Anaerobic Bacteriology

Susan M. Harrington, Ph.D.

2022

Anaerobic bacteria

- Dominate indigenous flora
- Commonly found in infection
- Special procedures required for collection and transport
- Most commonly overlooked bacterial infection
- Endogenous or exogenous sources

Major anaerobes encountered clinically

(see flow chart in text)

- Spore-forming GPR
  - *Clostridium* spp.
- Non-spore-forming GPR
  - *Actinomyces* spp.
  - *Cutibacterium (Proprionibacterium)*spp.
  - *Mobiluncus* spp.
  - *Lactobacillus* spp.
  - *Eubacterium* spp.
  - *Bifidobacterium* spp.
- Gram-positive cocci
  - *Peptostreptococcus* spp.
- Gram-negative bacilli
  - *Bacteroides fragilis* group
  - Other *Bacteroides* spp.
  - *Porphyromonas* spp.
  - *Prevotella* spp.
  - *Fusobacterium* spp.
  - *Bilophila* spp.
  - *Sutterella* spp.
- Gram-negative cocci
  - *Veillonella* spp.

Habitat of anaerobes

- Widespread: soil, marshes, lake and river sediments, ocean, sewage, foods, animals
- Humans
  - oral cavity around teeth
  - URT
  - GI tract
  - Orifices of GU tract
  - skin

Incidence of anaerobes as indigenous flora in humans

Gram Negative Anaerobes	Skin	URT	Mouth	Intestine	GU tract
B. fragilis group	**0**	**0**	**0**	**2**	**+/-**
Bilophila	**0**	**0**	**+/-**	**1**	**+/-**
Fusobacterium	**0**	**1**	**2**	**1**	**1**
Porphyromonas	**0**	**+/-**	**1**	**1**	**+/-**
Prevotella	**0**	**1**	**2**	**1**	**1**
Sutterella	**0**	**0**	**0**	**1**	**0**
Other GNR, GNC	**0**	**1**	**1**	**1**	**1**

Incidence of anaerobes as indigenous flora in humans

Gram Positive Anaerobes	Skin	URT	Mouth	Intestine	GU tract
Clostridium	**0**	**0**	**+/-**	**2**	**+/-**
Actinomyces	**0**	**1**	**2**	**+/-**	**+/-**
Bifidobacterium	**0**	**0**	**1**	**2**	**+/-**
Eubacterium	**+/-**	**+/-**	**1**	**2**	**+/-**
Lactobacillus	**0**	**0**	**1**	**1-2**	**1-2**
Cutibacterium	**2**	**1**	**+/-**	**+/-**	**+/-**
Peptostreptococcus	**1**	**2**	**2**	**2**	**2**

Events that lead to infection

Endogenous:

- Dental procedures
- GI surgery/traumatic abdominal injury
- Genital tract surgery, IUD use
- Antibiotic use

Exogenous:

- Human or animal bites
- Traumatic surface wounds
- Improperly prepared/stored food

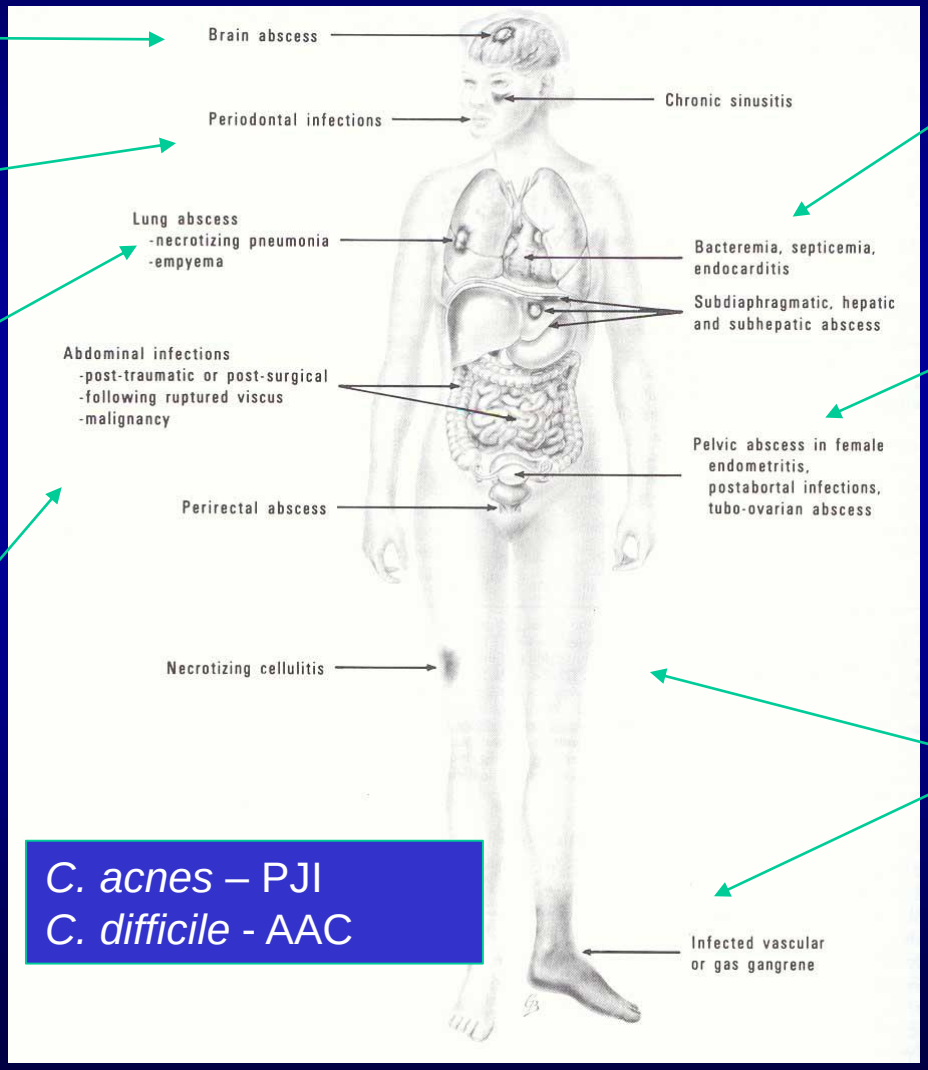
Anaerobic Infections
(often polymicrobial)

ANA GNB & *Clostridium spp*.

Peptrostreptococci, Fusobacterium spp., *Porphyromonas* spp.

Porphorymonas, Fusobacterium, Actinomyces, Finegoldia magna , Peptostreptococci, B. fragilis group

B. fragilis group, Fusobacterium spp., *Clostridium* spp., *Peptostreptococci*



C. acnes – PJI
C. difficile - AAC

Clostridium spp, B. fragilis group, Peptrostreptococci, Fusobacterium spp.

Peptostreptococci, Bacteroides spp., *Clostridium* spp, *Prevotella* spp., *Actinomyces israelii (IUD)*

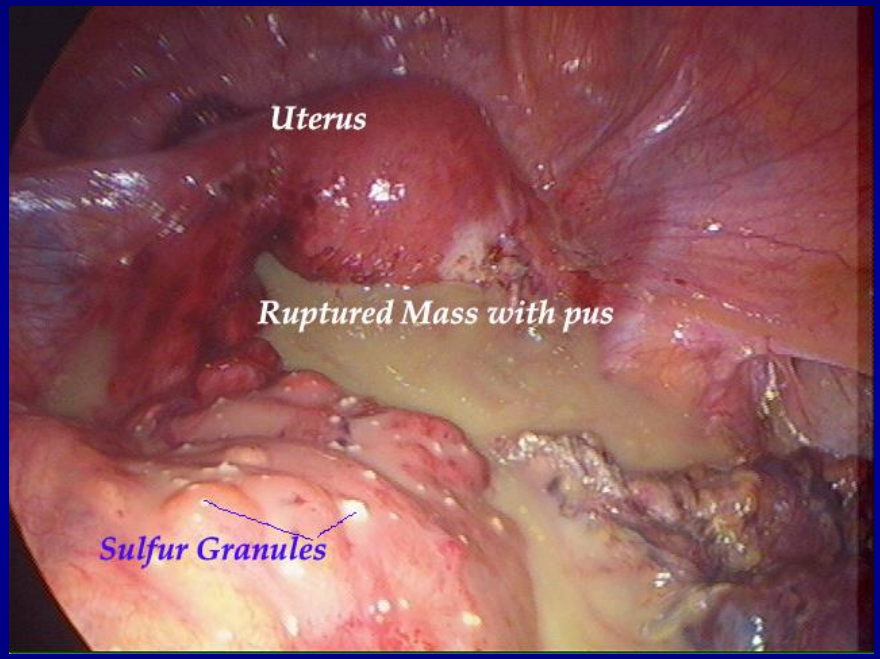
Clostridium spp.

