until proven otherwise.

Table 3.18.2-6 Biochemical characteristics of the nonmotile, yellow, nonfermenting, gram-negative rods that are catalase positive^a

Organism(s)	MAC	Oxidase	Yellow	Indole	Urea	Polymyxin B	Nitrate	Glucose	Mannitol	Sucrose	Maltose	Esculin	DNase	Other differential trait(s)
<i>Wecksella virosa</i>	—	+	Pale	—	S	—	n-o	—	—	—	—	—	—	Penicillin = S
<i>Chryseobacterium</i> <i>V</i>	+	Bright	+	V	R	V	O	—	V	+	+	+	—	—
<i>Chryseobacterium</i> <i>glaeum</i> and <i>Chryseobacterium</i> <i>indolo-</i> <i>genes</i>	(+)	+	Pale	—	R	—	O	+	—	+	+	+	+	—
<i>Chryseobacterium</i> <i>meningosepticum</i>	+	+	Pale	—	R	—	O, n-o	—	—	+	—	+	+	—
<i>Empedobacter brevis</i>	+	+	+	V	NA	—	O	V	—	V	—	—	+	NA
EO-3, EO-4 (EO-5)	V	+	+	—	—	—	—	—	—	—	—	—	—	Coccoid; EO-5 is oxidase negative—see Table 3.18.2-5
<i>Myroides odoratus/</i> <i>odoratimimus</i>	V	+	Green	—	R	—	n-o	—	—	—	—	—	—	+
<i>Sphingobacterium</i> <i>spiritivorum</i>	V	+	Pale	—	+	R	—	O	+	+	+	+	+	+
<i>Sphingobacterium</i> <i>multivorum</i>	+	+	Pale	—	+	R	—	O	—	+	+	+	+	V
<i>Sphingobacterium</i> <i>thaipophilum</i>	+	+	Pale	—	+	R	+	O	—	+	+	+	+	Grows at 42°C
<i>Sphingobacterium</i> <i>mizutaii</i>	—	+	Pale	—	R	—	O	—	+	+	+	+	—	—

^a For indole reaction, Ehrlich's method may be needed. See Table 3.18.2-4 for yellow-pigmented, catalase-negative *Elkenella* and *Capnocytophaga*. Data are from references 11, 13, and 14 and from G. L. Gilardi, unpublished identification tables. See footnote *b* to Table 3.18.2-1 and footnote *a* to Tables 3.18.2-3 and 3.18.2-5 for abbreviations and symbols.

Table 3.18.2-7 Biochemical differentiation of the motile, yellow, non-glucose-fermenting, gram-negative rods^a

Organism(s)	MAC	Oxidase	Yellow	Polymyxin B	Urea	Nitrate	Glucose	Mannitol	Sucrose	Maltose	Esculin	Arginine	Other differential traits
<i>Stenotrophomonas maltophilia</i>	+	-	+	V	-	V	O	-	V	+	+	-	Lysine +, DNase +
<i>Burkholderia cepacia complex/B. gladioli</i>	+	V	+	R	V	V	O	V	V	V	V	-	Lysine V, DNase - (See Table 3.18.2-11)
<i>Pseudomonas luteola</i>	+	-	+	S	V	V	O	+	V	+	+	V	Wrinkled colonies; PYR +
<i>Pseudomonas oryzihabitans</i>	+	-	+	S	V	-	O	+	V	+	-	-	Wrinkled colonies; PYR +
<i>Pseudomonas stutzeri</i>	+	+	Pale, V	S	V	+ , gas	O	V	-	+	-	-	Wrinkled colonies; PYR -
<i>Pseudomonas mendocina</i> (Vb-2)	+	+	S	V	+ , gas	O	-	-	-	-	-	-	Colonies buttery
CDC group Vb-3	+	+	S	V	+ , gas	O	V	-	+	+	-	-	Wrinkled colonies
<i>Balneatrix alpica</i>	-	+	Pale	S	-	+	O	(+)	-	+	-	-	Indole +, PDA +
<i>Acidovorax</i> spp.	V	+	+ -	NA	+ -	+	O	V	-	-	-	-	Rarely isolated
<i>Sphingomonas paucimobilis</i>	- +	V	Deep	V	-	-	W	-	+	+	+	-	Motility better by wet mount; vancomycin = S
O-1	- +	V	+	NA	V	-	O, n-o	-	-	-	+	-	Rarely isolated
O-2	-	+	Orange	NA	V	O, n-o	-	(+)	(+)	V	V	-	Rarely isolated
<i>Agrobacterium</i> yellow group	- +	+	V	(+)	-	O, n-o	-	(+)	+	V	-	-	Catalase +
<i>Brevundimonas vesicularis</i>	V	+	Orange	R	-	-	W	-	-	+	+	-	Catalase V, PYR -, vancomycin = S

