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**Medical Laboratory
Journeyman: Microbiology**

Volume 2. Bacteriology



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THIS VOLUME of CDC 4T051O, Medical Laboratory Journeyman: Microbiology, begins in unit 1 with the most medically important genera—aerobic gram-positive cocci and bacilli—that cause some of the most serious human diseases. Subsequently, unit 2 provides a description of the aerobic gram-negative cocci and coccobacilli organisms. This is followed by the aerobic gram-negative bacilli in unit 3, which includes a discussion of the enterics. The volume concludes with unit 4, which has brief discussions of the anaerobic gram-positive and gram-negative organisms. In all units in this volume our study of pathogens will consist of brief reviews of their general characteristics, clinical significance, and laboratory identification procedures. A glossary of terms, abbreviations, and acronyms used in this course is included at the end of this volume.

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This volume is valued at 21 hours and 7 points.

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NOTE:

In this volume, the subject matter is divided into self-contained units. A unit menu begins each unit, identifying the lesson headings and numbers. After reading the unit menu page and unit introduction, study the section, answer the self-test questions, and compare your answers with those given at the end of the unit. Then do the units review exercises.

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Unit 1. Aerobic Gram-Positive Organisms

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IN THIS unit, you'll learn about the gram-positive organisms of greatest clinical significance to humans. Our discussion begins by characterizing the organism in a concise manner as to its physical appearance and general physiologic characteristics. We'll conclude with clinical significance and laboratory identification.

Let's begin with the gram-positive cocci; because next to the *Enterobacteriaceae*, they're the organisms most frequently isolated from human infections. Our discussion is not limited to the gram-positive cocci; we'll also discuss gram-positive bacilli. Much of what you need to know to differentiate these organisms from the thousands of species that surround them is the knowledge they are gram-positive—linked to their cell wall structure. Gram-positive cell walls are higher in peptidoglycan and lower in lipid content than gram-negative cells. This characteristic allows them to retain crystal violet stain even when exposed to organic solvent such as alcohol.

Cell wall composition also accounts for differing susceptibility exhibited by gram-positive organism antibiotics, as compared to the patterns seen with gram-negative organisms. Antibiotics that interfere with cell wall structure (penicillins) are more effective against gram-positive organisms than antibiotics (aminoglycosides, gentamicin) that require entry into the cell cytoplasm in order to exert their antibacterial properties.

1–1. Gram-Positive Cocci

Gram-positive organisms are very much a part of our environment and grow on the skin and mucus membranes of humans and animals. Common objects are likely to be contaminated with them and may act as sources of infection. Penetration of the skin via trauma or surgical procedure always carries with it the possibility of infection with the ubiquitous gram-positive bacteria. Gram-positive bacteria may cause local or systemic infections and bacteremia. Examples are listed in the following table.

Gram-positive bacteria	Resulting systemic infections and bacteremia
Staphylococci	Abscesses. Carbuncles. Cellulitis. Folliculitis. Furuncles.
Streptococci	Cellulitis. Endocarditis. Erysipelas. Meningitis. Pharyngitis.
Pneumococci	Pneumonia.

201. *Staphylococcus* and *Micrococcus* species

Members of the genera *Staphylococcus* and *Micrococcus* are in the family *Micrococcaceae*. Also included in this family are *Planococcus* and *Stomatococcus*. *Planococcus* species have been isolated from seawater, brine tanks, and frozen seafood but have not been implicated in human infection. There is only one species in the genera *Stomatococcus*. *Stomatococcus mucilaginosus* is part of the human respiratory tract normal flora but has been associated with endocarditis, intravenous drug use, peritonitis, and bacteremia. *Planococcus* and *Stomatococcus* are rare. *Stomatococcus* are discussed in a later lesson, but *Planococcus* won't be discussed any further.

Staphylococcus species

Knowledge of staphylococci is essential to your job performance in microbiology. These organisms are of great clinical significance. They're second only to enteric organisms in rate of recovery from clinical samples and responsible for a wide variety of diseases ranging from superficial skin infection to deep tissue involvement. Continued research into this genus has identified additional species and revealed new clinical manifestations of known pathogenic species. Toxic shock syndrome (TSS), an acute illness primarily associated with *Staphylococcus aureus* phage group I, was first described in 1978. We may see other species of this genus identified as the causative agents of additional syndromes as our knowledge of this genus and its effect on the immunocompromised patient broadens. The genus *Staphylococcus* is currently comprised of 32 species. We'll only discuss the most common species.

General characteristics

Staphylococci are gram-positive, usually unencapsulated, nonspore-forming cocci from 0.5 to 1.5 μm in diameter, occurring singly, in pairs, tetrads, short chains (composed of three or four cells), and irregular grapelike clusters. They are nonmotile and usually catalase positive. Most staphylococci are facultative anaerobes. The exceptions are *S. saccharolyticus* and *S. aureus* subspecies *anaerobius* that grow more abundantly in the absence of oxygen and are, therefore, classified as anaerobes.

The table below categorizes these species into two groups: (1) staphylococci found in humans and other primates and (2) staphylococci found in other animals and in the environment. Some of these species may be found in both humans and animals, examples are *S. aureus*, *S. schleiferi*, and *S. intermedius*.

Human and Other Primate Staphylococci		Other Animal and Environmental Staphylococci	
<i>S. aureus</i>	<i>S. hominis</i>	<i>S. hyicus</i>	<i>S. delphini</i>
<i>S. epidermidis</i>	<i>S. lugdunensis</i>	<i>S. chromogenes</i>	<i>S. carnosus</i>
<i>S. capitis</i>	<i>S. auricularis</i>	<i>S. sciuri</i>	<i>S. caseolyticus</i>
<i>S. caprae</i>	<i>S. saprophyticus</i>	<i>S. gallinarum</i>	<i>S. kloosii</i>
<i>S. saccharolyticus</i>	<i>S. cohnii</i>	<i>S. intermedius</i>	<i>S. arlettae</i>
<i>S. warneri</i>	<i>S. xylosus</i>	<i>S. piscifermentans</i>	<i>S. vitulus</i>
<i>S. pasteurii</i>	<i>S. simulans</i>	<i>S. lentus</i>	<i>S. schleiferi</i>
<i>S. haemolyticus</i>		<i>S. felis</i>	<i>S. muscae</i>
		<i>S. equorum</i>	

Now, let's focus our attention on *S. aureus*, *S. epidermidis*, *S. saprophyticus*, *S. haemolyticus*, and *S. lugdunensis*—the species most commonly associated with human infections.

Clinical significance

The following material is quoted by permission from *Listen, Look, and Learn: Self-Study Microbiology Series, Clinical Microbiology*.

“*S. aureus* is well documented as an opportunistic pathogen in humans, and a variety of other mammals and birds. In most clinical settings, it is the species of primary concern, because it is a major cause of morbidity and mortality. Such infections are often pyogenic and acute and, if untreated, may spread contiguously (to surrounding tissue) or via bacteremia to metastatic sites of infection (involving other organs). Factors predisposing to *S. aureus* infection include: (1) injury to skin produced by abrasions, wounds, burns, surgical incisions, injections (without aseptic technique), and primary skin diseases, (2) prior viral infections, such as influenza and measles, (3) *leukocyte* defects, such as those brought about by immunosuppressive drugs, congenital or acquired *leukopenia*, serum opsonins or other serum factors, diabetes, and chronic granulomatous disease, (4) presence of foreign bodies such as sutures, prostheses, and catheters, (5) certain chronic diseases involving mucoviscidosis (cystic fibrosis), coronary artery disease, uremia, alcoholism, and malignant tumors, and (6) prior prophylactic or therapeutic use of antibiotics to which infecting *S. aureus* is resistant. Some of the major infections produced by *S. aureus* include: furuncles (boils), carbuncles, cellulitis, impetigo, staphylococcal scalded skin syndrome, pneumonia, osteomyelitis, meningitis, endocarditis, mastitis, bacteremia, various abscesses, enterocolitis, urogenital infections, TSS, and food poisoning (via enterotoxin).

“For many years, the coagulase negative *Staphylococcus* (CoNS) species indigenous to humans were generally regarded as contaminants when isolated from clinical specimens, but this view is rapidly changing. It is becoming clear that modern medical practices leading to the compromise of patients, such as the introduction of prostheses or catheters and immunosuppressive therapy, have greatly enhanced the risk of infection by resident coagulase negative species. Of the various coagulase negative species, *S. epidermidis* and *S. saprophyticus* are the best documented as opportunistic pathogens. *S. epidermidis* has been frequently implicated in prosthetic valve endocarditis (approximately 25% of cases, with mortality reaching as high as 70%) and infections of intravascular catheters, cerebrospinal fluid shunts, and orthopedic appliances (for example, hip replacement). Slime mediated adherence may be an important factor in the pathogenesis of *S. epidermidis* infections of these medical devices. *S. epidermidis* also has been implicated in peritonitis in patients receiving continuous peritoneal dialysis and urinary tract, eye, ear (otitis media) and wound infections.”

“*S. saprophyticus* has been frequently implicated in urinary tract infections, such as cystitis, urethritis, and pyelonephritis, usually accompanied with a significant bacteriuria. This species appears to be the predominant staphylococcal species in acute urinary tract infections of young, sexually active females. It has a higher capacity to adhere to uroepithelial cells than to buccal or skin cells and does so better than other staphylococcal species. Other members of the *S. saprophyticus* species group, such as *S. cohnii* and *S. xylosus*, have been much less frequently implicated in urinary tract infections.”

S. epidermidis has been isolated from 74 to 92% of patients with coagulase negative staphylococcal nosocomial bacteremia. But the second most frequently isolated CoNS implicated in human infections is *S. haemolyticus*. *S. haemolyticus* is associated with native valve endocarditis, peritonitis, UTIs, septicemia, and wound, joint, and bone infections. *S. lugdunensis* is associated with brain abscesses, septicemia, native and prosthetic valve, chronic osteoarthritis, and infections in bone and peritoneal fluid.

Most of the serious, invasive staphylococcal infections currently encountered are seen in patients who have been subjected to extensive surgery or have a serious underlying disease and are especially susceptible to infection with staphylococci. Hospital personnel and patients have significantly higher carrier rates than the general population. These organisms are generally resistant to penicillin and to other antibiotics. Direct person-to-person contact is the most common means of transmission.

Staphylococcal organisms exhibit a number of mechanisms of pathogenicity including the production of enzymes, hemolysins, and toxins. The enzymes produced include coagulase, phosphatase,

thermostable deoxyribonuclease and ribonuclease, lipase, gelatinase, hyaluronidase, protease, and fibrinolysin. The hemolysins produced include α (alpha), β (beta), γ (gamma) and δ (delta). These hemolysins produce their own unique toxins that act on different cell types and structures. The other toxins are leukocidins that act on red and white blood cell membranes, exfoliation, exotoxins, and enterotoxins. The toxins that exhibit their pathogenicity in such toxigenic diseases as food poisoning and antibiotic-induced pseudomembranous colitis are enterotoxins, in staphylococcal scalded skin syndrome are exfoliative toxins, and in TSS are exotoxins.

Types of enterotoxins

The enterotoxins are divided into five types designated A through E. Types A and D are most commonly associated with staphylococcal food poisoning—the most common type of food poisoning in the United States. They are heat-stable (resistant to boiling) enterotoxins that are produced in food, such as custard or cream-filled bakery products. Ham, processed meats, ice cream, cottage cheese, hollandaise sauce, and chicken salads are foods that are often implicated. Foods containing the enterotoxins are normal in appearance, taste, and odor. Sufficient toxin is produced in four to six hours at 30°C, but not at refrigerated temperature, to produce symptoms of food poisoning. The enterotoxin is rapidly absorbed by the intestinal mucosa, resulting in nausea, vomiting, diarrhea, and acute prostration within two to eight hours after the contaminated food is consumed. Staphylococcal food poisoning is not an infection, but an intoxication resulting from the ingestion of the preformed toxin. Hence, the implicated foods, rather than stool specimens, are more likely to yield the pathogen on bacterial analysis. The food poisoning is self-limited and only supportive therapy is needed.

Exfoliative toxin, called exfoliation, cleaves to the middle layers of the skin and allows the surface of the skin to peel. This is associated with staphylococcal scalded skin syndrome, which is often seen in newborns.

TSS

TSS is an acute illness usually affecting women of childbearing age. Headache, high fever, dizziness, diarrhea, vomiting, hypotension, and a diffuse rash mark the syndrome. The syndrome usually occurs during or shortly after menses in women who use superabsorbent tampons. The tampon's role in causing TSS seems to be twofold. (1) Tampons seem to promote the growth of large numbers of organisms in the vaginal canal, and (2) the fibers used in certain brands of tampons bind Mg^{++} . TSS is associated with toxic shock syndrome toxin-1 (TSST-1) that is an exotoxin produced and secreted by certain strains of *S. aureus*. *S. aureus* is the causative agent of TSS. All isolates to date have been penicillin-resistant and predominately from phage group I. TSS can be a serious disease with a 5 to 8% mortality rate among patients who acquire this infection. Today, TSS is not exclusively seen in young women during menstruation; it has been reported in both males and females. It is associated with osteomyelitis, postsurgical wound infections, staphylococcal abscesses, and postinfluenza pneumonia.

Laboratory identification

Laboratory identification consists of colony morphology, selective media, staining reactions, coagulase production, biochemical or carbohydrate fermentation tests, and commercial identification kits or instruments.

Colony morphology and selective media

Regardless of the source of the specimen, thioglycolate broth and blood agar (sheep blood) are the preferred primary or initial media for culture and isolation of all *Staphylococcus*. The species of staphylococci of medical importance usually produce abundant growth throughout the thioglycolate broth. On blood agar, most strains of *Staphylococcus* produce colonies 1 to 3 mm in diameter within 18 to 24 hours. Within three days, at room temperature (34 to 37°C), the colonies are 3 to 8 mm in diameter. These colonies are usually opaque, circular, raised or slightly convex, and smooth with a

creamy or butyrous consistency with an entire margin. Hemolysis and pigmentation are sometimes used to tentatively identify *S. aureus* on the basis of colony morphology; however, these two characteristics, although helpful, are not totally reliable. *S. aureus* pigments range from yellow to yellow-orange, while other strains may produce gray or off-white colonies. Several staphylococcal species produce yellow pigment besides *S. aureus*; however, some *S. aureus* do *not* produce this pigment. Pigment production may be more noticeable after incubation at room temperature. Some species of staphylococci other than *S. aureus* produce hemolysins, although the zones of hemolysis they produce are generally smaller than the zones surrounding *S. aureus* colonies.

Selective media are suggested for use in isolating staphylococci from highly contaminated specimens. Phenylethyl alcohol agar (PEA), Columbia colistin-nalidixic acid (CNA), and mannitol salt agar (MSA) are commonly used agars for this purpose. Both PEA and CNA inhibit the growth of gram-negative organisms while favoring the growth of staphylococci. The selectivity of MSA is based on its salinity, a characteristic that is tolerated by staphylococci but not by other organisms. On selective media incubation, extend the time to at least 48 hours for good colony development. Colonies that conform to the description above should be Gram-stained, subcultured, and tested for species-specific characteristics.

Staining reactions

Direct examination of Gram-stained material, in conjunction with colony morphology, can aid in the identification of staphylococci. Microscopic examination of Gram-stained material offers you the advantage of rapidly determining the presence of gram-positive organisms in the patient's sample. This can be of great value especially if the specimen is one that is normally sterile (cerebrospinal fluid (CSF), joint fluid, etc.). Direct examination of nonsterile specimens can yield valuable information as well, since you can note the presence of inflammatory cells versus epithelial cells. Make only presumptive reports of gram-positive cocci resembling staphylococci when the sample is from a source that is normally sterile or contains gram-positive normal flora. Microscopy by itself cannot differentiate staphylococci, micrococci, planococci, streptococci, or various anaerobic cocci from one another.

Coagulase production

A test of the ability to clot plasma is the most widely used test to identify pathogenic staphylococci. Either a tube or slide test may be used. The tube test is considered more reliable. It detects the presence of extracellular coagulase. Rabbit plasma containing EDTA or citrate is the preferred reagent for performing this test. Place 0.5 ml of rabbit plasma in a test tube and add one loopful of the organism to be tested. Incubate at 35°C, checking every half hour during the first four hours for clot formation. Clot formation indicates a positive test due to extracellular coagulase reacting with coagulase-reacting factor (CRF) to produce coagulase-CRF complex, a substance clinically indistinguishable from thrombin. The coagulase-CRF complex then reacts with fibrinogen to produce an insoluble fibrin clot. The slide test detects coagulase that is bound to the surface of staphylococcal cells and acts directly on fibrinogen to form a clot. The slide test is a rapid means of testing a suspected coagulase-positive organism. When a heavy saline suspension of the organism is mixed with a drop of plasma on a glass slide, a positive reaction is marked by the formation of a white, flaky fibrin precipitate. Since the slide test is not considered as definitive as the tube test, confirm all negative or delayed positives (those reacting in 20 to 60 seconds) with a tube test. Good laboratory technique also dictates that an autoagglutination control should be used to decrease the number of false-positive reactions.

Key tests for identification

Listed here are the key tests for identification of most clinically significant *Staphylococcus* species. These tests include colony morphology, coagulase test procedures, clumping factor, heat-stable thermonuclease (TNase), alkaline phosphatase, pyrrolidonyl arylamidase activity, ornithine

decarboxylase activity, urease activity, β -galactosidase activity, acetoin production, novobiocin resistance, polymyxin B resistance, and acid production from carbohydrates. The principles and procedures for each of the tests are beyond the scope of this career development course (CDC). Please consult the bibliography for references for additional information.

Commercial identification systems

There are several commercial kits and automated instruments available through various manufacturers for the identification of *Staphylococcus* species. The kits and instruments use a variety of methods which include conventional biochemical tests, modified carbohydrate fermentation tests, and fluorogenic or chromogenic enzyme substrate tests that are adapted to the manufacturer's specific format. Strips with small cupules, impregnated filter paper disks, microtiter trays, or plastic cards are format examples. These products need to include the key tests or the identification could be questionable with regard to certain species. Examples of commercial kits and instruments available are API STAPH-IDENT, STAPH Trac System, ID 32 STAPH, Vitek and Vitek Jr. fully automated microbiology systems that utilize a Gram-Positive Identification (GPI) Card (bioMérieux Vitek); MicroScan Pos ID panel read manually or by MicroScan instruments (Baxter Diagnostics, Inc., MicroScan Division); GP MicroPlate test panel read manually or using Biolog equipment (Biolog); and the Microbial Identification System (MIS) that uses cellular fatty-acid analysis with computerized high-resolution gas chromatography (MIDI). AccuProbe (Gen-Probe, Inc.) uses DNA probe technology for the rapid detection of *S. aureus* from cultures. Immunoenzymatic assays based on monoclonal antibodies prepared against *S. aureus* enzymes are being developed.

Additional comments on identification of staphylococcal species

Your final report to the requesting physician is based on data relating to the media utilized, colony morphology, environmental requirements, Gram-stain, and biochemical characteristics exhibited by the organism in question.

The following material is quoted by permission from *Listen, Look, and Learn: Self-Study Microbiology Series, Clinical Microbiology*.

"In most clinical laboratories, identification of *S. aureus* as an etiological agent or indicator of potential health risk is of primary importance. *S. aureus* can be distinguished from other staphylococcal species on the basis of the following character profile: positive coagulase activity, alkaline phosphatase activity, deoxyribonuclease activity (including a heat-stable nuclease), hemolysin activity, acetoin production, protein A, nitrate reduction, and acid from sucrose, trehalose, maltose, and mannose, and usually mannitol and lactose. *S. intermedius* can be distinguished from *S. aureus* on the basis of delayed, weak acid from maltose and mannitol, absence of protein A, no or very weak acetoin production, and strong β -galactosidase activity. Coagulase positive strains of *S. hyicus* can be distinguished from *S. aureus* and *S. intermedius* on the basis of no acid from maltose and usually no acid from mannitol, absence of acetoin production (distinguishing it from *S. aureus*), undetectable β -galactosidase activity (distinguishing it from *S. intermedius*), and usually absence of hemolysis. Also, many strains of *S. hyicus* demonstrate β -glucuronidase activity, a property not found in *S. aureus* and *S. intermedius*. *S. hyicus* subsp. *chromogenes* strains are usually pigmented; whereas strains of *S. hyicus* subsp. *hyicus* are unpigmented".