Koneman 1997; Fig 14-1

& Mahon Text of Diagnostic Micro 6th Ed

Specimen – Indications for Anaerobic Infection

- Infection close to mucosal surface (pyogenic)
- Foul odor
- Large quantity of gas
- Sulfur granules
- Black discoloration of tissue
  - Pigments of *Porphorymonas* spp. & *Prevotella* spp. may cause blackening



https://microbe-canvas.com/diseases.php?p=2398

Appropriate for Anaerobes (Deep Sites)

- Aspirates (by needle)
- Blood and bone marrow
- Bronchoscopy by protected specimen brush
- Transtracheal aspirate
- Nasal sinus aspirate
- Deep tissue (organs, placenta, surgically obtained)
- IUD for Actinomyces
- Suprapubic urine

Inappropriate for Anaerobes

- Cervical, vaginal & urethral secretions
- Prostatic or seminal fluid
- BAL & bronchial washes
- NP swabs & sinus washings
- Endotracheal aspirates
- Sputum
- Surface wounds or tissues
- Skin scrapings
- Stool or rectal swabs
- Urine (voided or catheter)

Relationship of bacteria to oxygen

- Obligate aerobes
  - *Micrococcus, Pseudomonas* spp.
  - Require O_2 as terminal electron acceptor; no fermentation
- Facultative anaerobes
  - *E. coli, S. aureus*
  - Grow under aerobic or anaerobic conditions
- Microaerophiles
  - *Campylobacter* – optimal growth in 5% O_2; 10% CO_2
  - Require O_2 as terminal electron acceptor, yet do not grow in aerobic incubator (21% O_2); grow minimally under anaerobic conditions

Anaerobes

- Strict obligate - cannot tolerate >0.5% O_2
  - *Clostridium haemolyticum, C. novyi type B*
- Moderate obligate - can tolerate 2-8% O_2
  - *Bacteroides fragilis, Prevotella-Porphyromonas groups, Fusobacterium nucleatum, C. perfringens*
- Aerotolerant anaerobe – show scant growth on agar in room air or 5-10% CO_2; good growth under anaerobic conditions
  - *Clostridrium carnis, C. histolyticum, C. tertium*
  - *C. acnes, Lactobacillus*

Culture for Anaerobes

- To preserve strict anaerobes culture within 20 min.
- Swabs are poor specimens.
- PRAS transport media for swabs, fluids & tissues is preferred. E swab.
- If specimen is small put the whole thing in transport & process for aerobes & anaerobes.
- Refrigerated/frozen unacceptable

Primary Anaerobic Media

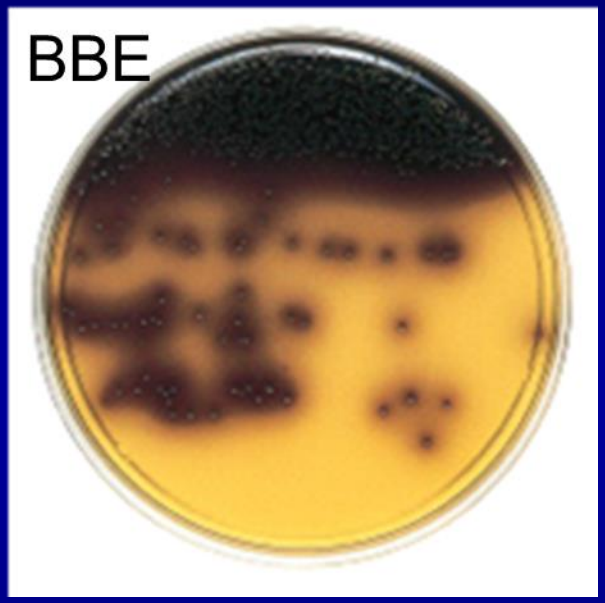
- Media should be RT; pre-reduced.

- Anaerobic Blood Agar (CDC) or Brucella Blood Agar
  - Supports growth of almost all obligate and facultative anaerobes
  - Vit K, hemin, yeast extract

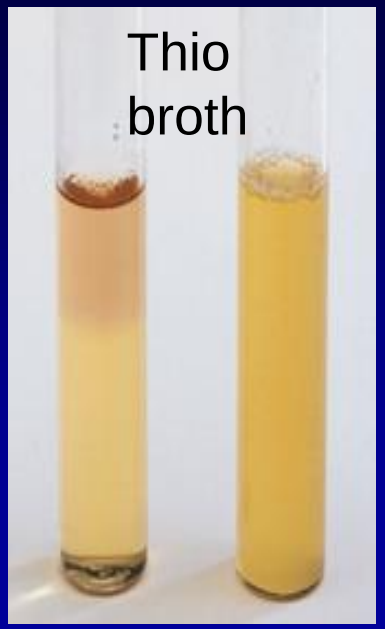
- Kanamycin-Vancomycin Laked Blood Agar (KVLB)
  - Selective isolation of *Bacteroides* and *Prevotella*
  - Pigmentation of Prevotella

Primary Anaerobic Media

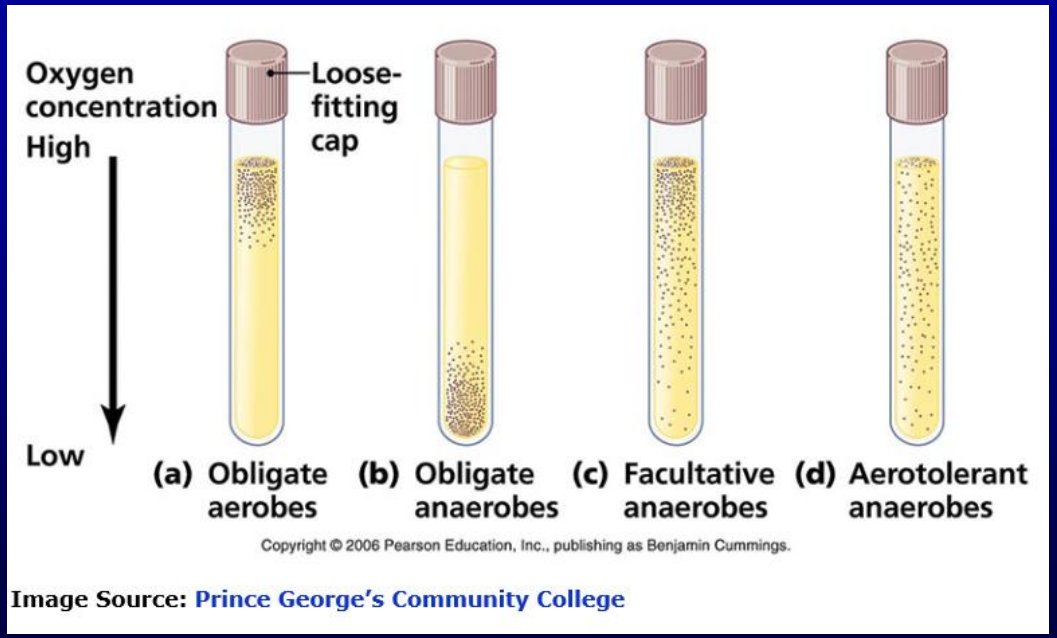
- Bacteroides Bile Esculin Agar (BBE)
  - Selective, differential for *B. fragilis* group
    - Bilophila wadsworthia grows
  - 20% bile salts & gentamicin
  - Bile resistant organisms grow
  - Hydrolysis of esculin – black/brown colony formation/halo
    - *B. vulgatus* is esculin neg



Anaerobic Media



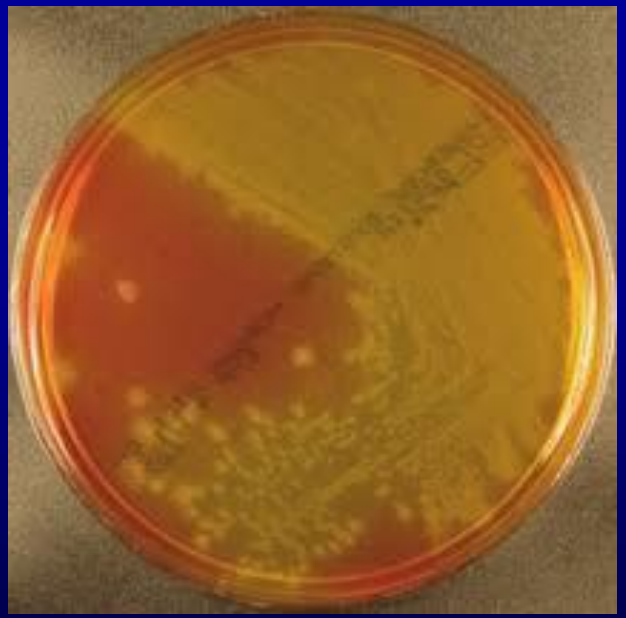
- Thioglycolate Broth
  - Provides enrichment
  - Non-selective
  - Thioglycolate & cystine – reducing agents
  - Low conc of agar
  - Resazurin indicator (pink if oxidized)



Anaerobic Media

- Phenylethyl Alcohol Blood Agar (PEA)
  - Usually w/sheep blood
  - Inhibits facultative GNBs
  - Inhibits swarming
- Cycloserine cefoxitin fructose agar (CCFA)
  - 500 µg/ml cycloserine; 16 µg/ml cefoxitin
  - *C. difficile;* yellow colony
  - Horse manure odor
  - UV light fluorescence

CCFA

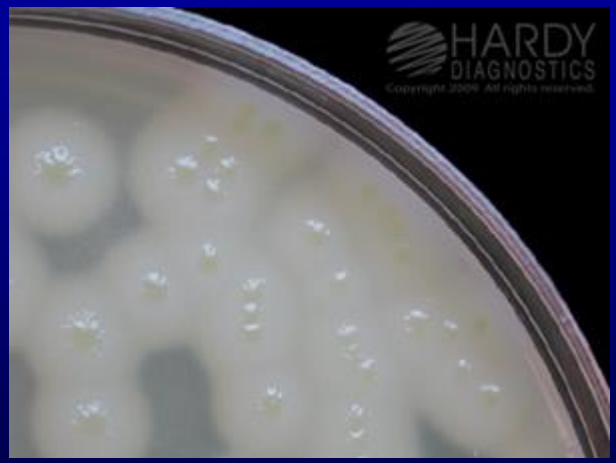


AnaerobeSystems.com

Differential Media

- Egg Yolk Agar
  - Egg emulsion
  - Identification
    - lecithinase (opaque) (Nagler reaction)
    - lipase (sheen)
    - protease (clearing)

EYA - Lipase



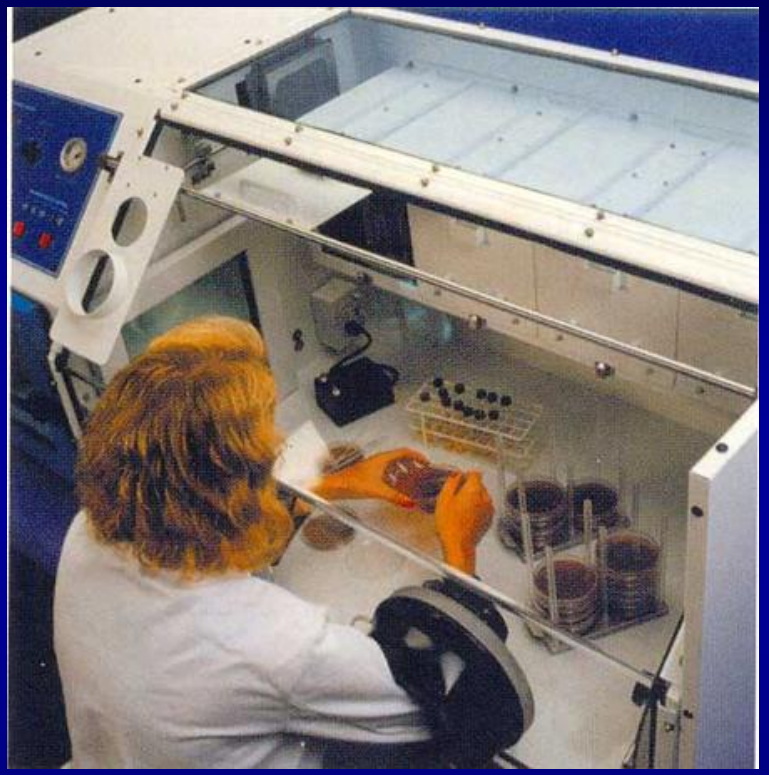
EYA - Lecithinase

Anaerobic incubation

- 5% H_2, 5-10% CO_2, 85-90% N_2
- Techniques
  - Systems that generate gas from envelope (pack):
    - jar
    - bag
    - box
  - Anoxomat
    - Gas generated by tank connected to jar
- Methylene blue indicator strip: white = ANA