^a Motility is best at 22°C. All are indole negative except *Balneatrix*. Data are from references 10, 11, 13, and 14 and from G. L. Gilardi, unpublished identification tables. —*, most strains are negative but a few are known to be positive. See footnote *b* to Table 3.18.2-1 and footnote *a* to Tables 3.18.2-3 and 3.18.2-5 for other abbreviations and symbols.

Table 3.18.2–8 Characteristics of the common pathogenic oxidase-positive, glucose-fermenting rods that grow on MAC and are not yellow pigmented^a

Organism	Oxidase	Indole	Lactose	Sucrose	Lysine	Arginine	Ornithine	O/129 ^b		Growth on ^c :	
								10 µg	150 µg	MH agar	MH agar + 4% salt
<i>V. cholerae</i>	+	+	—	+	+	—	+	S	S	V	V
<i>V. mimicus</i>	+	+	— ⁺	—	+	—	+	S	S	V	V
<i>V. parahaemolyticus</i>	+	+	—	—	+	—	+	R	V	—	+
<i>V. vulnificus</i>	+	+	+ [—]	— ⁺	+	—	V	S	S	+	V
<i>V. alginolyticus</i>	+	+ [—]	—	+	+	—	V	R	V	V	+
<i>V. fluvialis</i>	+	— ⁺	—	+	—	+	—	R	V	—	+
<i>Aeromonas</i>	+	+ [—]	V	V	+ [—]	+ [—]	— ⁺	R	R	+	—
<i>Plesiomonas</i>	+	+	—	—	+	+	+	R, S	S	+	—
<i>Chromobacterium violaceum</i> ^d	V	V	—	V	—	+	—	NA	NA	+ (violet)	—

^a 1% Salt may be required for sugar and decarboxylase reactions. All strains ferment glucose on TSI or KIA. CDC group II-g grows on MAC and is indole positive but is a non-glucose oxidizer; see Table 3.18.2–3. Data are from references 1, 8, 11, and 14. See footnote ^b to Table 3.18.2–1 and footnote ^a to Tables 3.18.2–5 and 3.18.2–7 for abbreviations and symbols.

^b Resistant *V. cholerae* strains have been isolated in India. Confirmation of arginine-negative *Aeromonas* may be needed to prevent misidentifications. Testing with O/129 is key to prevention of misidentification of *V. fluvialis* and *V. vulnificus* as *Aeromonas* spp.

^c Data on file at University of California, San Francisco. The importance of using both media is to get growth on at least one plate in order to observe the O/129 and other disk susceptibility and to observe that *Aeromonas* and *Plesiomonas* do not grow with salt added. Some *Vibrio* organisms do not grow on MH agar without salt added, and some do not grow with 4% salt.

^d The combination of polymyxin B resistance and indole positivity is associated with nonpigmented strains. The combination of sucrose negativity and growth on MH agar separates this organism from *V. fluvialis*. *C. violaceum* is lysine, maltose, and mannitol negative, which separates it from *Aeromonas*.

Table 3.18.2–9 Differentiation of *Yersinia pestis* from similar bacteria^a

Organism	Lysine	Ornithine	Urea ^b	Motility at:		Glucose	ONPG	Mannitol	Rhamnose	Other differential trait(s)
				35°C	25°C					
<i>Yersinia pestis</i>	—	—	5%	—	—	+	V	+	1%	VP — at 25°C
<i>Yersinia pseudotuberculosis</i>	—	—	+	—	+ [—]	+	V	+	70%	VP — at 25°C
<i>Yersinia enterocolitica</i>	—	+	+ [,] (+)	—	+	+	+	+	—	VP + at 25°C
<i>Klebsiella rhinoscleromatis</i>	—	—	—	—	—	+	—	+	+	Sucrose +
<i>Pantoea agglomerans</i> group	—	—	V	V	V	+	+	+	+	Citrate +
<i>Shigella</i> spp.	—	V	—	—	—	+	V	V	— ⁺	Serology

^a Reactions of *Yersinia* are faster at room temperature. Data are from references 2 and 6. See footnote ^b to Table 3.18.2–1 and footnote ^a to Tables 3.18.2–3 and 3.18.2–7 for abbreviations and symbols.