"The identification of coagulase negative staphylococcal species has been made practical by the selection of characters testable by simple procedures. Furthermore, the recent development of rapid identification, commercially available systems has reduced both the need for preparing a variety of test media and reagents and the time required for interpretation of results. These systems can identify coagulase negative and coagulase positive species within five to 24 hours, depending on the particular product."

“The two coagulase negative species of major medical interest are *S. epidermidis* and *S. saprophyticus*. They each form the nucleus of separate species groups composed of several related species. *S. epidermidis* can be identified on the basis of the following character profile: novobiocin susceptible (resistance is rare), rapid and strong anaerobic growth in thioglycolate (within 16 to 24 hours), acid from maltose, fructose, and sucrose, but no acid from mannitol, trehalose, xylitol, or xylose, and usually demonstrates ($\geq 80\%$ of strains) alkaline phosphatase and urease activity. Acid from turanose is variable and acid is usually produced slowly (48 to 72 hours) from mannose. *S. saprophyticus*, on the other hand, is novobiocin resistant, produces relatively slow anaerobic growth, usually does not demonstrate alkaline phosphatase activity and produces acid from turanose and usually xylitol, but not acid from mannose or xylose. Many strains of *S. saprophyticus* also produce acid from trahalose and mannitol. Most strains of this species demonstrate β -galactosidase activity; whereas, strains of *S. epidermidis* do not.”

“For those clinical laboratories interested in identifying most or all of the various staphylococcal species found in clinical specimens, it is recommended that rapid identification, commercially available systems be used whenever possible.”

***Micrococcus* species**

The genus *Micrococcus* is composed of nine species that can be readily identified with conventional biochemical tests, commercial kits, and instrumentation. The nine species are: *Micrococcus luteus*, *M. agilis*, *M. varians*, *M. roseus*, *M. lylae*, *M. kristinae*, *M. sedentarius*, *M. nishinomiyaensis*, and *M. halobius*.

General characteristics

Micrococci are gram-positive cocci, usually in pairs, tetrads, and irregular clusters, and are 0.5 to 2.0 μm in diameter. They are nonmotile, except for *M. agilis* that is the only species having flagella.

Clinical significance

Micrococci are found in nature and on the skin of humans and other mammals. Occasionally micrococci have been isolated from shellfish, fish, natural waters, algal blooms, sand, and plant products. *Micrococcus* species are usually considered harmless saprophytes except in the immunocompromised host, where it is an opportunistic pathogen. *Micrococcus* species have been implicated in intracranial abscesses, bacteremia, peritonitis, pneumonia, septic arthritis, and meningitis. They have also been associated with infections of prosthetic valve endocarditis and infections from cerebrospinal fluid shunts.

Laboratory identification

Staphylococcus and *Micrococcus* differ in their deoxyribonucleic acid (DNA) and cell wall chemical composition. However, no simple test is available to identify these organisms on the basis of these differences. A number of tests that may be routinely performed in the clinical laboratory to distinguish these organisms do exist. The modified oxidase and benzidine test along with resistance to lysozyme, lysostaphin, and bacitracin can be used to differentiate these organisms. Also, the oxidation fermentation test (OF) may be helpful since most staphylococci ferment glucose under anaerobic conditions, whereas most micrococci do not. All micrococci yield positive modified oxidase and benzidine tests, since these tests detect the presence of cytochrome C. All staphylococci with the exception of *S. sciuri*, *S. lentus*, and *S. caseolyticus* do not possess cytochrome C and are negative for both of these tests.

Lysozyme resistance is seen in most staphylococci, whereas most micrococci are susceptible. Lysozyme is an enzyme that cleaves the glycan strands between N-acetylmuramic acid and N-acetylglucosamine both of which are found in the cell walls of staphylococci and micrococci. However, the presence of certain concentrations of lysozyme inhibits micrococci, but not

staphylococci. *Micrococci luteus* is used as a positive control for this procedure and *S. aureus* that is not inhibited is used as a negative control. The lysostaphin susceptibility test is based on the presence of glycine-glycine linkages in the cell structure of staphylococci. Micrococci do not have glycine-glycine linkages. Lysostaphin is an enzyme that lyses glycine-glycine bonds. Many staphylococci are susceptible to lysis by this enzyme, whereas micrococci are not. Thus, lysostaphin inhibits the growth of staphylococci, but not the growth of micrococci. *S. aureus* is used as a positive control of the lysostaphin susceptibility test and *Micrococcus luteus*, that shows no visible inhibition, is used as a negative control.

Susceptibility to Taxo A bacitracin disks is yet another way to differentiate these genera. Staphylococci are resistant to the disks, whereas micrococci are susceptible, producing zones of inhibition from 10.5 to 25 mm in diameter.

Finally, the results of the OF test, in which most staphylococci ferment glucose under anaerobic conditions but most micrococci do not, are illustrated in the table on the following page along with the other tests.

Genus	Oxidase and Benzidine Test	Lysozyme Resistance	Lysostaphin Resistance	Bacitracin Resistance (Taxo A)	OF Test
<i>Staphylococcus</i>	–	+	–	+	Fermentative
<i>Micrococcus</i>	+	–	+	–	Oxidative
NOTE: Reactions above are true for the vast majority of <i>Staphylococcus</i> and <i>Micrococcus</i> species; however, certain species give variable results.					

By using a combination of these tests, you should be able to distinguish the coagulase negative staphylococci from the micrococci likely to be found in most clinical samples. Commercial kits available for differentiation include the Lysostaphin Test Kit and Microdase Disk. Also, they are included in the database of Staph-TRAC, StaphIDENT, and ID32 STAPH systems.

Micrococcus species are distinguished from each other by colony morphology, pigment production, nitrate reduction, acid production from glucose, lysozyme resistance, oxidase reaction, and growth on inorganic nitrogen agar, to name a few. Check with your microbiology laboratory's OI for identification and reporting policies or civilian technical references for additional information.

Self-Test Questions

After you complete these questions, you may check your answers at the end of the unit.

201. *Staphylococcus* and *Micrococcus* species

1. How many species does the genus *Staphylococcus* currently contain?
2. What species are most commonly associated with human infections?
3. What species is a major cause of morbidity and mortality?
4. What are six predisposing factors to a *S. aureus* infection?

5. List eight major types of infection produced by *S. aureus*.
6. What modern medical practices have greatly enhanced the risk of infection by coagulase negative species of *Staphylococcus*?
7. What are seven types of infection produced by *S. epidermidis*?
8. What is thought to be an important factor in the pathogenesis of *S. epidermidis* infections of medical devices?
9. What appears to be the predominant staphylococcal species in acute urinary tract infections of young women?
10. Which of the coagulase negative staphylococci is isolated from 74 to 92% of patients with coagulase negative staphylococcal nosocomial bacteremia?
11. Staphylococcal organisms exhibit a number of mechanisms of pathogenicity; what are three?
12. What role do tampons play in causing TSS?
13. What are two preferred primary or initial media for the culture and isolation of all staphylococci?
14. In what portion of a tube of thioglycolate broth will staphylococci of medical importance usually produce abundant growth?
15. What two colony characteristics are sometimes used to tentatively identify *S. aureus*?
16. What are three selective media used to isolate staphylococci from highly contaminated specimens?

17. What is the selectivity of MSA based upon?
18. How should Gram-stains exhibiting gram-positive cocci be reported to the requesting health care provider?
19. What is the most widely used test to identify pathogenic staphylococci?
20. Which version of the coagulase test is considered more reliable, and what does it detect?
21. What test can be used to distinguish *S. intermedius* from *S. aureus*?
22. *Micrococcus* species have been implicated in what eight disease processes?

202. *Streptococcus* and *Enterococcus* species

The genus *Streptococcus* is in the family *Streptococcaceae*, which is distinguished from the family *Micrococcaceae* by the catalase test. *Micrococcaceae* are catalase positive and *Streptococcaceae* are catalase negative. Streptococci are important pathogens for two reasons: (1) the severe acute infections they cause, and (2) the various complications that the patient may experience after the initial acute streptococcal infection has subsided. Some organisms in the previously considered Lancefield's group D streptococci are now in the new genus *Enterococcus*.

***Streptococcus* species**

Hemolytic activity, antigenic composition, and physiology have all been used to characterize and develop classification schemes for the genus *Streptococcus*. Sherman, Brown, and Lancefield are three names that are linked to the classification of the genus. We'll briefly discuss each of their systems of classification.

In the 1930s Sherman developed a classification system that had the greatest impact on the classification of the streptococci in the clinical laboratory. Sherman divided the streptococci into four major divisions: enterococci, lactic, viridans, and pyogenic/hemolytic. Through the years there have been new bacteria discovered with characteristics that cross these divisional lines. Some elements of this system still remain from a clinical standpoint but organizations that classify these organisms have largely abandoned this method in favor of others because of the inability to easily classify the organisms with traits that cross the divisional lines.

Brown was the originator of a system based on the hemolytic activity of streptococcal colonies grown on sheep blood agar. Four types of hemolysis were described: *alpha*, *beta*, *gamma*, and *alpha prime*.

Type of hemolysis	Characteristics
Alpha (α)	An indistinct zone of partially lysed red blood cells surrounds the colony, often accompanied by a greenish to brownish discoloration.
Beta (β)	A clear, colorless zone around the streptococci colonies in which the red blood cells have undergone complete discoloration. This type is best seen in deep colonies in a pour plate. On the other hand, surface colonies may appear as alpha or nonhemolytic due to inactivation of one of the hemolysins, streptolysin O, that is oxygen labile and streptolysin S, an oxygen-stable hemolysin that may be present in only small amounts in these strains that hemolyze the surface poorly.
Gamma (γ)	No apparent hemolytic activity or discoloration produced by the colony.
Alpha-prime (α') or Wide Zone Alpha (WZ α)	A small halo or envelope of intact or partially lysed red blood cells lying adjacent to the bacterial colony with a zone of complete hemolysis extending further out into the medium. This can be confused with a beta hemolytic colony when only surface growth is observed.

Lancefield's serological classification system was based on the reaction of C substances (antigenic material) found on the cell walls of various streptococci and specific antisera. The antisera were harvested from rabbits that had been inoculated with various strains of streptococci in order to elicit an immune response to these C substances in the form of the creation of their corresponding antibodies. In Lancefield's system, the streptococci are placed in group A through O based on their antigenic makeup. Sherman's system used as its basis physiologic characteristics to divide the streptococci into four groups: pyogenic, lactic, enterococci, and viridans.

Although the Lancefield system had the greatest impact on the classification of streptococci in the clinical laboratory, the other two system's contributions are still evident today. We still describe colonies using Brown's terminology, and the terms *enterococci* and *viridans* are still used to refer to particular groups of organisms.

Streptococcus species include *S. pyogenes* (group A streptococci), *S. agalactiae* (group B streptococci), "*S. milleri*" group (other beta-hemolytic, nongroup A or B, streptococci), *S. pneumoniae*, viridans streptococci, *S. bovis*, and nutritionally variant streptococci.

General characteristics

Streptococci are facultatively anaerobic, catalase-negative, gram-positive, spherical, oval, or occasionally elongated into rods and occur in pairs, or as short or long chains. Liquid cultures normally yield longer chains than cultures grown on agar. The cells are nonmotile and are less than 2 μm in diameter. Characteristically, streptococci are gram-positive but may become gram-negative as the cell ages. Capsules are usually noted in virulent forms and contain an abundance of hyaluronic acid.

Growth on agar surface is scanty; thus, enriched infusion medium, such as brain heart infusion, trypticase soy, or heart infusion agars is required. Streptococci are facultative with respect to oxygen. Many strains grow better anaerobically than aerobically. Streptococcal colonies are small, translucent to slightly opaque, circular, generally less than 1 mm in size, convex, and appear as minute beads of moisture on a moist agar surface. Colonies are less moist and almost opaque on drier surfaces. The Lancefield groups A, C, and G are divided into subgroups depending on their colony size—large colony (> 0.5 mm in diameter) and small colony (< 0.5 mm in diameter) forming groups. Pneumococcal colonies are flatter and translucent. Variations in colony morphology occur frequently showing the mucoid, smooth or glossy, and matte or rough forms. Virulence is indicated by the presence of the mucoid and matte forms that contain relatively large amounts of M protein; whereas, the glossy or smooth forms contain very little of this substance and are usually not virulent. After repeated transfer on laboratory media, virulent rough or smooth colonies eventually develop. Streptococci are oxidase-negative. *Neisseria* is oxidase-positive. This property with the Gram-stain characteristics and cellular morphology differentiates streptococci from *Neisseria*. Growth is best at

35 to 37°C, unless otherwise stated. Sheep blood, free from fermentable carbohydrates and a final pH of 7.3 to 7.4, is preferred. The presence of dextrose in the basal medium for blood plates results in the inhibition of hemolysis by beta-hemolytic streptococci. All plates should be incubated aerobically in the presence of 5 to 10% CO₂. The conditions achieved in candle-jar incubation permit the detection of hemolytic activity by streptococci on streaked blood plates if the hemolytic activity is read from the growth in the stabbed areas of the plate as described previously. This permits the detection of both O and S hemolysins. Characteristically, after 18 to 24 hours of growth on blood agar, streptococci produce varying degrees of hemolytic activity (α , β , and γ). This is used as one criterion for their identification.

Clinical significance

Streptococci are usually found as normal flora in the respiratory and genital tracts and as parasites of humans and other animals. However, humans are the most susceptible of all animals to streptococcal infections, and streptococci than any other genus of bacteria cause a wider variety of clinical manifestations. Among the diseases of great importance caused by these organisms are streptococcal pharyngitis, glomerulonephritis, scarlet fever, pyoderma (a purulent skin infection), endocarditis, meningitis, pneumonia, and the newly emerging streptococcal TSS caused by an old organism (group A streptococcus) that has become more virulent.

Streptococcus pyogenes (group A streptococci or GAS)

This species of streptococci is the most dangerous of all. *S. pyogenes* is most commonly responsible for infections of the upper respiratory tract and the skin. It is not only the initial infection at these sites that is of concern to the health care provider, but also the sequelae or complications that may follow the acute infection. Acute rheumatic fever and post-streptococcal glomerulonephritis are two secondary sequelae of infections caused by *S. pyogenes*. Streptococcal TSS is associated with the severely invasive groups of *S. pyogenes* that cause shock, organ failure, and necrotizing infections or what have been coined as flesh-eating bacterial infections. Streptococcal pharyngitis (strep throat) occurs most frequently during winter and spring in school age children. Exposure to droplets of respiratory secretions is the most common route of infection. However, contaminated food or beverages, especially milk, may be at fault. M protein, lipoteichoic acid, enzymes, and toxins are the virulence factors produced by *S. pyogenes* that cause the wide array of serious infections. One example is the erythrogenic toxin produced by scarlet fever-associated strains that is responsible for the characteristic rash.

Complications following streptococcal pharyngitis and skin infections fall into two categories—suppurative, and nonsuppurative. Suppurative (purulent or pus producing) infections in which the organism itself may be found in the affected tissues include cellulitis, scarlet fever, erysipelas, and pyoderma. The two most significant nonsuppurative complications are rheumatic fever and glomerulonephritis. The exact way in which *S. pyogenes* causes rheumatic fever (RF) is unknown. RF seems to be an autoimmune disease in which antibodies developed against streptococcal cell membrane antigens cross-react with myosin in the heart muscle causing chronic progressive damage to the heart, and possibly death, several years after the acute attack. Likewise, post-streptococcal acute glomerulonephritis (AGN) is an acute inflammatory disease of the renal glomerulus that may follow a streptococcal skin infection and whose pathogenesis is not clearly understood. Evidence to date indicates that AGN is an immune complex disease resulting from the disposition of performed complexes of antigens and antibodies in the kidney. Both RF and AGN are considered nonsuppurative because the organism itself and a purulent inflammatory response are not present in the affected organs (heart, joints, blood vessels, and kidneys). Either of these nonsuppurative complications may follow streptococcal pharyngitis, but of the two, only glomerulonephritis is a complication of skin infections.

Epidemics due to this organism are not uncommon in populations where individuals live in close quarters, such as in military barracks and school dormitories. This is due to the fact that there is a good likelihood of the organism being introduced into this type of environment by a carrier. It is estimated that from 5 to 20% of the general population are asymptomatic carriers. The organism is usually carried in the nasopharynx, but anal carriage is also possible. Epidemics have also been linked to the ingestion of certain contaminated foods, especially unpasteurized dairy products.

Streptococcus agalactiae (group B streptococci or GBS)

Postpartum women, immunosuppressed individuals with cancer or HIV, and adults with underlying conditions, such as diabetes and genitourinary disorders are at risk from infection by this organism. Adult infections include osteomyelitis, endocarditis, skin and soft tissue infections, and bacteremia. However, it is the greatest threat to neonates, especially premature infants born to infected mothers. Neonate infections fall into two patterns designated as early and late onset infections. Early onset infections are acquired in utero or during passage through the birth canal. The point of entry for GBS is most likely to be the upper respiratory tract. As a result, the infant displays symptoms of pneumonia and bacteremia within seven days of birth resulting in a mortality rate of between 50 and 70%. Several factors centering on various obstetric complications cause an infant to be at greater than average risk in developing an infection. However, the risk factor of greatest importance is prematurity. Premature infants are not as immunocompetent as full-term infants, and they cannot deal with the large numbers of organisms they encounter during birth. Late onset infections occur anywhere from the seventh day after birth to eight to 12 weeks after birth and the infant experiences meningitis or osteomyelitis, either with or without bacteremia. Late onset infections typically are less common and result in lower mortality rates of between 10 to 20%. The route and source of infection for late onset infections is unclear; however, the hands of infected mothers or the contaminated hands of hospital workers are likely avenues of infection.

Other β -hemolytic streptococci

The other beta-hemolytic streptococci are divided into two groups—those that form large colonies and those that form small colonies. The large-colony-forming groups C and G are similar to *S. pyogenes* because they are also associated with serious infections, such as meningitis, bacteremia, endocarditis, septic arthritis, and infections of the skin and respiratory tract. These streptococci are usually identified with the “*S. milleri*” group of species.

The group C *Streptococcus S. equisimilis* is the species most frequently encountered in human infections, but infections with this organism are still listed as uncommon. Other species in this group include *S. zooepidemicus* and *S. equi*. Clinically, the disease produced by this organism is similar to those produced by group A streptococci, and the organism is known to cause pharyngitis, tonsillitis, pneumonia, endocarditis, bacteremia, osteomyelitis, brain abscess, and skin infections. Some reports have associated outbreaks of group C pharyngitis with post-streptococcal glomerulonephritis.

Group G streptococci are most commonly seen in patients that are compromised due to some underlying condition, or who engage in activities that place them at risk of acquiring an infection with this organism. Predisposing medical conditions, treatments, or high-risk activities include malignancies (especially of the nervous system), lymphatic obstruction, diabetes mellitus, surgery, alcoholism, and intravenous drug use. Group F streptococci have been associated with cellulitis, deep tissue abscesses, osteomyelitis, endocarditis, and bacteremia.

Streptococcus pneumoniae

This organism is the number one causative agent of community-acquired pneumonia, and the second most common cause of bacterial meningitis. It is passed from person-to-person in aerosols of mucus secretions and is not found on objects in the environment. Up to 50% of the population has been found to have *S. pneumoniae* as normal flora in their upper respiratory tract. The polysaccharide

capsule that surrounds the *S. pneumoniae* cells and the presence of a predisposing host condition are both important factors in the pathogenesis of this organism. The capsule inhibits phagocytosis of the organism—the body's main defense against the establishment of the initial infection. A preexisting debilitating condition such as a viral respiratory tract infection prior to being exposed to the organism serves to increase the chances of an infection developing. Lung abscesses, pericardial infections, pleural effusions, and empyema, are complications of pneumococcal pneumonia. Mortality from streptococcal pneumoniae, even when treated early with appropriate antibiotics, still ranges between 5 and 7%. Other *S. pneumoniae* infections include endocarditis, sinusitis, meningitis, and otitis media. Vaccines are now available to protect against pneumococcal infections.