Anaerobic Incubation

- Anaerobic chamber (glove box)



gloveless

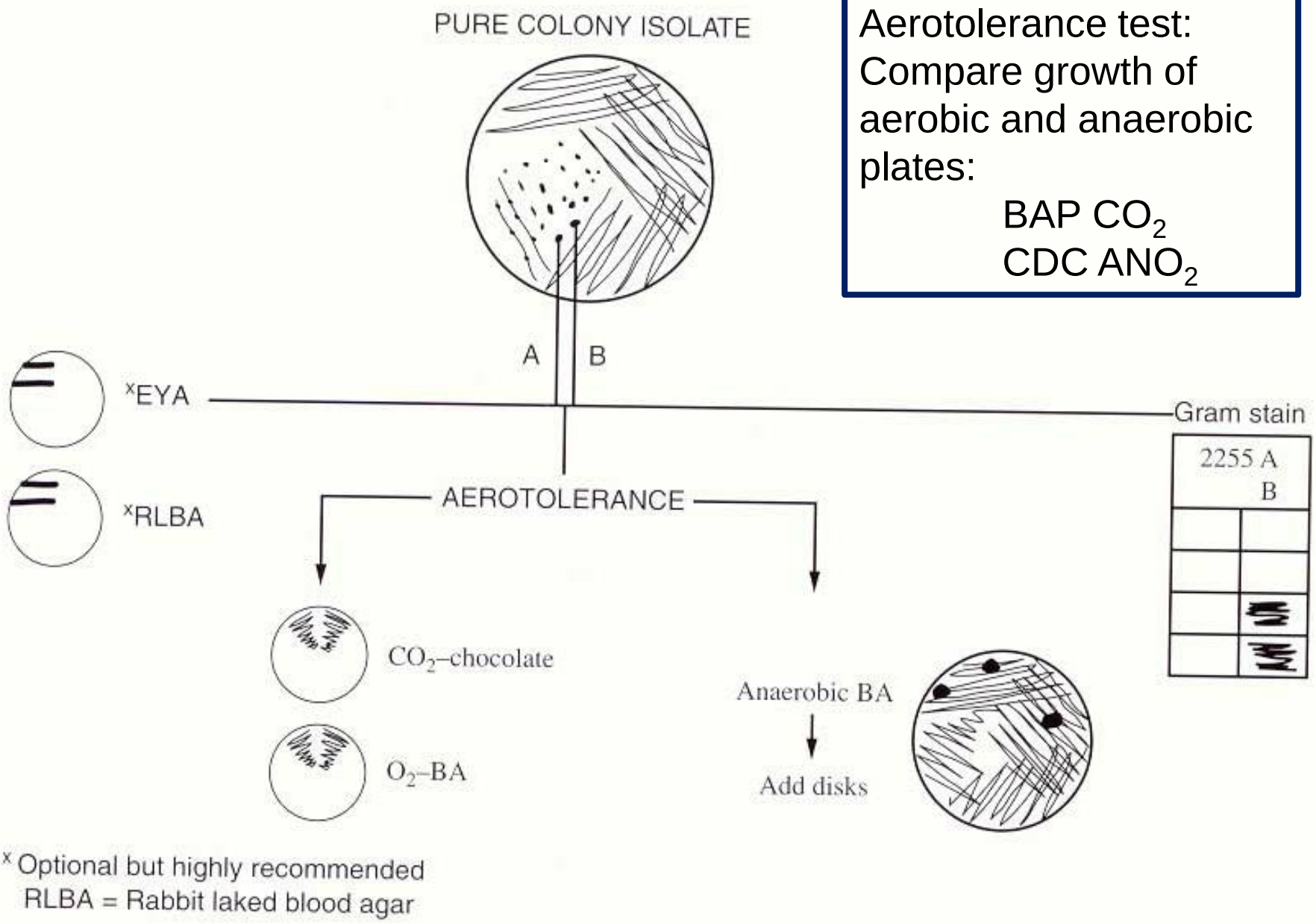
Incubation Times: Anaerobic Cultures

- Incubated for 5 days
- Media usually includes: CDC & BBE or LKV in ANO_2
  - BAP O_2; Thio for selected sites.
- Incubated for 48 hours before first examination.
- Extended incubation:
  - *C. acnes* (7 – 14 days)
  - Actinomyces (14 days)

Identification of Anaerobes

Preliminary Tests

- Gram stain
- Colony morphology/differential growth
- Pigment
- Fluorescence
- Catalase (15% H_2O_2)
- Spot indole
- Nitrate
- Aerotolerance



Aerotolerance test:
Compare growth of aerobic and anaerobic plates:

BAP CO_2
CDC ANO_2

Wadsworth manual; Fig 3-11

Identification of anaerobes

- Special potency antibiotic disks
- Commercial biochemical systems
  - PRAS biochemicals (std method, exp, labor intensive)
  - API 20A (24-48 h)
    - Saccharolytic, rapid growing (*B. frag, Clostridium* spp.)
  - RapID ANA II (2-4 h) - detect preformed enzymes

- Gas liquid chromatography (GLC)
  - Cell wall fatty acids
- MALDI-TOF MS
- 16S rRNA gene sequencing

Special potency disk patterns

S ≥10 mm R <10 mm	Vancomycin 5 µg	Kanamycin 1000 µg	Colistin 10 µg
B. fragilis group	R	R	R
Prevotella spp.	R	R	V
Porphyromonas spp.	S	R	R
Fusobacterium spp.	R	S	S
Veillonella spp.	R	S	S

Anaerobes of Medical Importance

Pathogenic *Clostridrium* and Assoc . Human Diseases

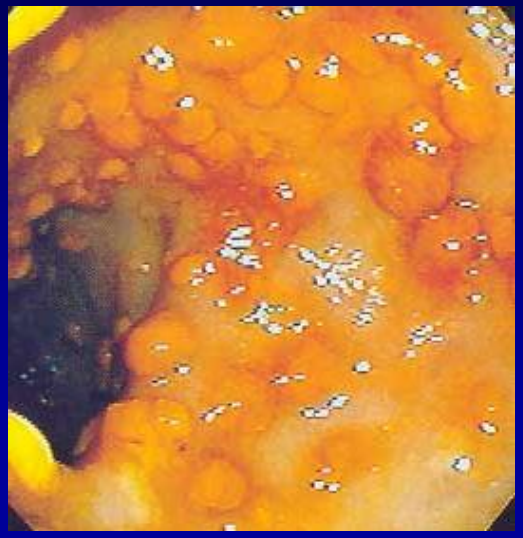
Species	Human Disease	Frequency
C. difficile	Abx assoc diarrhea, pseudom colitis	Common
C. perfringens	Soft tissue infections, food poisoning, enteritis necroticans, septicemia	Common
C. septicum	Gas gangrene, septicemia	Uncommon
C. tertium*	Opportunistic infections	Uncommon
C. botulinum	Botulism	Uncommon
C. tetani	Tetanus	Uncommon
C. barati, C. butyricum	Botulism	Rare
C. histolyticum*, C. novyi, C. sordellii	Gas gangrene	Rare

*aerotolerant

Clostridioides (*Clostridium*) *difficile*

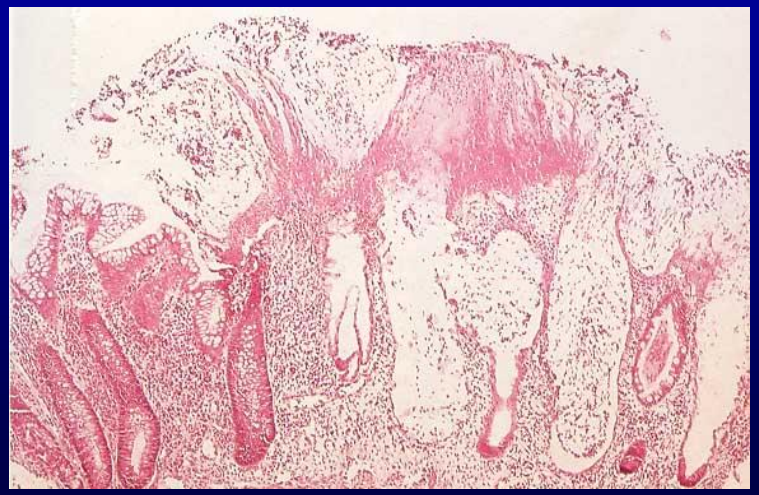
- Asymptomatic colonization
  (~50% healthy neonates 1st yr)

- Disease is toxin-mediated **All *C. diff* don't make toxin
  - Toxin A (enterotoxin)
  - Toxin B (cytotoxin)



Disease:

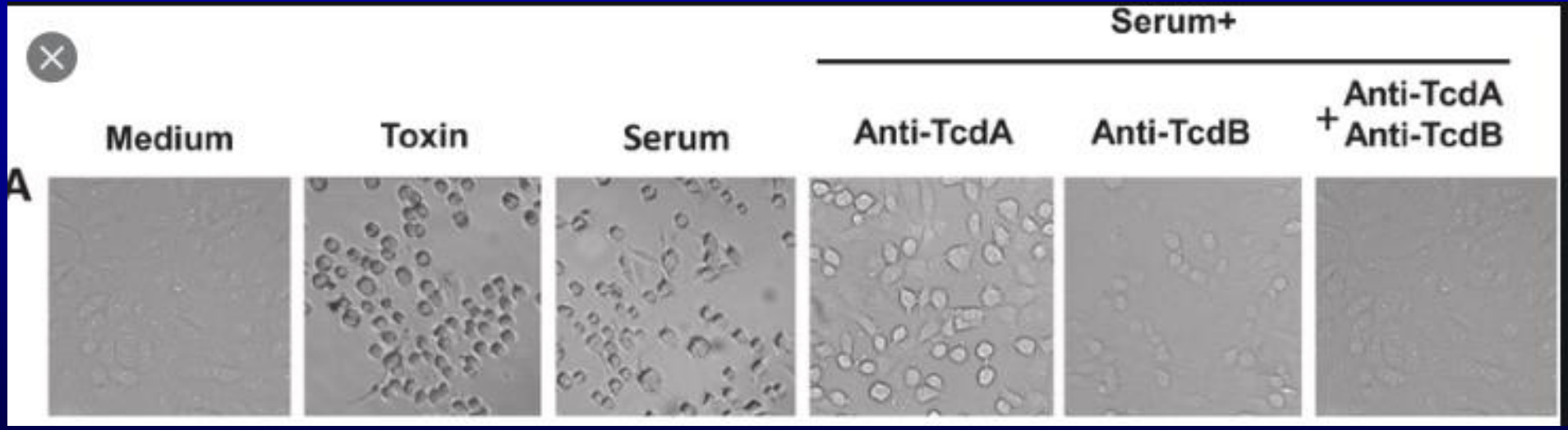
- Antibiotic associated colitis
  - diarrhea
- Pseudomembranous colitis
- Toxic dilatation, perforation of colon and death



Koneman 1997

C. difficile Toxin Detection

- Cell Culture Neutralization Assay
  - Add filtered stool + antitoxin to cells in culture
  - Toxin causes cytopathic rounding effect (CPE)
  - Antitoxin neutralizes CPE, showing specificity
- Expensive, 48 h TAT – not used routinely



Yu H. PLoS ONE 10(4):e0124235 (2015)

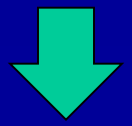
Clostridioides difficile Culture

- Toxigenic culture
  - Most sensitive (96%), long TAT
  - Yellow-white, ground glass colonies
  - Horse manure odor
  - Fluoresce chartreuse - UV light
  - Cytotoxin assay on 24 h broth cxs to show toxin is present

Koneman 1997; color plate14-4 F

Algorithms for *C. difficile* Detection

- Detection of *C. difficile* organism: GDH (glutamate dehydrogenase) Ag screen
  - If NEG, no further testing, saves $$ (>98% NPV)
  - If POS



  - Toxin antigen assay
  - Toxin assay, arbitrated by NAAT

- Antigen assays lower sensitivity if used w/o NAAT

Allgorithms for *C. difficile* Diagnosis

- Test for toxin by NAAT alone.
  - excellent sensitivity
  - poor specificity/PPV; over-diagnosis.
  - may detect colonization; non-viable bacilli

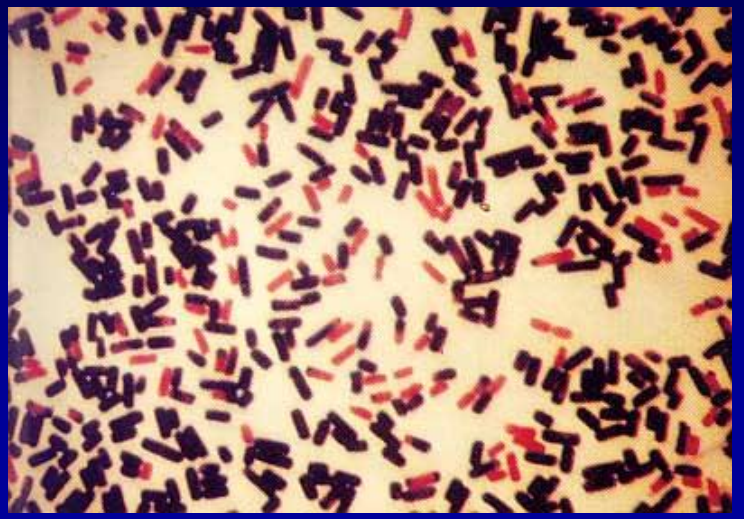
- NAAT, if positive do Toxin Ag assay
  - NAAT + Ag + : C diff present, making toxin
  - NAAT + Ag - : clinical correlation

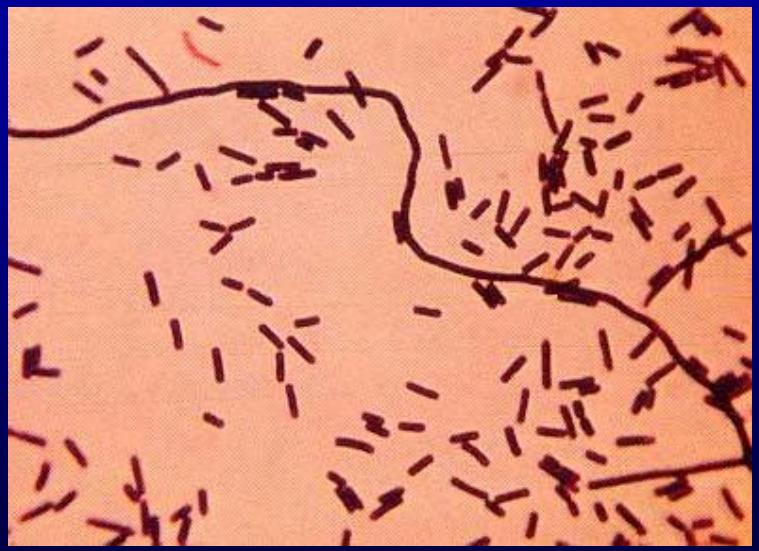
C. difficile Therapy

- Discontinue antibiotics, if possible
- Antibiotic therapy
  - Oral vancomycin
  - Fidaxomicin
- 10-20% relapse (persist spores, reinfect from environment)
- Fecal transplant
- Probiotics

Clostridium perfringens

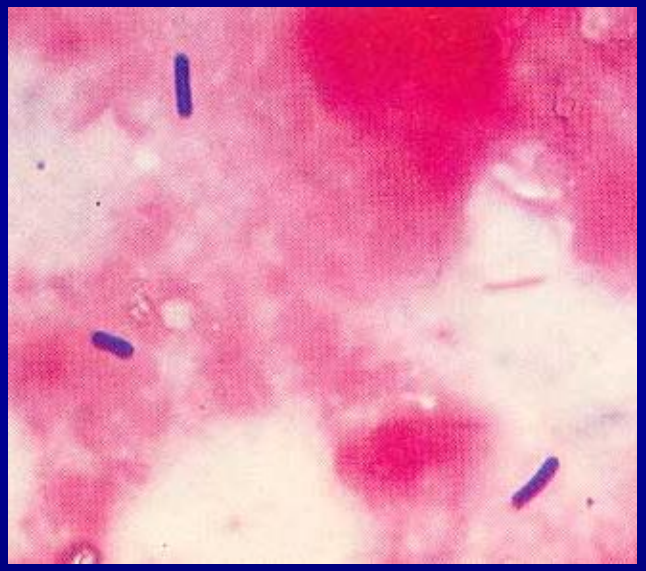
- Most common *Clostridium* spp. (20%) isolated in clinical specimens
- Common GI flora, transient skin flora
- Ubiquitous -intestinal tract animals, humans, type A can survive in soil
- Boxcar-shaped GPR
- Spores rarely seen





Clostridium perfringens

- >16 toxins and enzymes
  - Types A-E based on production of ≥1 lethal toxin (α,β,ε,ι)
  - Type A (alpha toxin) - most human infections
- Spectrum of disease
  - Soft tissue infections (cellulitis, suppurative myositis, myonecrosis or gas gangrene)
  - Food poisoning - heat-labile enterotoxin
  - Enteritis necroticans (ß toxin)
  - Septicemia



C. perfringens Food Poisoning

- Usually watery diarrhea with severe crampy abdominal pain.
- Uncommon: fever, chills, vomiting, headache.
- Incubation - 8-30 hours

- Associated with beef or chicken that has been stewed, roasted or boiled and allowed to stand and cool slowly.
- Type A enterotoxin – most common
- Self-limiting. Not treated.
- Stool not cultured in lab.

Gas Gangrene (Myonecrosis)

- *Clostridium spp.*
  - *C. perfringens*, *C. histolyticum*, *C. septicum*, also *C. novyii, C. sordellii,* others


Connor, Path Infect Dis, p. 524

- Organisms contaminate wounds through trauma or surgery
  - Painful, foul-smelling, gas-filled, serosanguinous discharge.
  - Rapid necrosis
  - Shock, organ failure, 50% CFR if bacteremic

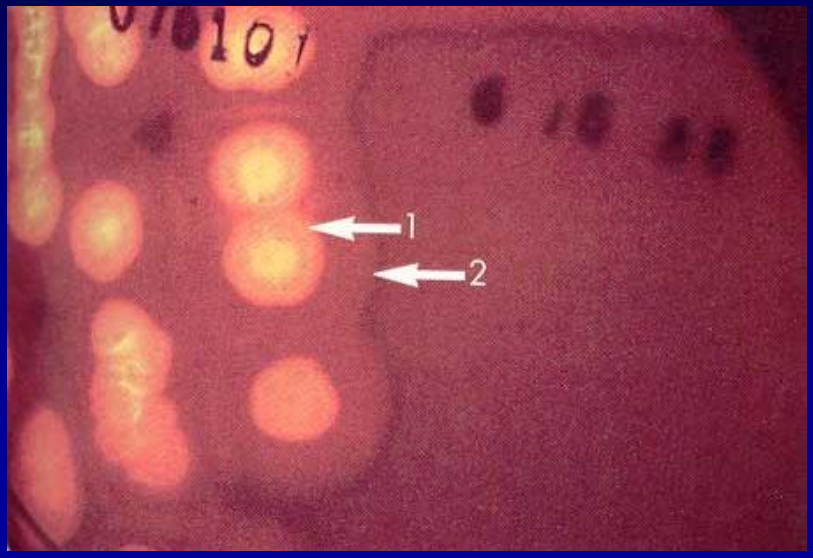
- Non-traumatic abdominal myonecrosis often due to *C. septicum*
  - Carcinoma, diverticulitis, surgery, chemo and other risk factors

