

**EXERCISE****54**Mc  
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Labs

## The Streptococci and Enterococci: Isolation and Identification

### Learning Outcomes

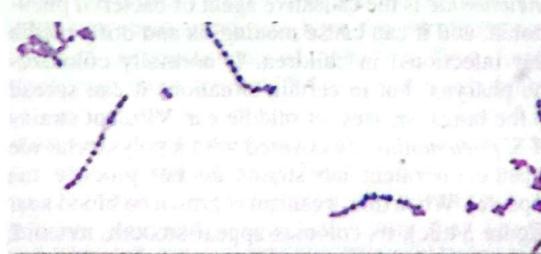
After completing this exercise, you should be able to

1. Isolate streptococci from a mixed culture or from the human throat using selective media and culturing techniques.
2. Identify unknown streptococci that you have isolated using selective media and biochemical tests specific for the streptococci.

The streptococci and enterococci differ from the staphylococci discussed in Exercise 53 in two significant characteristics: (1) Most isolates occur in chains rather than in clusters (figure 54.1), and (2) they lack the enzyme catalase, which degrades hydrogen peroxide to form water and oxygen.

The streptococci and enterococci comprise a large and varied group of gram-positive cocci. They are facultative anaerobes and generally considered non-motile. They can occur singly or in pairs, but they are best known for their characteristic formation of long chains (figure 54.1). At one time, streptococci and enterococci because of their similarities were placed in the same genus. However, molecular genetic analysis has determined that they are different enough to be placed in separate families, *Streptococcaceae* and *Enterococcaceae*.

Initial identification of these bacteria is often based on their hemolytic pattern when grown on blood agar. Some species of streptococci and enterococci produce exotoxins that completely destroy red



**Figure 54.1** Gram stain of *Streptococcus*.  
Centers for Disease Control and Prevention

### Why It Matters

How can you apply this exercise to the real world?

The *Streptococcus* and *Enterococcus* genera contain a large number of species, and a few of them are medically important human pathogens. There are several physiological tests that can be used to distinguish between the species. The type of hemolysis exhibited when grown on a blood agar plate and the species' antibiotic sensitivity are commonly used in their differentiation.

For example, you visit the doctor with a sore throat, and strep throat is suspected. The doctor will likely order a throat culture on blood agar to determine if beta-hemolytic Group A streptococci are causing an infection.

blood cells in blood agar. Complete lysis of red blood cells around a colony is known as **beta-hemolysis** and results in a clear zone surrounding the colonies. Other species of streptococci and enterococci partially break down the hemoglobin inside red blood cells on a blood agar plate, producing a greenish discoloration around the colonies known as **alpha-hemolysis**. Species of bacteria that do not exhibit any hemolysis of blood display **gamma-hemolysis**, that is, they have no effect on the red blood cells in a blood agar plate. The three kinds of hemolysis on blood agar are shown in figure 54.2. After patterns of hemolysis have been identified, species of streptococci and enterococci can be further differentiated based on their cell wall carbohydrates. A method developed by Rebecca Lancefield in the 1930s uses an alphabetic system (A, B, C, etc.) to designate different groups of bacteria. Serological tests are used to differentiate antigenic differences in cell wall carbohydrates that occur in these bacteria. Along with hemolytic patterns and serologic grouping, physiologic and biochemical characteristics are also used to identify streptococcal and enterococcal isolates.

### Beta-Hemolytic Groups

The Lancefield serological groups that fall into the beta-hemolytic category are groups A, B, and C.

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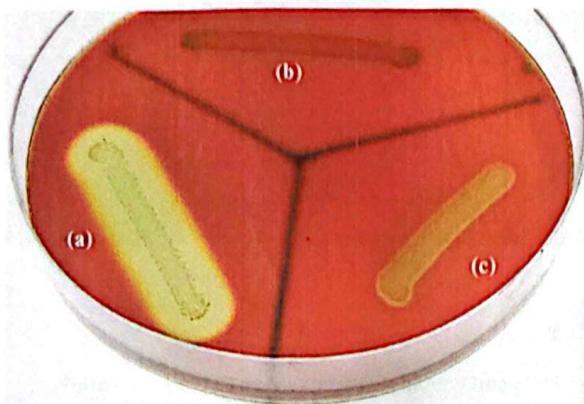


Figure 54.2 The three kinds of hemolysis produced by streptococci growing on blood agar plates: (a) beta-hemolysis, (b) gamma-hemolysis, and (c) alpha-hemolysis.

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### Group A Streptococci

*Streptococcus pyogenes*, the main representative of group A streptococci, is by far the most serious streptococcal pathogen. Humans are the primary reservoir of *S. pyogenes*. Although the pharynx is the most likely place to find this species, it may be isolated from the skin and rectum. Infections from *S. pyogenes* range from pharyngitis and skin infections to scarlet fever, rheumatic fever, and acute glomerulonephritis. *S. pyogenes* also causes child-birth fever (puerperal sepsis), a serious infection that occurs in women after giving birth. When grown on blood agar, colonies are small (0.5 mm diameter), transparent to opaque, and domed. *S. pyogenes* produce hemolysins which rapidly injure cells and tissues. These hemolysins result in complete lysis of red blood cells around each *S. pyogenes* colony when grown on a blood agar plate, producing a clear zone usually two to four times the diameter of the colony (figure 54.2a). These bacteria are spherical cocci, arranged in short chains in clinical specimens and longer when grown in broth. To differentiate *S. pyogenes* from other streptococci and enterococci, isolates are tested for resistance to bacitracin. If a bacterial isolate is beta-hemolytic and sensitive to bacitracin, it is presumed to be *S. pyogenes*.

### Group B Streptococci

*S. agalactiae* is the only recognized species of Lancefield group B. Like *S. pyogenes*, this pathogen may be found in the pharynx, skin, and rectum; however, it is more likely to be found in the genital and intestinal tracts of healthy adults and infants. This organism is an important cause of a serious neonatal infection involving sepsis and meningitis. Colonization of the

maternal genital tract is associated with colonization of infants and risk of disease. In the adult population, *S. agalactiae* infections consist of abscesses, endocarditis, septicemia, bone and soft tissue infections, and pneumonia. *S. agalactiae* colonies are large, with a narrow zone of beta-hemolysis, in contrast to *S. pyogenes* colonies, which are small with a large zone of hemolysis. *S. agalactiae* cells are spherical to ovoid and occur in short chains in clinical specimens and long chains in culture. Preliminary identification of this species relies heavily on a positive CAMP reaction.

### Group C Streptococci

Group C streptococci are uncommon human pathogens but may be involved in zoonoses (infections transmitted from animals to humans). The organism of importance in this group is *S. dysgalactiae*, and infections from this species account for less than 1% of all bacteremias. Members of this group can cause pharyngitis, endocarditis, and meningitis; however, most clinical infections from group C streptococci occur in patients with underlying illness. *S. dysgalactiae* produce large colonies with a large zone of beta-hemolysis on blood agar. Presumptive differentiation of *S. dysgalactiae* from other beta-hemolytic streptococci (*S. pyogenes* and *S. agalactiae*) is based primarily on resistance to bacitracin and a negative CAMP test.

### Alpha-Hemolytic Groups

The grouping of streptococci and enterococci on the basis of alpha-hemolysis is not as clear-cut as it is for beta-hemolytic groups. Note in table 54.1 that some groups exhibit weak alpha-hemolysis, and a few alpha-hemolytic types may exhibit variable hemolysis, highlighting the notion that hemolysis can be a misleading characteristic in identification.

### *Streptococcus pneumoniae*

Although *S. pneumoniae* does not possess a Lancefield antigen, it is a significant human pathogen. *S. pneumoniae* is the causative agent of bacterial pneumonia, and it can cause meningitis and otitis media (ear infections) in children. It normally colonizes the pharynx, but in certain situations it can spread to the lungs, sinuses, or middle ear. Virulent strains of *S. pneumoniae* are covered with a polysaccharide capsule (avirulent lab strains do not produce the capsule). When this organism is grown on blood agar (figure 54.2c), its colonies appear smooth, mucoid, and surrounded by a zone of greenish discoloration (alpha-hemolysis). In culture these cells usually grow as diplococci, but they can also occur singly

**Table 54.1** Physiological Tests for Streptococci and Enterococci Differentiation

		BERGEY'S GROUP	LANCEFIELD GROUP	HEMOLYSIS	BACITRACIN SUSCEPTIBILITY	CAMP REACTION	SXT SENSITIVITY	BILE ESCULIN HYDROLYSIS	TOLERANCE TO 6.5% NaCl	OPTOCIN SUSCEPTIBILITY
<i>S. pyogenes</i>	Pyogenic	A	β	+	-	R	-	-	-	
<i>S. agalactiae</i>		B	β	-	+	R	-	±	-	
<i>S. pneumoniae</i>		none	α	-	-		-	-	+	
<i>S. dysgalactiae</i>		C	β	-	-	S	-	-	-	
<i>E. faecalis</i>	Enterococci	D	γ or α <sup>1</sup>	-	-	R	+	+	-	
<i>E. faecium</i>		D	γ or α	-	-	R	+	+	-	
<i>S. bovis</i>	Other	D	γ or α	-	-	R/S	+	-	-	
<i>S. mitis</i> <sup>2</sup>	Oral (Viridans)	none	α <sup>1</sup>	-	-	S	-	-	-	
<i>S. salivarius</i> <sup>2</sup>		none	α <sup>1</sup>	-	-	S	-	-	-	
<i>S. mutans</i> <sup>2</sup>		none	none	-	-	S	-	-	-	

Note: R = resistant; S = sensitive; blank = not significant.

<sup>1</sup>Weakly alpha.<sup>2</sup>Oral streptococci commonly isolated from throat swabs. Differentiation is based on additional biochemical tests not performed in this exercise.

or in short chains. Presumptive identification of *S. pneumoniae* can be made with a positive optochin susceptibility test.

### Viridans Streptococci Group

The viridans streptococci are a heterogeneous cluster of alpha-hemolytic and nonhemolytic streptococci. These organisms colonize the oral cavity, pharynx, gastrointestinal tract, and genitourinary tract. Most of the viridans are opportunists, and they are usually regarded as having low pathogenicity. Two species in the viridans group are thought to be the primary cause of dental caries (cavities). Although these organisms have few virulence factors and are constituents of the normal flora, introduction of these species into tissues through dental or surgical means can cause severe infections. The most serious complication of all viridans infections is subacute endocarditis. When grown on blood agar, viridans colonies appear very small, gray to whitish gray, and opaque. In culture they appear rod-like and grow in chains. The viridans group can be differentiated from the pneumococci and enterococci by a negative result in the bile esculin

hydrolysis test, the salt-tolerance test, and the optochin susceptibility test.

### Group D Enterococci

The enterococci of serological group D are part of the *Enterococcaceae* family and are considered variably hemolytic. There are two principal species of this enterococcal group, *E. faecalis* and *E. faecium*. They are predominantly inhabitants of the gastrointestinal tract; however, they have also been isolated from the genitourinary tract and oral cavity. Although the enterococci do not possess many virulence factors, they are important pathogens in hospitalized patients where they can cause urinary tract infections, bacteraemia, and endocarditis. When grown on blood agar, enterococci form large colonies that can appear nonhemolytic (figure 54.2b), alpha-hemolytic, or rarely beta-hemolytic. In culture, they grow as diplococci in short chains. The enterococci can grow under extreme conditions, and this phenotype can be exploited to help differentiate them from various streptococcal species. Isolates that are able to grow in the presence of 6.5% NaCl and are able to hydrolyze bile esculin are presumed to be enterococci in this exercise.

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### **Group D Nonenterococci**

*S. bovis* is the only clinically relevant nonenterococcal species of group D. It is found in the intestinal tracts of humans, as well as cows, sheep, and other ruminants. Although it is found in many animals, *S. bovis* is a human pathogen and has been implicated as a causative agent of endocarditis and meningitis, and is associated with malignancies of the gastrointestinal tract. Colonies of *S. bovis* appear large, mucoid (many strains have a capsule), and are either nonhemolytic or alpha-hemolytic. In culture *S. bovis* grows in pairs and short chains. Key reactions for this group are a positive bile esculin test and negative salt broth test.

The purpose of this exercise is twofold: (1) to learn the standard procedures for isolating streptococci and enterococci and (2) to learn how to differentiate the medically important species of these families. Figure 54.3 illustrates the overall procedure to be followed in the exercise. To broaden the application of the tests for identifying streptococci and enterococci, your instructor may supply you with unknown cultures to be identified along with your pharyngeal isolates. Keep in mind as you complete this exercise that these tests result in presumptive identification of your isolates. Commercial kits are available, such as the RapID Streptococci panel, which can be used to confirm the identification of your isolates.

### **Caution**

Please review the safety information concerning human microbiota discussed in the introduction section for Part 12 before proceeding with this exercise. It is also imperative that you follow your institution's regulations and all directions given by your instructor. Some institutions may not allow the isolation of student samples, and therefore, you may be asked to complete this lab on the provided unknown only.

### **First Period**

#### (Making a Streak-Stab Agar Plate)

During this period, a plate of blood agar is swabbed and streaked in a special way to determine the type of hemolytic bacteria that are present in the pharynx and in an unknown mixture.

Since swabbing one's own throat properly can be difficult, it will be necessary for you to work with your laboratory partner to swab each other's throats. Once your throat has been swabbed, you will use the swab and a loop to inoculate a blood agar plate according to a special procedure shown in figure 54.4.

### **Materials**

- 1 tongue depressor
- 1 sterile cotton swab
- Inoculating loop
- 2 blood agar plates
- Unknown mixture

1. With the subject's head tilted back and the tongue held down with the tongue depressor, rub the back surface of the pharynx up and down with the sterile swab.

Also, look for white patches in the tonsillar area. Avoid touching the cheeks and tongue.