Viridans Streptococcus

Viridans was derived from the Latin word “viridis” that means green. Members of this group are normal flora of the genitourinary tract, gastrointestinal tract, and respiratory tract, and are frequently linked to bacterial endocarditis. They are the most common cause of subacute bacterial endocarditis (SBE). Patients who develop this disease often had previous damage to their heart valves due to rheumatic fever or some other condition. Viridans streptococci often enter the blood stream after dental work has been performed on the patient. These organisms are very proficient at adhering to cells, and this is thought to be a key factor in their ability to cause disease. This is certainly true of *S. mutans*, one member of this group that has been definitively linked to tooth decay in addition to being responsible for a good number of cases of endocarditis. The production of extracellular sugars, called dextrans, that serve as attachment mediators, undoubtedly allow these organisms to establish colonies on the surface of teeth as well as heart valves. Commonly isolated viridans streptococci include *S. mutans* group, *S. salivarius* group, *S. sanguis* group, and *S. mitis*.

Nutritionally variant streptococci

Nutritionally variant streptococci are viridans streptococci that require thiol compounds, pyridoxal (active form of vitamin B₆) or pyridoxamine, and cysteine for growth. They are normally found in the oral cavity but have been isolated from patients with ophthalmic infections and identified as agents in endocarditis involving both native and prosthetic valves. Standard 5% sheep blood agar cannot support the growth of these organisms; however, they grow well in blood cultures where human erythrocytes provide the necessary growth factors. In order to subculture a blood culture, the agar plate must be able to support growth of the nutritionally variant streptococci. An alternative method includes inoculating a blood agar plate with the suspected specimen and “cross-streaking” with *S. aureus* that provides the necessary growth factors. “Satelliting” colonies of these streptococci grow in a zone around the staphylococci. There are only two nutritionally variant streptococci described; they are *S. adjacens* and *S. defectivus*.

Group D streptococci

Group D streptococci are β -hemolytic on rabbit blood, but they are normally nonhemolytic or α -hemolytic on sheep blood agar that is used in most clinical laboratories. This group of organisms possesses the group D lipoteichoic acid antigen in their cell walls. *S. bovis* and *S. equinus* are members of the group D streptococci, with *S. bovis* being the most clinically significant. The recovery of *S. bovis* from the blood has been associated with the development of colon cancer or gastrointestinal tract malignancies, so the patient should undergo a thorough gastrointestinal tract evaluation. Isolating *S. bovis* from the blood is of great significance since it is indicative of a loss of integrity of the gastrointestinal mucosa. It has also been isolated from patients with endocarditis and meningitis.

Laboratory identification

The laboratory identification of streptococci is based on hemolytic reaction on SBAP, inhibition of growth by various substances, and the biochemical characteristics of the isolate. Numerous

serological tests (for example latex agglutination) for the Lancefield group A, B, C, D, F, and G are commercially available.

Colony morphology and hemolysis on sheep blood agar

The starting point in the identification of streptococci is observing the hemolytic reaction and colony morphology of the organism in question. Carefully noting the type of hemolysis and morphology exhibited by the organisms indicates which of the following tests is most helpful in determining the identity of any isolate you are likely to encounter in the clinical laboratory.

Bacitracin disk test

Use of low-concentration bacitracin (Taxo A disk) is the method most commonly used in clinical laboratories to distinguish between Lancefield's group A and other groups of beta hemolytic streptococci. The test depends on the selective inhibition of group A streptococci on a blood agar plate by a paper disk containing 0.04 units of bacitracin. The users of bacitracin disk should be aware of several important factors:

- Disks sold and used for bacitracin susceptibility testing have too high a concentration of bacitracin to differentiate between group A and other or nongroup A streptococci. Use *differential* (0.04 units of bacitracin), and not *sensitivity* (10 units of bacitracin) disks. There is a high degree of correlation between bacitracin and serological tests with group A streptococci.
- A heavy inoculum of a pure culture is advisable. The test has been designed for use with pure cultures, not mixed cultures.
- The test is designed for differentiating beta-hemolytic streptococci. Determine the hemolysis correctly before doing this differential test. Many alpha-hemolytic streptococci, including pneumococci, are sensitive to bacitracin differential disks.
- There is some variation in lots of commercial disks; thus, each new lot of disks should be tested with known strains of group A and nongroup A streptococci.
- Any zone of inhibition, regardless of diameter, is positive. No zone of inhibition means the culture is resistant. Users of the differential disks should realize that growth of some strains of beta-hemolytic streptococci, other than group A, are inhibited by the bacitracin disks. These false positive results may occur in approximately 8% of isolates; this group usually includes organisms that belong to serogroups B, C, and G. False negative results may occur in up to 4% of isolates that are serologically confirmed as group A streptococci. The relatively high rate of false positives may be reduced by carefully evaluating the type of hemolysis produced by isolates. The false negative results are clinically of greater concern to you, the technician, because they may cause a group A streptococcal infection to go unrecognized, and the patient may not receive the appropriate treatment.

Trimethoprim-sulfamethoxazole (SXT) susceptibility

Streptococcal groups A and B can be separated from groups C, F, and G on the basis of the susceptibility of beta-hemolytic streptococci to SXT. This test is performed just like the bacitracin disk testing. The SXT disk that is used for antibiotic testing is placed on the streaked surface of a blood agar plate and incubated overnight at 35°C. Groups A and B streptococci are resistant to SXT; whereas, other beta-hemolytic streptococci are susceptible.

Sodium hippurate hydrolysis

Hydrolysis of sodium hippurate is used for the presumptive identification of group B streptococci and the separation of some of the viridans streptococci. The test is based on the ability of bacteria to produce the enzyme hippuricase to hydrolyze a 1% aqueous solution of sodium hippurate to benzoic acid and glycine. Either of these by-products of hydrolysis can then be detected. Ferric chloride is

used to detect benzoic acid within 18 to 24 hours, and ninhydrin reagent can detect glycine within four hours. The ninhydrin glycine reaction is the more sensitive of the two reactions. A large loopful of organism is suspended in the test solution and incubated at 35°C for two to four hours. Ninhydrin reagent is then added and the test is read after an additional incubation of 10 to 30 minutes. The development of a deep purple color is interpreted as a positive reaction; whereas, colorless or faintly purple reactions are considered negative. Some group D streptococci, including enterococci, may also react positively. The bile esculin test, that is discussed shortly, can be used to differentiate group B and D streptococci. Group D streptococci, including enterococci, are bile-esculin positive while group B streptococci are negative.

L-pyrrolidonyl-β-naphthalamide (PYR) test

This test uses an enterococcal-selective media containing PYR. The test measures the production of free β-naphthalamide by beta-hemolytic group A streptococcus and enterococcal organisms that are capable of hydrolyzing L-pyrrolidonyl-β-naphthalamide. Free β-naphthalamide is detected by the addition of a reagent that consists of an acid solution of N, N-dimethylamino-cinnamaldehyde. A positive test is indicated by the formation of a cherry-red color within two minutes. Beta-hemolytic streptococci, other than group A, are negative as are nonenterococcal and viridans streptococci. Rapid disk PYR test and other methods that determine the activity of pyrrolidonyl arylamidase are commercially available. Pyrrolidonyl arylamidase is an enzyme produced by *S. pyogenes* but not by the other beta-hemolytic streptococci.

Voges-Proskauer (VP) test

The VP test for acetoin production can be used to differentiate the small-colony-forming β-hemolytic “*S. milleri*” group strains from the larger-colony-forming pyogenic strains with the same Lancefield antigens. Various versions of the test are commercially available.

CAMP test

Like the sodium hippurate hydrolysis test, the CAMP test is used for the presumptive identification of group B streptococci. The test is based on the production by group B streptococci of the CAMP factor, an extracellular substance produced by group B streptococci that enhances the action of β-hemolysin produced by staphylococcal cells. The test is performed by placing a single streak of American Type Culture Collection #25923 *S. aureus* on a blood agar plate and then streaking the streptococcal isolate perpendicular to it but not touching the staphylococcal inoculate. The plate is incubated overnight at 35°C. A positive CAMP test is observed as an arrowhead-shaped zone of enhanced beta hemolysis at the junction of the two streaks. The CAMP test plate should not be incubated anaerobically because some strains of group A streptococci produce positive CAMP reactions in the absence of oxygen.

The following table is used by permission from *Manual of Clinical Microbiology* and Kathryn Ruoff. Differentiation tests for β-hemolytic streptococci are shown.

Lancefield group	Colony size	Species	PYR	VP	CAMP ^a	Trehalose ^c	Sorbitol ^f
A	Large	<i>S. pyogenes</i>	+	–	–		
A	Small	“ <i>S. milleri</i> ” group	–	+	–		
B		<i>S. agalactiae</i>	–	–	+		
C	Large	<i>S. equi</i> ^b	–	–	–	–	–
		<i>S. equisimilis</i> ^b	–	–	–	+	–
		<i>S. zooepidemicus</i> ^b	–	–	–	–	+
C	Small	“ <i>S. milleri</i> ”	–	+	–		
F	Small	“ <i>S. milleri</i> ” group	–	+	–		

Lancefield group	Colony size	Species	PYR	VP	CAMP^a	Trehalose^c	Sorbitol^c
G	Large ^b		–	–	–		
G	Small	" <i>S. milleri</i> " group	–	+	–		
Non-groupable	Small	" <i>S. milleri</i> " group	–	+	–		

^aTest for synergistic hemolysis.
^bTraditional classification of large-colony group C is presented here.
^cThese carbohydrate fermentation tests are used to differentiate the traditional large-colony group C species.

Optochin disk sensitivity test

Most strains of pneumococcus are sensitive to ethylhydroxycupreine hydrochloride (optochin); whereas, most strains of alpha-hemolytic streptococci are not. Optochin destroys the integrity of the pneumococcal cell membrane causing cell death. This test is the most widely used test for differentiating pneumococci from other alpha streptococci. The 6 mm optochin disks are placed on the heavily inoculated area of a blood agar plate and incubated at 35°C for 18 to 24 hours in a candle jar or CO₂ incubator. A clear zone of ≥ 14 mm indicates a positive test.

Bile solubility test

Bile salts such as sodium desoxycholate, considered a surface-active agent, acts on the cell wall of pneumococci and results in lysis of the cell. For the test, use a 24- to 48-hour old pure culture broth. Prepare two tubes each containing a light suspension (0.5 to 1.0 McFarland standard) of the sample in buffered broth, pH 7.4. To one tube, add a few drops of 10% solution of sodium desoxycholate and comparable volume of sterile physiological saline solution to the second tube. If the cells are "bile soluble", the tube containing the bile salt should lose its turbidity in five to 15 minutes, showing an increase in viscosity along with the clearing.

Bile esculin hydrolysis

The hydrolysis of the glycoside esculin to its product esculetin in the presence of 40% bile is another test used in the presumptive identification of group D streptococci. Bile esculin agar containing ferric citrate as an indicator of esculetin production is inoculated and incubated 18 to 24 hours at 35°C. A positive test is indicated by a dark brown to black discoloration of the media.

The table below is used by permission from *Manual of Clinical Microbiology* and Kathryn Ruoff. Differentiation of nonbeta-hemolytic streptococci^a is reviewed.

Type or species of streptococcus	Optochin	Bile solubility	Bile esculin	Satelliting behavior
<i>S. pneumoniae</i> ^b	+	+	–	–
Viridans	–	–	–	–
<i>S. bovis</i>	–	–	+	–
Nutritionally variant ^c				+

^aStrains of group B streptococci may be nonbeta-hemolytic. Serological methods or the CAMP test should be used to rule out possible group B strains.
^bStrains may be PYR positive.
^cPYR positive.

Commercial identification systems

There are several commercial kits and automated instruments available through various manufacturers for the identification of *Streptococcus* species. The kits and instruments use a variety of methods—conventional biochemical test or modified carbohydrate fermentation tests and fluorogenic or chromogenic enzyme substrate tests—that are adapted to the manufacturer's specific format. Strips

with small cupulae, impregnated filter paper disks, microtiter trays, or plastic cards are format examples. These products should include the key tests or the identification could be questionable with regard to certain species. Examples of commercial kits and instruments available are API Rapid Strep System; Vitek and Vitek Jr. fully automated microbiology systems that utilize a GPI Card (bioMérieux Vitek); MicroScan Pos ID panel read manually or by MicroScan instruments (Baxter Diagnostics, Inc., MicroScan Division); and AccuProbe (Gen-Probe, Inc.) that uses DNA probe technology for the rapid detection of group A streptococci, group B streptococcus, and *S. pneumoniae* from cultures. Although these kits are available, they are not as reliable for identification of streptococcal, enterococcal, and other rare gram-positive cocci except for DNA techniques. The above biochemical methods are the most reliable and most often used. Latex agglutination procedures are used almost exclusively for the identification of beta-hemolytic streptococci. Next, you'll study another important gram-positive cocci, *Enterococcus* species.

***Enterococcus* species**

The genetic evidence that *Streptococcus faecalis* and *Streptococcus faecium* were significantly different from the other members of the genus *Streptococcus* merited a separate genus. The genus *Enterococcus* was proposed in 1984 and to date has been generally accepted. *S. faecalis* and *S. faecium* were renamed to *Enterococcus faecalis* and *E. faecium* in 1984; since then, numerous other species have been proposed. The most commonly isolated *Enterococcus* is *E. faecalis* (80 to 90% of the isolates) with *E. faecium* (5 to 10%) ranking second.

General characteristics

Enterococci are gram-positive cocci seen singular, in pairs, or in short chains and are generally catalase-negative. Some strains of enterococci, especially *E. faecalis*, produce a "pseudocatalase" that is responsible for weak-positive catalase reactions usually from primary isolation. When Gram-staining from agar plates, the cells may appear coccobacillary. They are more oval and in-chains appearing when Gram-stains are from thioglycolate broth. They are facultative anaerobes and optimum growth is at 35°C, although most strains can grow at 10 and 45°C. Some strains are motile, and the majority possess the group D teichoic acid antigen that is identified as the streptococcal group D antigen and can cause false positive results with some serological procedures. Additional testing may need to be accomplished in order to distinguish between the two organisms.

Clinical significance

Enterococci are normal flora in the gastrointestinal tract of humans and animals, and can be found in soil, water, birds, insects, and food. They are becoming more important as agents of human disease because of their resistance to antimicrobial agents. *Enterococcus* species are often found to be the causative agents of urinary tract, intra-abdominal, soft tissue, and wound infections. They are the third most common etiological agent of nosocomial bacteremia behind *S. aureus* and CoNS in the United States. They are second only to *E. coli* as agents of nosocomial urinary tract infections. These infections occur in elderly patients with underlying medical problems, immunocompromised patients, and those who have received antimicrobial therapy. Endocarditis caused by enterococci is also serious but less common than enterococcal bacteremia.

Laboratory identification

Laboratory identification for *Enterococcus* species include colony morphology, catalase test, bile esculin hydrolysis test, ability to grow in the presence of 6.5% sodium chloride, and the various manual, semi-automated, and automated methods. Species identification is based on the formation of acid in carbohydrate broths; arginine hydrolysis; ability to tolerate tellurite, and utilization of pyruvate, motility test, and pigmentation.

Colony morphology

Colonies are nonbeta-hemolytic, 0.5 to 1 mm in diameter, circular with an entire edge, convex, smooth, transparent to translucent, and gray or slightly milky in color. On rabbit or horse blood agars, some strains of *E. faecalis* are beta-hemolytic but are nonbeta-hemolytic on sheep blood agar.

Tolerance to 6.5% sodium chloride

Used in conjunction with the bile esculin hydrolysis test (see above), the 6.5% NaCl test can help differentiate enterococcal and nonenterococcal streptococci. All enterococci are capable of hydrolyzing esculin in the presence of 40% bile salts, growing in media containing a concentration of 6.5% NaCl, and most are penicillin resistant; whereas, nonenterococcal streptococci are sensitive to penicillin and cannot tolerate 6.5% NaCl. A liquid media, such as Tryptic soy broth, Todd-Hewitt broth, or brain heart infusion supplemented with NaCl to a concentration of 6.5%, is suitable for this procedure. Once inoculated, tubes of media are incubated up to 48 hours at 35°C; a positive test is indicated by turbidity within the tube.

Other *Enterococcus* species are *E. avium*, *E. malodoratus*, *E. raffinosus*, *E. pseudoavium*, *E. casseliflavus*, *E. mundtii*, *E. flavescens*, *E. gallinarum*, *E. durans*, *E. hirae*, *E. dispar*, *E. faecalis* (var.), and *E. sulfureus*. The differentiation of these species includes the acid formation from mannitol, sorbitol, sorbose, sucrose, ribose, arabinose, and raffinose; hydrolysis of arginine; ability to tolerate tellurite and utilize pyruvate, motility test, and production or nonproduction of a yellow pigment.

Self-Test Questions

After you complete these questions, you may check your answers at the end of the unit.

202. *Streptococcus* and *Enterococcus* species

- Match each of the given systems for classification of the streptococci, the hemolytic reactions on blood agar, and related characteristics in column B with the statements in column A by placing the letter of the column B item beside the number of the column A item that most nearly describes it. Each element in column B may be used once, more than once, or not at all.

Column A

- Based on the reaction of C substances (antigenic material) found on the cell walls of various streptococci and specific antisera.
- System with the greatest impact on the classification of streptococci in the clinical laboratory.
- Based on the hemolytic activity of streptococci on red blood cells.
- An indistinct zone of partially lysed red blood cells, often accompanied by a greenish to brownish discoloration, surrounds the colony.
- A clear, colorless zone around the streptococci colonies, in which the red blood cells have undergone complete discoloration.
- Produces acute rheumatic fever and post-streptococcal glomerulonephritis.
- Is oxygen labile.
- Is oxygen stable.

Column B

- Alpha-prime or Wide Zone Alpha.
- Beta.
- Alpha.
- Gamma.
- The Sherman classification.
- Streptolysin O.
- Viridans streptococci.
- The Lancefield classification.
- Streptolysin S.
- The Brown classification.
- S. pyogenes*.
- S. agalactiae*.
- S. pneumoniae*.
- S. bovis*.

- ___ 9. The most common cause of subacute bacterial endocarditis.
- ___ 10. No apparent hemolytic activity or discoloration produced by the colony.
- ___ 11. This reaction can be confused with a beta hemolytic activity colony when surface growth is observed.
- ___ 12. A small halo or envelope of intact or partially lysed red blood cells lying adjacent to the bacterial colony with a zone of complete hemolysis extending further out into the medium.
- ___ 13. Designations of four physiological divisions (pyogenic, viridans, lactic, and enterococci).
- ___ 14. The greatest threat to neonates, especially premature infants born to infected mothers.
- ___ 15. Third most common cause of meningitis.

2. Match each test in column B with the statements in column A by placing the letter of the column B item beside the number of the column A item that most nearly describes it. Each element in column B may be used once, more than once, or not at all.

Column A

- ___ 1. Sodium desoxycholate acts upon the cell wall of pneumococci and results in lysis of the cell.
- ___ 2. Starting point in the identification of streptococci.
- ___ 3. Method most commonly used for the presumptive identification of group A streptococci.
- ___ 4. Any zone of inhibition, regardless of diameter, is positive.
- ___ 5. Used to detect glycine in the sodium hippurate hydrolysis test.
- ___ 6. Extra cellular substance produced by group B streptococci.
- ___ 7. Positive test observed as an arrowhead-shaped zone of enhanced hemolysis.
- ___ 8. Basis of this test is the susceptibility of beta-hemolytic group A and B streptococci to trimethoprim-sulfamethoxazole.
- ___ 9. Ferric citrate acts as an indicator in this test.
- ___ 10. Positive test indicated by turbidity within the tube.
- ___ 11. Free β -O detected.
- ___ 12. A positive test indicated by a clear zone of ≥ 14 mm.