^b Use rapid urea test method to increase sensitivity. See procedure 3.17.48.

Table 3.18.2-10 Biochemical reactions of non-glucose-fermenting, gram-negative rods that are catalase positive, oxidase negative, or delayed and grow well on MAC within 48 h^a

Organism(s)	Motility	Pigment	Polymyxin B	Lysine	Glucose	Urea	Nitrate	Arginine	Mannitol	Maltose	Other differential trait(s)
<i>Burkholderia gladioli/Pandoraea</i>	+	Variable yellow	R	—	O (O)	V	V	—	V	V	Rod; see Table 3.18.2-11 for species identification; DNase —
<i>Stenotrophomonas maltophilia</i>	+	Variable yellow	V	+	O	—	V	—	—	+	Rod; DNase +, PYR —
<i>Pseudomonas oryzihabitans/luteola</i>	+	Yellow	S	—	O	V	V	+	+	+	Wrinkled colonies; PYR +
<i>Burkholderia mallei</i>	—	—	R	—	O	V	+	V	V	V	Coccobacilli, hazardous; MAC and oxidase V, citrate —; no growth at 42°C
<i>Acinetobacter</i> spp. Saccharolytic	—	—	S	—	O	—	—	V	—	NA	Coccobacilli; most are <i>A. baumannii</i> , the only one that grows at 42°C
Asaccharolytic	—	—	S	—	n-o	—	—	V	—	—	Coccobacilli; not all grow on MAC; nitrate negativity and rod shape separates them from NO-1
<i>Bordetella trematum</i>	+	—	NA	—	n-o	—	V	—	—	—	Coccobacilli
<i>Roseomonas</i> spp.	V	Pink	R	NA	n-o	+	V	NA	—	—	Coccoid, mucoid, delayed positive oxidase; does not absorb long-wave UV light

^a Reactions from references 10 and 14 and from G. L. Gilardi, unpublished identification tables. All strains are catalase positive. For oxidase-negative, yellow-pigmented organisms, see Table 3.18.2-7. See footnote *b* to Table 3.18.2-1 and footnote *a* to Tables 3.18.2-3 and 3.18.2-5 for abbreviations and symbols.

Table 3.18.2-11 Characteristics of *Burkholderia cepacia* complex and related polymyxin B-resistant organisms^a

Organism(s)	Catalase	Oxidase ^c	Oxidation of:					Lysine decarboxylase	Ornithine decarboxylase	ONPG	PYR
			Glucose	Maltose	Lactose	Xylose	Sucrose				
<i>B. cepacia</i> geno-movars I and III	I is -; III is +	+	+	V	V, (+)	V, (+)	+	+	V	+	-
<i>B. multivorans</i>	-	+	+	+	+	-	-	+	V	-	+
<i>B. stabilis</i>	-	+	+	+	V	-	-	V, (+)	+	-	-
<i>B. vietnamensis</i>	-	+	+	+	V	+	-	-	-	-	-
<i>B. gladioli</i>	+	-	+	-	+	-	+	-	-	-	+
<i>Pandoraea</i>	+	V	-, (+)	-	-	-	-	-	-	-	+
<i>Ralstonia pickettii</i> / <i>R. mannitolytica</i> ^b	+	+	V	V	V	-	-	-	-	-	+

^a Data are from references 7 and 11. PYR data are from reference 10. All strains are arginine negative. See footnote *b* to Table 3.18.2-1 and footnote *a* to Tables 3.18.2-3 and 3.18.2-5 for abbreviations and symbols.

^b *Ralstonia mannitolytica*, formerly known as *Ralstonia pickettii* bv. 3, is unique among the *Ralstonia* organisms in that it is mannitol positive. *R. mannitolytica* is nitrate positive.

^c Oxidase reactions may be slow, up to 30 s.