2. Since streptococcal hemolysis is most accurately analyzed when the colonies develop anaerobically beneath the surface of the agar, it will be necessary to use a streak-stab technique as shown in figure 54.4. The essential steps are as follows:

- Roll the swab over an area approximating one-fifth of the surface. The entire surface of the swab should contact the agar.
- With a wire loop, streak out three areas, as shown, to thin out the organisms.
- Stab the loop into the agar to the bottom of the plate at an angle perpendicular to the surface to make a clean cut without ragged edges.
- Be sure to make the stabs in an unstreaked area so that streptococcal hemolysis will be easier to interpret with a microscope.

### **Caution**

Dispose of swabs and tongue depressors in a beaker of disinfectant or biohazard bag.

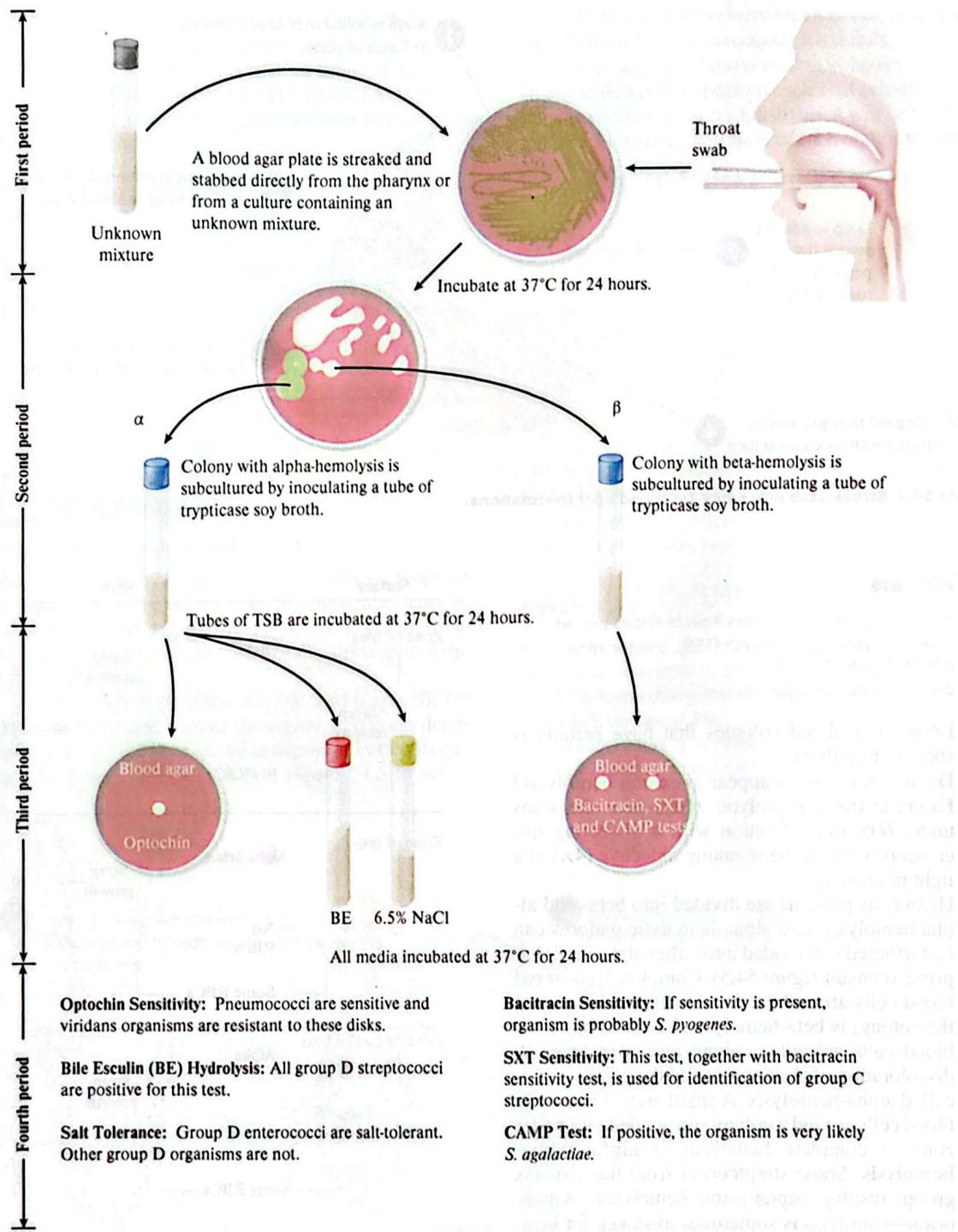
3. Repeat the inoculation procedure for the unknown mixture.
4. Incubate the plates in a candle jar at 37°C for 24 hours. Do not incubate the plates longer than 24 hours.

### **Second Period**

#### (Analysis and Subculturing)

During this period, two objectives must be accomplished: first, the type of hemolysis must be correctly determined, and second, well-isolated colonies must be selected for making subcultures. The importance of proper subculturing cannot be overemphasized; without a pure culture, future tests are certain to fail. Proceed as follows:

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**Figure 54.3 Media Inoculations for the presumptive identification of streptococci and enterococci.**

For any two media inoculations, either both should be used or individual media should not be used because one could give a false-positive result and the other could give a false-negative result. For example, if a streptococcus gives a positive result on the CAMP test but a negative result on the Optochin test, it would be considered to be a *Streptococcus* rather than a *Streptococcus* because the CAMP test is more specific than the Optochin test.

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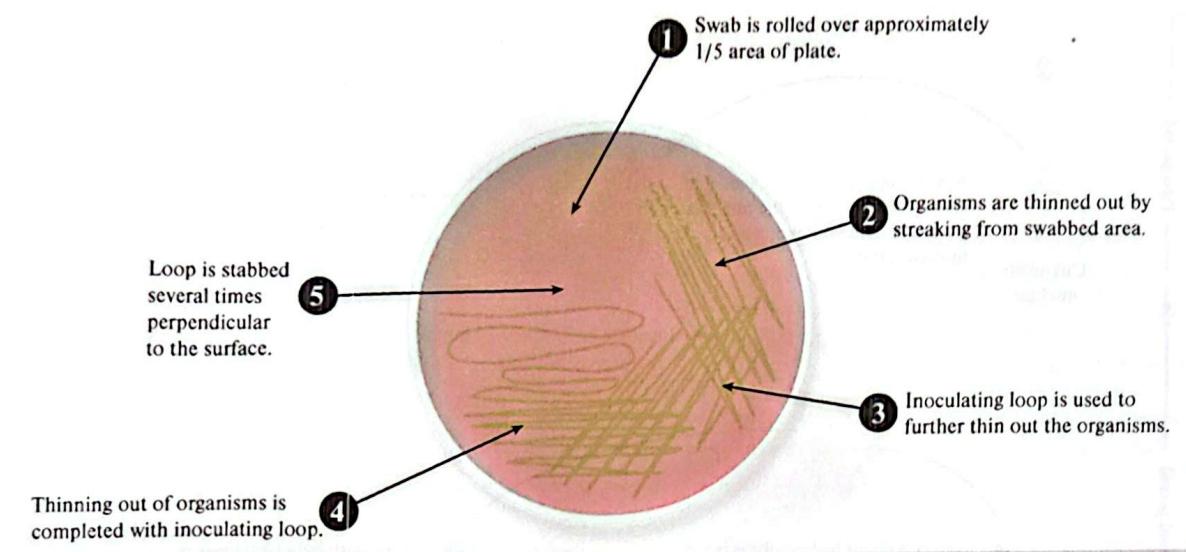


Figure 54.4 Streak-stab procedure for blood agar inoculations.

### Materials

- incubated blood agar plates from previous period
- tubes of trypticase soy broth (TSB), one for each different type of colony
- dissecting microscope

- Look for isolated colonies that have hemolysis surrounding them.
- Do any of the stabs appear to exhibit hemolysis? Examine these hemolytic zones near the stabs under 60 $\times$  magnification with a dissecting microscope or with the scanning objective (4 $\times$ ) of a light microscope.
- Hemolysis patterns are divided into beta- and alpha-hemolysis, and alpha-hemolytic patterns can be furthered subdivided into either alpha or alpha-prime (consult figure 54.5). Complete lysis of red blood cells around a colony (a clear area around the colony) is beta-hemolysis. Partial lysis of red blood cells around a colony results in greenish discoloration of the area around the colony and is called alpha-hemolysis. A small area of intact red blood cells around a colony surrounded by a wider zone of complete hemolysis is alpha-prime-hemolysis. Some streptococci from the viridans group display alpha-prime-hemolysis. Alpha-prime-hemolysis is sometimes mistaken for beta-hemolysis, which is why it is recommended the hemolytic colonies/stabs be viewed under either the dissecting scope or the lowest power of a light microscope. Colonies that do not affect the red blood cells surrounding them are said to exhibit nonhemolysis or gamma-hemolysis.

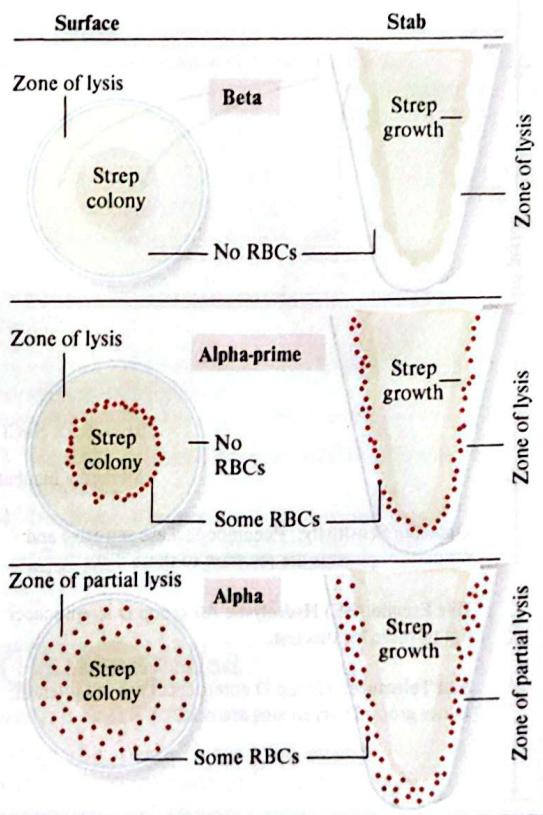


Figure 54.5 Comparison of the types of hemolysis seen on blood agar plates. The illustrations on the left indicate the appearance of red blood cells surrounding a bacterial colony on the surface of a blood agar plate. The illustrations on the right indicate the appearance of red blood cells surrounding stabs into the blood agar.

4. Record your observations in Laboratory Report 54.
5. Select well-isolated colonies that exhibit hemolysis (alpha, beta) for inoculating tubes of TSB. Be sure to label the tubes ALPHA or BETA. Whether or not the organism is alpha or beta is crucial in identification.

### Caution

Consult with your instructor before proceeding. The chances of isolating beta-hemolytic streptococci from the pharynx are usually quite slim, but it is important that your instructor is aware if you think you have isolated one. Depending on the laboratory's equipment and policies, you may be asked not to proceed with further study of this isolate.

6. Incubate the tubes at 37°C for 24 hours.

### ⌚ Third Period

#### (Inoculations for Physiological Tests)

Presumptive identification of the various groups of streptococci is based on the physiological tests in table 54.1. Note that groups A, B, and C are all beta-hemolytic. The remainder are usually alpha-hemolytic or nonhemolytic.

Since each of the physiological tests is specific for differentiating only two or three groups, it is not desirable to do all the tests on all unknowns. For economy and precision, carefully follow figure 54.3 to select

which tests you will perform on an isolate or unknown based on the type of hemolysis it exhibits.

Before any inoculations are made, however, it is desirable to do a purity check on each TSB culture from the previous period. To accomplish this, it will be necessary to make a Gram-stained slide of each of the cultures.

### Gram Stained Slides (Purity Check)

### Materials

- TSB cultures from previous period
- Gram-staining kit

1. Make a Gram stained slide from your isolates and examine them under an oil immersion lens. Do they appear to be pure cultures?
2. Draw the organisms in the appropriate circles in Laboratory Report 54.

### Beta-Type Inoculations

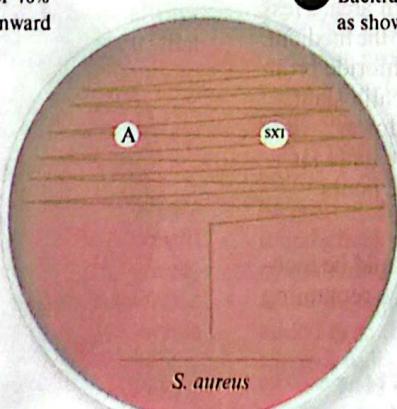
Use the following procedure to perform tests on each isolate that has beta-type hemolysis:

### Materials

for each isolate:

- 1 blood agar plate
- 1 bacitracin differential disk (0.04 units)
- 1 SXT sensitivity disk
- 1 broth culture of *S. aureus*
- dispenser or forceps for transferring disks

**Unknown** is heavily streaked out over 40% of the area and brought straight downward in a single line.



**A loopful of *S. aureus*** is streaked perpendicular to unknown streak. A gap of 1 cm should separate the two streaks.

**Bacitracin and SXT differential disks** are placed as shown in area streaked by the unknown.

Figure 54.6 Blood agar inoculation technique for the CAMP, bacitracin, and SXT tests.

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1. Label a blood agar plate with the proper identification information of each isolate and unknown to be tested.
2. Follow the procedure outlined in figure 54.6 to inoculate each blood agar plate with the isolate (or unknown) and *S. aureus*.  
Note that a streak of the unknown is brought down perpendicular to the *S. aureus* streak, keeping the two organisms about 1 cm apart.
3. With forceps or dispenser, place one bacitracin differential disk and one SXT disk on the heavily streaked area at points shown in figure 54.6. Press down lightly on each disk.
4. Incubate the blood agar plates at 37°C, aerobically, for 24 hours.

#### Alpha-Type Inoculations

As shown in figure 54.3, three inoculations will be made for each isolate or unknown that is alpha-hemolytic.