Column B

- a. Optochin disk sensitivity test.
- b. Bile solubility test.
- c. Camp test.
- d. Bacitracin disk test.
- e. Hemolysis and morphology on blood agar.
- f. Ninhydrin reagent.
- g. Camp factor.
- h. SXT susceptibility.
- i. Bile esculin hydrolysis.
- j. Tolerance to 6.5% NaCl.
- k. PYR test.

203. Miscellaneous gram-positive cocci

In this lesson, we'll discuss various organisms that are taxonomically diverse and are infrequent or rare clinical isolates that act as opportunistic pathogens. These bacteria are normally only pathogenic in the compromised host and appear to be of low virulence. They have been misidentified as staphylococci, streptococci, and enterococci. These organisms include, but are not limited to, *Aerococcus*, *Gemella*, *Leuconostoc*, *Pediococcus*, and *Stomatococcus* species.

***Aerococcus* species**

Aerococcus species include *A. viridans* and *A. urinae* that have been isolated from the urinary tract.

General characteristics

Aerococcus species are gram-positive cocci in pairs and tetrads in broth medium. They can be catalase-negative or produce a "pseudocatalase" that is responsible for weak-positive catalase reactions. They are microaerophilic and have sparse or no growth under anaerobic conditions.

Clinical significance

Aerococci are found in the environment, are usually associated with water, and can be found on human skin. At times they appear to be contaminants in clinical cultures; however, they have been associated with endocarditis, bacteremia, wound, and urinary tract infections.

Laboratory identification

Aerococcus colonies are circular, convex, gray-white in color, and may be mistaken for streptococci. *Aerococcus viridans* produce a zone of alpha hemolysis as the name indicates. *Aerococcus* species show a zone of inhibition to 30 µm vancomycin disk that indicates they are susceptible to vancomycin. They are negative for gas production from glucose in sealed MRS (de Man, Rogosa, and Sharpe) broth, positive for growth in 6.5% NaCl, and variable for esculin hydrolysis. *A. urinae* is PYR negative and LAP positive, and *A. viridans* PYR positive and LAP negative. The LAP test detects the presence of the enzyme leucine aminopeptidase (LAP).

***Gemella* species**

Gemella haemolysans and *G. morbillorum* are in the genus *Gemella*. *G. morbillorum* was first isolated from blood cultures of patients with the measles; hence, the original name was *Diplococcus rubeolae*. It has also been called *Diplococcus morbillorum*, *Peptostreptococcus morbillorum*, and *Streptococcus morbillorum*. In 1988, it was renamed into the genus *Gemella*; also included is *G. haemolysans* that was originally classified as *Neisseria* species because of its gram-variable and sometimes gram-negative staining reaction and cellular morphology of flattened adjacent sides. Cell wall studies demonstrated that the organisms had a gram-positive type cell wall that was thinner than the other gram-positive organisms. This could explain the gram-variable staining reactions. Also, DNA analysis showed no relatedness to the *Neisseria* species.

General characteristics

Gemella, like *Aerococcus*, resemble streptococci on gross inspection of colonies, but Gram-staining from broth reveals cocci in pairs, tetrads, or clusters similar to staphylococci. They are catalase negative and resemble viridans streptococci on blood agar; although *G. haemolysans* can produce beta-hemolysis on rabbit or horse blood agars. *G. haemolysans* grow poorly under anaerobic conditions, but *G. morbillorum* flourishes under anaerobic conditions.

Clinical significance

G. haemolysans can be found in the upper respiratory tract as normal flora and *G. morbillorum* is found in the human respiratory and gastrointestinal tracts. They have been isolated from blood,

wounds, abscesses, and urine specimens. They have also been involved in endocarditis and meningitis.

Laboratory identification

Gemella species are vancomycin susceptible, esculin hydrolysis negative, *nongrowing* in 6.5% NaCl, variable for LAP, and PYR positive.

***Leuconostoc* species**

Leuconostoc genus contains four species at this time; however, the taxonomy of *Leuconostoc* is in a state of flux. For this study, we'll look at the genus and not the individual species.

General characteristics

Leuconostoc species are nonmotile, nonspore-forming, gram-positive cocci that appear in pairs, chains, and as coccobacilli. They are facultatively anaerobic and catalase negative.

Clinical significance

These organisms are found in the environment on plants and in soil; they have economic importance because of their use in winemaking, dairy, and pickling industries. In immunocompromised hosts, they have been associated with bacteremia, meningitis, intra-abdominal abscesses, fistulas, gastrostomy and tracheostomy site infections, and urinary tract infections. *Leuconostoc* have been isolated from blood cultures of neonates suggesting that the infants may have been infected during delivery by *Leuconostoc* in the birth canal.

Laboratory identification

Leuconostoc species also resemble streptococci morphologically and biochemically. They produce variable esculin hydrolysis results and growth in 6.5% NaCl is variable; however, they are resistant to vancomycin; whereas, streptococci are susceptible. The *Leuconostoc* are PYR and LAP negative, but positive for gas production from glucose in MRS broth.

***Pediococcus* species**

Seven species are in the genus *Pediococcus*. *P. acidilactici* is the most common isolate in this genus.

General characteristics

Predominately gram-positive cocci (occasionally coccobacilli) that appear in pairs, tetrads, or clusters, *Pediococcus* species are nonmotile and nonspore forming. They are facultatively anaerobic and catalase negative.

Clinical significance

Pediococcus species are found naturally on plants and are important in food and brewing industries for processing and preservation. They can be found in sausage, cheese, beers, and ales. They are also used as flavor enhancers in processed vegetables and soy products. Recently, they have been recovered from blood cultures from severely compromised patients and are thought to be opportunistic organisms. They have also been isolated from saliva, urine, stool, wounds, and abscesses in patients with various underlying conditions such as diabetes, chronic lung disease, cardiovascular disease, and malignancy.

Laboratory identification

Their biochemical reactions are identical to the *Leuconostoc* with regards to vancomycin resistance, esculin hydrolysis, growth in 6.5% NaCl, and PYR results. The only difference is that *Pediococcus* is LAP positive and negative for gas production from glucose in MRS broth.

***Stomatococcus* species**

As mentioned earlier, *Stomatococcus* species are classified with *Micrococcus* and *Staphylococcus* species in the family *Micrococcaceae*. *S. mucilaginosus* is the most common isolate in this genus.

General characteristics

Stomatococcus species are facultative, encapsulated, large gram-positive cocci arranged in pairs or clusters, with strong or weak catalase positive reactions, and with some strains as catalase negative. Colonies are generally mucoid, clear to white, and adherent to the agar surface and sometimes known as “sticky staph”.

Clinical significance

Stomatococcus is part of the normal human upper respiratory tract. *Stomatococcus* is associated with bacteremia, endocarditis, meningitis, peritonitis, and intravascular catheter infections. It has also been noted in patients with a serious underlying disease, neutropenia, cardiac-valve disease, the presence of a foreign body, intravenous drug usage, and those with destroyed oral mucous membranes due to chemo- or radiotherapy.

Laboratory identification

Stomatococcus are PYR positive (most strains), vancomycin susceptible, LAP and esculin hydrolysis positive, and negative for gas production from glucose in MRS broth and growth in 6.5% NaCl. They are also included in the database of Staph-TRAC, StaphIDENT, and ID32 STAPH systems. Although these kits are available, they are not as reliable for identification as the biochemical methods.

Self-Test Questions

After you complete these questions, you may check your answers at the end of the unit.

203. Miscellaneous gram-positive cocci

1. Match the organisms in column B with the statements in column A by placing the letter of the column B item beside the number in column A item that best describes it. Each element in column B may be used once, more than once, or not at all.

Column A

- ____ (1) Was first isolated from blood cultures of patients with the measles.
- ____ (2) Classified with *Staphylococcus* and *Micrococcus*.
- ____ (3) Was originally classified as a *Neisseria*.
- ____ (4) A new species that has been isolated from the urinary tract.
- ____ (5) A “pseudocatalase” is produced.
- ____ (6) Isolated from blood cultures from neonates suggesting they are infected during delivery.
- ____ (7) They are used as flavor enhancers in processed vegetables and soy products.
- ____ (8) Sometimes known as the “sticky staph”.

Column B

- a. *Aerococcus viridans*.
- b. *Aerococcus urinae*.
- c. *Gemella haemolysans*.
- d. *Gemella morbillorum*.
- e. *Leuconostoc* species.
- f. *Pediococcus* species.
- g. *Stomatococcus* species.

1-2. Gram-Positive Bacilli

This group of aerobic, facultatively anaerobic, and anaerobic, gram-positive, rod-shaped bacteria is taxonomically heterogeneous, and its lines of relatedness are not clearly defined at present, but progress is being made. With recent DNA and rRNA studies, taxonomic changes have occurred in the gram-positive rods or bacilli. The gram-positive bacilli can be divided into different groups based on endospore-forming, nonendospore-forming, morphologically regular or straight rods, and morphologically irregular or coryneform (“club foot” or “Chinese letter”) bacilli. When you encounter an organism with such morphology, the genera that need to be considered are *Bacillus*, *Corynebacterium*, *Listeria*, *Erysipelothrix*, *Lactobacillus*, and, if the organism is cultured anaerobically, *Clostridium*. Selected members of all of these genera are known pathogens. In this section, we’ll discuss the aerobic and facultatively anaerobic gram-positive rods or bacilli; anaerobic gram-positive bacilli are discussed in unit 4.

204. *Bacillus* and *Corynebacterium* species

The most common hospital laboratory isolated aerobic or facultatively anaerobic gram-positive bacilli are of the genera *Bacillus* and *Corynebacterium*. The gram-positive-bacilli virulence is highly variable, ranging from *Bacillus anthracis*—one of the most highly pathogenic microorganisms—to *Corynebacterium* (except *C. diphtheriae*)—common laboratory contaminants.

Bacillus species

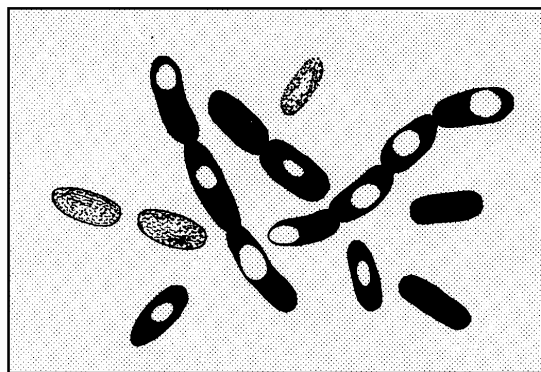
Bacillus species are in the endospore-forming group. The genus *Bacillus* contains over 60 species, 51 substantially described species, and many other species of uncertain taxonomic status. The genus *Bacillus* is in the family *Bacillaceae* that comprises a highly diverse group of aerobic and anaerobic endospore-forming bacteria. The two principle genera are *Bacillus* and *Clostridium*.

General characteristics

Members of the genus *Bacillus* are catalase-positive, large, gram-positive, aerobic, endospore-forming rods or bacilli usually occurring in chains, but sometimes singly, as shown in figure 1-1. Individual cells range between 1 to 3 μm in width and 5 to 10 μm in length. *Bacillus anthracis* is encapsulated and nonmotile, but the many saprophytic forms lack capsules and are usually actively motile; however, the extent of motility is variable. Most species are catalase-positive. The encapsulated cells of *B. anthracis* found in direct smears of clinical specimens are rarely observed in smears from laboratory cultures, unless grown on sodium bicarbonate medium with increased CO_2 .

Most bacilli appear as long, regular or straight-sided rods with rounded ends. The cells of *B. anthracis* are usually described as straight-sided rods with square ends when observed on direct smears. The other members of the genus *Bacillus* may produce endospores that are

round, oval, cylindrical, or occasionally kidney shaped. In Gram-stains of *B. anthracis* taken from routine culture media, such as blood agar, the cells are described as being straight-sided bacilli with pointed ends often containing spores. When the cells are arranged in chains, the pointed ends adjacent cells form a small, oval-shaped empty area between cells. These cellular configurations are referred to as bamboo-pole formations. The spores usually are elliptical and occur in the center of the cell. The spores may be the same size as the diameter of the vegetative wall and not distort the bacilli, or they may be large enough to distort it. On Gram-stains, the spores do not stain well and appear as clear



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Figure 1-1. Microscopic morphology of *Bacillus anthracis*.

areas within the cell. When stained with spore stains, they appear green. *B. anthracis* does not sporulate in living tissue, but highly resistant spores that can remain viable for decades are formed when the organism is exposed to the external environment.

Clinical significance

Bacillus species are mostly saprophytes found throughout nature, especially in soil, airborne dust, water, and on materials of plant and animal origins. Because of their spore forms, they can survive environmental extremes. *Bacillus* species have a wide range of facultative variants, such as mesophilic, facultative and obligate thermophiles; psychrophiles; acidophiles; and halophiles. They are usually considered contaminants, normal flora, or representatives of superficial contamination in single specimens. Interpretation, to say the least, is difficult; therefore, repeat specimens and clinical symptoms should be considered.

Bacillus anthracis, the chief human pathogen in the genus *Bacillus*, is rarely encountered in the average hospital or public health laboratory in the United States. Anthrax is primarily a disease of herbivorous animals incurred during grazing on pastures contaminated by anthrax spores. Viable spores germinate in the intestinal tract or buccal mucosa, and the bacilli are disseminated via the lymphatics to the bloodstream and deeper tissues. Infections in humans are usually of animal origin occurring in butchers, herdsman, wool-handlers, tanners, and other occupational groups dealing with infected animals or their products.

How Bacillus enters the body

The organisms may enter through the skin, the respiratory tract, or the intestinal mucosa. Cutaneous anthrax, the most common form of human infection, results from direct contact with infected tissue, hides, hairs, bones, or bristles. Skin lesions or papule may liberate bacilli resulting in septicemia and widespread involvement of internal organs. Primary pulmonary anthrax arises from inhalation of spores or disseminated into the air during the processing of infected materials, especially animal hides and fleece. Symptoms of pneumonia often progress to a fatal septicemia in untreated cases. Intestinal anthrax results from consumption of insufficiently cooked meat from infected animals or from ingestion of food contaminated with spores. Pulmonary anthrax and infections of the intestinal tract are very rare in humans, but the latter are the most common form of the disease in animals.

Potent exotoxins

Bacillus anthracis produces two potent exotoxins: (1) lethal factor (LF) and (2) edema factor (EF). Both of these factors are dependent upon a third factor, protective antigen (PA) for their biological activity. PA appears to control the ability of LF and EF to pass through the cell membranes of infected tissues. In combination, LF and EF act to further the infectious process by increasing host susceptibility to infection by suppressing the production of polymorphonuclear neutrophil function. *B. cereus* produces an enterotoxin complex responsible for the diarrheal type of food poisoning.

The vegetative cells of *Bacillus* species are no more resistant to disinfectants or heat than other bacteria, but the spores are highly resistant. Anthrax spores have been known to survive for decades in soil. The spores ordinarily survive boiling for several minutes, and exposure to most disinfectants must be prolonged to be effective. Standard sterilization temperatures and the usual periods of heating in the autoclave successfully destroy all bacillus spores.

In the past, *B. anthracis* was considered the only significant pathogen in the genus *Bacillus*, but other members of the genus were suspect. Suspicion that other members of the genus were potential pathogens has been proven to be well founded. *Bacillus cereus* is now the most frequently isolated pathogenic member of the genus, having been linked to food poisoning. *B. cereus* is the etiologic agent of two different types of food poisoning syndromes: (1) the emetic type, that causes nausea and vomiting and (2) the diarrheal type. Both types are due to the fact that the spores can survive cooking procedures, then with improper storage the spores germinate. At this time no toxins or other virulence

factors have been identified for *B. cereus* or the other *Bacillus* species, although, the toxins of *B. anthracis* are well characterized. Other members of the genus such as *B. subtilis*, *B. pumilus*, *B. alvei*, *B. brevis*, *B. circulans*, *B. coagulans*, *B. licheniformis*, *B. macerans*, *B. thuringiensis*, and *B. sphaericus*, have been known to be opportunistic pathogens associated with endocarditis, bacteremia, abscesses, wound infections (necrotic or gangrenous), osteomyelitis, empyema, meningitis, peritonitis, eye and ear infections, and pneumonia in humans.

Laboratory identification

The laboratory identification of *Bacillus* species is based on colony morphology, hemolytic reaction on SBAP, spore characteristics, inhibition of growth by various substances, and the biochemical characteristics of the isolate.

Colony morphology

The organisms grow well on most common laboratory media, but in order to demonstrate characteristic colony morphology, inoculate the specimens on 5% sheep blood agar. The optimal temperature for maximum growth is 37°C. Within 18 to 24 hours the anthrax bacilli produce colonies that are 3 to 5 mm in diameter, gray-white, opaque, raised with irregular margin and having a dull, frosted-glass appearance. *B. cereus* colonies range from small, shiny, and compact to large, spreading, feathery types. The other species have round or irregular, raised or flat, usually large colonies with entire to undulate or rhizoid edges. They have a frosted-glass appearance initially but may become opaque and have a variety of colors. Some may produce smooth or mucoid colonies on blood agar. Hemolysis is an important basis for differentiation. Anthrax colonies are nonhemolytic on blood agar, while a definite zone of alpha- or beta-hemolysis usually surrounds *cereus* and other saprophytic forms.

Staining

When performing the microscopic examination of specimens from a suspected case of anthrax, one air-dried, heat-fixed smear from the patient must be Gram-stained. Reserve the second for fluorescent-antibody staining. Perform additional stains for spores and capsules with organisms isolated from cultures. When cultures are collected after initiation of antibiotic therapy, the fluorescent antibody technique may prove extremely important. If the organisms are to be examined by this technique, they must be grown on sodium bicarbonate media under a CO₂ atmosphere to allow capsules to develop.

Other key tests for identification

Motility testing is useful, although this property is variable to some extent among the saprophytic species. The test may be performed on a hanging drop or by inoculating a motility medium and incubating for four days at 37°C. *B. anthracis* is nonmotile while 99% of *B. cereus* strains are motile, as are most of the other *Bacillus* species. Additional tests include growth under anaerobic conditions, at various temperatures, in 7% NaCl, and at different pH levels; Voges-Proskauer (V-P) reaction; production of acid from different sugars; starch hydrolysis; citrate utilization; nitrate reduction; and casein and tyrosine decomposition. The serological test for identification of *Bacillus* species has been plagued by problems of cross-reacting antigens and autoagglutination because of hydrophobic surface properties of the spores. Toxin and antitoxin detection procedures utilizing enzyme immunoassay systems are being perfected for use in identification of *B. anthracis* and *B. cereus*.

***Corynebacterium* species**

Current literature describes over 30 species or groups of corynebacteria and other coryneform organisms isolated from humans and animals. The classification of the coryneform bacteria is an ongoing process and no doubt taxonomic changes occur in this genus as the powerful tools of molecular biology continue to increase our knowledge. Initially, inclusion in the genus *Corynebacterium* is based on morphological features. This classification criteria resulted in a

heterogeneous collection of morphologically similar organisms comprised of three major groups—human and animal pathogens, plant pathogens, and saprophytic species. Organisms with *Corynebacterium*-like characteristics: pleomorphic (irregular), gram-positive, nonspore-forming, and nonacid fast bacilli were all lumped together under the general heading of “coryneform” or diphtheroid organisms. As stated earlier, most corynebacteria are opportunistic pathogens or contaminants, except for *C. diphtheriae*, the causative agent of diphtheria that can be fatal.