Table 3.18.2-12 Biochemical reactions of nonyellow gram-negative rods that are oxidase positive and grow well on MAC within 48 h^a

Organism(s)	Polynyxin B	Glucose	Nitrate	Arginine	Urea ^b	PYR	Xylose	Mannitol	Sucrose	Maltose	Other differential trait(s)
<i>Burkholderia cepacia</i> complex, <i>Ralstonia</i> , <i>Pandoraea</i>	R	O (O)	V	-	V	V	V	V	V	V	See Table 3.18.2-11 for species identification.
<i>Burkholderia pseudomallei</i> ^b	R	O	+ , gas	+	V	-	+	+	V	+	White opaque colonies with sheen, then wrinkled, not beta-hemolytic.
<i>Rhizobium radiobacter</i>	V	O	V	-	+	+	+	+	V	+	PDA +; see reference 11, p. 760
<i>Ochrobactrum anthropi</i> and unnamed <i>Achromobacter</i> groups B, E, and F	V	O	V, gas V	V	+	+	+	V	V	+	PDA +; see reference 11, p. 760
<i>Achromobacter xylosoxidans</i> subsp. <i>xylosoxidans</i>	V	O	+ , gas V	V	-	+	+	-	-	-	Oxidizes xylose better than glucose
<i>Pseudomonas</i> fluorescent group	S	O	V	+	V	V	+	V	V	V	See Fig. 3.18.2-6 for species separation.
CDC group Ic	NA	O	+ , gas	+	V	NA	-	-	-	-	+
<i>Pseudomonas stutzeri</i>	S	O	+ , gas	-	V	-	+	V	-	-	Wrinkled colonies H ₂ S +, ornithine +, brown, foul smelling
<i>Shewanella putrefaciens</i> <i>slagae</i>	S	O	+	-	V	+	-	-	+	+	Coccoid but large cells with vacuoles; mucoid colonies
<i>Paracoccus yeeii</i> (EO-2)	S	O	+	-	V	NA	+	-	-	-	Beta-hemolytic; turns OF base +
OFBA-1	S	NA	+ , gas	+	-	NA	NA	NA	NA	NA	<i>P. pseudoalcaligenes</i> is nitrate + but no gas and 42°C +; <i>P. alcaligenes</i> is nitrate V and 42°C -; <i>P. pseudomonas</i> CDC group 1 is nitrate + with gas.
<i>Pseudomonas alcaligenes</i> , <i>Pseudomonas</i> CDC group 1, <i>Pseudomonas</i> <i>pseudoalcaligenes</i>	S	n-o	V	-	-	W	-	-	-	-	<i>Achromobacter piechauensis</i> is similar but nitrite - and no gas from nitrate.
<i>Achromobacter xylosoxidans</i> subsp. <i>denitrificans</i>	S	n-o	+ , gas	-	-	+	-	-	-	-	Fruity odor; nitrite +, acetamide +
<i>Alcaligenes faecalis</i> ^c	S	n-o	-	-	-	-	-	-	-	-	Orange color of colony with Kovács' indole
<i>Comamonas</i> spp.	S	n-o	+	-	-	+ -	-	-	-	-	Brown on MH agar
<i>Deffia acidovorans</i>	R	n-o	+	-	-	+	-	+	-	-	<i>B. avium</i> is beta-hemolytic, nitrite - PDA +
<i>Brevundimonas diminuta</i>	R	n-o/O	-	-	V	-	-	-	-	-	No growth on SS agar
<i>Bordetella avium/hinzii</i>	S	n-o	-	-	-	+	-	-	-	-	Grows on SS agar
<i>Oligella ureolytica</i>	NA	n-o	+ , gas V	NA	+	NA	-	-	-	-	
<i>Ralstonia paucula</i> (IVc-2)	V	n-o	-	-	+	-	-	-	-	-	
<i>Bordetella bronchiseptica</i>	S	n-o	+	-	+	-	-	-	-	-	

^a All strains are motile and indole negative. Also see Table 3.18.2-5 for nonmotile, gram-negative rods that are MAC variable. Verify that strains are nonfermenting rods using TSI or KIA. For fermenting rods, see Table 3.18.2-8. Data are from references 10, 11, 13, and 14 and from G. L. Gilardi, unpublished identification tables. SS, salmonella-shigella. See footnote b to Table 3.18.2-1 and footnote a to Tables 3.18.2-3, 3.18.2-5, and 3.18.2-7 for other abbreviations and symbols for reaction key.

^b *B. mallei* can have similar reactions but is nonmotile, has no odor, and does not produce gas from nitrate.

^c To separate *Alcaligenes faecalis* from other related nonoxidizers: *Ralstonia gilardii* is nitrite negative; nonyellow *Myxoides* is urea and PYR positive but nonmotile and polymyxin B resistant, and Gilardi rod group 1 is nonmotile and PDA positive.