#### Materials

- 1 blood agar plate (for up to 4 unknowns)
- 16.5% sodium chloride broth tube per unknown
- 1 bile esculin (BE) slant per unknown
- 1 optochin disk per unknown
- candle jar setup or CO<sub>2</sub> incubator

1. Mark the bottom of a blood agar plate to divide it into halves, thirds, or quarters, depending on the number of alpha-hemolytic organisms to be tested. Label each space with the code number of each test organism.
2. Completely streak over each area of the blood agar plate with the appropriate test organism, and place one optochin disk in the center of each area. Press down slightly on each disk to secure it to the medium.
3. Inoculate one tube of 6.5% sodium chloride broth and streak the surface of one bile esculin slant.
4. Incubate all media at 35–37°C as follows:

Blood agar plates: 24 hours in a candle jar  
6.5% NaCl broths: 24, 48, and 72 hours  
Bile esculin slants: 48 hours

**Note:** While the blood agar plates should be incubated in a candle jar or CO<sub>2</sub> incubator, the remaining cultures can be incubated aerobically.

#### Inoculations of Colonies That Do Not Exhibit Hemolysis (Gamma-Type)

Refer to table 54.1. Isolates of this nature are probably members of group D or viridans. Perform an SXT sensitivity test, a bile esculin hydrolysis test, and a salt-tolerance test as described under Alpha-Type Inoculations.

**Note:** Optochin susceptibility can be evaluated as well if an optochin disk is placed on the streaked area of the SXT sensitivity BAP, in the same manner as the SXT disk is placed.

#### 1 Fourth Period

(Evaluation of Physiological Tests)

Once all of the inoculated media have been incubated for 24 hours, begin to examine the plates and tubes and add test reagents to some of the cultures. Some of the tests will also have to be checked at 48 and 72 hours.

After the appropriate incubation period, assemble all the plates and tubes from the last period, and examine the blood agar plates first that were double-streaked with the unknowns and *S. aureus*. Note that the CAMP reaction, bacitracin susceptibility test, and SXT sensitivity test can be read from these plates. Proceed as follows:

#### CAMP Reaction ( $\beta$ test)

If you have an unknown that produces an enlarged arrowhead-shaped hemolytic zone at the juncture where the unknown meets the *Staphylococcus aureus* streak, as shown in figure 54.7, the organism is *Streptococcus agalactiae*. This phenomenon is due to what is called the *CAMP factor*, named for the developers of the test, Christie, Atkins, and Munch-Peterson. The CAMP factor produced by *Streptococcus agalactiae* acts synergistically with staphylococcal

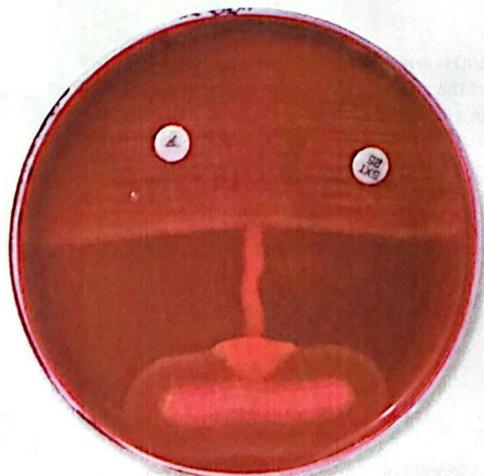


Figure 54.7 Note positive SXT disk on right, negative bacitracin disk on left, and positive CAMP reaction (arrowhead). Organism: *S. agalactiae*.

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hemolysins, causing an enhanced breakdown of red blood cells and therefore producing the arrowhead zone of clearing. The only problem that can arise from this test is that if the plate is incubated anaerobically, a positive CAMP reaction can occur on *S. pyogenes* inoculated plates.

Record the CAMP reactions for each of your isolates or unknowns in Laboratory Report 54.

#### Bacitracin Susceptibility ( $\beta$ test)

Any size zone of inhibition seen around the bacitracin disks should be considered to be a positive test result. Note in table 54.1 that *S. pyogenes* is positive for this characteristic.

This test has two limitations: (1) the disks must be of the *differential type*, not sensitivity type, and (2) the test should not be applied to alpha-hemolytic streptococci. Reasons: Sensitivity disks have too high a concentration of the antibiotic, and many alpha-hemolytic streptococci are sensitive to these disks.

Record the results of this test in the table under number 2 of Laboratory Report 54.

#### SXT Sensitivity Test ( $\beta$ test)

The disks used in this test contain 1.25 mg of trimethoprim and 27.75 mg of sulfamethoxazole (SXT). The purpose of this test is to distinguish groups A and B from other beta-hemolytic streptococci. Note in table 54.1 that both groups A and B are uniformly resistant to SXT.

If a beta-hemolytic streptococcus proves to be bacitracin resistant and SXT susceptible, it is classified as being a **non-group A or B beta-hemolytic streptococcus**. This means that the organism is probably a species within group C. *Keep in mind that an occasional group A streptococcal strain is susceptible to both bacitracin and SXT disks.* One must always remember that exceptions to most tests do occur; that is why this identification procedure leads us only to *presumptive conclusions*.

Record any zone of inhibition (resistance) as positive for this test.

**Note:** A few strains of *E. faecalis* are beta-hemolytic. A beta-hemolytic isolate of *E. faecalis* would have a negative CAMP reaction and would appear resistant to bacitracin and SXT. Although this exercise does not outline performing a bile esculin hydrolysis test or salt-tolerance test on a beta-hemolytic colony, these physiological tests would help confirm identification of a beta-hemolytic *E. faecalis* strain.

#### Bile Esculin (BE) Hydrolysis ( $\alpha$ test)

This is the best physiological test that we have for the identification of group D streptococci. Both

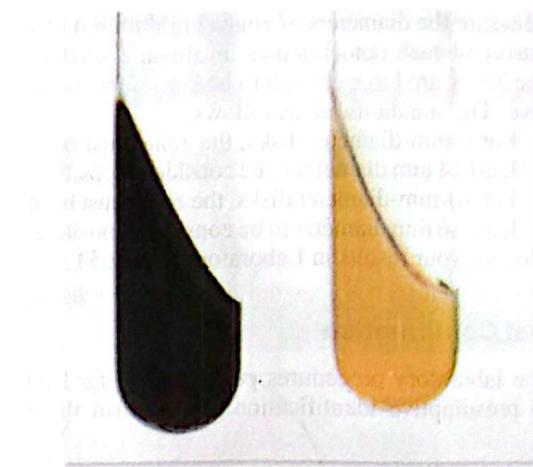


Figure 54.8 Positive bile esculin hydrolysis on left; negative on right.

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enterococcal and non-enterococcal species of group D are able to hydrolyze esculin in the agar slant, causing the slant to blacken.

A positive BE test tells us that we have a group D streptococcus; differentiation of the two types of group D streptococci (*Enterococcus* and *S. bovis*) depends on the salt-tolerance test.

Examine the BE agar slants, looking for **blackening of the slant**, as illustrated in figure 54.8. If less than half of the slant is blackened, or if no blackening occurs within 24 to 48 hours, the test is negative.

#### Salt-Tolerance (6.5% NaCl) (Group D) ( $\alpha$ test)

All enterococci of group D produce heavy growth in 6.5% NaCl broth. As indicated in table 54.1, *S. bovis* does not grow in this medium. This test, then, provides a good method for differentiating the organisms of group D.

A positive result shows up as turbidity within 72 hours. A color change of **purple to yellow** may also be present. If the tube is negative at 24 hours, incubate it and check it again at 48 and 72 hours. *If the organism is salt tolerant and BE positive, it is considered to be an enterococcus.* Parenthetically, it should be added here that approximately 80% of group B streptococci will grow in this medium.

#### Optochin Susceptibility ( $\alpha$ test)

Optochin susceptibility is used for differentiation of the alpha-hemolytic viridans streptococci from the pneumococci. The pneumococci are sensitive to these disks; the viridans organisms are resistant.

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1. Measure the diameters of zones of inhibition that surround each optochin disk, evaluating whether the zones are large enough to be considered positive. The standards are as follows:
  - For 6-mm-diameter disks, the zone must be at least 14 mm diameter to be considered positive.
  - For 10-mm-diameter disks, the zone must be at least 16 mm diameter to be considered positive.
2. Record your results in Laboratory Report 54.

#### **Final Confirmation**

All the laboratory procedures performed so far lead us to presumptive identification. To confirm these

conclusions, it is necessary to perform serological tests on each of the unknowns. If commercial kits are available for such tests, they should be used to complete the identification procedures.

#### **Laboratory Report**

Complete Laboratory Report 54 adding data from all group isolates.

**EXERCISE****56**

## A Synthetic Epidemic

### Learning Outcomes

After completing this exercise, you should be able to

1. Define terminology related to the field of epidemiology.
2. Differentiate between common source and propagated epidemics.
3. Perform a simple test, using detectable reagents as a "microbe," to demonstrate how an infectious agent can be passed from person to person.
4. Describe how epidemiology is used to trace the source and spread of communicable diseases.
5. Explain how herd immunity can reduce the spread of communicable diseases through a population and protect susceptible individuals from infection.

A disease caused by microorganisms that enter the body and multiply in the tissues at the expense of the host is said to be an **infectious disease**. Infectious diseases that are transmissible from one person to another are considered to be **communicable**. The transfer of communicable infectious agents between individuals can be accomplished by direct contact, such as in handshaking, kissing, and sexual intercourse, or these agents can be spread indirectly through food, water, objects, animals, and so on.

**Epidemiology** is the study of how, when, where, what, and who are involved in the spread and distribution of diseases in human populations. An epidemiologist is, in a sense, a medical detective who searches out the sources of infection so that the transmission cycle can be broken.

Whether an epidemic actually exists is determined by comparing the number of new cases with previous records. If the number of newly reported cases in a given period of time in a specific area is excessive, an **epidemic** is considered to be in progress. Notable epidemics in the United States today include chlamydia and pertussis. If the disease spreads to one or more continents, a **pandemic** is occurring. An example of a pandemic disease is HIV/AIDS. According to the World Health Organization (WHO), over 36.9 million people are living with HIV worldwide. Tuberculosis, caused by *Mycobacterium tuberculosis*,

is also considered a pandemic, with an estimated one-third of the world's population now infected with the causative bacterium. An infectious disease that exhibits a steady frequency over a long period of time in a particular region is considered **endemic**. In tropical regions of the world, malaria is endemic.

Epidemics fall into two categories: common source epidemics and host-to-host epidemics (see figure 56.1). Common source epidemics occur rapidly and have many new cases immediately after the initial case. This type of epidemic usually involves a contaminated fomite (inanimate object) or contaminated food or water. After the infected source has been identified and removed in a common source epidemic, the number of new cases of disease drops rapidly, and the epidemic quickly subsides. In late 2019, 167 people across 27 states were infected with Shiga toxin-producing *E. coli* O157:H7 after eating contaminated romaine lettuce from the Salinas Valley in California. The identification of this source led to recalls of the vegetable and a quick end to the epidemic.

Propagated, or host-to-host, epidemics grow much more slowly and are also slower to subside. These epidemics involve the transmission of the infectious agent through direct contact with the infected

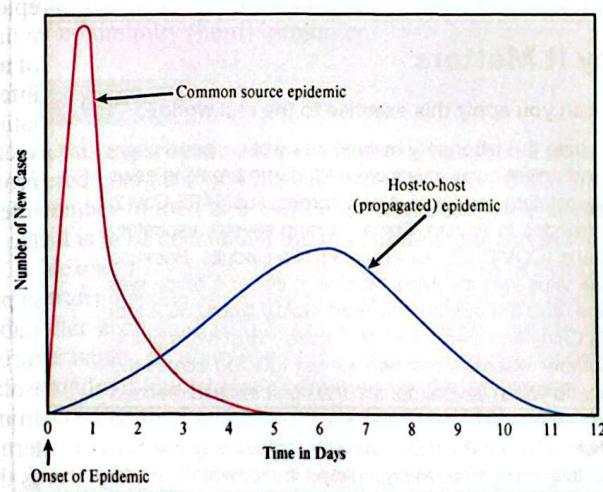


Figure 56.1 Comparison of common source and host-to-host (propagated) epidemics.

## EXERCISE 56 A Synthetic Epidemic

individual, a carrier, or a vector. A **vector** is a living carrier, such as an insect or rodent, which transmits an infectious agent. Control of propagated epidemics can involve education, vaccination, administration of antivirals or antibiotics, improved hygiene, and voluntary quarantine. The 2009 outbreak of H1N1 influenza was an example of a host-to-host epidemic and was eventually declared a pandemic as it spread from person to person across continents. Many of these control measures were implemented to slow the spread of the infection.

Human history has been shaped by epidemics. Population growth has been curbed, wars have been lost and won, and migrations have occurred all due to past outbreaks of disease. In the Middle Ages, “The Black Death,” or bubonic plague caused by *Yersinia pestis*, killed one-third of the European population, redistributing wealth and property. In 1918, the flu pandemic affected one-third of the world population. The rapid spread of the influenza virus during this epidemic is attributed to the living conditions and travel of soldiers during World War I. Smallpox was brought to Mexico by European explorers, and the spread of this disease through the native population of Mexico is thought to be the reason they were defeated by the Spaniards. AIDS has wreaked havoc on the sub-Saharan African population and has slowed economic growth throughout Africa. Continual changes in human behavior and the threat of bioterrorism make pandemics a concern for the future. Today, it is common to travel across continents and expand our presence into environments that historically have been untouched. This creates opportunities for the spread of infection throughout the world as well as interactions

with microbes and animals that may transmit diseases to humans.