General characteristics

The corynebacteria are slender, gram-positive rods measuring from 1 to 6 μm in length and 0.3 to 0.8 μm in width. They usually exhibit considerable pleomorphism; however, corynebacteria do not branch and are not acid fast. In addition to straight or slightly curved rods, club- or dumbbell-shaped forms are common. The diversity of shapes stems from the fact that the cell wall of some species is weaker at the ends, allowing the organism to assume club and various other shapes, and suggesting the genus name (coryne means “club” in Greek). Many of these organisms also contain metachromatic granules especially when grown on Loeffler’s slant or Pai’s media. In smears stained with Loeffler’s alkaline methylene blue and similar dyes, the metachromatic granules appear as deeply stained bodies against a lighter cytoplasm. This contrast gives the cell a banded, barred, or beaded appearance. Corynebacteria are characteristically arranged in palisades, but L-, V-, or Y-shaped forms may also occur. Microscopic groupings have been compared to “Chinese letters” or “piles of matches”. Although the appearance of *C. diphtheriae* in stained smears is highly characteristic, it should not be identified by morphology alone because many diphtheroids and actinomycetes stain in the same irregular fashion and are pleomorphic. Confirmation requires that *C. diphtheriae* be isolated in pure culture and identified by means of cultural, biochemical, and toxigenicity tests.

Clinical significance

Corynebacterium species can be found in nature in soil, in water, and on plants. They can reside on the skin and mucous membranes and as normal flora in the gastrointestinal tract of humans, mammals, and animals. *Corynebacterium* species that are associated with human disease or infections, especially in the immunocompromised host, are reviewed in the following table.

Organism	Disease	Comments
<i>C. diphtheriae</i>	Diphtheria, septicemia, endocarditis, pharyngitis	Exotoxin mediated disease. A vaccination (DPT) does exist.
<i>C. ulcerans</i>	Pharyngitis (mild diphtheria-like disease)	Can be considered in the <i>C. diphtheriae</i> group.
<i>C. jeikeium</i> (formerly CDC group JK)	Septicemia, endocarditis, skin and soft tissue, rarely; meningitis, peritonitis, and pneumonia	Most common corynebacterial pathogen isolated in the laboratory.
<i>C. urealyticum</i> (formerly CDC group D2)	Urinary tract infections; rarely, endocarditis and wound infections	Can be isolated in urine cultures from asymptomatic patients.
<i>C. pseudodiphtheriticum</i>	Endocarditis, pneumonia, lung abscess	Usually seen in the immunocompromised host.
<i>C. minutissimum</i>	Septicemia, skin graft infections	Usually seen in the immunocompromised host.
<i>C. xerosis</i>	Septicemia, endocarditis, pneumonia	Usually seen in the immunocompromised host.
<i>C. striatum</i>	Pneumonia and lung abscess	Usually seen in the immunocompromised host.
<i>C. pseudotuberculosis</i>	Human necrotizing lymphadenitis	Usually found in sheep, goats, and horses, where it can cause horse abortion and abscesses.

Because of its clinical significance, *Corynebacterium diphtheriae* is discussed in detail here and in laboratory identification. *C. diphtheriae* is found in the upper respiratory tract of infected individuals and asymptomatic carriers. The organism is rarely isolated from the skin or wounds but cutaneous diphtheria does occur in the northwestern United States. The bacilli are spread by nasal or oral droplets, or by direct contact. The virulent bacilli invade the mucous membranes, multiply rapidly, and produce a powerful exotoxin—one of the most well studied bacterial toxins. Absorption of the toxin by the mucous membranes yields an acute inflammatory response and destruction of the epithelium. The accumulation of fibrin, red blood cells, and white blood cells results in the formation of a gray, clotted film or pseudomembrane that covers the tonsils, pharynx, or larynx. As the disease progresses, the toxin is absorbed by other tissues. The organisms remain localized in the upper respiratory tract. In clinically typical diphtheria, lesions and pseudomembranes in the throat usually yield large numbers of the characteristic bacilli upon direct microscopic examination of smears. It is the exotoxin, disseminated by the blood to deeper tissues that accounts for the symptoms of systemic involvement.

Laboratory identification

Most *Corynebacterium* are catalase positive, nonmotile, do not hydrolyze esculin or gelatin, and are negative for utilization of lactose, xylose, and mannitol. Differentiation of *Corynebacterium* species is in-depth and complex; consult technical references for further study. Let's look at some of the general characteristics and tests for *C. diphtheriae*.

Cultural characteristics and colony morphology

Typical specimens for the isolation of *C. diphtheriae* or other corynebacteria are throat swabs or swabs and aspirated material from wounds or cutaneous lesions collected in such a way as to avoid normal skin flora. Coryneform bacteria are hardy organisms and good growth is usually obtained on blood agar plates that also serve to differentiate corynebacteria from *S. pyogenes* that may mimic early diphtheria. A selective tellurite-containing media such as cystine tellurite agar or Modified Tinsdale should also be used to inhibit normal flora and to demonstrate the typical morphology of *C. diphtheriae*. Both of these tellurite-containing agars have their supporters and detractors. Modified Tinsdale has the advantage of allowing you to detect the production of hydrogen sulfide by the presence of brown to black halos around colonies after 24 to 48 hours of incubation. The following material is quoted by permission from *Listen, Look, and Learn: Self-Study Microbiology Series, Clinical Microbiology*:

“Very few organisms that can grow on this medium produce hydrogen sulfide, so that growth and halos around the colonies are strong presumptive evidence of diphtheria bacilli. The primary disadvantage of Modified Tinsdale is that it has an extremely short shelf life of only four days, and for this and other reasons cystine tellurite, agar or serum tellurite agars are usually preferred for the isolation of *C. diphtheriae*. Typical strains of *C. diphtheriae* can usually be readily distinguished from one another on serum tellurite medium after 48 hours incubation. The distinctive colony morphology of *C. diphtheriae* on Modified Tinsdale medium is a convenient starting point in its identification.”

Only two other species of *Corynebacterium* besides *C. diphtheriae* form a brown halo on this medium—*C. pseudotuberculosis* and *C. ulcerans*, both of which are closely related to *C. diphtheriae* and capable of producing a diphtheriae-like toxin. Although presumptive identification of *C. diphtheriae* can be made based on its colony morphology on selective and differential media and its microscopic morphology, biochemical and toxigenicity tests are still required for confirmation.

Toxigenicity test

Not all strains of *C. diphtheriae* are toxinogenic, and other species besides diphtheriae may produce diphtheriae-like toxins. Therefore, in order to determine and isolate the cause of diphtheriae, you

must demonstrate toxinogenicity. The Elek gel diffusion precipitin test is used for the determination of toxin production. Isolates to be tested are streaked perpendicular to a paper strip impregnated with antitoxin that has been embedded in the agar. Run known positive and negative control organisms with each isolate. The plate is incubated in CO₂ at 35°C for 24 to 48 hours. Figure 1-2 illustrates typical test results, with a positive reaction being marked by the formation of a white precipitate where there is an optimum proportion of antigen (toxin) and antibody (antitoxin). The agar used in this procedure should have a low iron concentration, a characteristic that enhances toxin production.

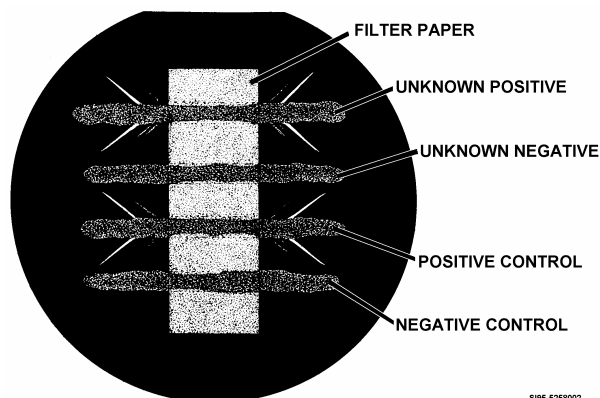


Figure 1-2. Method for in vitro demonstration of toxigenicity of *C. diphtheriae*.

Self-Test Questions

After you complete these questions, you may check your answers at the end of the unit.

204. *Bacillus* and *Corynebacterium* species

1. How does *B. anthracis* compare with the many saprophytic forms in terms of the capsule production and motility?
2. Under what conditions are the encapsulated cells of *B. anthracis* usually found?
3. What is the cellular morphology of most bacilli?
4. How well are spores observed in Gram-stained preparations? If seen, how do they appear?
5. When does *B. anthracis* sporulate in living tissue?
6. What is the chief human pathogen in the genus *Bacillus*? Is this organism commonly encountered in the clinical laboratory?

7. What is the most common form of *B. anthracis* infection in man?
8. What two potent exotoxins are produced by *B. anthracis*?
9. What role does PA play in *B. anthracis* infections?
10. What organism is the most frequently isolated pathogenic member of the *Bacillus* genus and has been linked to food poisoning?
11. What two types of food poisoning does this organism cause?
12. What medium should be inoculated in order to demonstrate characteristic colony morphology of *Bacillus* species?
13. After 18 to 24 hours of incubation, how do typical colonies of the anthrax bacillus appear on sheep blood agar?
14. What is the typical colony morphology of *B. cereus* and the other species after 18 to 24 hours of incubation?
15. What type of hemolysis is produced by the colonies of these organisms?
16. What two staining procedures are initially done on the air-dried, heat-fixed smears from the patient for the microscopic examination?
17. What type of additional stains should be performed with organisms isolated from cultures?
18. If the organisms are to be examined by the fluorescent antibody-technique, they must be grown on what media? Why?

19. What two methods may be used for motility testing of *Bacillus* species?
20. What causes the pleomorphism observed on examining a Gram-stain of corynebacteria?
21. What stain(s) is/are used to demonstrate the characteristic appearance of corynebacteria?
22. With the type of staining indicated in the question above, how do the metachromatic granules appear?
23. Characteristically, corynebacteria are arranged in what morphological form?
24. *C. diphtheria* is most likely to be isolated from what part of the body of infected individuals and asymptomatic carriers?
25. How is *C. diphtheriae* spread?
26. How does this virulent bacilli cause cell destruction?
27. How is the pseudomembrane formed, and what organs are affected?
28. As the disease progresses, what happens to the toxin?
29. In clinically typical diphtheria, what specimens yield large amounts of the characteristic bacilli upon direct microscopic examination of smears?
30. What type of toxin is produced?
31. What are the two selective medias used for *C. diphtheriae* identification?

32. What is the primary disadvantage of Modified Tinsdale agar?
33. What three species of *Corynebacterium* produce halos on Modified Tinsdale agar?
34. What test is used to determine toxin production by *Corynebacterium*?

205. Miscellaneous gram-positive bacilli

For the purpose of this study, the miscellaneous gram-positive bacilli are a group of organisms that can be clinically significant but are usually rare isolates, except for the *Lactobacillus* that are common isolates but rarely cause disease.

***Lactobacillus* species**

The genus *Lactobacillus* consists of numerous species belonging to the family *Lactobacillaceae*. The species in this genus range from facultative to strictly anaerobic organisms with most strains preferring to grow microaerobically.

General characteristics

Lactobacillus species are nonspore forming, long and slender gram-positive rods to short coccobacilli. They'll commonly form chains and occasionally exhibit spiral forms. They are catalase negative, and most species are nonmotile.

Clinical significance

The lactobacilli are widely distributed in nature and are normal flora in the oral cavity, gastrointestinal tract, and female genital tract in humans. They are rarely pathogenic, so species level identification is usually not necessary. However, they have been implicated in clinically significant bacteremia, pneumonia, endocarditis and meningitis.

Laboratory identification

Lactobacillus grows on sheep blood agar and on chocolate media with excellent growth on Rogosa's selective tomato juice media. On blood agar colonies range from pinpoint, alpha-hemolytic to large, rough, gray colonies. They produce large amounts of lactic acid as a byproduct of metabolism that helps maintain the acid pH in which lactobacilli thrive. *Lactobacillus* species are usually identified by Gram-stain, a negative catalase test, and by gas chromatography showing a major lactic-acid peak from glucose.

***Listeria* species**

Listeria is another interesting gram-positive bacilli with its own unique physiological characteristics and a rather obscure epidemiology. Seven species of *Listeria* are listed in *Bergey's Manual of Systematic Bacteriology*: *L. monocytogenes*, *L. innocua*, *L. welshimeri*, *L. seeligeri*, *L. grayi*, *L. ivanovii* and *L. ivanovii* subsp. *londoniensis*. Only the *L. monocytogenes* species of the genus has been clearly linked to pathogenesis in humans, and for this reason we will center our discussion on this species. It is not a common isolate; only a couple of hundred cases are reported annually in the United States. It is estimated that many more cases of listeriosis go undetected in healthy adults in whom flu-like symptoms may occur.

General characteristics

L. monocytogenes are gram-positive, regular, nonspore-forming, short rods with rounded ends. They usually occur singly or in short chains, as indicated in figure 1-3(A) and may resemble diplococci. No capsules or spores are formed. One cell measures 0.5 to 2.0 μm . The bacilli often form “palisades” on Gram-stained smears as shown in figure 1-3(A). Metachromatic granules are not observed. The organisms are actively motile by means of peritrichous flagella. Also, observe in figure 1-3(B) the morphological appearance of *Listeria monocytogenes* after two days of incubation at 37°C. End over end tumbling motility can also be seen in the wet or hanging drop preparations of this organism and can be used as presumptive evidence of its presence.

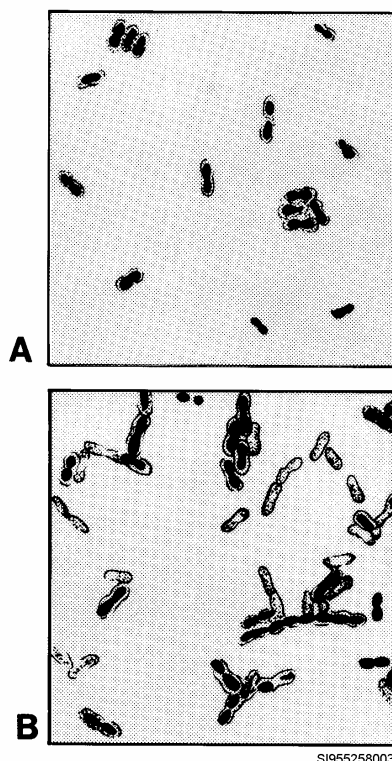


Figure 1-3. Microscopic morphology of *L. monocytogenes* (A) young culture (B) after two days incubation at 37°C.

Clinical significance

L. monocytogenes are widely distributed in nature and in a variety of animal reservoirs including mammals, birds, fish, crustaceans, and insects. It has been isolated from soil and decaying vegetable matter. In nonpregnant humans, it produces infection with a variety of manifestations. Meningitis is the most frequent disease caused by the organism, especially in newborns (\leq one month old) and patients over 50. The pregnant, immunocompromised or immunosuppressed patient, as well as those suffering from major underlying diseases, such as malignancies, endocrine disorders, liver cirrhosis, or cardiac disease are at increased risk from infection with this organism. Both pathogenic and nonpathogenic strains of *L. monocytogenes* abound in nature and can be isolated from humans and animals as well as the environment. Pathogenic and nonpathogenic varieties isolated from disease processes in humans and animals can generally be separated on the basis of hemolysis. Pathogenic strains are generally hemolytic while nonpathogens are not.

Listeriosis infections include, but are not limited to, the following disorders: meningitis, encephalitis, septicemia, vaginal infections, metritis (inflammation of the uterus), and perinatal infection. The perinatal infections may lead to serious consequences for the fetus such as spontaneous abortion, fetal

damage, or meningitis of the newborn. Human infections generally fall into three categories: (1) neonatal sepsis, or meningitis, (2) sepsis or meningitis in immunocompromised patients, and (3) sepsis or nonspecific flu-like illness in healthy women during pregnancy. The epidemiology of listeriosis is poorly understood, but the organism is thought to be ingested rather than inhaled. A 1985 outbreak that claimed more than 30 lives seems to support the theory of ingestion since the deaths were all associated with eating cheese contaminated with *L. monocytogenes*. Since 1981, more than five major epidemics of listeriosis have occurred that were linked to dairy products, cabbage, and no source for one outbreak. Studies have shown raw vegetables, sausages, processed and unprocessed foods, and frozen dairy products can be contaminated with *L. monocytogenes*.

Laboratory identification

Isolation of the organism from cerebrospinal fluid, blood, amniotic fluid, respiratory secretions, placental swabs, gastric aspirate or meconium (material from the intestines of a fetus) can be made primarily by inoculating trypticase soy with 5% sheep, horse, or rabbit blood agar, tryptose agar, and thiol or brain-heart broths.

Colony morphology

L. monocytogenes grow well on sheep blood agar. After incubation at 37°C, examine blood agar plates for small, round, translucent, slightly raised, gray colonies with a fine-textured surface that vary from 0.3 to 1.5 mm in diameter with a narrow zone of complete hemolysis. Optimal temperature ranges from 30 to 37°C; however, growth temperature ranges from 3 to 45°C. If clear media such as lithium chloride-phenylethanol-moxalactam (LPM) agar is used, after 18 to 24 hours incubation they should be examined by unfiltered oblique illumination with a scanning microscope. Colonies are recognized by their blue-green color as seen with oblique (45° angle) light on the LPM agar. These colonies are translucent, round, slightly raised, watery in consistency, and 0.2 to 0.8 mm in diameter. This selective enrichment media is used by the United States Department of Agriculture (USDA) for recovery of *L. monocytogenes* for epidemic studies.

Motility and other key test

Umbrella-like motility is seen in semi-solid agar that is stabbed and incubated at room temperature (20 to 25°C). The motility is best demonstrated by the stab inoculation of two tubes containing 0.1% dextrose semi-solid agar. Incubate one at 36°C and the other at 25°C. Motility in the agar is more pronounced at 25°C. A ring forms just beneath the surface of the agar; a cloud-like growth then extends downward from the ring. *L. monocytogenes* colonies are often confused with the colonies of *S. agalactiae* (group B streptococci), but they may be differentiated on the basis of Gram-stain, motility and catalase (*Listeria* are catalase positive and are motile).

Commercial identification systems

The API-*Listeria* (bioMérieux Vitek, Inc.) is a miniaturized biochemical test kit that can be used for species identification. Rapid identifications are available through enzyme immunoassay kits using monoclonal antibodies, DNA probes and techniques, and pulsed-field gel electrophoresis is available. These different procedures are used for initial identification or to differentiate the strains and serotypes of *L. monocytogenes*.

Erysipelothrix species

The genus *Erysipelothrix* is composed of two species, *E. rhusiopathiae* and *E. tonsillarum* (a recently isolated organism from swine). *Erysipelothrix* species are similar to *Lactobacillus* species in morphology and catalase negative result.

General characteristics

This genus is a facultatively anaerobic, regular gram-positive short, and, at times, long filamentous rod. They are also nonspore forming, nonacid fast, and nonmotile. *E. rhusiopathiae* grows on sheep

blood-Trypticase soy agar, chocolate agar, and other gram-positive selective media at 36°C in 5 to 10% CO₂. In blood cultures, the organism produces bizarre and irregular shapes.

Clinical significance

E. rhusiopathiae is a very hardy organism that is widely distributed in the environment that is thought to occur secondary to contamination by animal products. It is carried by a variety of animals and is the causative agent of the disease in animals known as *erysipelas*. It has considerable economic impact in the agricultural community since it infects turkeys, ducks, sheep, cattle, and especially swine. It has also been isolated from other birds, fish, seafood, and mammals. *E. tonsillarum* has been isolated from the tonsils of healthy swine and from water and animal sources. These infections may be acute or chronic and result in septicemia, endocarditis, etc. In humans, the most common form of infection is a painful, spreading cellulitis-like lesion called *erysipeloid* that usually appears on the hands or arms, two to seven days after contact with the infected source. However, the organism occasionally disseminates, causing septicemia, endocarditis, and arthritis. Erysipeloid is an occupational hazard for slaughterhouse workers, veterinarians, fish handlers, and others who frequently are exposed to animals and animal products.