Alpha toxin of *Clostridium perfringens*

- Lecithinase activity
- Produced by all *C. perfringens*
- Lyses RBCs, plt, WBCs, endothelial cells
- Platelets & PMNs occlude blood vessels
- Increases vascular permeability

Clostridium perfringens

- Rapid growth - detected after ≤1 day; divide every 8-10 min
- Nonmotile
- Double-zone ß-hemolysis
  - Inner zone (complete): theta toxin
  - Outer zone (incomplete): alpha toxin (lecithinase)

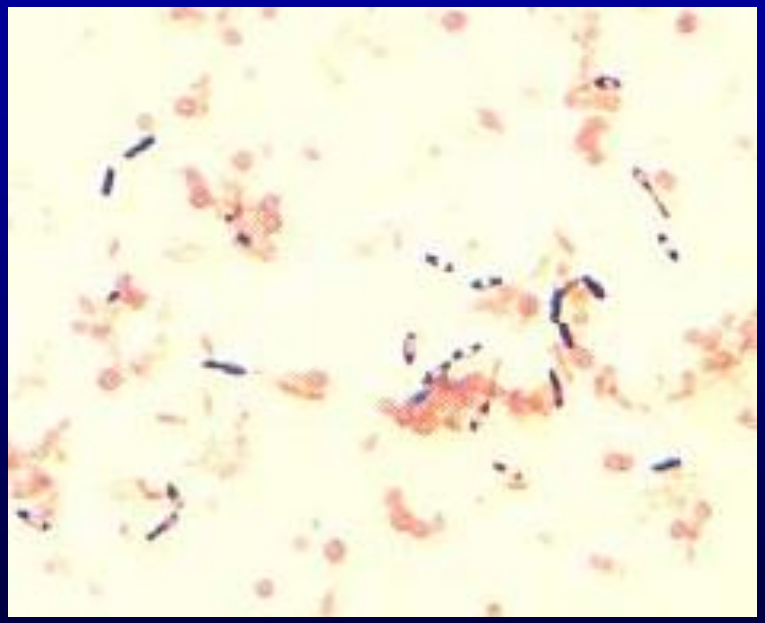


Clostridium septicum

- Gas gangrene, septicemia
- 70-85% w/ pos blood cx have underlying malignancy
- ≥ 68% mortality

- Medusa-head colony morph on BBA 4-8 h; swarms by 24-48 h

- Ovoid (citron) subterm spores

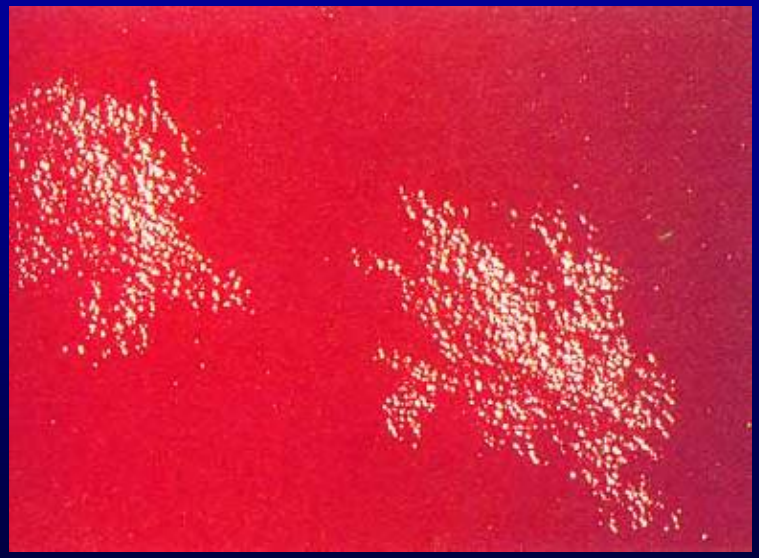


Clostridium tetani

- Tetanus
  - Puncture wounds
  - Tetanospasmin blocks release of neurotransmitters - inhibitory impulses are blocked in spinal cord & brain stem
  - Prolonged muscle spasms
  - Spasm of facial muscles
  - Spinal rigidity & spasm
  - Dx - clinical presentation
  - Rx - debride wound, MTZ, human tetanus IgG, vaccination

Clostridium tetani

- Microscopy - poor sensitivity
- Prominent terminal spores (drumstick)
- Strict anaerobe - cx of wnd 30% sensitive
- Colony (slowly) spreading
- Tetanus toxin can be detected in serum



Clostridium botulinum

- Botulism: life-threatening neuroparalytic disease
  - also *C. baratii, C. butyricum*

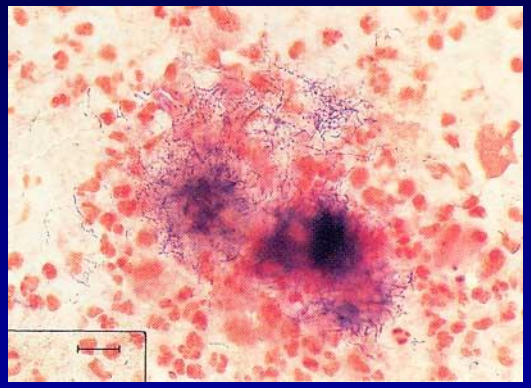
  - 7 neurotoxins; A, B & E most common.
  - Prevents release of acetylcholine at neuromuscular junction
  - Blurred vision, impaired speech, flaccid paralysis
  - Foodborne, infant, wound
  - Home-canning; honey (infant)
  - Public health reporting; bioterrorism concern.
  - Dx: isolate org or detect toxin in food or stool, serum
  - Rx: vent, antitoxin, elim org (gastric lav, MTZ or Pen)

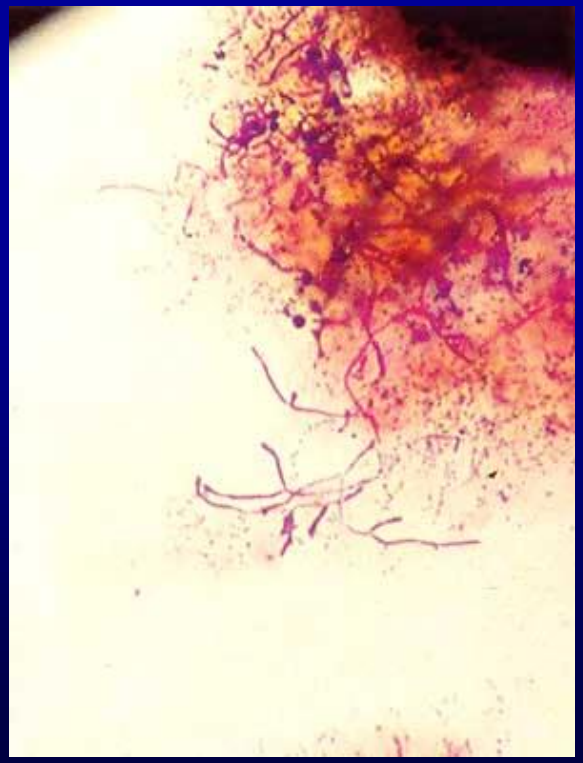
Actinomyces spp.

- Gram pos branching rods; variable
- Colonies vary: white, grayish, red, pink, tan, yellow
- All facultative anaerobic except *A. meyeri*
- Actinomycosis, PID assoc w/ IUD, and pyogenic liver abscesses

Actinomycosis

- Chronic granulomatous lesion forms abscess and draining sinus tracts
- Macroscopic sulfur granules
- Beaded, branching - usually in clinical specimens



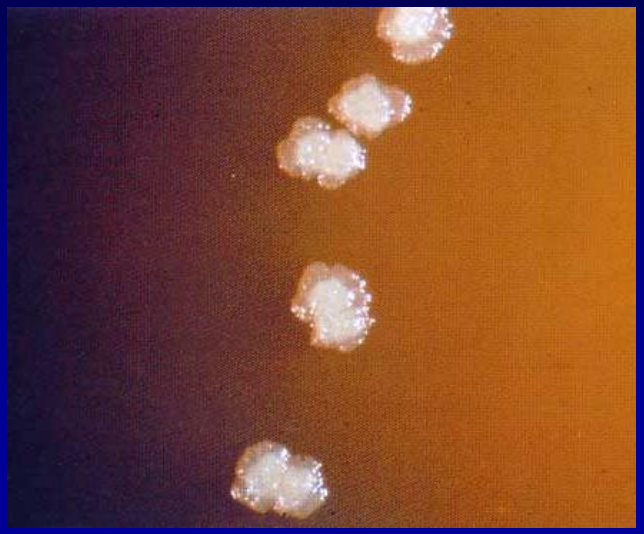


Connor, Path Infect Dis, p. 392

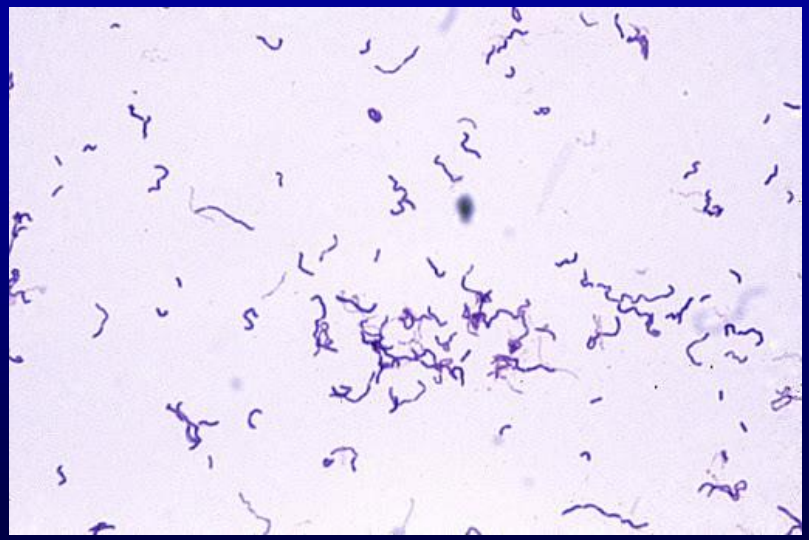
Actinomyces spp.