The ability of infectious diseases to become epidemic and pandemic depends on the transmission cycle of the disease. The transmission cycles of infectious pathogens can be greatly affected by environmental changes. Vacations in the healthcare field, travel, lifestyles, and crowded living arrangements can increase the likelihood that a person will come in contact with an infectious agent. Vaccination, quarantine, and improved hygiene can decrease susceptibility to infectious agents. Extreme weather events can alter contact between humans and vectors and can increase reservoirs, the natural hosts or habitats for pathogens. Another important consideration in the transmission of infectious pathogens is the length of time an individual remains infectious. The span of time from the onset of symptoms to death during infection with Ebola (a hemorrhagic fever virus) is usually 2–21 days. Although highly contagious, Ebola is often confined to small regions because the length of time between the onset of symptoms and death is short, thus restricting the amount of time an infected individual can act as a carrier/host. In the case of HIV/AIDS, patients are infectious for the remainder of their lifetimes. The length of time they can act as carriers is much longer, thus greatly enhancing the number of individuals to whom they can transmit the disease. Finally, some infections, such as chlamydia and hepatitis B, can be asymptomatic. Carriers can unknowingly transmit the infection to others, resulting in significant spread of the causative agent through the population.

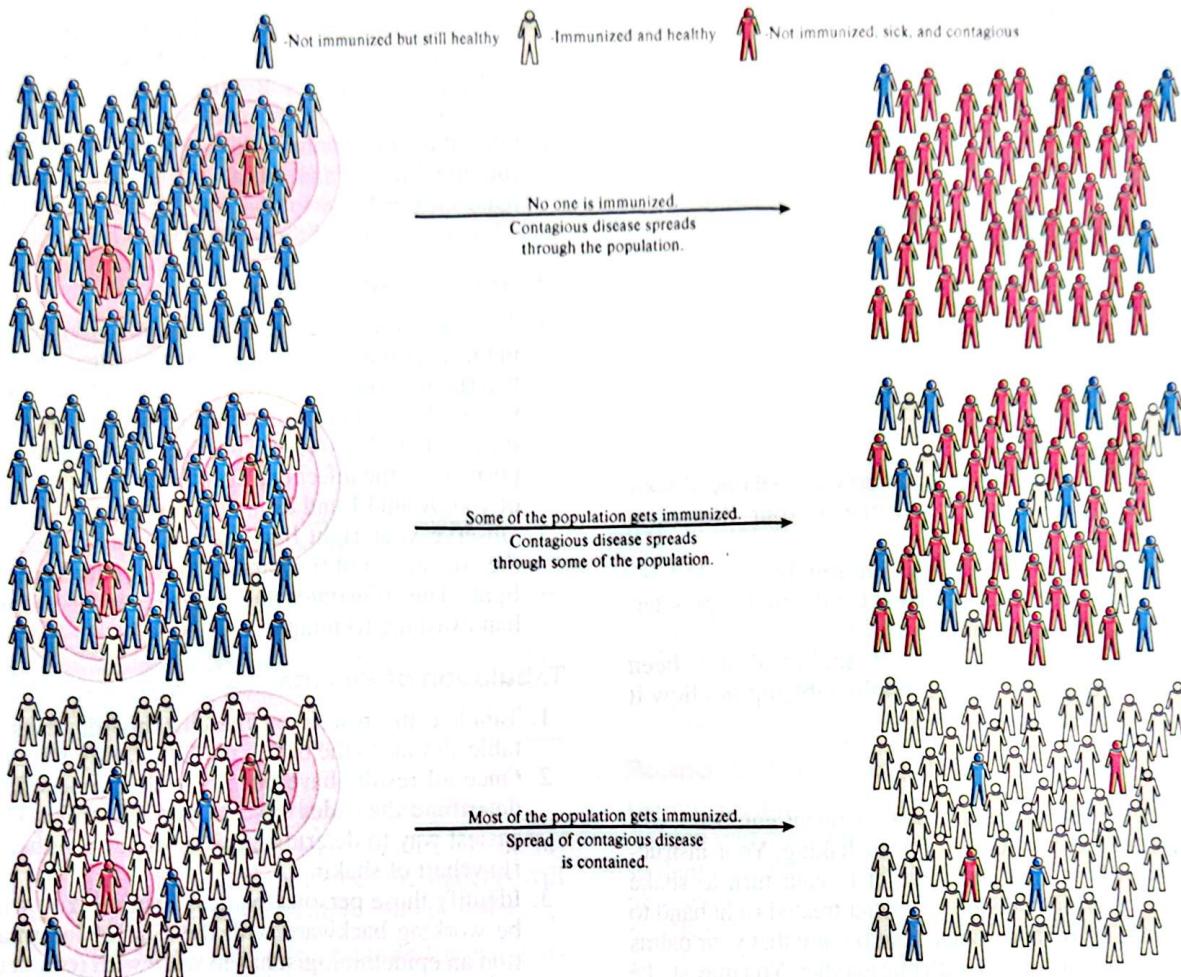
Another principle that affects the spread of an epidemic through a population is referred to as community, or herd, immunity. When a certain proportion of a population is vaccinated or already immune to an infectious agent, an outbreak is unlikely in the population. Even individuals who cannot be vaccinated, such as infants, pregnant women, and immunocompromised individuals, are somewhat protected from infection because the spread of the agent is limited in the community (figure 55.2).

The microbiology laboratory plays a crucial role in preventing an outbreak from becoming an epidemic. Laboratory support involves, but is not limited to, culturing of infectious agents and environmental sites, isolate identification, and serological typing. Results from the microbiology lab help epidemiologists determine the first incident in a given outbreak, known as the **index case**. Identification of the index case aids in determining the original source of infection in an outbreak. Additionally, the microbiology lab is responsible for reporting suspicion or identification of infectious diseases to health departments, the Centers for Disease Control and Prevention (CDC), and the WHO.

### Why It Matters

#### How can you apply this exercise to the real world?

At the time this laboratory manual was written, news channels and online publications were filled with breaking news of a devastating pandemic. A new coronavirus (SARS-CoV-2) had emerged in Wuhan, China, causing severe respiratory infections (COVID-19), especially in older adults. Because a novel virus was involved, no one in the population was immune, and the epidemic spread rapidly (figure 56.2 top) across China and then across the globe. In just two months, the number of cases reached almost 100,000 across the world, and within seven months, the numbers had reached almost five million in the United States and eighteen million worldwide. Over half a million people across the globe died during this short time. Many nations implemented drastic shutdown measures, including the closure of schools and businesses, and the words “social distancing” and “quarantine” became household terms. If you were taking classes during the spring 2020 semester, you likely remember a rapid shift to remote learning to finish the semester!



**Figure 56.2 Community or herd immunity.**

Source: National Institute of Allergy and Infectious Diseases

In this experiment, we will have an opportunity to approximate, in several ways, the work of the epidemiologist. Each member of the class will take part in the spread of a “synthetic infection” with simple reagents used to simulate the spread of an imaginary disease.

Two different experiments will be conducted: Procedures A and B. In Procedure A, students transfer a detectable agent by handshaking. The agent used is visible under ultraviolet (UV) light. Procedure A represents an epidemic in which the infectious agent is transferred from person to person by physical contact, and class data will be analyzed to determine the index case. In Procedure B, students transfer a detectable agent by the exchange of fluid in a test tube. The detectable agent in Procedure B is NaOH, which causes a change in pH that can be detected by the addition of a pH indicator. Procedure B represents an epidemic in which the infectious agent is transferred by the exchange of body fluid (saliva, sweat, urine, secretions). This procedure will be slightly modified

and repeated three times to demonstrate the concept of community (herd) immunity.

#### Procedure A

In this experiment, each student will be given a numbered container of white unknown powder. Only one member in the class will be given a detectable agent that is to be considered the infectious agent and is visible under UV light. The other members will be issued a transmissible agent that is considered noninfectious. After each student has spread the powder on his or her hands, all members of the class will engage in two rounds of handshaking, directed by the instructor. A record of the handshaking contacts will be recorded on a chart similar to the one in the Laboratory Report. After each round of handshaking, the hands will be rubbed on a Kimwipe or tissue that will later be placed under UV light to determine the presence or absence of the infectious agent.

## EXERCISE 56 A Synthetic Epidemic

Once all the data are compiled, an attempt will be made to determine two things: (1) the original source of the infection, and (2) who the carriers are. The type of data analysis used in this experiment is similar to the procedure that an epidemiologist would employ. Proceed as follows:

### Materials

- 1 numbered petri dish containing an unknown white powder (either powder that is detectable using a UV light, such as GloGerm, or powder that is undetectable under UV light, such as baking soda)
- 2 Kimwipes or tissues

### Preliminaries

1. After assembling your materials, label one of your Kimwipes Round 1 and one of your Kimwipes Round 2.
2. Wash and dry your hands thoroughly.
3. Thoroughly coat your right hand with the powder, focusing on the palm surface.

**IMPORTANT:** Once the hand has been prepared, do not rest it on the tabletop or allow it to touch any other object.

### Round 1

1. On the cue of the instructor, student number 1 will begin the first round of handshaking. Your instructor will inform you when it is your turn to shake hands with someone. Use your treated right hand to make firm hand contact and be sure that your palms are fully in contact with one another. You may shake with anyone, but it is best not to shake your neighbor's hand. *Be sure to use only your treated hand, and avoid touching anything else with that hand.*
2. In each round of handshaking, you will be selected by the instructor *only once* for handshaking; however, due to the randomness of selection by the handshakers, it is possible that you may be selected as the "shaker" several times. The names of each "shaker" and "shakee" should be recorded on the board as the round progresses.
3. After every member of the class has shaken someone's hand, you need to assess just who might have picked up the "microbe." To accomplish this, rub your right hand thoroughly on the Kimwipe labeled Round 1. Set the Kimwipe aside until after the second round of handshaking.

**IMPORTANT:** Don't allow your hand to touch any other object. A second round of handshaking follows.

### Round 2

1. On cue of the instructor, student number 1 will again select a person at random to shake hands

with, proceeding as in Round 1 until everyone has had a turn to initiate a handshake. Avoid contact with any other objects. Be sure to keep a record of each handshake on the board.

2. Once the second handshaking episode is finished, rub the fingers and palm of the contaminated hand on the Kimwipe labeled Round 2. Set the Kimwipe aside and thoroughly wash hands.

### Chemical Identification

1. The powder that is considered the infectious agent in this lab exercise is comprised of synthetic beads that fluoresce under UV light. Place your Kimwipes under a UV light in a darkened room to determine if they glow. A glowing Kimwipe is interpreted as positive for the infectious agent. Record the results of your Round 1 and Round 2 Kimwipes.
2. Observe your right hand, which was washed at the conclusion of the handshaking, under the UV light. This illustrates the importance of proper handwashing technique.

### Tabulation of Results

1. Tabulate the results on the chalkboard, using a table similar to the one in the Laboratory Report.
2. Once all results have been recorded, proceed to determine the index case in this epidemic. The easiest way to determine this is to put together a flowchart of shaking.
3. Identify those persons that test positive. You will be working backward with the kind of information an epidemiologist has to work with (contacts and infections). Eventually, a pattern will emerge that shows which person or persons may have started the epidemic.
4. Complete Laboratory Report 55.

### Procedure A: Results and Analysis

**Note to instructors:** If GloGerm and a UV light source are unavailable, you can use corn starch or baking powder as safe, inexpensive alternatives. In this case, iodine is used to determine those students who have been "infected." Because student safety takes the highest priority in this manual, microorganisms were not used during this exercise.

### Procedure B

In this experiment, you will be investigating the benefit of community immunity for a population. In each round, students will be given a test tube containing water (representing a susceptible individual), 0.1 M NaOH (representing an infected individual), or a pH buffer solution (representing a vaccinated individual).

After receiving their tubes, students will exchange a portion of the fluid in the tubes with one another as directed by the instructor. During each round, each student will exchange fluid with three different students. Once all of the exchanges have been completed for each round, a pH indicator will be added to all tubes to determine the presence or absence of the “infectious agent.”

Proceed as follows for each round, recording class data in the Laboratory Report.

### Materials

- sterile gloves
- 1 numbered test tube with lid containing liquid (either distilled water, a pH buffer solution such as a sodium phosphate buffer pH = 6.8, or 0.1 M NaOH)
- 1 dropper
- phenolphthalein solution, dissolved in alcohol and diluted in water (pH indicator) by instructor

### Caution

Sodium hydroxide (NaOH) and phenolphthalein can irritate the eyes and skin. Wear gloves, and alert your instructor if any spills occur.

### Round 1: 100% Susceptible Population

For this round, one student will unknowingly be infected with the agent and the rest of the students will be susceptible to infection (sample fluid of water).

1. On cue from the instructor, each student will participate in the first fluid exchange by selecting another student at random to swap fluid with. Each participant will use a dropper to trade a few drops of fluid.
2. After the class has conducted the first exchange, your instructor will announce that each student

needs to find a second random student to exchange fluid with. Complete the fluid exchange with this second classmate.

3. Repeat with a third exchange when indicated by your instructor.
4. Add one drop of phenolphthalein to your test tube. If the fluid turns pink, you are positive for the infectious agent.
5. Record the class results in Laboratory Report 56, Procedure B: Results and Analysis.

### Round 2: 50% Susceptible Population

For this second round, you will receive a new test tube sample of fluid. One student will unknowingly be infected with the agent, 50% of the students will be susceptible to infection (sample fluid of water), and 50% will represent vaccinated individuals (sample fluid of pH buffer).

1. Repeat the same procedure as above with your new fluid sample, exchanging with three individuals as your instructor leads.
2. Use the indicator to test for infection, and record the class results in Laboratory Report 56, Procedure B: Results and Analysis.

### Round 3: 10% Susceptible Population

For this final round, repeat the preceding experiment starting with one infected individual, 90% vaccinated students (sample fluid of pH buffer), and 10% susceptible students (sample fluid of water). Record the class results in Laboratory Report 56, Procedure B: Results and Analysis.

### Laboratory Report

Complete Laboratory Report 56.

## Bacterial Counts of Foods

### Learning Outcomes

After completing this exercise, you should be able to

1. Understand the role that microorganisms have in food production and in food spoilage.
2. Perform a standard plate count on a food sample to determine the number of bacteria in the sample.