Laboratory identification

Like the microscopic examination of short or long filamentous rods, the macroscopic colony morphology on the primary plate may reveal two or more different morphologic types. Large, flat, rough colonies as well as smaller, smooth, translucent colonies may be present, since *E. rhusiopathiae* is capable of producing both. What you may, in fact, be viewing microscopically is not a polymicrobial infection but rather organisms from both types of colonies. The cells from the rough colonies are slender and filamentous, while those from the smaller, translucent colonies appear as small slender bacilli or coccobacilli. Both colony types are a translucent gray and can cause an alpha-hemolysis type of greening of blood-containing media. Incubation at 35°C favors the development of smooth colonies. Growth is better on blood agar plates than on chocolate agar and better anaerobically or in CO₂ than in air. Trypticase soy broth should also be inoculated and subcultured to blood agar or chocolate after two and seven days of incubation to enhance recovery of the organism. One of the most definitive biochemical tests for the identification of *E. rhusiopathiae* is that it is the only catalase negative, gram-positive, aerobic, bacillus that produces H₂S when inoculated into triple sugar iron (TSI) or Kligler's iron agar (KIA) slants. This reaction is accompanied by acid production throughout the tube. *A note of caution is warranted here to remind you that you should never base the identification of any organism solely on the basis of one biochemical reaction.* Keep in mind that you are working with living, possibly changing, organisms that may not always react true to form. Other tests such as gelatin motility or carbohydrate fermentation, although not necessary to identify *E. rhusiopathiae*, should be run to confirm your identification. Cystine trypticase agar (CTA) supplemented with appropriate sugars may be used to gain more data on which to base your identification of *E. rhusiopathiae*. Glucose and lactose are fermented while maltose, mannitol, salicin, sucrose, trehalose, and most other sugars are negative. *E. tonsillarum* differs biochemically from *E. rhusiopathiae* only by the ability of *E. tonsillarum* to ferment sucrose. There are no serological tests for *E. rhusiopathiae* because of the apparent little serological response to the infection.

Gardnerella species

Gardnerella vaginalis is the only species in this genus. Some recent DNA studies show it is closely related to *Bifidobacterium* species that is an anaerobic gram-positive rod.

General characteristics

G. vaginalis is facultatively anaerobic, nonspore-forming, nonencapsulated, pleomorphic gram-variable rod. It is nonmotile, catalase negative, and oxidase negative. Acid is produced from a wide variety of sugars.

Clinical significance

G. vaginalis is normal vaginal flora in most women of reproductive age. It is associated with bacterial vaginosis. In women with symptomatic bacterial vaginosis, it produces a “fishy” or malodorous vaginal discharge. The male partners of women with bacterial vaginosis commonly have urethral colonization with *G. vaginalis*. The exact mode(s) of transmission of bacterial vaginosis is not clear. Some suggest it is sexually transmitted because of the direct relationship to the number of sexual partners a woman has had, and others suggest it is colonization from rectal organisms. In pregnant women, it has been associated with preterm birth, premature rupture of the membranes, postpartum and postabortal fevers. In the infant, it can cause a fatal or nonfatal septicemia and infections in the umbilical cord and in lesions from the fetal scalp monitor.

Laboratory identification

Isolation is best from a semiselective human blood bilayer Tween agar (HBT, commercially available) on which the tiny, gray, convex, opaque colonies produce a distinct beta-hemolysis. Incubate plates at 37°C in 5 to 7% CO₂ for 48 hours. Colony morphology on the selective media, Gram-stain, and a negative catalase result is used for a presumptive identification. If a more accurate identification is needed, a α -glucosidase, β -glucosidase, hippurate hydrolysis, and starch hydrolysis tests can be performed. Direct detection by fluorescent-antibody tests and an oligonucleotide probe is commercially available.

Other gram-positive bacilli

Other aerobic or facultatively anaerobic, gram-positive, nonspore-forming bacilli are listed here with a preliminary identification flow chart. Some of these organisms we have already talked about; others we’ll talk about in other more appropriate lessons, and some other organisms are only mentioned here because they are rare isolates. These organisms are *Actinomyces*, *Agromyces*, *Actinomadura*, *Arcanobacterium*, *Arthrobacter*, *Aureobacterium*, *Brochothrix*, *Brevibacterium*, *Caryophanon*, *Cellulomonas*, *Caseobacter*, *Clavibacter*, *Corynebacterium*, *Curtobacterium*, *Dermatophilus*, *Erysipelothrix*, *Gardnerella*, *Jonesia*, *Kurthia*, *Lactobacillus*, *Listeria*, *Microbacterium*, *Nocardia*, *Nocardiosis*, *Oerskovia*, *Propionibacterium*, *Renibacterium*, *Rhodococcus*, *Rothia*, *Streptomyces*, and *Tsukamurella*. Use the following flow chart for preliminary identification (fig.1-4); however, check with your laboratory’s operating instructions for further testing or information.

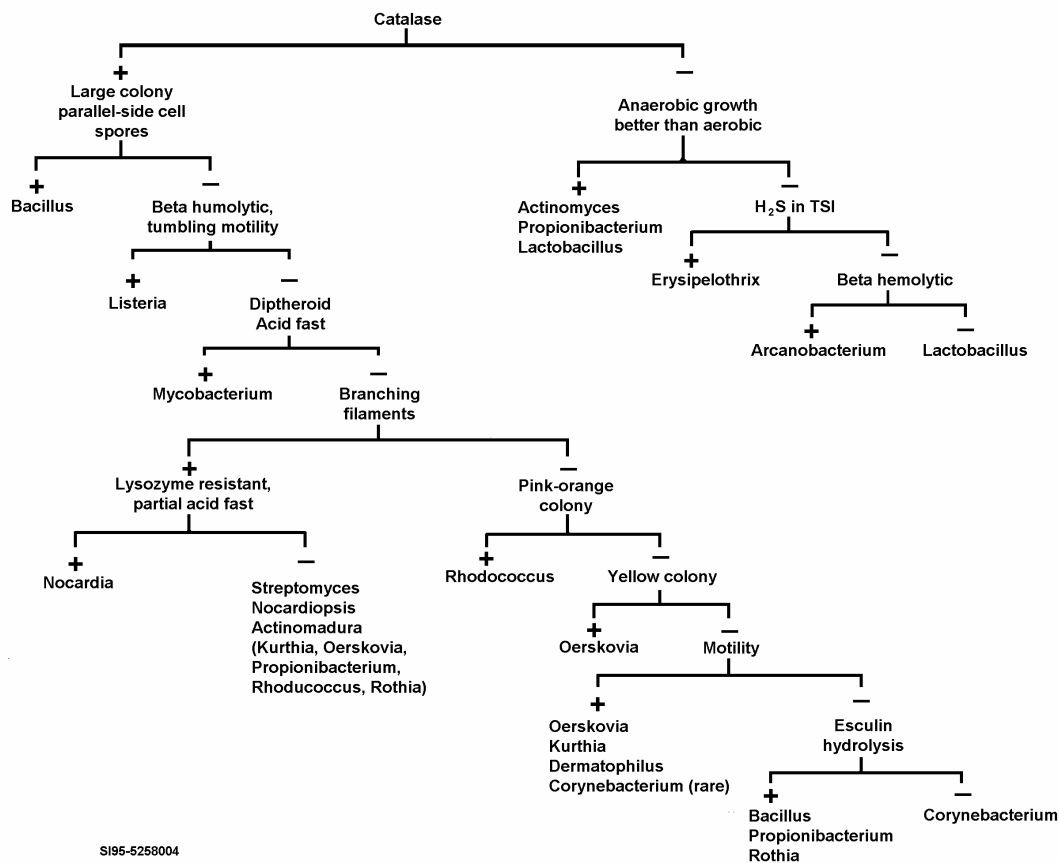


Figure 1-4. Flow chart for preliminary identification of aerobically growing, gram-positive, nonspore-forming bacilli.

Self-Test Questions

After you complete these questions, you may check your answers at the end of the unit.

205. Miscellaneous gram-positive bacilli

- Under what conditions do most strains of *Lactobacillus* species prefer to grow?
- What is the cellular morphology of the *Lactobacillus*?
- On which media does the *Lactobacillus* produce excellent growth?
- Describe *Lactobacillus* colony morphology on blood agar.

5. What microscopic technique can provide presumptive evidence of the presence of *L. monocytogenes* in an unstained preparation?
6. What is the most frequent disease caused by *L. monocytogenes*?
7. What characteristic can be used to separate pathogenic *Listeria* from nonpathogenic strains?
8. What are the three general categories of listeriosis in which most human disease can be placed?
9. What is thought to be the route of infection by *Listeria*?
10. What type of hemolysis does *L. monocytogenes* exhibit on blood agar?
11. What type of motility is associated with *L. monocytogenes* that has been stab inoculated into semisolid media?
12. What types of shapes may be seen in blood cultures of *E. rhusiopathiae*?
13. What is the most common type of infection with *E. rhusiopathiae* seen in man?
14. What complications may arise if *E. rhusiopathiae* is disseminated throughout the body?
15. How may the macroscopic and microscopic morphology of *E. rhusiopathiae* confuse you, the technician, when you are attempting to identify this organism?
16. What incubation temperature favors the development of smooth colonies?
17. What is one of the most definitive biochemical tests for the identification of *E. rhusiopathiae*?

18. With what is *G. vaginalis* associated?
19. What has *G. vaginalis* been linked with in pregnant women?
20. What is the best media for isolation, and what is the colony morphology of *G. vaginalis*?

Answers to Self-Test Questions

201

1. Thirty-two.
2. *S. aureus*, *S. epidermidis*, *S. saprophyticus*, *S. haemolyticus*, and *S. lugdunensis*.
3. *S. aureus*.
4. (1) Injury to the skin.
(2) Prior viral infections.
(3) Leukocyte defects.
(4) Presence of foreign bodies.
(5) Chronic diseases.
(6) Prior use of antibiotics to which infecting *S. aureus* is resistant.
5. Any eight of the following:
(1) Furuncles (boils).
(2) Carbuncles.
(3) Cellulitis.
(4) Impetigo.
(5) Staphylococcal scalded skin syndrome.
(6) Pneumonia.
(7) Osteomyelitis.
(8) Meningitis.
(9) Endocarditis.
(10) Mastitis.
(11) Bacteremia.
(12) Various abscesses.
(13) Enterocolitis.
(14) Urogenital infections.
(15) TSS.
(16) Food poisoning (via enterotoxin).
6. Introduction of prostheses or catheters and immunosuppressive therapy.
7. (1) Prosthetic valve endocarditis.
(2) Infections of intravascular catheters, cerebrospinal fluid shunts, and orthopedic appliances.
(3) Peritonitis.
(4) Urinary tract infections.

- (5) Eye infections.
- (6) Ear infections.
- (7) Wound infections.
- 8. Slime-mediated adherence.
- 9. *S. saprophyticus*.
- 10. *S. epidermidis*.
- 11. Production of enzymes, hemolysins, and toxins.
- 12. They promote the growth of large numbers of organisms in the vaginal canal and lower the concentration of the Mg^{++} ion that enhances the production of TSST-1.
- 13. Thioglycollate broth and blood agar.
- 14. Throughout the tube.
- 15. Hemolysis and pigmentation.
- 16. PEA, CNA, and MSA.
- 17. Its salinity—a characteristic that is tolerated by staphylococci but not by other organisms.
- 18. Only a presumptive report of gram-positive cocci resembling staphylococci should be made.
- 19. Coagulase test.
- 20. Tube; extracellular coagulase.
- 21. Acid production from maltose and mannitol, protein A, acetoin production, and β -galactosidase activity.
- 22. (1) Intracranial abscesses.
 - (2) Bacteremia.
 - (3) Peritonitis.
 - (4) Pneumonia.
 - (5) Septic arthritis.
 - (6) Meningitis.
 - (7) Infections of prosthetic valve endocarditis.
 - (8) Infections from cerebrospinal fluid shunts.

202

- 1. (1) h.
 - (2) h.
 - (3) j.
 - (4) c.
 - (5) b.
 - (6) k.
 - (7) f.
 - (8) i.
 - (9) g.
 - (10) d.
 - (11) a.
 - (12) a.
 - (13) e.
 - (14) l.
 - (15) m.
- 2. (1) b.
 - (2) e.

- (3) d.
- (4) d.
- (5) f.
- (6) g.
- (7) c.
- (8) h.
- (9) i.
- (10) j.
- (11) k.
- (12) a.

203

- 1. (1) d.
- (2) g.
- (3) c.
- (4) b.
- (5) a, b.
- (6) e.
- (7) f.
- (8) g.

204

- 1. *Bacillus anthracis* is encapsulated and nonmotile; whereas, the saprophytic forms lack capsules and are usually actively motile; however, the extent of motility is variable.
- 2. In direct smears of clinical specimens.
- 3. Long, regular or straight-sided rods with rounded ends that may produce endospores that are round, oval, cylindrical, or occasionally kidney shaped.
- 4. Do not stain well; appear as clear areas within the cell.
- 5. It does not, but spores are formed when the organism is exposed to the external environment.
- 6. *B. anthracis*. No.
- 7. Cutaneous.
- 8. Lethal factor and edema factor.
- 9. PA appears to control the ability of LF and EF to pass through cell membranes of infected tissues.
- 10. *Bacillus cereus*.
- 11. The emetic type and the diarrheal type.
- 12. 5% sheep blood agar plates.
- 13. They are 3 to 5 mm in diameter, gray-white, opaque, raised with irregular margin, and have a dull, frosted-glass appearance.
- 14. *B. cereus* colonies range from small, shiny, and compact to large, spreading, feathery types. The other species have round or irregular, raised or flat, usually large colonies, with entire to undulate or rhizoid edges.
- 15. Anthrax colonies are nonhemolytic on blood agar, while a definite zone of alpha- or beta-hemolysis usually surrounds cereus and other saprophytic forms.
- 16. Gram-stain and fluorescent-antibody staining.
- 17. Stains for spores and capsules.
- 18. Sodium bicarbonate media under increased CO₂ atmosphere; this allows the capsules to develop.
- 19. A hanging drop, or by inoculating a suitable motility medium and incubating for four days at 37°C.

20. The diversity of shapes stems from the fact that the cell wall of some species is wider at the ends, allowing the organism to assume club and various other shapes.
21. Loeffler's alkaline methylene blue.
22. Banded, barred, or beaded.
23. Palisades, but L, V, or Y shaped, branching forms may also occur.
24. Upper respiratory tract.
25. By nasal droplets, oral droplets, or direct contact.
26. By invading the mucous membranes, multiplying rapidly, and producing a powerful exotoxin that, in turn, yields an acute inflammatory response and destruction of epithelium.
27. By the accumulation of fibrin, red blood cells, and white blood cells resulting in the formation of a gray, clotted film or pseudomembrane that covers the tonsils, pharynx, or larynx.
28. It is absorbed by other tissues.
29. Lesions and pseudomembranes of the throat.
30. An exotoxin.
31. Cystine tellurite agar and Modified Tinsdale.
32. Its extremely short shelf life of only four days.
33. *C. diphtheriae*, *C. pseudotuberculosis*, and *C. ulcerans*.
34. Elek gel diffusion precipitin test.

205

1. Microaerobically.
2. They are nonspore forming, long and slender gram-positive rods to short coccobacilli and commonly form chains and occasionally exhibit spiral forms.
3. Rogosa's selective tomato juice media.
4. Colonies range from pinpoint, alpha-hemolytic to large, rough, gray colonies.
5. Hanging drop motility test.
6. Meningitis; especially in newborns (\leq one month old) and patients over 50.
7. Hemolysis.
8. (1) Neonatal sepsis or meningitis, (2) sepsis or meningitis in immunocompromised patients, and (3) sepsis or nonspecific flu-like illness in healthy women during pregnancy.
9. Ingestion.
10. Narrow zone of complete hemolysis.
11. Umbrella-like motility when incubated at room temperature.
12. Bizarre and irregular shapes.
13. A painful, spreading cellulitis-like lesion called erysipeloid.
14. Septicemia, endocarditis, and arthritis.
15. What you may be viewing microscopically is not a polymicrobial infection but rather organisms from large, flat, rough colonies and smaller, smooth, translucent colonies. The cells from the rough colonies are slender and filamentous while those from the smaller, translucent colonies appear as small slender bacilli or coccobacilli.
16. 35°C.
17. When inoculated into TSI or KIA, it is the only catalase negative, gram-positive, aerobic, bacillus that produces H₂S.
18. Bacterial vaginosis.
19. It has been associated with preterm birth, premature rupture of the membranes, postpartum, and postabortal fevers.

20. From a semi-selective human blood bilayer Tween agar (HBT, commercially available) on which the tiny, gray, convex, opaque colonies produce a distinct beta-hemolysis.

Do the unit review exercises before going to the next unit.

Unit Review Exercises

Note to Student: Consider all choices carefully, select the *best* answer to each question, and *circle* the corresponding letter. When you have completed all unit review exercises, transfer your answers to ECI (AFIADL) Form 34, Field Scoring Answer Sheet.

Do not return your answer sheet to AFIADL.

1. (201) Which *Staphylococcus* species is a major cause of morbidity and mortality?
 - a. *S. aureus*.
 - b. *S. epidermidis*.
 - c. *S. haemolyticus*.
 - d. *S. saprophyticus*.
2. (201) Which of the CoNS are the *best* documented opportunistic pathogens?
 - a. *S. cohnii* and *S. xylosum*.
 - b. *S. intermedius* and *S. hyicus*.
 - c. *S. haemolyticus* and *S. warneri*.
 - d. *S. epidermidis* and *S. saprophyticus*.
3. (201) Members of the genus *Micrococcus* may be distinguished from CoNS by all of the following tests *except*
 - a. OF.
 - b. Taxo A.
 - c. Taxo O.
 - d. Oxidase.
4. (202) Who was the originator of a system based on the hemolytic activity of streptococcal colonies grown on sheep blood agar?
 - a. Brown.
 - b. Sherman.
 - c. Lancefield.
 - d. Spallanzani.
5. (202) "A clear, colorless zone around the streptococci colonies, in which the red blood cells have undergone complete discoloration" describes
 - a. beta (β) hemolysis.
 - b. alpha (α) hemolysis.
 - c. gamma (γ) hemolysis.
 - d. alpha-prime or wide zone alpha hemolysis.
6. (202) *S. agalactiae* (group B streptococcus) is the greatest threat to
 - a. diabetics.
 - b. neonates.
 - c. postpartum women.
 - d. immunosuppressed individuals.

7. (202) Which *Streptococcus* species is the *second most* common cause of bacterial meningitis?
 - a. *S. pyogenes*.
 - b. *S. agalactiae*.
 - c. *S. equisimilis*.
 - d. *S. pneumoniae*.
8. (202) Which *Streptococcus* species is the *most* common cause of SBE?
 - a. *S. pyogenes*.
 - b. *S. agalactiae*.
 - c. *S. pneumoniae*.
 - d. Viridans streptococcus.
9. (202) Isolating *S. bovis* from the blood has been associated with the development of
 - a. hepatitis.
 - b. bacteremia.
 - c. colon cancer.
 - d. peptic ulcers.
10. (202) The purpose of the bacitracin disk test is to distinguish between Lancefield's Group A and
 - a. *Streptococcus bovis*.
 - b. *Streptococcus pneumoniae*.
 - c. other groups of beta hemolytic streptococci.
 - d. other groups of pneumococcus type III colonies.
11. (202) What are the two *most common* isolated *Enterococcus*?
 - a. *E. faecalis* and *E. faecium*.
 - b. *E. avium* and *E. malodoratus*.
 - c. *E. flavescens* and *E. gallinarum*.
 - d. *E. raffinosus* and *E. pseudoavium*.
12. (203) Which miscellaneous gram-positive cocci were *first* isolated from blood cultures of patients with the measles?
 - a. *A. viridans*.
 - b. *G. morbillorum*.
 - c. *Pediococcus* species.
 - d. *Leuconostoc* species.
13. (203) Which miscellaneous gram-positive cocci are important to the food and brewing industries, and used as a flavor enhancer in processed vegetables and soy products?
 - a. *A. urinae*.
 - b. *G. haemolysans*.
 - c. *Pediococcus* species.
 - d. *Leuconostoc* species.
14. (203) Which miscellaneous gram-positive cocci are classified with *Micrococcus* and *Staphylococcus* species in the family *Micrococcaceae*?
 - a. *Aerococcus* species.
 - b. *Leuconostoc* species.
 - c. *Pediococcus* species.
 - d. *Stomatococcus* species.