- *A. israelii* most common cause of actinomycoses in humans
- Smooth or molar-tooth colony
- Can be difficult to isolate - especially if specimen rich in microflora
- Also difficult to ID

Cutibacterium (*Propionibacterium*) spp.



- Small GPR in short chains or clumps; diphtheroidal
- Catalase positive & indole pos = *C. acnes*
- *C. acnes*
  - Acne
  - Opportunistic infections assoc w/ prosthetic devices, IV lines

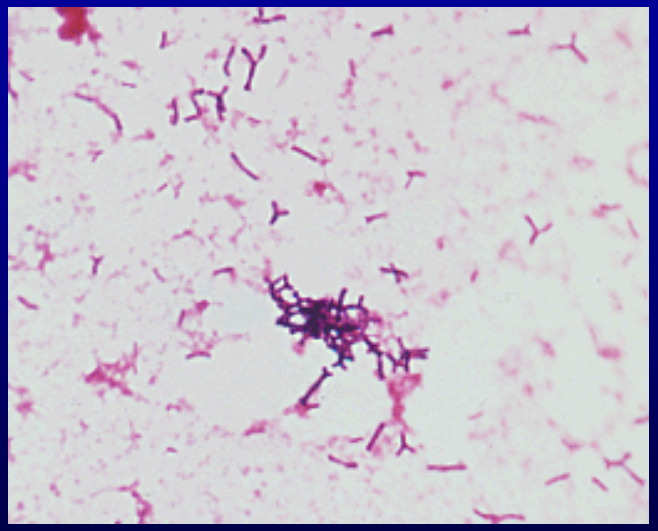


Bifidobacterium and *Eubacterium* spp.

- Oropharynx, large intestine, vagina
- Low virulence
- Usually contaminants

- Bifidobacterium: pleomorphic GPR with bifurcated ends;
  - cocco-bacilli to long, branching rods
  - Colony is white to cream, convex, smooth, glistening

Eubacterium Gram Stain from thio broth;

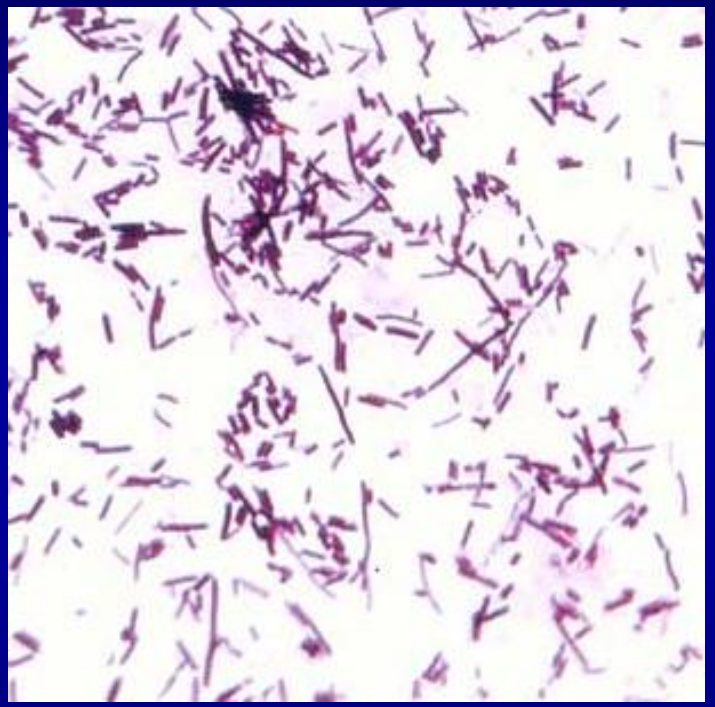


Bifidobacterium (www.uaz.edu)

Lactobacillus spp.

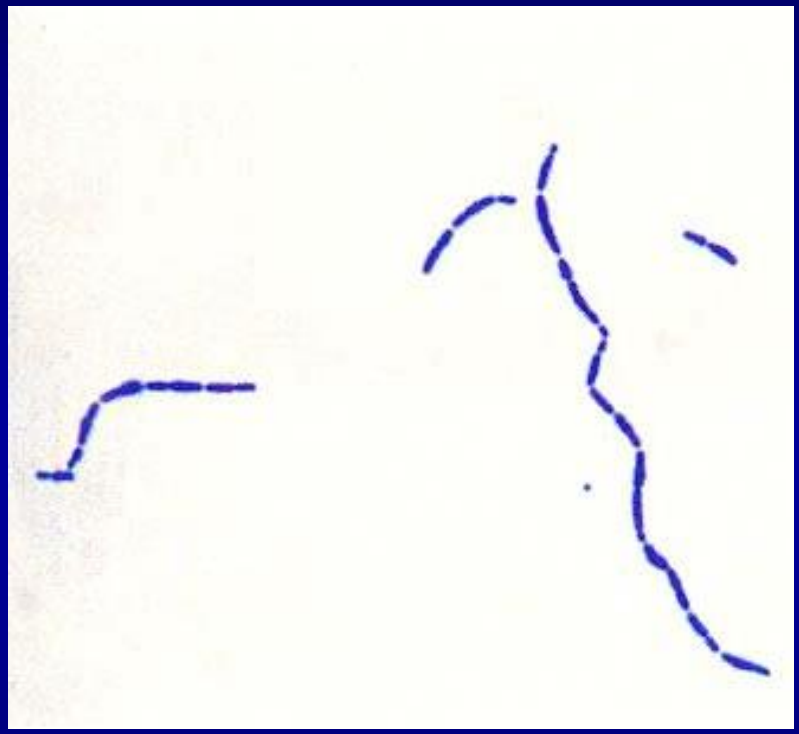
- Most facultative anaerobes; 20% obligate
- Some grow well aerobically

- Flora mouth, GI, GU tracts, foods
- Usually not clinically significant
- Rare cases of bacteremia, endocarditis



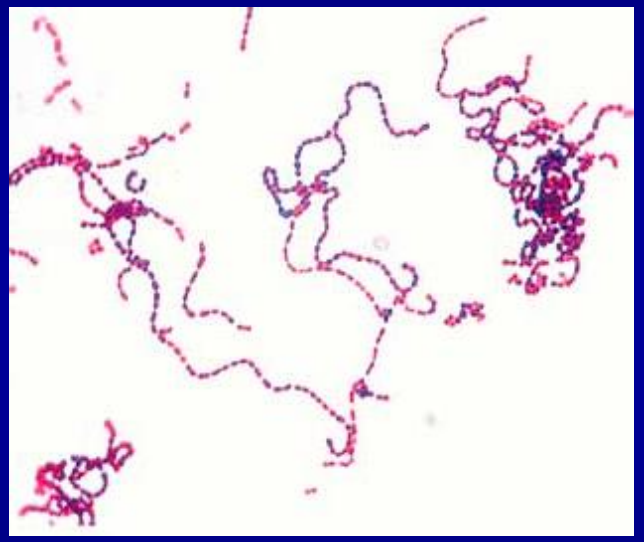
Lactobacillus spp.

- Long and slender rods
- Often in chains
- Most nonmotile
- Catalase negative
- Colony alpha-hemolytic to gray, pinpoint to medium-sized.
- Many species are vancomycin resistant



Gram-pos cocci: *Peptostreptococcus et al*

- Colonize oral cavity, GI tract, GU tract, skin
- Infection when spread to sterile sites
- Recently named genera
  - *Finegoldia magna*
    - Most pathogenic
  - *Parvimonas micra*
  - *Anaerococcus* spp.
  - *Peptoniphilus* spp.



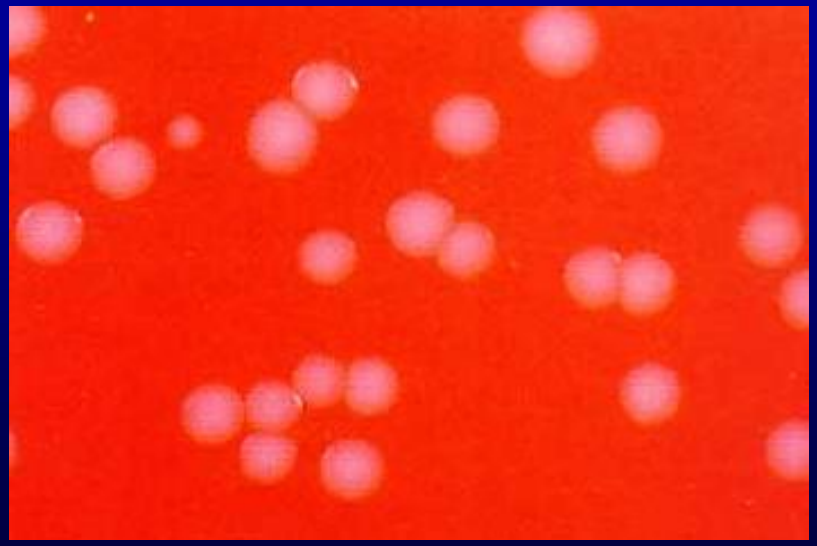
- See fig 22.28 in text for simple ID scheme
- Vanc S, GLC, sequencing, MALDI-TOF MS for accurate ID

Common Gram-negative Aanaerobic Rods

- *Bacteroides fragilis* group (24 spp)
  - Most common
  - Bile resistant
  - More virulent and resistant than most other anaerobes
  - *Bacteroides* once included >50 spp
- *Parabacteroides,* other *Bacteroides* spp.
- Bile-sensitive species:
  - *Porphyromonas*
    - Pigmented, asaccharolytic; most indole pos
    - may fluoresce brick red
  - *Prevotella*
    - saccharolytic, bile sensitive
    - pigmented and nonpigmented
    - May fluoresce brick red