The presence of microorganisms in food does not necessarily indicate that the food is spoiled or that it has the potential to cause disease. Some foods can have high counts because microorganisms are used in their production. Yogurt, sauerkraut, and summer sausage are examples of foods prepared by microbial fermentation and, therefore, they have high bacterial counts associated with them during production. However, postproduction treatments such as pasteurization or smoking will significantly reduce the number of bacteria present. During processing and preparation, food can become contaminated with bacteria, which naturally occur in the environment. These bacteria may not be necessarily harmful or pathogenic. Bacteria are naturally associated with some foods when they are harvested. For example, green beans, potatoes, and beets have soil bacteria associated with them when harvested. Even after washing, some bacteria can remain and will be preserved with the food when it is frozen. The chalky appearance of grapes is due to yeasts that are naturally associated with grapes. This is also true of other fruits. Milk in the udders of healthy cows is sterile, but bacteria such as *Streptococcus* and *Lactobacillus* are introduced during milking and processing because they are bacteria that are associated with the animal, especially on the outside of the udder. Pasteurization kills many of the bacteria that are introduced during processing and any pathogens that may be present. However, it does not kill all the bacteria in milk as some bacteria can survive pasteurization temperatures, and these bacteria can eventually cause spoilage and souring of milk.

Although high bacterial counts in food do not necessarily mean that the food is spoiled or that it harbors disease-causing organisms, it can suggest the

### Why it Matters

How can you apply this exercise to the real world?

The Centers for Disease Control estimates that 48 million people per year in the United States become sick, 128,000 are hospitalized, and 3,000 people die from foodborne illnesses. Foodborne illnesses usually result because pathogenic bacteria or their toxins are introduced into food products during processing, handling, or preparation. Food handlers can transmit pathogens associated with the human body, like *Staphylococcus aureus* or intestinal bacteria, such as *Salmonella*. Transmission occurs because of unsanitary practices such as failure to wash their hands before preparing or handling food. Botulism food poisoning results from ingesting a toxin produced by *Clostridium botulinum* when its endospores grow in improperly home-canned foods. The endospores occur in the soil and the environment and contaminate the prepared vegetables. *Salmonella* and *Campylobacter* are associated with poultry and eggs and can cause illness if these foods are not properly prepared. *Escherichia coli* O157:H7 is found in the intestines of cattle and can become associated with meat if fecal material from the animal's intestines contaminates meat during the butchering process. This pathogen is then incorporated into hamburger during grinding and processing (figure 43.1). Serious illness results from eating improperly cooked hamburger because cooking temperatures are insufficient to kill the organism. Transmission of this pathogen has also occurred when fecal material of cattle contaminated fruits and vegetables such as apples, lettuce, and spinach.

potential for rapid spoilage of the food. Thus, high counts can be important indicators of potential problems. One method to ascertain if food is contaminated with fecal bacteria and, therefore, has the potential to spread disease is to perform coliform counts. Coliforms are bacteria such as *E. coli* that normally occur in the intestines of humans and warm-blooded animals. Their presence in food or water indicates that fecal contamination has occurred and therefore, there is a high potential for the spread of serious diseases

### EXERCISE 43 Bacterial Counts of Foods

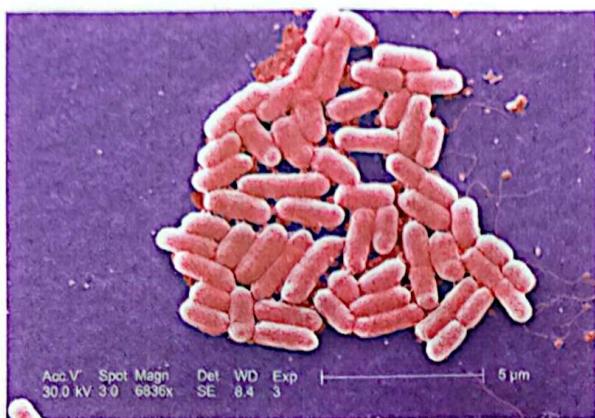


Figure 43.1 *E. coli* O157:H7 is a potential contaminant of ground beef and can cause illness if undercooked meat is consumed.

left: Janice Haney Carr/CDC; right: Tetra Images/Alamy Stock Photo

such as typhoid fever, bacillary dysentery, cholera, and intestinal viral diseases.

The standard plate count and the coliform count can be used to evaluate foods in much the same manner that they are used for milk and water to determine total bacterial counts and the number of coliforms. In this exercise, samples of ground beef, dried fruit, and vegetables will be tested for total numbers of bacteria. This procedure, however, will not determine the numbers of coliforms (a similar process with selective media would be required to isolate these species). Your instructor will indicate the specific kinds of foods to be tested and make individual assignments. Figure 43.2 illustrates the general procedure.

### Materials

per student:

- 3 nutrient agar plates
- one 99 ml sterile water blank
- two 1.0 ml dilution pipettes
- sterile cell spreaders

per class:

- food blender
- sterile blender jars (one for each type of food)
- sterile weighing paper
- 180 ml sterile water blanks (one for each type of food)
- samples of ground meat, dried fruit, and thawed frozen vegetables or prewashed lettuce

1. Using aseptic technique, weigh out on sterile weighing paper 20 grams of food to be tested.
2. Add the food and 180 ml of sterile water to a sterile blender jar. Blend the mixture for 5 minutes. This suspension will provide a 1:10 dilution.

3. With a sterile 1.0 ml pipette, dispense 0.1 ml from the blender to plate I and 1.0 ml to the water blank. Use a sterile cell spreader to disperse the sample across the surface of the plate (see figure 43.2).
4. Shake the water blank 25 times in an arc for 7 seconds with your elbow on the table, as done in Exercise 19 (Enumeration of Bacteria).
5. Using a fresh pipette, dispense 1.0 ml to plate II and 0.1 ml to plate III. Use sterile cell spreaders to disperse the samples across the surface of each plate.
6. Incubate the plates at 35–37°C for 24 hours.
7. Count the colonies on the best plate and record the results in Laboratory Report 43.

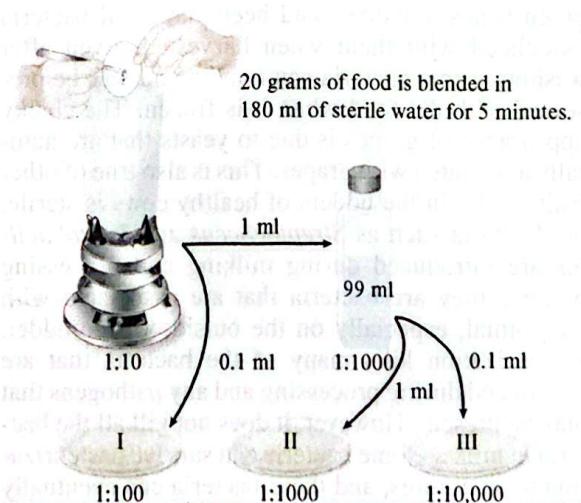


Figure 43.2 Dilution procedure for bacterial counts of food.

**EXERCISE****60**

## Enzyme-Linked Immunosorbent Assay (ELISA)

### Learning Outcomes

After completing this exercise, you should be able to

1. Understand the basis of enzyme-linked immunosorbent assays (ELISA).
2. Perform an indirect ELISA to detect anti-HIV antibodies in patient serum as an example of disease detection using this assay.

Immunological testing involves *specific* interactions between antigens and antibodies and is highly *sensitive*, detecting even minute amounts in a patient sample. **Immunoassays** are *in vitro* tests that use labeled antibodies to visualize these antigen–antibody interactions. Antibodies may be labeled with fluorescent dyes, radioactive isotopes, or enzymes. **Direct** immunoassays use the labeled antibodies to detect unknown antigens in a clinical specimen, whereas **indirect** immunoassays detect specific antibodies in a patient's serum.

Enzyme-linked immunosorbent assays (ELISA) use enzyme-labeled antibodies to detect either unknown antigens or antibodies in a patient sample. Specifically, how does an indirect ELISA work? First, known antigens are bound to the wells of a clear microtiter plate. Then, test serum from a patient is added to the wells. If the patient has detectable levels of antibodies to that known antigen, they will bind to the antigen in the well. Next, a labeled antibody that can specifically bind to those test antibodies is added to the well. This secondary antibody is linked to an enzyme. If the patient's serum contains the antibodies to the known antigen, these secondary antibodies will bind to them and remain in the well after washing. Finally, when the enzyme's substrate is added to that well, the enzyme catalyzes a color reaction that is easily visible. If the patient's serum does not contain the antibodies to the antigen, the secondary antibody with the enzyme is removed from the well by washing, and no visible reaction occurs.

An ELISA can be used to diagnose many different types of viral infections, including influenza, measles, hepatitis B and C, as well as West Nile virus. Bacterial infections such as syphilis, strep throat, and salmonellosis from food-borne outbreaks can also be detected using immunoassays.

A specific example of the use of an ELISA is the initial screening test for human immunodeficiency virus (HIV), a fourth-generation immunoassay aimed at detecting antibodies to HIV-1 and HIV-2 (two strains of HIV found in different parts of the world). If this immunoassay is positive, follow-up tests such as the Western blot, an indirect immunofluorescence assay, or a nucleic acid test are used to confirm HIV infection.

A successful vaccine for HIV has been elusive despite considerable efforts over the four decades since this infectious agent was discovered. Instead, prevention education and advanced methods of diagnostic testing have been the keys to slowing the spread of HIV. Identifying HIV-positive individuals increases their access to healthcare and antiretroviral therapy, which improves patient outcomes and overall public health. Early detection and treatment can lessen the severity of the disease, slow its progression, and reduce the rates of transmission to others.

### Why It Matters

How can you apply this exercise to the real world?

HIV infection is still a major global health concern, affecting millions across all inhabited continents. Recent World Health Organization (WHO) statistics estimate 37.9 million people are living with HIV around the world. Thirty-two million have died of AIDS since the pandemic began in the early 1980s. In the United States, the Centers for Disease Control and Prevention (CDC) estimates more than 1.1 million individuals are infected with HIV, and up to 40,000 new cases develop each year. Even though the number of new HIV infections and AIDS deaths are decreasing, HIV remains a major cause of death worldwide.

## EXERCISE 60 Enzyme-Linked Immunosorbent Assay (ELISA)

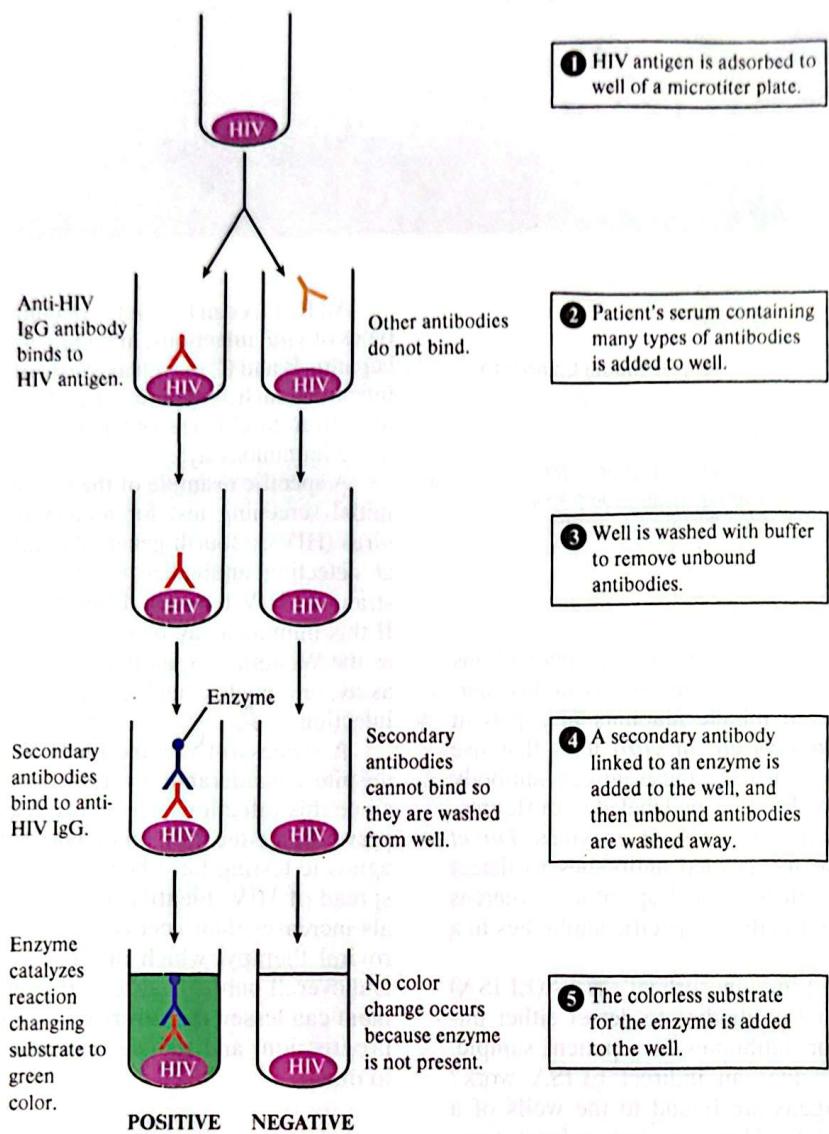


Figure 60.1 The steps of the indirect ELISA test for HIV Infection.

Data from Edvotek, AIDS Kit 1: Simulation of HIV-1 Detection, [www.edvotek.com/site/pdf/271.pdf](http://www.edvotek.com/site/pdf/271.pdf)

### ELISA Procedure

In this exercise, you will use an indirect ELISA to detect the presence of anti-HIV IgG antibodies in hypothetical patient serum samples, with a positive result indicating likely infection with HIV. Figure 60.1 illustrates and explains each step of this process.

### Materials

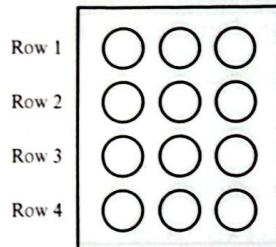
- microtiter plate (4 rows of three wells)
- micropipette and sterile tips
- phosphate buffered saline (PBS) in small beaker
- small waste beaker
- biohazard bag for used tips
- reagents in labeled microcentrifuge tubes: HIV antigen, positive (+) control, patient serum #1, patient serum #2, secondary antibody, substrate

Carefully follow the steps below to minimize the risk of contamination between test wells. In a real testing lab, micropipette tips would be changed consistently throughout the process; however, in this simulated ELISA test, the use of new tips at specific intervals is indicated to minimize cost and materials but still provide accurate results.