15. (204) The encapsulated cells of *B. anthracis* found in direct smears of clinical specimens are rarely observed in smears from laboratory cultures, unless grown in increased CO₂ on media supplemented with
 - a. chloral hydrate.
 - b. sodium chloride.
 - c. potassium tellurite.
 - d. sodium bicarbonate.
16. (204) What is the *most* common form of human *B. anthracis* infection in man?
 - a. Pulmonary.
 - b. Cutaneous.
 - c. Intestinal.
 - d. Renal.
17. (204) Which *Bacillus* species is the etiologic agent of two different types of food poisoning syndromes?
 - a. *B. alvei*.
 - b. *B. brevis*.
 - c. *B. cereus*.
 - d. *B. subtilis*.
18. (204) In typical colonies of the anthrax bacillus, which characteristics on sheep blood agar will show after 18 to 24 hours incubation?
 - a. 1 to 3 mm in diameter, dull frosted glass, with beta hemolysis.
 - b. 3 to 5 mm in diameter, dull frosted glass, with beta hemolysis.
 - c. 1 to 3 mm in diameter, dull frosted glass, with no zone of hemolysis.
 - d. 3 to 5 mm in diameter, dull frosted glass, with no zone of hemolysis.
19. (204) *Corynebacterium* is *more* likely to exhibit metachromatic granules when grown on
 - a. Loeffler's slant or Pai's media.
 - b. Loeffler's slant or blood agar.
 - c. Pai's media and blood agar.
 - d. Pai's media and chocolate.
20. (204) Which *Corynebacterium* species is the *most* virulent and produces a powerful exotoxin?
 - a. *C. jeikeium*.
 - b. *C. diphtheriae*.
 - c. *C. urealyticum*.
 - d. *C. pseudodiphtheriticum*.
21. (204) What three species of *Corynebacterium* form a brown halo around colonies grown on Modified Tinsdale medium?
 - a. *C. xerosis*, *C. striatum*, and *C. ulcerans*.
 - b. *C. diphtheriae*, *C. striatum*, and *C. xerosis*.
 - c. *C. diphtheriae*, *C. pseudotuberculosis*, and *C. xerosis*.
 - d. *C. diphtheriae*, *C. pseudotuberculosis*, and *C. ulcerans*.
22. (205) *Lactobacillus* grow *excellent* on
 - a. mannitol salt agar.
 - b. bismuth sulfite agar.
 - c. thiosulfate citrate bile salts agar.
 - d. Rogosa's selective tomato juice agar.

23. (205) Colonies of *Listeria monocytogenes* are recognized by their blue-green color as seen with oblique light on
- a. lithium chloride-phenylethanol-moxalactam agar.
 - b. potassium tellurite agar.
 - c. tryptose agar.
 - d. blood agar.
24. (205) How can the colonies of *L. monocytogenes* and *S. agalactiae* (group B streptococci) be differentiated?
- a. Hemolysis.
 - b. Site of infection.
 - c. Colony morphology.
 - d. Catalase and motility.
25. (205) What is the *most* common form of *E. rhusiopathiae* infection seen in man?
- a. Arthritis.
 - b. Septicemia.
 - c. Erysipeloid.
 - d. Endocarditis.
26. (205) Which characteristic is typical for *Erysipelothrix rhusiopathiae*?
- a. Ferments maltose and mannitol.
 - b. Motility at room temperature.
 - c. H₂S positive in KIA.
 - d. Hydrolyzes esculin.

Please read the unit menu for unit 2 and continue ➔

Unit 2. Aerobic Gram-Negative Cocci and Coccobacilli

2–1. Gram-Negative Cocci	2–1
206. <i>Neisseria</i> species	2–1
207. <i>Moraxella</i> species	2–6
2–2. Gram-Negative Coccobacilli	2–10
208. <i>Haemophilus</i> species	2–10
209. <i>Bordetella</i> species	2–14
210. <i>Francisella</i> and <i>Brucella</i>	2–19
211. Other gram-negative coccobacilli	2–25

THIS UNIT contains a number of taxonomically unrelated bacterial species, some of which are rarely encountered in isolates from human clinical sources. Some of these organisms cause specific diseases, such as gonorrhea, meningitis, and endocarditis, while others are associated with a wide range of human infections. Some of these organisms are endemic in mammals other than humans and may or may not cause disease in these animals. They may represent classic examples of zoonoses.

2–1. Gram-Negative Cocci

Bacteriologists have long been familiar with members of the family *Neisseriaceae*, the true gram-negative cocci, because two species of the aerobic genus *Neisseria gonorrhoeae* and *Neisseria meningitidis* have proven to be persistent challenges to the medical profession.

206. *Neisseria* species

The genus *Neisseria* is one of five genera in the family *Neisseriaceae*, sharing membership in this family along with *Kingella*, *Moraxella*, *Acinetobacter*. The pathogen *Moraxella catarrhalis* was formerly known as *Branhamella catarrhalis* and is listed as such in numerous texts. For the purpose of these career development courses (CDC), the name *M. catarrhalis* will be used.

There are 10 species in the genus *Neisseria* that are of human origin and six of animal origin. Of the 10 species that are of human origin, there are two primary pathogens commonly encountered in the clinical laboratory: *N. gonorrhoeae* and *N. meningitidis*. *N. gonorrhoeae* is the causative agent of the sexually transmitted disease gonorrhea, which is still prevalent around the world despite intensive efforts to eradicate it. The meningococcus, *N. meningitidis*, poses a constant threat in the form of outbreaks of cerebrospinal meningitis, even in the most advanced nations. Most of the other members of the genus are considered nonpathogenic and normal inhabitants of the oro- and nasopharyngeal mucous membranes of humans. These are *N. lactamica*, *N. cinerea*, *N. polysaccharea*, *N. subflava*, *N. sicca*, *N. mucosa*, *N. flavescens*, and *N. elongata*. The animal origin *Neisseria* species should be considered when *Neisseria* is isolated from infections due to animal bites. The *Neisseria* of animal origin are *N. canis*, *N. weaverii*, *N. caviae*, *N. ovis*, *N. cuniculi* and *N. iguanae*.

General characteristics

All members of the genus *Neisseria*, with the exception of *N. elongata*, share a common microscopic morphology being described as gram-negative, diplococci with the indented or flattened side adjacent to one another, approximately 0.6 by 0.8 microns in size. In stained smears of pus or body fluids, the cells are often paired and joined together on their indented or flattened sides. The indented or flattened sides give the characteristic coffee or kidney bean appearance. *N. elongata*, on the other

hand, is described as a short, slender rod, but once exposed to antibiotics they become long, stringy rods. They are nonspore forming and nonmotile.

All members of the genus are oxidase positive and catalase positive except for *N. elongata*. However, they differ in the various growth requirements (media required, optimum temperature), acid production from carbohydrates by oxidation (not fermentation), and biochemical reactions. The special selective media involved in culturing are Thayer-Martin (TM), modified Thayer-Martin (MTM), and New York City (NYC) medium. TM medium is a chocolate agar with an enrichment supplement, and the antibiotics vancomycin (to inhibit gram-positive organisms), colistin (to inhibit gram-negative bacilli), and nystatin (to inhibit yeast). MTM is the same as TM with trimethoprim lactate added to inhibit the swarming of *Proteus* species and anisomycin (which has a longer half-life) as a substitute for nystatin. NYC medium contains the same antibiotics as TM but is a transparent medium with buffered proteose-peptone and other supplements.

Clinical significance

The clinical significance of the *Neisseria* is directly related to the species isolated; therefore, we will look at the two primary pathogens separately. The other species will be reviewed briefly.

N. gonorrhoeae

The gonococcus is the causative agent of gonorrhea, a disease of great public health importance. Since the mid 1960s, it has had the distinction of being the most commonly reported communicable disease in the United States with over one million cases reported annually. This detection level, though impressive, does not clearly delineate the size of the problem since it is estimated that an additional one million cases that are detected go unreported to public health officials. What may be even more alarming is that a sizable number of infected females are asymptomatic and, therefore, seek no medical attention and go completely undiagnosed and unreported. Estimates of what percentage of infected females are asymptomatic carriers of *N. gonorrhoeae* range from 25 to 80%, and in males, 1 to 5%.

What is apparent, however, is that complicated infections, for example, gonococcal infections that spread from the original site of infection to other areas of the body, occur much more frequently in women than in men. This is most likely due to the initial infection going undetected for much longer periods in women. Studies indicate that in 8 to 12% of infected women, these complicated gonococcal infections give rise to such conditions as endometritis, salpingitis, and peritonitis that are collectively referred to as pelvic inflammatory disease (PID). Men normally display signs of infection one to seven days after contracting the infection.

Another condition involving the spread of the gonococcus via the blood stream has been labeled as disseminated gonococcal infection or DGI, whose most common manifestations are a maculopapular rash, skin lesions, tenosynovitis, and arthritis. In the case of a child delivered to an infected mother, it's possible to have an infection of the conjunctiva of the eye (ophthalmia neonatorum) because the child was exposed to the organism as it passed through the birth canal. With the routine application of silver nitrate drops or erythromycin ointment to the eyes of newborns, the blindness caused by gonococcal infections has almost been eliminated in industrial countries. Oropharyngeal and anorectal infections due to *N. gonorrhoeae* may be seen in those who participate in oral or anal intercourse. Women may acquire anorectal infections by contamination from cervical secretions.

N. meningitidis

The meningococcus is the other commonly isolated pathogen in the genus *Neisseria* and is one of the primary agents of bacterial meningitis. It may also be isolated from other body sites, such as the nasopharynx. In fact, a significant percentage of healthy individuals are asymptomatic carriers of this organism. These asymptomatic carriers seem to be the primary source of infection for susceptible

individuals with whom they have close contact. The usual route of transmission is by aerosols, and the portal of entry for the meningococci is the nasopharynx.

The organisms may constitute part of the transient flora in immune individuals, producing no symptoms, or they may set up a local nasopharyngeal infection in susceptible persons. The infection can extend to the bloodstream, resulting in septicemia (meningococcemia), or to the meninges, causing meningitis. Other complications of meningococcemia include septic monarthrititis and disseminated intravascular coagulation known as DIC. Meningococcal meningitis is considered an acute bacterial disease characterized by sudden onset with fever, intense headache, nausea, vomiting, stiff neck, and frequently a petechial rash. Delirium and coma often appear, and the patient may suddenly lose consciousness and go into shock.

Meningococcal disease is primarily a disease of young children with the highest rate of attack being in children under one year of age. However, young adults are susceptible, and it is more common in males than females. The incidence of disease is high when individuals are gathered together in close living quarters since overcrowding and fatigue seem to be conducive to epidemic outbreaks of meningitis. The above conditions are prevalent in various institutional settings including military barracks, and from time to time epidemic outbreaks occur at military training bases. At one time, fatality rates exceeded 50%, but with early diagnosis and modern treatment the fatality rate dropped to less than 10%.

Other Neisseria species

N. lactamica has, on occasion, been isolated from cerebrospinal fluid (CSF), blood, and the genitourinary tract. Also on rare occasions, *N. sicca*, *N. mucosa*, and *N. flavescens* which are all normal flora of the upper respiratory tract have been shown to be the causative agents of bacteremia, meningitis, endocarditis, pneumonia, and otitis media. *N. cinerea* has been isolated from endocervical and rectal infections, lymphadenitis, and conjunctivitis in neonates, and has been misidentified as *N. gonorrhoeae* because of its growth on selective media.

Laboratory identification

Once the specimen is properly collected and cultured onto a selective or an enrichment media, it is incubated under increased CO₂ and humidity at 35 to 37°C for 18 to 24 hours. Various identification procedures can be employed depending on the circumstances of the patient. A quick preliminary report from a CSF may be needed in order to start antimicrobial therapy when *N. meningitidis* is suspected. Serious social and medicolegal issues are involved with the misdiagnosis of gonorrhea or misidentification of strains of *N. gonorrhoeae*. The Centers for Disease Control and Prevention has recommended criteria for reporting diagnoses of gonorrhea. The three levels include clinical findings, presumptive identification, and definitive identification. The last two levels include laboratory results. Your laboratory's OI should address the appropriate procedures. Only general identification schemes will be briefed in this course.

Colony morphology

After incubation, colony morphology may be observed and a Gram-stain performed followed by a cytochrome oxidase test. On primary isolation media, *N. gonorrhoeae* isolates from genital sites will usually appear as tiny to small (0.5 to 1 mm in diameter), gray, convex, shiny, translucent colonies with entire or undulate margins, although other larger, flatter colony types can be present. These colonies tend to lift completely off the agar surface when touched with a loop and may appear to be mucoid or sticky. *N. meningitidis* colonies can grow on blood agar and are usually 1 to 2 mm in diameter and flatter than those of *N. gonorrhoeae*. They are smooth, circular, raised, have a slight tan color, and may produce a greenish cast underneath on blood agar. The other *Neisseria* species resemble *N. gonorrhoeae* and *N. meningitidis* but can usually be differentiated by their growth or no growth on selective agar and production of yellow pigment and opaque colonies. Most *Neisseria*

isolates will not survive longer than 48 hours in culture; therefore, subculture them every 18 to 24 hours to maintain maximum viability.

Stained smears

Take extra care in preparing smears from clinical materials containing these cocci. Lightly roll swab specimens (rather than rub) on glass slides to accomplish an even distribution and prevent destruction of white cells or tissue cells. This technique will give smears of even thickness that are easily scanned for the presence of intracellular organisms. The preparation of joint fluids for direct examination and culturing of *N. gonorrhoeae* is best accomplished by centrifuging the specimen and using the sediment as an inoculum. In the later, chronic stages of gonorrhea, few cocci are present in clinical materials. In pus or body fluids the characteristic “kidney bean” organisms are seen within polymorphonuclear leukocytes. Leukocytes may contain 20 to 50 cocci or more. In stained smears from cultures, the typically flattened border of adjacent cell may still be observed. It is important to note that *N. gonorrhoeae* cannot be distinguished from *N. meningitidis* on the basis of microscopic morphology, and since they both may inhabit common sites of infection, biochemical and other tests must be used to differentiate them.

In males, a diagnosis of gonorrhea is usually based on history, characteristic clinical signs, and symptoms. The classic symptom of gonorrhea in males is the usually profuse urethral discharge that occurs one to seven days after infection. Urethral discharge is taken directly from the penile orifice. When there is no discharge at the time of examination or the male is asymptomatic, it is necessary to obtain a specimen from within the urethral canal for culture. Prostatic fluid may be inoculated directly onto TM medium. In the past, microscopic demonstration of gram-negative intracellular diplococci (GNID) on a smear of urethral discharge exudate from a male patient constituted sufficient basis for a diagnosis of gonorrhea. However, recent evidence suggests an increase of gonorrhea-like infections in genital specimens caused by *N. meningitidis*, indicating that the reporting of gonococcal disease based on presumptive criteria is no longer valid.

Although the Gram-stain of urethral discharge is highly effective in demonstrating gonococcal infections in males, in women this technique will detect only 40 to 70% of endocervical, gonococcal infections. To diagnose gonorrhea in women, obtain culture specimens from the cervix and anal canal, and inoculated on separate TM—or MTM—culture plates or in separate Transgrow bottles. Ideally, specimens from suspected gonococcal lesions or infected sites should be inoculated directly upon selected media when collected and placed under increased CO₂ at 35 to 37°C.

Oxidase test

The oxidase test can be used to gather additional information on suspected colonies. The oxidase test is performed by touching an isolated colony with a platinum loop (nichrome loops may give false positive reactions) and transferring the material onto filter paper. A drop of Kovac’s reagent (tetramethyl-p-phenylenediamine dihydrochloride) is then placed on the paper. When the respiratory enzyme cytochrome oxidase is present, it will oxidize the Kovac’s reagent resulting in the formation of a dark blue to black compound within 10 seconds. Do *not* conduct the oxidase test beyond 10 seconds; which may also result in false positive reactions. At this point, any genitourinary specimen which demonstrates colony morphology compatible with that of *N. gonorrhoeae*, a Gram-stain showing gram-negative diplococci, and a positive oxidase test may be presumptively identified as *N. gonorrhoeae*. Likewise, an organism isolated from CSF meeting the same criteria of colony and microscopic morphology and displaying a positive oxidase test may be tentatively identified as *N. meningitidis*. However, a more definitive (confirmatory) identification is still necessary since both pathogenic species of *Neisseria* can be found in either type of specimen. Also, certain other *Neisseria* species, such as *N. lactamica* or *N. cinerea*, can grow on the media suggested for the isolation of the two pathogens and are oxidase positive.

Key test for identification

The definitive identification of *Neisseria* can be accomplished using four categories of tests: (1) immunologic, (2) carbohydrate utilization, (3) chromogenic enzymatic substrate, and (4) nucleic acid probes. The carbohydrate utilization test involves placing a 3 mm loopful of growth from a subculture of the primary plate a few millimeters below the surface of tubes containing cystine trypticase agar (CTA) supplemented with phenol red and a 1% concentration of an appropriate carbohydrate. Lactose, glucose, sucrose, maltose, and fructose may all be used.

After incubation, a positive test is detected by a change in color of the phenol red indicator from red to yellow. *N. meningitidis* uses both glucose and maltose; whereas *N. gonorrhoeae* uses only glucose. One disadvantage of this method is it is designed to detect acid production from fermentation and is relatively insensitive to the detection of acid production by oxidation. Another disadvantage of this method of detecting the utilization of carbohydrates is that it requires the suspected organism to grow. Some fastidious *Neisseria* species may not comply or may require prolonged incubation periods before they produce enough acid to be detected.

Nongrowth dependent methods were developed to alleviate these growth-dependent problems. These methods center on changing the composition of the media used. Some of these newer methods also simplify the addition of carbohydrates to the media by using paper discs impregnated with various carbohydrates which can be dropped into the tubes of CTA media just prior to testing. These methods, collectively referred to as rapid-fermentation tests (RFT), do not require growth of the organism being tested and usually have two traits in common: (1) a higher concentration of carbohydrate is incorporated into the media, and (2) a much heavier suspension of organism is used to inoculate the modified CTA media. Although these tests are referred to as rapid-fermentation tests because they can be read within four hours after being inoculated, they still require that the inoculum used comes from a subculture of the primary plate just like the original grow-dependent CTA carbohydrate utilization test. The following table is a review of carbohydrate reactions and growth on different mediums.

Species	MTM or NYC media	CAP or SBAP at 22°C	Nutrient agar at 35°C	Glu.	Mal.	Lac.	Suc.	Fru.
<i>N. gonorrhoeae</i>	+	—	—	+	—	—	—	—
<i>N. meningitidis</i>	+	—	V	+	+	—	—	—
<i>N. lactamica</i>	+	V	+	+	+	+	—	—
<i>N. cinerea</i>	V	—	+	—	—	—	—	—
<i>N. polysaccharea</i>	V	—	+	+	+	—	—	—
<i>N. subflava</i>	V	+	+	+	+	—	V	V
<i>N. sicca</i>	—	+	+	+	+	—	+	+
<i>N. mucosa</i>	—	+	+	+	+	—	+	+
<i>N. flavescens</i>	—	+	+	—	—	—	—	—
<i>N. elongate</i>	—	+	+	—	—	—	—	—
+ = most strains positive for acid from sugars, — = most strains negative, V = strain dependent Glu. = glucose, Mal. = maltose, Lac. = lactose, Suc. = sucrose, Fru. = Fructose								

To further accelerate the identification of *N. gonorrhoeae*, various immunological tests were developed, such as the coagglutination test which uses as a carrier particle—killed *S. aureus* cells coated with antigonococcal antibodies. A suspected colony is smeared on two separate areas of a slide. A test control which contains *S. aureus* organisms without antigonococcal antibodies attached is then added to one area and a test reagent containing *S. aureus* cells coated with antigonococcal antibodies is added to the other test area. The slide is then observed for agglutination. A positive test is indicated by agglutination of the area containing the test reagent and no agglutination in the control

area. However, some strains of *N. gonorrhoeae* do not react with this type of test, and cross-reactions are common with other *Neisseria* species; therefore, use other tests for confirmation.