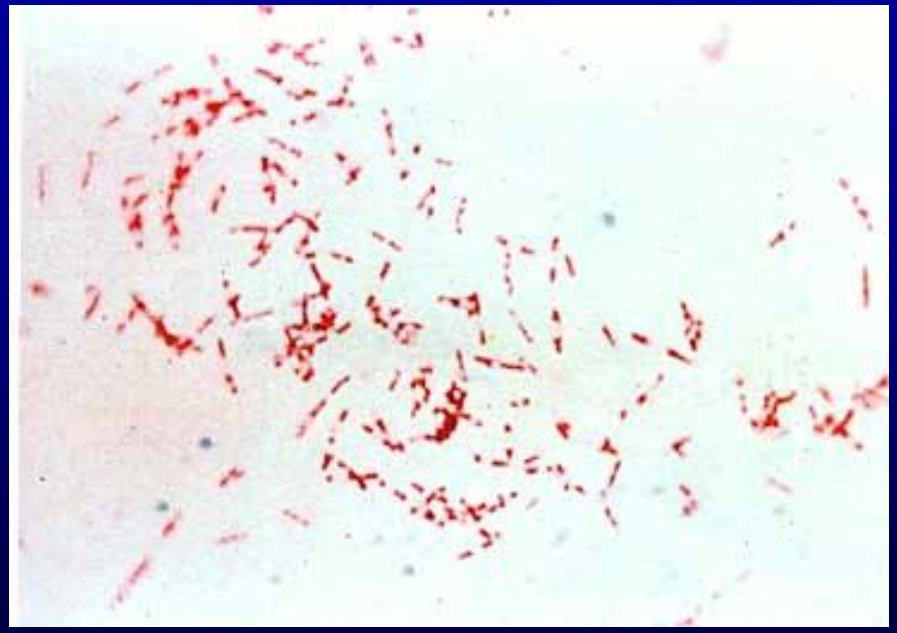
Bacteroides fragilis group

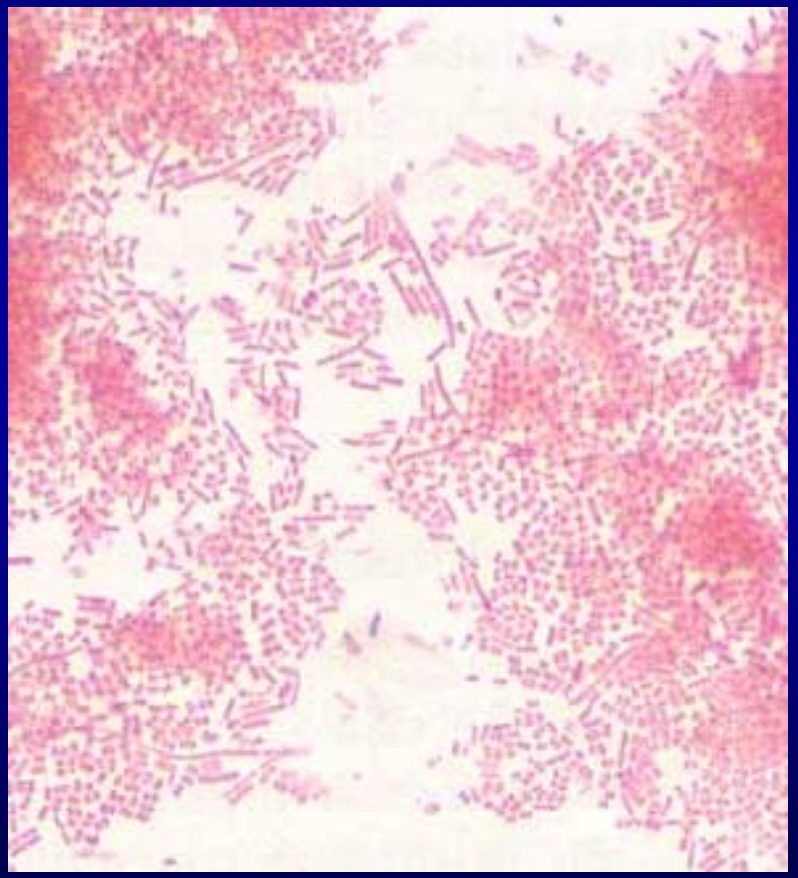
- Grows well on BBE
- R to Van, Kan, Coli
- Circular, convex, grey-white colonies on BA
- Hydrolyze esculin (except *B. vulgatus*)



Bacteroides fragilis group

- Pleomorphic, faintly staining gram-neg bacilli (may be vacuolized)





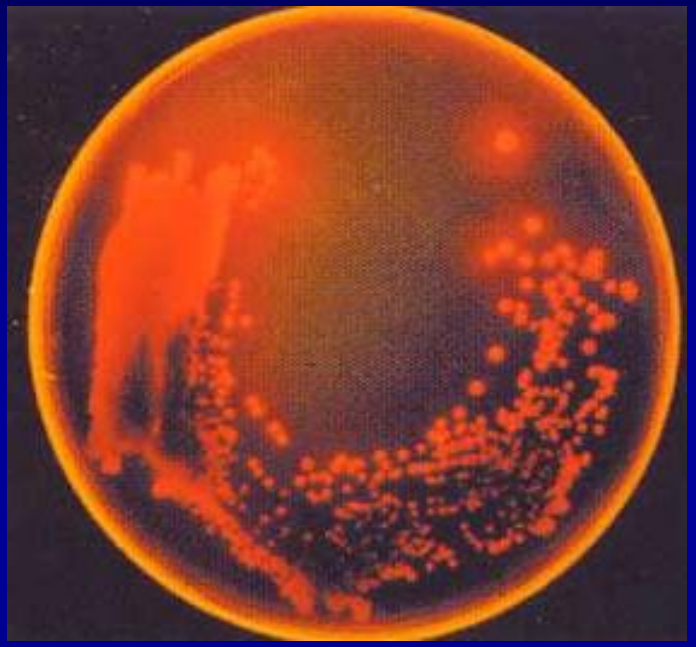
Campylobacter (Bacteroides) urealyticus

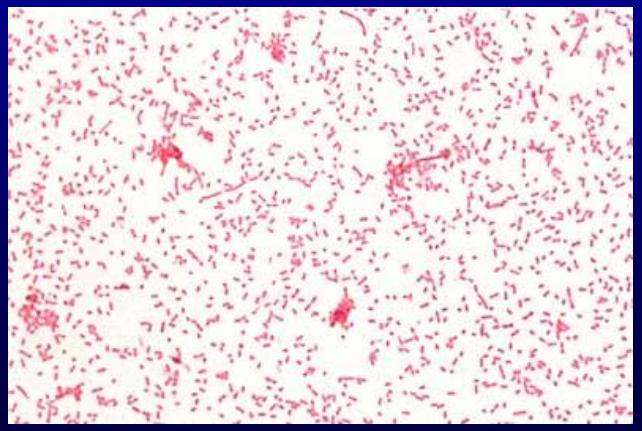
- Pitting colony on BA
- Catalase neg
- Urease pos
- Bile sensitive; BBE neg

- Vanc R
- Kanamycin S
- Colistin S

Pigmented *Prevotella* spp.- *Porphyromonas* spp. group

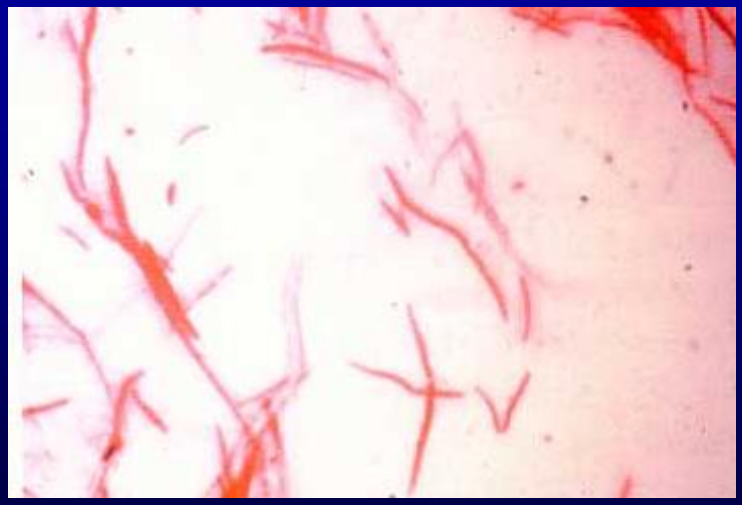
- GN coccobacilli
- (Brick-red) fluorescence under long-wave UV light
- Black pigmented colonies
- *Porphyromonas* spp.: vanc S no growth on LKV; spot indole +





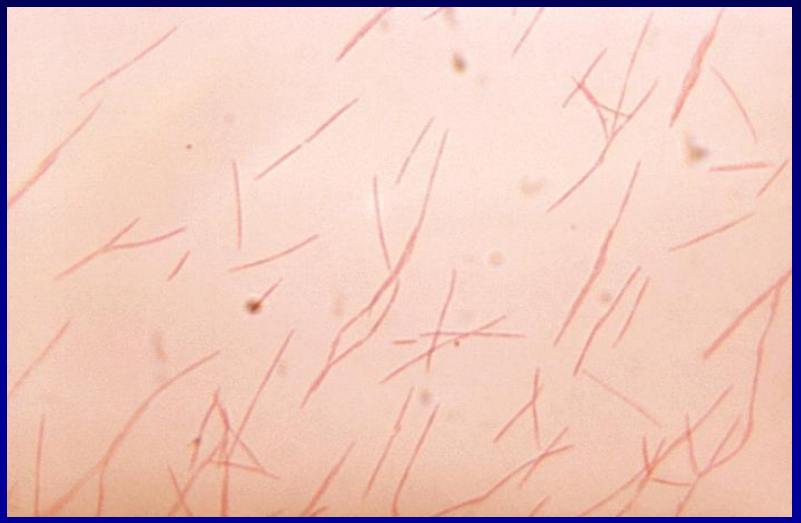
Fusobacterium spp.