### Note

Gloves should be worn throughout this exercise, replicating the test process as it would be conducted in the medical laboratory.

1. Label your microtiter plate with a Sharpie, as shown below. Also, label the plate with your table number. Each student at the table should assume responsibility for one row of the ELISA throughout the exercise.



2. Using one micropipette tip for all wells, add 50 $\mu$ l of HIV antigen to all 12 wells. Discard the tip into the biohazard receptacle.
3. Incubate the plate for 5 minutes at room temperature.
4. Using one micropipette tip for all wells, remove all the liquid in each well and discard into waste beaker. Discard your tip when finished with this step.
5. Using one micropipette tip for all wells, wash each well with 200 $\mu$ l of PBS. This means that you will add the liquid and remove it immediately. Discard the PBS wash into the waste beaker, and discard the tip when finished with this step.
6. Using a new tip for each row, add the following reagents into each:

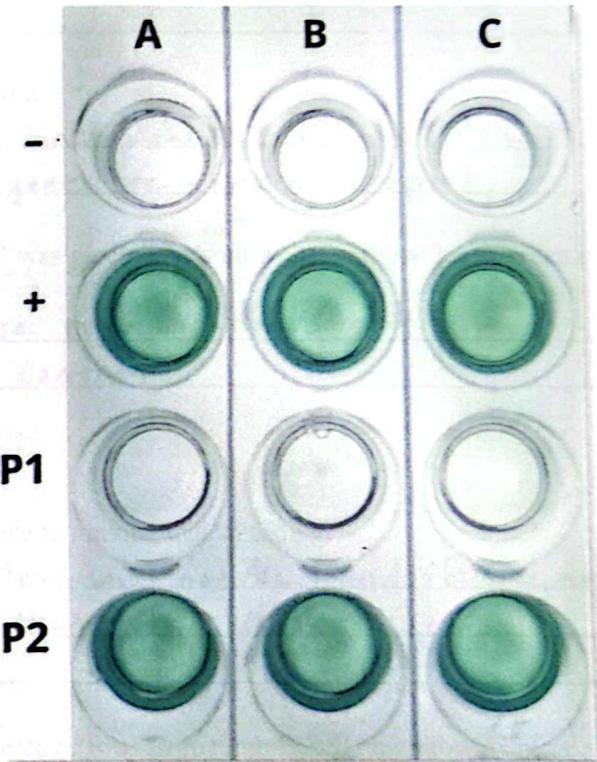
  - Row 1: Add 50 $\mu$ l of PBS (negative control).
  - Row 2: Add 50 $\mu$ l of + (positive control).
  - Row 3: Add 50 $\mu$ l of PS1 (patient serum #1).
  - Row 4: Add 50 $\mu$ l of PS2 (patient serum #2).

7. Incubate at 37°C for 15 minutes.
8. Using a new tip for each row, remove the liquid from each well and discard into the waste beaker.

9. Using a new tip for each well, wash all of the wells with PBS buffer as in step 5.
10. Using a new tip for each row, add 50 $\mu$ l of the anti-IgG with peroxidase (secondary antibody) to all 12 wells.
11. Incubate at 37°C for 15 minutes.
12. Using a new tip for each row, remove the liquid from each well and discard into the waste beaker.
13. Using a new tip for each well, wash all of the wells with PBS buffer.
14. Using a new tip for each row, add 50 $\mu$ l of substrate to all 12 wells.
15. Incubate at 37°C for 5 minutes.
16. Remove the plate for analysis, and record your observations in the Laboratory Report. Figure 60.2 shows an example of a microtiter plate with both positive (green) and negative (colorless) wells.

### Laboratory Report

Complete Laboratory Report 60.



**Figure 60.2 Sample results of Indirect ELISA test for HIV. Green wells indicate anti-HIV IgG antibodies were present in the sample, whereas colorless wells indicate these test antibodies were not present.**

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**EXERCISE****61**

## Blood Grouping

### Learning Outcomes

After completing this exercise, you should be able to

1. Understand the basis of blood grouping in the ABO and Rh systems.
2. Determine the ABO and Rh types of human blood.

Blood typing is an example of a medical procedure that relies on the agglutination of antigens and antibodies. The procedure for blood typing was developed by Karl Landsteiner around 1900. He is credited with having discovered that human blood types can be separated into four groups on the basis of two antigens that are present on the surface of red blood cells. These antigens are designated as A and B. The four groups (types) are A, B, AB, and O, which are named based on the antigens that are present on each type (figure 61.1). The last group, type O, which is characterized by the absence of A or B antigens, is the most common type in the United States (45% of the population). Type A is next in frequency, found in 39% of the population. The frequencies of types B and AB are 12% and 4%, respectively.

### Why It Matters

How can you apply this exercise to the real world?

Type O has been called the "universal donor" because cells lack the A and B antigens. Type AB has been called the "universal recipient" because individuals with this blood type do not have anti-A or anti-B antibodies in their serum. However, blood is always specifically typed to avoid errors.

Blood typing is performed with antisera containing high titers of anti-A and anti-B antibodies. The test is usually performed by either slide or tube methods. In both instances, a drop of each kind of antiserum is added to separate samples of a saline suspension of red blood cells. If agglutination occurs only in the suspension to which the anti-A serum was added, the blood is type A. If agglutination occurs only in the anti-B mixture, the blood is type B. Agglutination in both samples indicates that the blood is type AB. The absence of agglutination indicates that the blood is type O. Figure 61.2 illustrates an example of the results for type A blood. Notice that

Antigens	A	B	AB	O
Antibodies	Anti-B	Anti-A	Anti-A and Anti-B	
Blood Type	A	B	AB	O

**Figure 61.1 Understanding ABO blood groups.**

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## EXERCISE 61 Blood Grouping

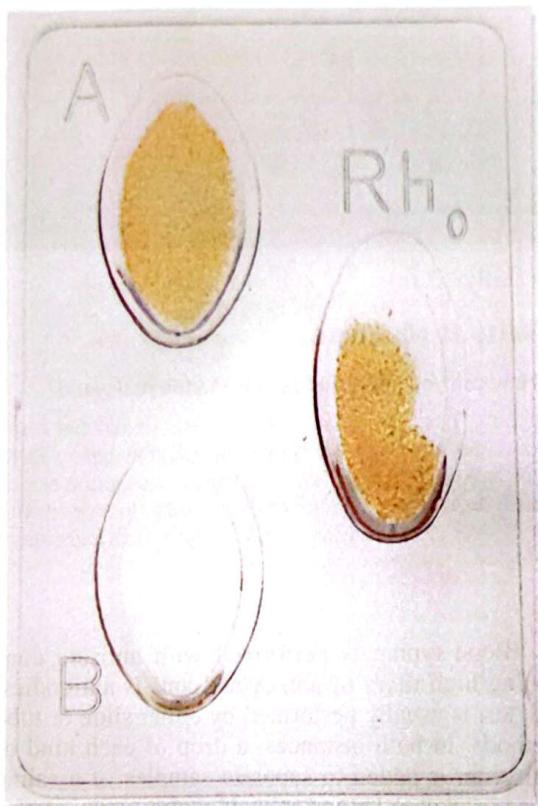


Figure 61.2 Sample blood typing test, indicating type A+ blood.

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agglutination occurs in the A well of this tray but not in the B well.

Between 1900 and 1940, a great deal of research was done to uncover the presence of other antigens in human red blood cells. Finally, in 1940, Landsteiner and Wiener reported that rabbit sera containing antibodies against the red blood cells of the rhesus monkey would agglutinate the red blood cells of 5% of humans. This antigen in humans, which was first designated as the **Rh factor** (for the rhesus monkey), was later found to exist as six antigens: C, c, D, d, E, and e. Of these six antigens, the D factor is responsible for the Rh-positive condition and is found in 85% of Caucasians, 94% of African-Americans, and 99% of Asians. Rh typing is also important to prevent incompatibilities and serious illness.

The Rh factor is important in a disease called **erythroblastosis fetalis**, or hemolytic disease of newborns. The condition occurs when an Rh-negative mother is pregnant with an Rh-positive fetus. Blood cells from the fetus cross the placenta into the mother, causing her to produce antibodies against the Rh D antigen on fetal blood cells. During the first

pregnancy, the mother produces IgM antibodies, which do not readily pass back into the fetus. At this point, the mother is sensitized to the Rh-positive antigen. In a second pregnancy with an Rh-positive fetus, however, fetal blood cells will pass into the mother, causing her to form IgG antibodies. These antibodies can then pass through the placenta back into the fetal blood supply, where they react with the D antigen on fetal blood, causing the blood cells to lyse and break up. This causes jaundice and a severe anemia in the fetus, sometimes requiring transfusion of the child at birth. To prevent this condition, the mother can be given an Rh immune globulin (RhoGAM) shot at week 28 and week 34 during every pregnancy and immediately after giving birth. RhoGAM reacts with the D antigen on fetal blood cells and prevents the mother from forming any antibodies to the D antigen. It is also important to note that this condition can result in cases where there is an ABO incompatibility between the mother and the fetus, but it is usually rarer than the Rh-induced condition.

### Note

In the exercise, you will be working with synthetic blood that simulates human blood in typing reactions for the major blood groups A, B, O, and the Rh factor. When reacted with the "antisera" provided for typing, the same type of agglutination that would be seen with authentic blood is observed.

## ABO and Rh Blood Typing

### Materials

- blood typing kit\* containing 4 synthetic blood samples and A, B, and Rh antisera
- plastic trays with circular wells
- colored mixing sticks

1. Add one drop of a blood sample to each of the three wells on the plastic tray.
2. Add one drop of correct antisera to each well based on its label (for example, anti-A into the well labeled A).
3. Use a different colored stick for each well, and gently mix the synthetic blood and antisera in each well for at least 30 seconds.
4. Observe each well for a granular appearance, indicating agglutination has occurred.
5. Record your results in the data table in Laboratory Report 61.
6. Wash the plastic tray and repeat the preceding steps for the rest of the blood samples.

\*Data from Carolina Biological Supply, ABO-RhBlood Typing with Synthetic Blood.

**EXERCISE****15**

## Endospore Staining

### Learning Outcomes

After completing this exercise, you should be able to

1. Prepare an endospore stain of bacterial cells and observe endospores in the stained preparation.
2. Differentiate between vegetative cells and endospores.

When species of bacteria belonging to the genera *Bacillus* and *Clostridia* face nutrient depletion, they undergo a complex developmental cycle that produces dormant structures called **endospores**. Endospores survive environmental conditions that are not favorable for normal bacterial growth. If nutrients once again become available, the endospore can go through the process of germination to form a new vegetative cell, and growth will resume. Endospores are very dehydrated structures that are not actively metabolizing. Furthermore, they are resistant to heat, radiation, acids, and many chemicals, such as disinfectants, that normally harm or kill vegetative cells. Their chemical resistance is due in part to the fact that they have a protein coat that forms a protective barrier around the spore. Heat resistance is associated with the water content of endospores. The higher the water content of an endospore, the less heat resistant the endospore will be. During sporulation, the water content of the endospore is reduced to 10–30% of the vegetative cell. During endospore formation, calcium dipicolinate and spore-specific proteins form a cytoplasmic gel that reduces the protoplasmic volume of the endospore to a minimum. In addition, a thick cortex forms around the endospore, and contraction of the cortex results in a smaller dehydrated structure. Calcium dipicolinate is not present in vegetative cells. The gel formed by this chemical and the spore-specific proteins controls the amount of water that can enter the endospore, thus maintaining its dehydrated state. Figure 15.1 illustrates a typical sporulation cycle.

Since endospores are not easily destroyed by heat or chemicals, they define the conditions necessary to establish sterility. For example, to destroy endospores by heating, they must be exposed for 15–20 minutes to steam under pressure, which generates

temperatures of 121°C. Such conditions are produced in an **autoclave**.