REFERENCES

1. Abbott, S. L., L. S. Seli, M. Catino, Jr., M. A. Hartley, and J. M. Janda. 1998. Misidentification of unusual *Aeromonas* species as members of the genus *Vibrio*: a continuing problem. *J. Clin. Microbiol.* **36**:1103–1104.
2. Bockemühl, J., and J. D. Wong. 2003. *Yersinia*, p. 672–683. In P. R. Murray, E. J. Baron, J. H. Jorgensen, M. A. Pfaffer, and R. H. Yolken (ed.), *Manual of Clinical Microbiology*, 8th ed. ASM Press, Washington, D.C.
3. Catlin, B. W. 1975. Cellular elongation under the influence of antibacterial agents: a way to differentiate coccobacilli from cocci. *J. Clin. Microbiol.* **1**:102–105.
4. D'Amato, R. F., L. A. Eriques, K. N. Tomforde, and E. Singerman. 1978. Rapid identification of *Neisseria gonorrhoeae* and *Neisseria meningitidis* by using enzymatic profiles. *J. Clin. Microbiol.* **7**:77–81.
5. Evangelista, A. T., A. L. Truant, and P. Bourbeau. 2002. Rapid systems and instruments for the identification of bacteria, p. 22–49. In A. L. Truant (ed.), *Manual of Commercial Methods in Microbiology*. ASM Press, Washington, D.C.
6. Farmer, J. J., III, B. R. Davis, F. W. Hickman-Brenner, A. McWhorter, G. P. Huntley-Carter, M. A. Asbury, C. Riddle, H. G. Wathen-Grady, C. Elias, G. R. Fanning, A. G. Steigerwalt, C. M. O'Hara, G. K. Morris, P. B. Smith, and D. J. Brenner. 1985. Biochemical identification of new species and biogroups of *Enterobacteriaceae* isolated from clinical specimens. *J. Clin. Microbiol.* **21**:46–76.
7. Henry, D. A., E. Mahenthiralingham, P. Vandamme, T. Coeyne, and D. P. Speert. 2001. Phenotypic methods for determining genomovar status of the *Burkholderia cepacia* complex. *J. Clin. Microbiol.* **39**:1073–1078.
8. Janda, J. M., C. Powers, R. G. Bryant, and S. L. Abbott. 1988. Current perspectives on the epidemiology and pathogenesis of clinically significant *Vibrio* spp. *Clin. Microbiol. Rev.* **1**:245–267.
9. Janda, W. M., and J. S. Knapp. 2003. *Neisseria* and *Moraxella catarrhalis*, p. 585–608. In P. R. Murray, E. J. Baron, J. H. Jorgensen, M. A. Pfaffer, and R. H. Yolken (ed.), *Manual of Clinical Microbiology*, 8th ed. ASM Press, Washington, D.C.
10. Laffineur, K., M. Janssens, J. Charlier, V. Avesani, G. Wauters, and M. Delmée. 2002. Biochemical and susceptibility tests useful for identification of nonfermenting gram-negative rods. *J. Clin. Microbiol.* **40**:1085–1087.
11. Murray, P. R., E. J. Baron, J. H. Jorgensen, M. A. Pfaffer, and R. H. Yolken (ed.). 2003. *Manual of Clinical Microbiology*, 8th ed. ASM Press, Washington, D.C.
12. NCCLS. 2002. *Abbreviated Identification of Bacteria and Yeast*. Approved guideline M35-A. NCCLS, Wayne, Pa.
13. Schreckenberger, P. C. 2000. *Practical Approach to the Identification of Glucose-Non-fermenting Gram-Negative Bacilli*, 2nd ed. University of Illinois at Chicago, Chicago.
14. Weyant, R. S., C. W. Moss, R. E. Weaver, D. G. Hollis, J. G. Jordan, E. C. Cook, and M. I. Daneshvar. 1995. *Identification of Unusual Pathogenic Gram-Negative Aerobic and Facultatively Anaerobic Bacteria*, 2nd ed. Williams & Wilkins, Baltimore, Md.
15. Yajko, D. M., A. Chu, and W. K. Hadley. 1984. Rapid confirmatory identification of *Neisseria gonorrhoeae* with lectins and chromogenic substrates. *J. Clin. Microbiol.* **19**:380–382.