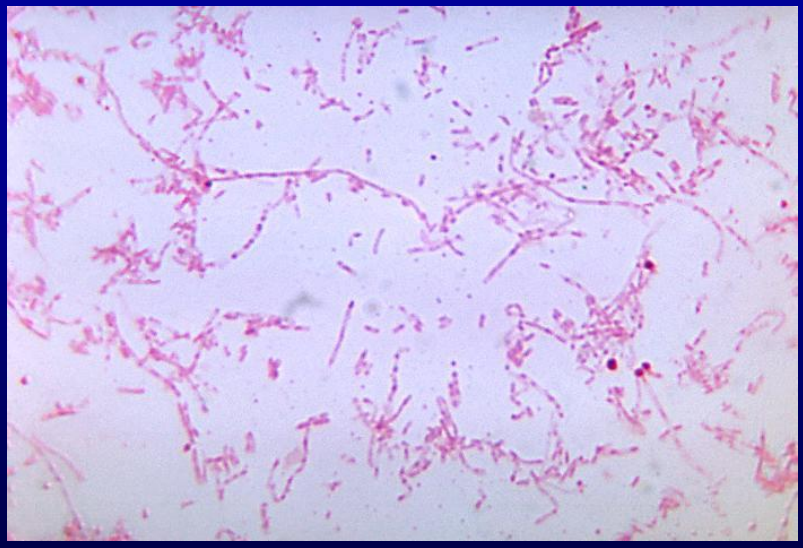
- Disk
  - Vanc R
  - Kanamycin S
  - Colistin S
- *F. nucleatum & F. necrophorum* indole pos, nitrate neg
- *F. nucleatum*: thin GNR with pointed ends
- *F. necrophorum & F. mortiferum*: pleomorphic and swollen forms



Fusobacterium spp.

- *F. nucleatum*
  - Common cause of abscess & BSI
- *F. necrophorum*
  - Lemierre's Disease – post-Strep infection. Septic thrombophlebitis, fever, chills, pain, swelling of throat
  - lipase pos on EYA





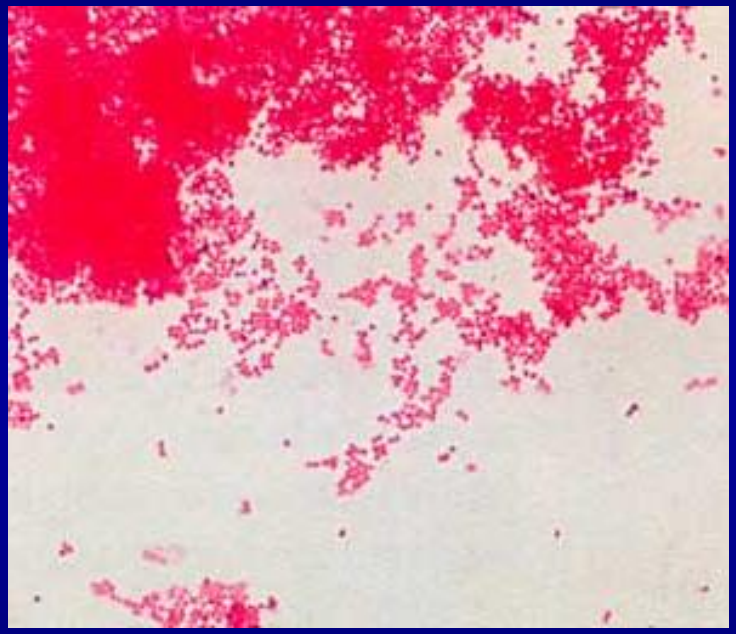
Koneman Image Atlas

Fusobacterium spp.

	Indole	Lipase	ONPG	BBE	Gram
F. necrophorum	+	+	-	-	Pleomorph
F. nucleatum	+	-	-	-	Slender, Pointed ends
F. varium	+	-	-	+	Regular shaped
F. mortiferum	-	-	+	+	Pleomorph swollen

Veillonella spp.

- Small (<1.5 µm) GN diplococci
- Predominant anaerobe in human pharynx
- <1% of all anaerobes isolated
- Fluoresce red under UV, slow
- Disk
  - Vanc R
  - Kanamycin S
  - Colistin S



Indications for susceptibility testing of anaerobes

- Serious infections: Brain abscess, endocarditis, osteomyelitis, joint infection, prosthetic device, bacteremia
- Normally sterile site
- Organisms considered highly virulent or unpredictable susceptibility profile
  - e.g., *B. fragilis* gp, *Prevotella*, *Fusobacterium*, *Clostridium*, *Bilophila, Sutterella* spp.
- Treatment failure (any anaerobe)

M100, Appx D: Anaerobic Antibiogram: 2013-16

Bacteroides fragilis group (7 spp.), n=2580.
% Susceptible, depends on species

- Ampicillin-sulbactam, 45-84%
- Piperacillin-tazobactam, 87-96%
- Cefoxitin, 13-100%
- Ertapenem, 82-84%
- Imipenem, 97-100%
- Meropenem, 93-100%
- Metronidazole, 100%
- Clindamycin, 26-53%
- Moxifloxacin 31-62%

CLSI M100, Appx D: Anaerobic Antibiogram: 2013-16

- Other anaerobes; %Susceptible

[*Prevotella,* ANA GPC*, Fusobacterium, Cutibacterium, Clostridium* spp., not C. diff]

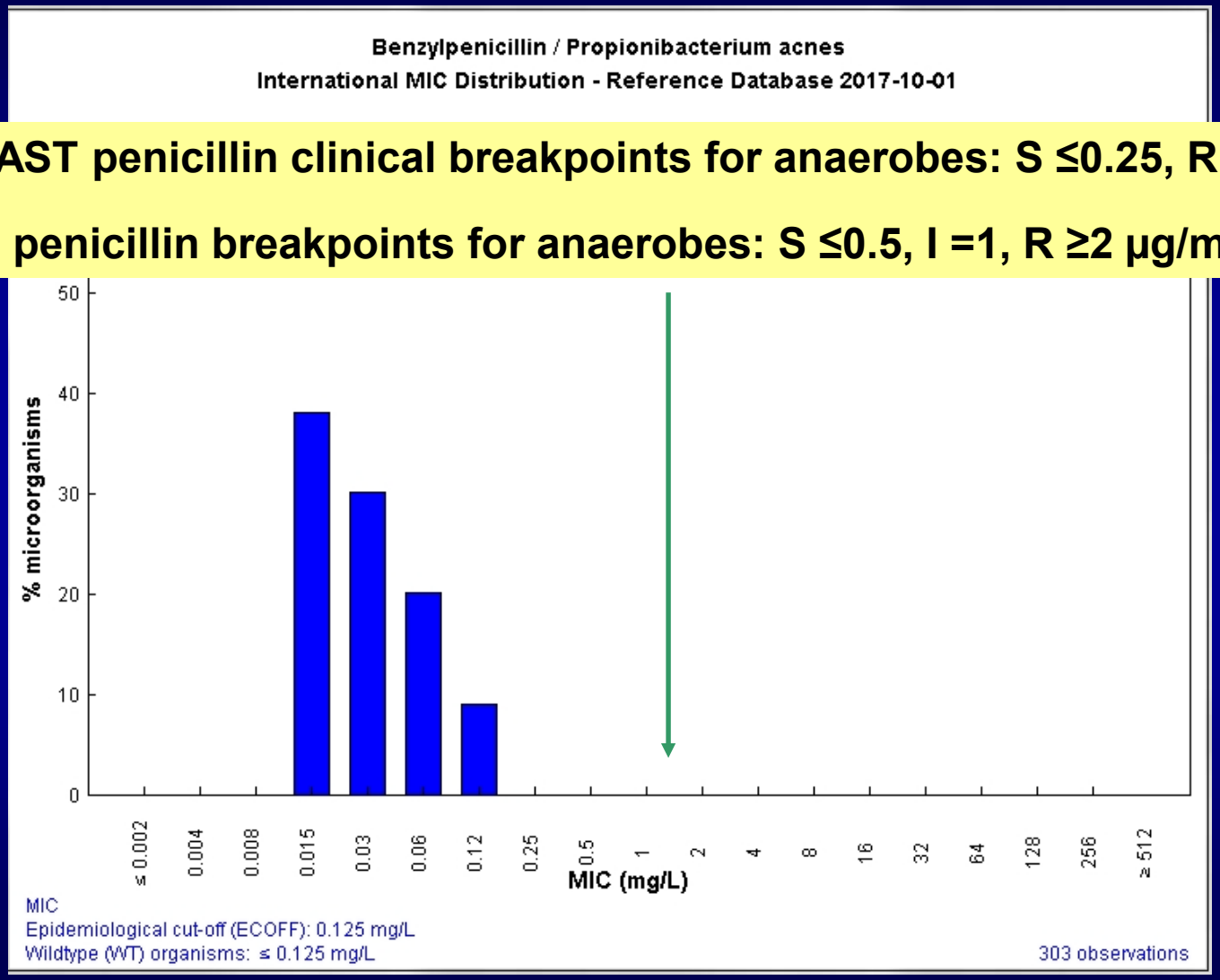
  - Ampicillin-sulbactam, 97-100%
  - Piperacillin-tazobactam, 94-100%
  - Imipenem, 94-100%
  - Meropenem, 98-100%
  - Metronidazole, 95-100% (*C. acnes* 0% Susc)
  - Clindamycin, 53-97%
  - Moxifloxacin, 62-95%

Anaerobic gram-positive cocci include *Peptococcus, Peptostreptococcus, Finegoldia, Peptoniphilus,* and *Anaerococcus* sp

C. acnes should be susceptible to Penicillin

EUCAST penicillin clinical breakpoints for anaerobes: S ≤0.25, R >0.5 mg/L

CLSI penicillin breakpoints for anaerobes: S ≤0.5, I =1, R ≥2 µg/mL



European Committee on Antimicrobial Susceptibility Testing. Data from the EUCAST MIC distribution website, last accessed 10/1/2017 http://www.eucast.org

Acknowledgement

- Dr. Sandra Richter

Toxicity of Oxygen

$O_2 + e^- \rightarrow O_2^-$ (superoxide anion)

$O_2 + e^- + 2H^+ \rightarrow H_2O_2$ (hydrogen peroxide)

$2O_2^- + 2H^+ \xrightarrow{\text{superoxide dismutase}} H_2O_2 + O_2$

$2\ H_2O_2 \xrightarrow{\text{catalase}} 2H_2O + O_2$

Clostridium difficile

- Most common cause of healthcare associated diarrhea
  - Doubled incidence since 1996; outbreaks common
  - Cost >$1 billion/yr
  - 027/NAP1 strain quinolone R outbreak strain
  - Risk factors: Clinda, amp, amox, ceph, quinolones (up to 8 wks later)
- Most toxigenic isolates A+/B+
  - Enterotoxin (toxin A)
  - Cytotoxin (toxin B)