### Why it Matters

#### How can you apply this exercise to the real world?

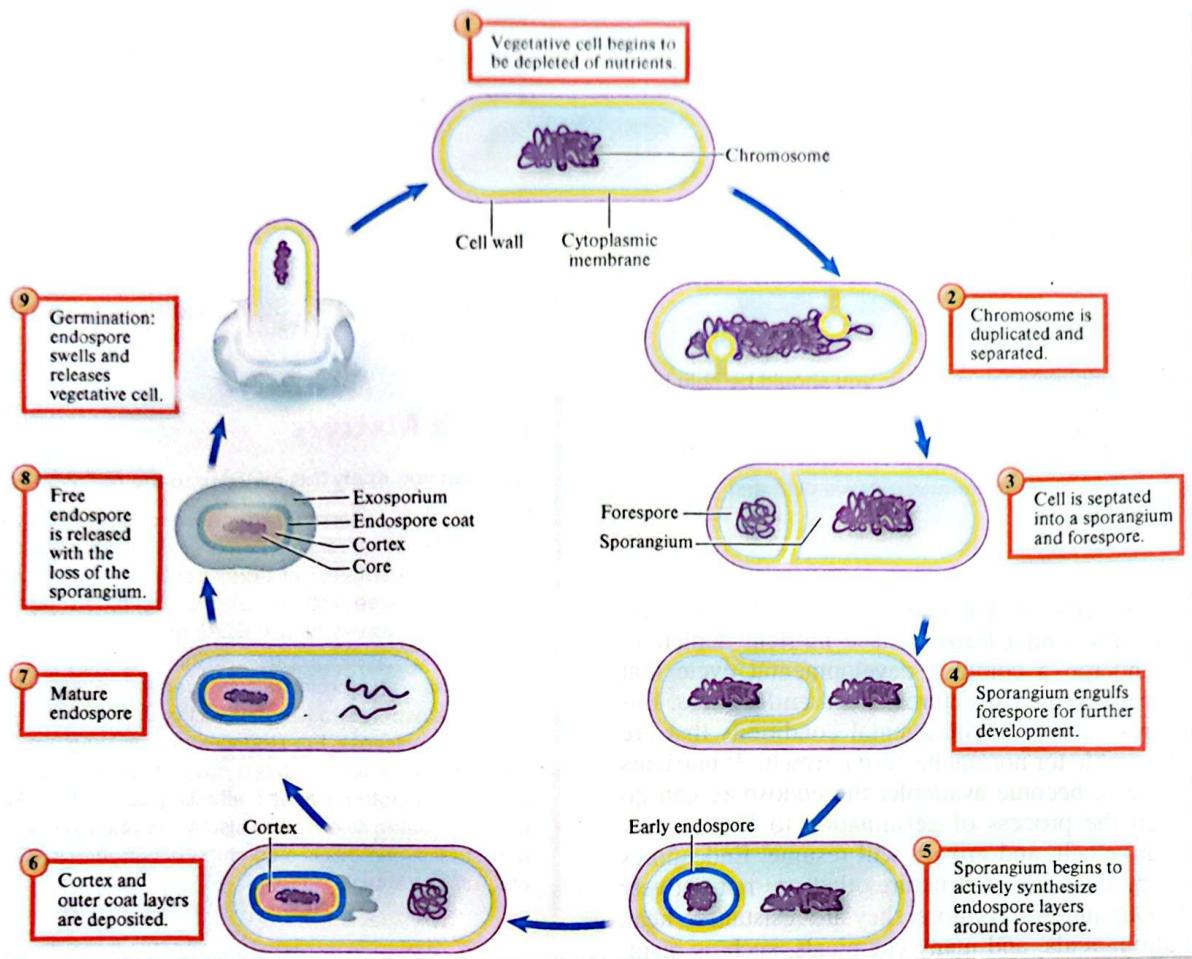
Many medically relevant pathogens produce endospores, which makes their transmission difficult to control because of the high resistance of endospores to regular control measures. These include *Bacillus anthracis* (anthrax), *Clostridium tetani* (tetanus), *Clostridium botulinum* (botulism), and *Clostridioides difficile* (diarrhea and colitis). In some cases, it is the inhalation, injection, or ingestion of these endospores rather than active bacterial cells that can lead to disease. For example, powdered *B. anthracis* spores were mailed to various government offices in 2001 as part of a bioterror attack, affecting 22 people. Recurrence of treated disease can also occur due to endospore persistence, and this is a common occurrence in 15–30% of *C. diff* patients.

The resistant properties of endospores also mean that they are not easily penetrated by stains. If endospore-containing cells are stained by basic stains such as crystal violet, the spores appear as clear, unstained areas in the vegetative cell. However, if heat is applied while staining with malachite green, the stain more readily penetrates and becomes entrapped in the endospore. In this instance, heat is acting as a mordant to facilitate the uptake of the stain into the endospore. The malachite green is not removed from the endospore by subsequent washing with decolorizing agents or water, but it is easily removed from the vegetative cell.

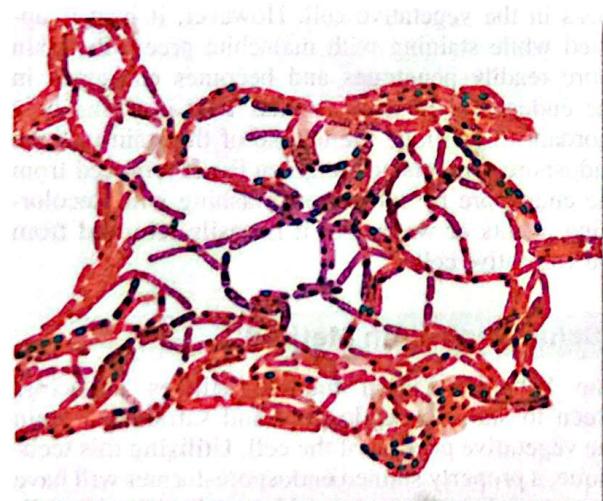
### Schaeffer-Fulton Method

The Schaeffer-Fulton method utilizes malachite green to stain the endospore and safranin to stain the vegetative portion of the cell. Utilizing this technique, a properly stained endospore-former will have a green endospore contained in a red vegetative cell. Figure 15.2 reveals what *Bacillus* and *Clostridium* should look like when correctly stained with this staining method.

## EXERCISE 15 Endospore Staining

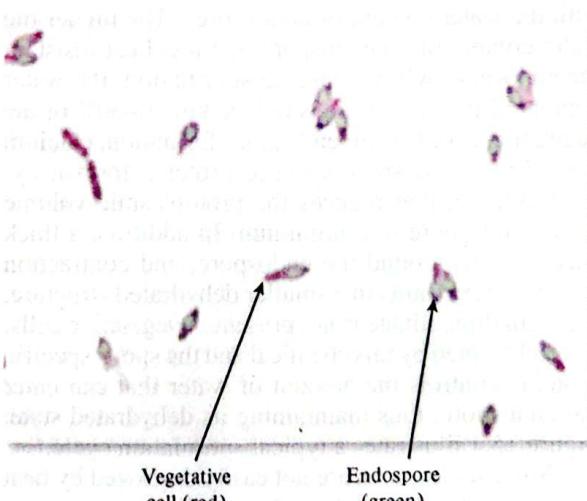


**Figure 15.1 Sporulation cycle.**



(a) Endospore stain of *Bacillus* sp. Note the dark red color of the vegetative cells and the bright green color of the endospores.

**Figure 15.2 Endospore stain of (a) *Bacillus* sp. and (b) *Clostridium* sp.**



(b) Endospore stain of *Clostridium* sp. Note the dark red color of the vegetative cells and the bright green color of the endospores.

**Materials**

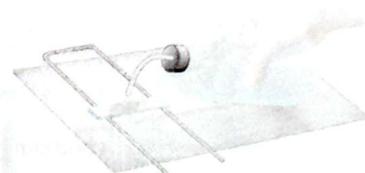
- 24–36 hour nutrient agar slant culture of *Bacillus cereus* (or other *Bacillus* sp.)
- electric hot plate and small beaker (50 ml)
- endospore-staining kit consisting of a bottle each of 5% malachite green and safranin
- wash bottle
- bibulous paper

Prepare a smear of *Bacillus cereus* and allow the smear to air-dry. Heat-fix the dried smear and follow the steps for staining outlined in figure 15.3.

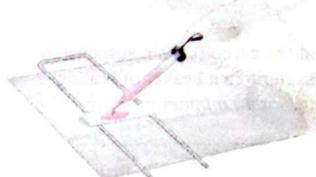
Note: A variation of the Schaeffer-Fulton method that does not require steaming may be used on 36-hour cultures of *Bacillus cereus*. In this variation, a heat-fixed smear is immediately flooded with malachite green and allowed to stand for at least 10 minutes. After washing the smear with water, it is then stained with safranin for 1 minute. Results will be green spores and red vegetative cells, but may be lighter than with the original method that uses steam.



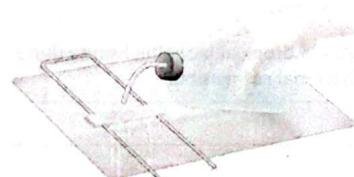
(1) Cover smear with small piece of bibulous paper and saturate it with malachite green. Steam over boiling water for 7 minutes. The bibulous paper should be small enough that it does not hang over the edges of the slide, and additional stain should be added as needed to keep the paper saturated.



(2) After the slide has cooled sufficiently, remove the paper and rinse with water for 30 seconds.



(3) Counterstain with safranin for about 30 seconds.



(4) Rinse briefly with water to remove safranin.



(5) Blot dry with bibulous paper, and examine the slide under oil immersion.

**Figure 15.3 The Schaeffer-Fulton spore stain method.**

Step 1: Steam the smear. (1) Place a small piece of bibulous paper over the smear. Add a few drops of malachite green to the paper. Place the slide on a hot plate and steam over a small beaker of boiling water for 7 minutes. Add more stain to the paper if necessary to keep it saturated. Step 2: Cool the slide. After the slide has cooled sufficiently, remove the paper and rinse with water for 30 seconds. Step 3: Counterstain with safranin. (3) Place a few drops of safranin onto the smear. Let it sit for about 30 seconds. Step 4: Rinse the slide. (4) Rinse the slide briefly with water to remove the excess safranin. Step 5: Examine the slide. (5) Blot the slide dry with a piece of bibulous paper and examine it under oil immersion.

## EXERCISE 15 Endospore Staining

### Alternative Method: Dorner Method

If your instructor chooses, you may instead use the Dorner method for staining endospores. This older method of endospore staining produces a red spore within a colorless cell. Nigrosin stains the background of the slide for contrast. The six steps involved in this technique are shown in figure 15.4. Although both the vegetative cell and endospore are stained during boiling in step 3, the cell is decolorized by the diffusion of carbolfuchsin molecules into the nigrosin.

### Materials

- carbolfuchsin
- nigrosin

- electric hot plate and small beaker (25 ml)
- small test tube (10 × 75 mm size)
- test-tube holder
- 24–36 hour nutrient agar slant culture of *Bacillus cereus*

Prepare a slide of *Bacillus cereus* that utilizes the Dorner method. Follow the steps in figure 15.4.

### Laboratory Report

After examining the organisms under oil immersion, draw a few cells in the appropriate circles in Laboratory Report 15.

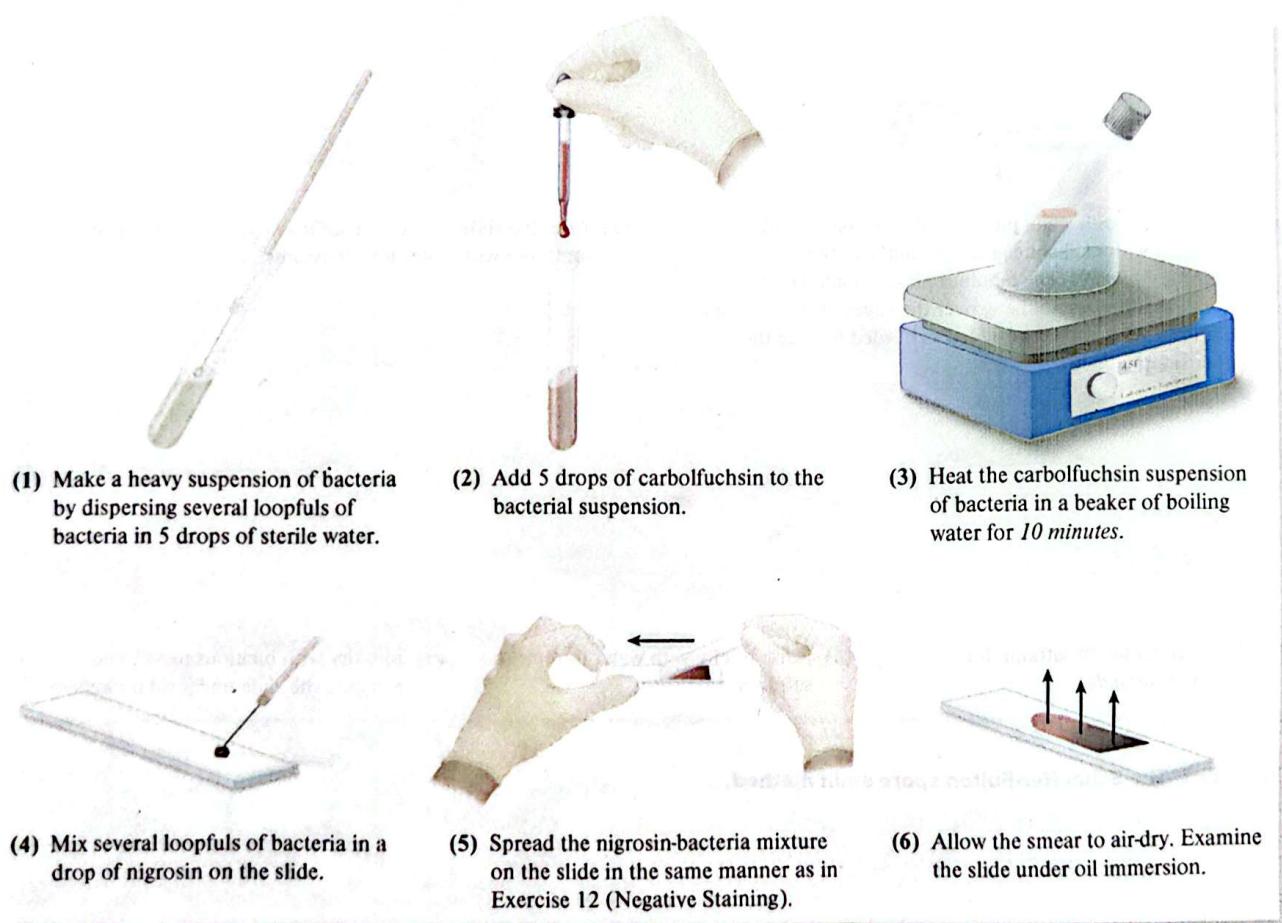


Figure 15.4 The Dorner spore stain method.

## Acid-Fast Staining: Kinyoun Method

### Learning Outcomes

After completing this exercise, you should be able to

1. Prepare an acid-fast stain of bacterial cells.
2. Differentiate between acid-fast and non-acid-fast cells in a mixed stain.
3. Explain the basis for the stain and why the stain is important in clinical microbiology.

### Why it Matters

How can you apply this exercise to the real world?

Bacteria such as *Mycobacterium* and some *Nocardia* have cell walls with high lipid content. One of the cell wall lipids is a waxy material called **mycolic acid**. This material is a complex lipid that is composed of fatty acids and fatty alcohols that have hydrocarbon chains up to 80 carbons in length. It significantly affects the staining properties of these bacteria and prevents them from being stained by many of the stains routinely used in microbiology. The acid-fast stain is an important diagnostic tool in the identification of *Mycobacterium tuberculosis*, the causative agent of tuberculosis, and *Mycobacterium leprae*, the bacterium that causes Hansen's disease (leprosy) in humans.

To facilitate the staining of acid-fast bacteria, it is necessary to use methods that make the cells more permeable to stains because the mycolic acid in their cell walls prevents the penetration of most stains. In the Ziehl-Neelsen staining method, the primary stain, carbolfuchsin, contains phenol, and the cells are heated for 5 minutes during the staining procedure. Phenol and heat facilitate the penetration of the carbolfuchsin into the cell. Heat is acting as a mordant to make the mycolic acid and cell wall lipids more permeable to the stain. Subsequent treatment of the cells with acid-alcohol, a decolorizer, does not remove the entrapped stain from the cells because of the mycolic acid in the walls. Hence, these bacteria are termed **acid-fast**. In order for non-acid-fast bacteria to be visualized in the acid-fast procedure, they must be counterstained with methylene blue, as the primary

stain is removed from these bacteria by the acid-alcohol. In the Ziehl-Neelsen method, the application of heat to cells during staining with carbolfuchsin and phenol is not without safety concerns. Phenol can vaporize when heated, giving rise to noxious fumes that are toxic to the eyes and mucous membranes.

The **Kinyoun acid-fast method** is a modification in which the concentrations of primary stain, basic fuchsin (substituted for carbolfuchsin), and phenol are increased, making it unnecessary to heat the cells during the staining procedure. The increased concentrations of basic fuchsin and phenol are sufficient to allow the penetration of the stain into acid-fast cells, and the basic fuchsin is not removed during destaining with acid-alcohol. This procedure is safer because phenol fumes are not generated during staining of the cells.

In the acid-fast staining method, acid-fast bacteria such as *Mycobacterium* are not decolorized by acid-alcohol and are therefore stained pink to red by the basic fuchsin. Because non-acid-fast bacteria such as *Staphylococcus* are decolorized by the acid-alcohol, a secondary stain, methylene blue, must be applied to visualize these cells in stained preparations. These appear blue after staining is completed (figure 16.1).

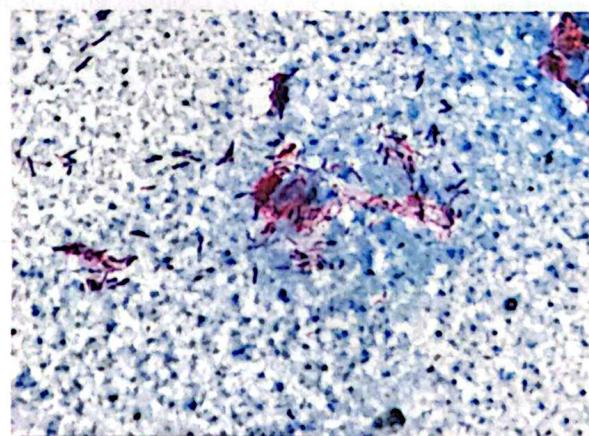


Figure 16.1 Acid-fast stain of *Mycobacterium smegmatis* (red) and *Staphylococcus aureus* (blue).

Auburn University Research Instrumentation Facility/Michael Miller/McGraw-Hill Education

## EXERCISE 16 Acid-Fast Staining: Kinyoun Method

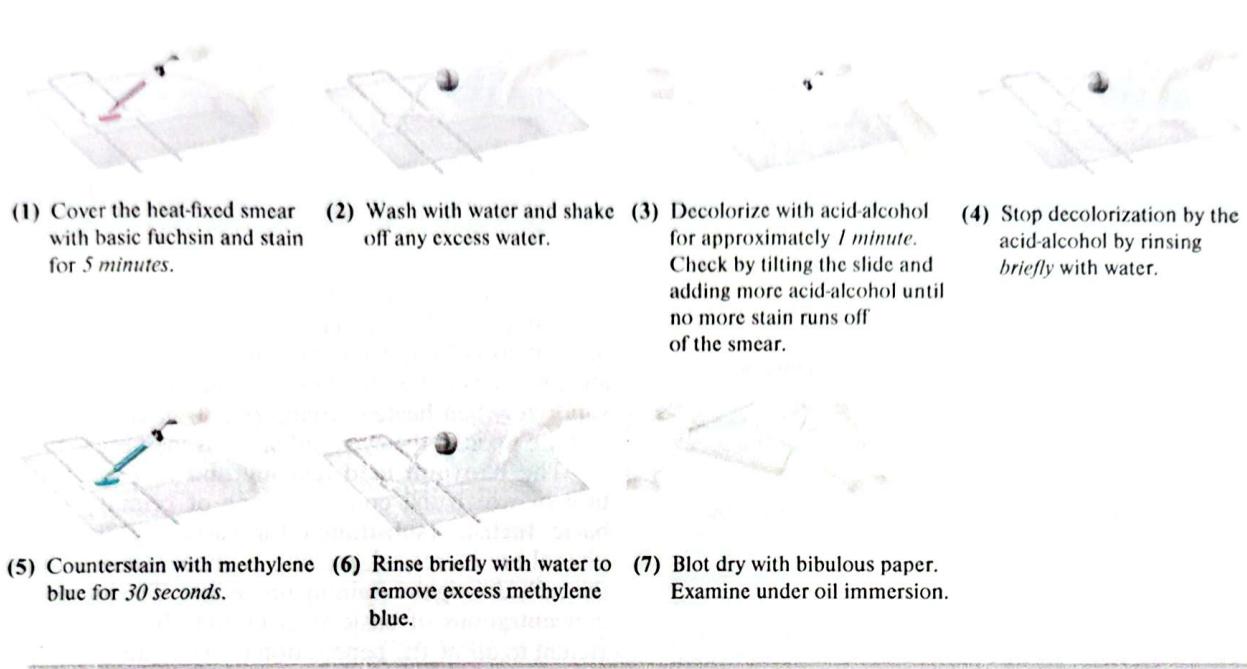


Figure 16.2 Kinyoun acid-fast staining procedure.

In the following exercise, you will prepare an acid-fast stain of a mixture of *Mycobacterium smegmatis* and *Staphylococcus epidermidis* using the Kinyoun method for acid-fast staining. *M. smegmatis* is a non-pathogenic, acid-fast rod that occurs in soil and on the external genitalia of humans. *S. epidermidis* is a non-acid-fast coccus that is part of the normal microbiota of human skin but is also known to be a common cause of hospital-acquired infections.

### Materials

- slant culture of *Mycobacterium smegmatis* (48-hour culture)
- broth culture of *S. epidermidis*
- acid-fast staining kit (basic fuchsin, acid-alcohol, and methylene blue)

**Smear Preparation** Prepare a mixed culture smear by placing two loopfuls of *S. epidermidis* on a slide

and transferring a small amount of *M. smegmatis* to the broth on the slide with an inoculating needle. Since the mycobacteria are waxy and tend to cling to each other in clumps, break up the masses of organisms with the inoculating needle. After air-drying the smear, heat-fix it.

**Staining** Follow the staining procedure outlined in figure 16.2. **Note:** If conducting the Ziehl-Neelsen method, continuously add carbolfuchsin with the slide over steam for 5 minutes in step 1.

**Examination** Examine under oil immersion and compare your slide with figure 16.1.

### Laboratory Report

Record your results in Laboratory Report 16.

## Capsular Staining

### Learning Outcomes

After completing this exercise, you should be able to

1. Prepare and stain a smear of an encapsulated bacterium using the Anthony capsule staining method.
2. Visualize the capsule and differentiate it from the cell body.

Many bacterial cells are surrounded by an extracellular gel-like layer that occurs outside of the cell wall. If the layer is distinct and gelatinous, it is referred to as a **capsule**. If the layer is diffuse and irregular, it is called a **slime layer**. The capsule or slime layer can vary in its chemical composition. If it is made up of polysaccharides, it is known as a **glycocalyx**, which literally means “sugar shell.” However, the capsule found in *Bacillus anthracis* is composed of a proteinaceous matrix. Evidence supports the view that probably all bacterial cells have some amount of slime layer, but in most cases, the amount is not enough to be readily discerned.

### Why It Matters

How can you apply this exercise to the real world?

Capsules or slime layers perform very important functions for some disease-causing organisms. In pathogens such as *Streptococcus pneumoniae*, they are protective structures because they prevent phagocytic white blood cells from engulfing and destroying the pathogen, enabling the organism to invade the lungs and cause pneumonia. Another function for capsules or slime layers is the attachment of the bacterial cell to solid surfaces in the environment. For example, *Streptococcus mutans* produces a capsule that facilitates the attachment of the organism to the tooth surface, resulting in the formation of dental plaque. If not removed, plaque will contribute to the formation of dental caries.

Staining of the bacterial capsule cannot be accomplished by ordinary staining procedures. If smears are heat-fixed prior to staining, the capsule shrinks or is destroyed and therefore cannot be seen in stains. In the Anthony method (figure 13.1), smears are prepared from cultures grown in skim milk broth. These smears

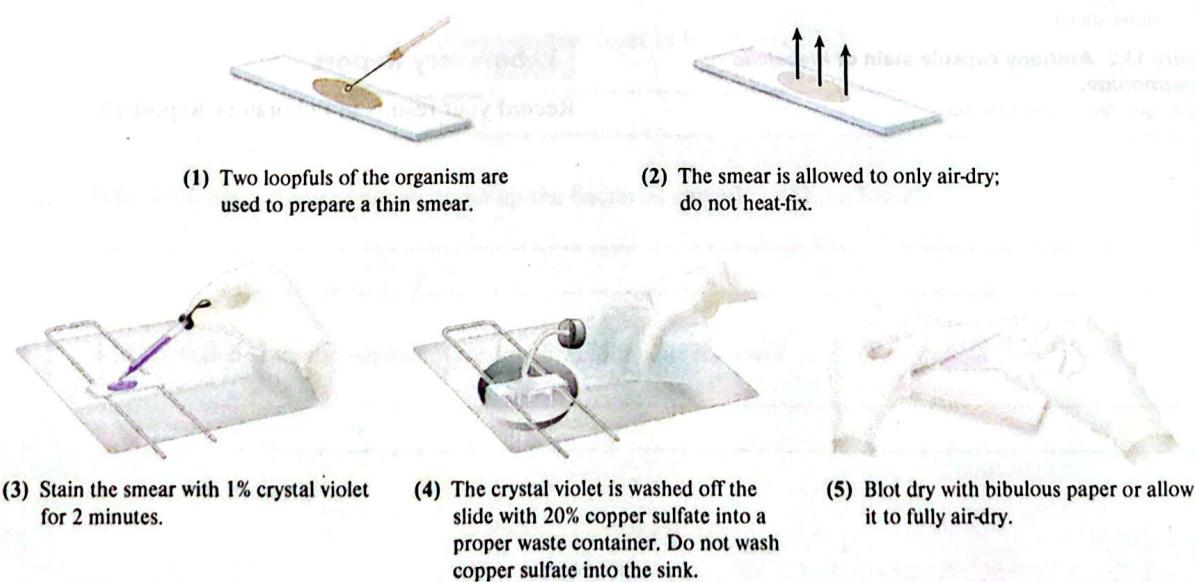
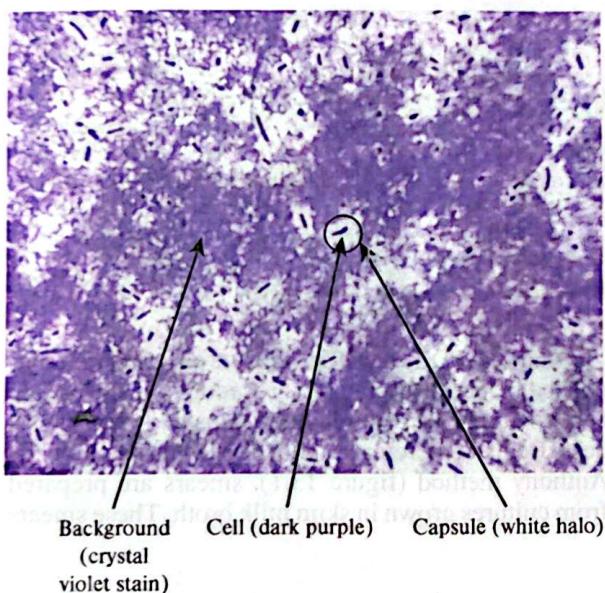


Figure 13.1 Steps of the Anthony capsule staining method.

### EXERCISE 13 Capsular Staining

are air-dried but not heat-fixed. Then, they are stained with crystal violet for 2 minutes. The crystal violet not only stains the cells, but it also binds to the milk proteins from the culture medium, causing the cells and the background of the slide to stain purple. The stain is then washed off with an aqueous solution of 20% copper sulfate. This reagent functions as a decolorizer, removing the crystal violet from the capsules. It also serves as a counterstain, staining the capsules a very light blue. Under oil immersion, the capsules will appear as clear or light blue halos around the cells, and the cells will be dark purple (figure 13.2). You will use this procedure to stain the capsules of *Klebsiella pneumoniae*.



**Figure 13.2** Anthony capsule stain of *Klebsiella pneumoniae*.

Lisa Burgess/McGraw-Hill Education

### Materials

- 36–48 hour skim milk culture of *Klebsiella pneumoniae*
- 1% (wt/vol) crystal violet
- 20% (wt/vol) aqueous copper sulfate
- waste containers for the copper sulfate
- container with disinfectant solution for used slides

### Procedure

1. Prepare a thin smear of *Klebsiella pneumoniae* on a microscope slide.
2. Allow the smear to air-dry only. **Do not heat-fix as this will cause the capsule to shrink or be destroyed.**
3. Apply 1% crystal violet and allow it to remain on the slide for 2 minutes.
4. With the slide over the proper waste container provided, gently wash off the crystal violet with 20% copper sulfate. **Caution: Do not wash the copper sulfate and stain directly into the sink.**
5. Gently blot the slide dry with bibulous paper or allow it to fully air-dry.
6. Observe with the oil immersion lens and compare your stain with figure 13.2.

### Caution

Be sure to dispose of your used slide in a disinfectant container when you are finished. Do not hand wash these slides.

### Laboratory Report

Record your results in Laboratory Report 13.