Enzyme immunoassay tests are available which are, in some cases, not only capable of identifying *N. meningitidis* and *N. gonorrhoeae*, but a variety of other *Neisseria* species plus *M. catarrhalis*, *Kingella* species, and *Moraxella*. These tests are based on the enzyme hydrolysis of chromogenic substrates. They have the advantages of using an inoculum taken directly from the primary plate and generating results in only four hours after inoculation. They are not growth-dependent, nor do they require the use of carbohydrates. Instead, these tests detect enzymes produced by various organisms that are capable of liberating chromogens (colored products) from various substrates. A disadvantage is that some strains of *N. cinerea*, *N. polysaccharea*, *N. subflava*, and *K. denitrificans* are also capable of liberating chromogens and may be confused with *N. gonorrhoeae*.

Monoclonal-fluorescent-antibody tests are commercially available for culture confirmation, but cross-reactivity with nongonococcal isolates and failure to react with some gonococcal strains have been noted. The *advantage* is a single colony from a mixed culture can be used. A *disadvantage* is a fluorescent microscope must be accessible. Also available is a DNA probe test (AccuProbe, Gen-Probe) which is highly sensitive and specific. Other tests include nitrate and nitrite reduction, DNase, β -lactamase production, and superoxol test. Commercial multitest, identification systems incorporate the acid production test and a combination of these other tests.

Thirteen serogroups of *N. meningitidis* have been designated A, B, C, D, 29E, H, I, K, L, W-135, X, Y, and Z. Grouping is based on agglutination reactions with commercially available typing sera. Agglutination is the most reliable procedure for routine serogrouping of the meningococci, although other methods such as immunodiffusion, coagglutination, and radioimmunoassay have been used. The sera react with the capsular polysaccharides or outer membrane protein antigens of the organisms. Each serogroup may contain a number of serotypes, some of which are more pathogenic than others. For example, serotype 2 found in both serogroup A and C has been associated with epidemic meningococcal disease in the United States. Serogroup B was the most common *N. meningitidis* serogroup recovered (51.1%), followed by group C (22.3%), group Y (5.8%), and group Z (4.7%). The most frequently isolated strains of *N. meningitidis* implicated in systemic disease belong to the groups A, B, C, Y, and W-135.

207. *Moraxella* species

At this time there is only one species in the genus *Moraxella* and that is *M. catarrhalis*.

Members of the genus *Moraxella* are *M. canis*, *M. catarrhalis*, *M. lacunata*, *M. lincolni*, *M. osloensis*, *M. atlantae*, *M. phenylpruvica*, and—the most common isolate—*M. nonliquefaciens*. We will look at the genus in general and then closely at *M. catarrhalis* as a pathogen.

General characteristics

The genus *Moraxella* is comprised of eight species of nonfermentative, strictly aerobic, catalase positive, nonmotile, oxidase-positive, plump coccobacilli, appearing in pairs, and sometimes short chains; they have a tendency to resist decolonization.

Clinical significance

Members of the genus *Moraxella* are rarely found on inanimate objects in the environment, but are considered normal flora of the skin and mucous membranes of humans and many animals. The species of *Moraxella* are considered opportunistic pathogens causing nosocomial infections, infecting wounds, or causing pneumonia. They have been associated with endocarditis, arthritis, septicemia, meningitis, and conjunctivitis; however, they are rare isolates. *M. lacunata* has generally been associated with conjunctivitis and keratitis. Its prevalence in causing these types of infections seems to be declining based on current studies. However, it is still found in significant infections of the eye

in the malnourished alcoholic population. In this population, these infections can become serious and at times do not respond well to treatment. *M. nonliquefaciens*, on very rare occasions, has proven to be the causative agent of meningitis, endocarditis, bronchitis, and conjunctivitis. However, usually it is isolated as a harmless commensal of the respiratory tract. *M. osloensis* is considered as part of the normal flora of the skin and genitourinary tract. It is, however, a rare agent of disease. Rarely, it can cause septic arthritis, meningitis, urethritis, mouth lesions, bacteremia, osteomyelitis, and endocarditis. *M. phenylpyruvica*, whose natural environment is unknown, is an opportunistic pathogen that occasionally has been isolated from infections in various body fluid specimens (pus, blood, CSF). *M. atlantae* has been recovered from blood and CSF, but its pathogenicity is uncertain.

Laboratory identification

On blood agar, colonies are 0.1 to 0.5 mm in diameter; round, smooth, and translucent to semi-opaque. At times, the colonies will spread and pit the agar. The genus *Moraxella* is rather inert biochemically, and its members do not produce acid from carbohydrates. Some of the enzymatic test kits for the identification of *Neisseria* are capable of identifying *Moraxella* species. With the advancement and application of computer technology to microbiology, microsystems for the identification of members of the genus *Moraxella* are available. These systems offer a quick, convenient, cost effective way to identify numerous organisms that might otherwise prove troublesome and time consuming to identify. However, if you do not have access to computer-assisted identification systems, *Moraxella* can be identified by means of manual tests. You can apply the following criteria to any organism suspected of belonging to the genus *Moraxella*: (1) Gram-stain morphology—tiny gram-negative coccobacilli, (2) growth on MacConkey (MAC) agar, (3) cytochrome oxidase test—all species are positive, and (4) asaccharolytic—does not ferment or oxidize carbohydrates in triple sugar iron (TSI) or oxidation fermentation test (OF) media. Other tests include growth at 42°C, urease, gelatin hydrolysis, nitrate and nitrite reduction, and DNase test.

Moraxella catarrhalis

M. catarrhalis are gram-negative diplococci with the characteristic kidney-bean appearance. They can grow on blood agar, and some strains can tolerate lower temperatures and grow well at 28°C. This organism was formerly known as *Branhamella catarrhalis* and is listed this way in many references.

Clinical significance

M. catarrhalis for years has been considered normal flora in the lower respiratory tract. This is being reevaluated in part because it has been misidentified as *N. cinerea* and because it was not isolated from oropharyngeal cultures of healthy individuals. It is now thought to be part of the normal flora of the upper respiratory tract. *M. catarrhalis* may also be the causative agent of a variety of infections from acute, localized infections to life-threatening, systemic infections. These infections include otitis media, sinusitis, bronchopneumonia, chronic bronchitis, endocarditis, and meningitis. *M. catarrhalis* has been increasingly recognized as the causative agent of otitis media (inflammation of the inner ear) and maxillary sinusitis in youngsters. Also, in the elderly and immunocompromised patient, it has been shown to cause bronchitis and pneumonia. It is less frequently isolated in cases of endocarditis, septicemia, and meningitis in patients whose pulmonary function is compromised.

Laboratory identification

Sinus aspirates for sinusitis and tympanocentesis fluid for otitis media are excellent specimens but are rarely collected due to cost. Also, they are invasive procedures that cause patient discomfort. Sputum and transtracheal aspirates are satisfactory for the isolation of *M. catarrhalis* from patients with lower respiratory tract infections. Inoculate specimens for the isolation of *M. catarrhalis* to 5% sheep blood agar and chocolate agar plates. Plates are incubated in increased CO₂ at 35 to 37°C for 18 to 24 hours. The colonies of *M. catarrhalis* are smooth, opaque, raised, have an entire edge and can produce a grayish-pink pigment or be gray to white in color. They usually stay intact when moved across the

plate or when picked up by a loop. The genus *Moraxella* is oxidase positive, catalase positive, and negative for acid production from glucose. Acid production from carbohydrates, chromogenic enzyme substrate tests, and multitest identification systems can be used to identify *M. catarrhalis*. A rapid method for confirming *M. catarrhalis* employs the hydrolysis of tributyrin which is positive for *M. catarrhalis* but negative for the other human *Neisseria* species. Several tests use this principle and are commercially available.

Self-Test Questions

After you complete these questions, you may check your answers at the end of the unit.

206. *Neisseria* species

1. Which of the 10 human origin species of the genus *Neisseria* is *not* a diplococci?
2. What is the most commonly reported communicable disease in the United States?
3. What percentage of females infected with gonorrhoeae are asymptomatic?
4. Normally, how long after infection with *N. gonorrhoeae* do men become symptomatic?
5. What member of the family *Neisseriaceae* usually causes ophthalmia neonatorum?
6. What is the usual route of transmission of *N. meningitidis*?
7. What factors favor epidemic outbreaks of meningitis?
8. What is the fatality rate for patients with meningitis?
9. How are the colonies of *N. gonorrhoeae* defined?
10. How often should *Neisseria* isolates be subcultured in order to maintain maximum viability?

11. In preparing smears for clinical materials containing possible *Neisseria gonorrhoeae*, for what two reasons should swab specimens be lightly rolled, rather than rubbed on glass slides?
12. Why is a diagnosis of gonorrhea based solely on the microscopic observation of GNID no longer valid?
13. What percentage of endocervical gonococcal infections can be tentatively diagnosed microscopically?
14. To diagnose gonorrhea in women, culture specimens should be obtained from what two sites and inoculated to what two separate media?
15. What type of loop should be used to perform the oxidase test and why?
16. What four categories of tests are used for the definitive identification of *Neisseria*?
17. What medium is used in the carbohydrate utilization test?
18. What carbohydrate utilization pattern distinguishes *N. gonorrhoeae* from *N. meningitidis*?
19. What are two disadvantages of the carbohydrate utilization test?
20. What are two traits of the most rapid fermentation test for the identification of *Neisseria*?
21. What is the principle of the coagglutination tests for *N. gonorrhoeae*?
22. What is the basis of enzyme immunoassay test for the identification of members of the family *Neisseriaceae*?

23. What is the most reliable procedure for routine serotyping of the meningococci?

207. *Moraxella* species

1. What is the most common isolate of the genus *Moraxella*?
2. Describe the general characteristics of *Moraxella* species.
3. What species of *Moraxella* is likely to be recovered from the eye of an alcoholic?
4. What is the natural habitat or environment of *M. phenylpyruvica*?
5. What are the general characteristics of *M. catarrhalis*?
6. How are the colonies of *M. catarrhalis* described?
7. What is the rapid method for confirming *M. catarrhalis*?

2-2. Gram-Negative Coccobacilli

This section encompasses a group of bacteria that have a common microscopic morphology but are quite different biochemically and in the diseases or infections they produce. Most of these organisms are rare isolates while others are isolated almost daily in the laboratory.

208. *Haemophilus* species

The genus *Haemophilus* is composed of a group of small, gram-negative, pleomorphic bacteria that require enriched media—usually containing blood or its derivatives—for isolation. *H. influenzae* type B is the most prominent pathogen of the genus. There are three species of *Bordetella*. *B. pertussis* is the primary pathogen of the genus and causes whooping cough. *B. parapertussis* and *B. bronchiseptica* are much less common causes of disease.

***Haemophilus* species**

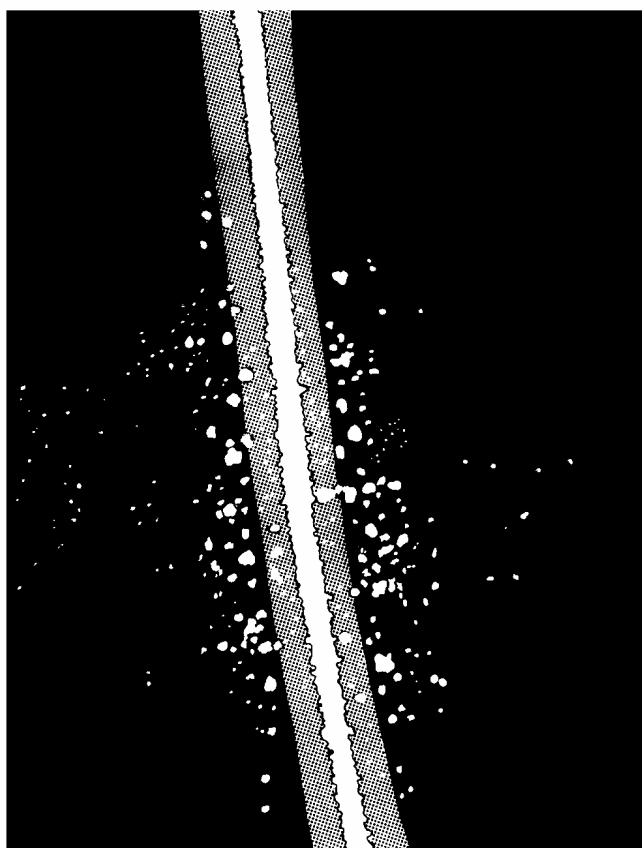
The genus *Haemophilus* is in the family *Pasteurellaceae* along with the genera *Actinobacillus* and *Pasteurella*. The genus *Haemophilus* contains eight human species and seven animal species. The species of greatest concern are the human species: *H. influenzae*, *H. parainfluenzae*, *H. ducreyi*, *H. aphrophilus*, *H. paraphrophilus*, *H. haemolyticus*, *H. parahaemolyticus*, and *H. segnis*.

General characteristics

These organisms are facultatively anaerobic, minute gram-negative rods that often take on a coccobacillary or threadlike, filamentous form. They are nonmotile, nonspore-forming cells which frequently display bipolar staining. Those isolated from cases of meningitis and bacteremia are encapsulated. The cells measure about 0.2 to 0.5 μm by 0.3 to 0.2 μm and have fastidious growth requirements. Whole blood contains the two factors—X factor and V factor—that are necessary for the growth of the genus *H. influenzae*.

Whole blood factors	Descriptions
X factor	Heat-stable substance, hemin, which is associated with hemoglobin.
V factor	Heat-labile substance that is coenzyme I, nicotinamide-adenine-dinucleotide (NAD); it may be supplied by yeast, potato extract, and certain bacteria (staphylococci), in addition to being found in blood.

The preferred medium for the isolation of *Haemophilus* is supplemented chocolate agar incubated in a humid atmosphere containing 5 to 10% CO_2 at 35 to 37°C. If enrichment supplements are not available, a plain blood agar plate can be streaked with the specimen presumed to hold *Haemophilus* species. Immediately thereafter, one or two streaks of a *Staphylococcus aureus* culture are applied at right angles to the primary inoculum. After 24 to 48 hours of incubation, *H. influenzae* and other species requiring V factor will appear as small colonies growing in close proximity to the staphylococci. This phenomenon is referred to as “satellitism” and results from factor V production by the staphylococci. We can see in figure 2-1 how diffusion of V factor into the surrounding medium provides a readily available, enrichment source for the *Haemophilus* species.



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Figure 2-1. *Haemophilus influenzae* satellitism with staphylococci on blood agar.

Clinical significance

Members of the genus *Haemophilus* are strict parasites that comprise part of the normal flora of the respiratory tract of humans and many animal species. Members of this genus are isolated from normal mucous membranes and various types of lesions and secretions of vertebrates.

Haemophilus influenzae

Some species, for example *H. influenzae*, are particularly troublesome, causing everything from respiratory tract infections to infections of the conjunctiva, otitis media, and meningitis. Of the eight species of *Haemophilus*, *H. influenzae* is by far the most commonly isolated pathogen responsible for an estimated 20,000 invasive infections per year. These numbers are declining due to a combination of vaccine administration, rifampin prophylaxis of disease contacts, and use of more effective therapeutic agents. There are six serotypes of *H. influenzae* designated *a* through *f*. This serological classification is based on the antigenic polysaccharide capsule surrounding the cells. *H. influenzae* and *H. parainfluenzae* contain eight biotypes, I through VIII. A biotype is a variant strain of a bacterial species differing in identifiable physiologic characteristics.

The serotype of *H. influenzae* recovered from serious, invasive infections such as meningitis, epiglottitis, and pneumonia, is almost invariably serotype *b*. Of the nontype-*b* typeable strains, *f* is the most common. The majority of these infections occur in children three months to five years of age; however, immunocompromised patients, as well as those suffering from chronic pulmonary disease, are at increased risk of developing pneumonia. These invasive infections usually have their origin in the nasopharynx. The organism then spreads by either of two routes: (1) localized infections of the surrounding tissues causing epiglottitis and pneumonia, or (2) systemic infection via the bloodstream, which results in infection of distant sites causing meningitis, septic arthritis, or osteomyelitis. The acute epiglottitis associated with the type *b* form of *H. influenzae* can develop rather quickly into a life-threatening infection. The epiglottis becomes highly inflamed and dramatically enlarged to the point of blocking the airway and suffocating the patient. Establishing an airway by nasotracheal intubation or tracheostomy frequently becomes necessary in order to save the patient's life.

Other *Haemophilus* species

Other members of the genus are responsible for conjunctivitis, sexually transmitted diseases, endocarditis, brain abscesses, and bacteremia. *H. influenzae* biogroup *aegypticus*, formerly *Haemophilus aegyptius*, is associated with the highly communicable form of purulent bacterial conjunctivitis. *H. ducreyi* is the cause of a sexually transmitted genital ulceration known as chancroid. *H. ducreyi* may be responsible for up to 10% of all the venereal diseases in civilian populations, but the frequency of chancroid may be much higher in military units in tropical countries. In smears from the genital ulcer, the small gram-negative rods occur as long strands. The pleomorphic organisms in fresh smears may appear gram-positive. They may be seen intracellularly or extracellularly. *H. ducreyi* grows on meat infusion of blood-enriched medium with increased CO₂, but primary isolation may be very difficult. Patients infected with this organism develop a hypersensitivity to it that may be detected by intradermal injection of heat-killed cells. The test is positive from one to two weeks after infection.

Haemophilus aphrophilus infections usually occur in patients who have underlying, serious health problems. Most strains are cultured from patients with a damaged endocardium, congenital heart disease, or secondary brain abscess. There is also an association between the isolation of this organism and malignancy, and cancer chemotherapy, or both. *H. aphrophilus* can cause serious infections such as endocarditis, brain abscesses, pneumonia, meningitis, and secondary bacteremia. *H. parainfluenzae* is associated with endocarditis, secondary bacteremia, and urethritis in adults. *H. haemolyticus* has been isolated from patients with urogenital, neonatal, and mother-infant infections.

Laboratory identification

The media or factor requirements of *Haemophilus* have been historically used as a clue in the identification process. Other characteristics that have been used for identification include its hemolytic properties, CO₂ requirements, carbohydrate fermentation, and biochemical tests.

Colony morphology

Colonies of *Haemophilus* species are small and translucent after overnight growth on chocolate agar. Most *Haemophilus* species do not grow at all on 5% sheep blood agar unless the V factor is added (it usually already contains enough of the X factor). On throat cultures, the colonies of *H. haemolyticus* should be differentiated from those of beta hemolytic streptococci. A Gram-stain will readily differentiate the two groups. They require only the V factor for growth. The colony morphology of *H. parainfluenzae* resembles that of *H. influenzae*. It does not produce hemolysis and shows satellitism around staphylococcal colonies. *H. parahaemolyticus* resembles *H. haemolyticus* even though it produces larger colonies on blood agar. These colonies produce zones of beta hemolysis.

Factor requirements

Two methods available for demonstrating the factor requirements of a *Haemophilus* species employ filter paper strips or discs. The most frequently used method for determining factor requirements uses paper strips impregnated with the X, V, and X+V factors. In this method a McFarland 0.5 standard suspension of the organism to be identified is inoculated onto a Mueller-Hinton trypticase soy, or brain heart infusion agar plate, and then the strips are applied to the surface of the agar. After 18 to 24 hours of incubation at 35 to 37°C in a 5 to 10% CO₂ atmosphere, the plates are examined for growth around the strips. Growth around a strip indicates a positive growth factor requirement. For example, if the suspected organism is *H. influenzae*, which requires both the X and V factors, growth can be seen around the X+V strip. On the other hand, if the inoculum contains *H. parainfluenzae*, growth only occurs around the V strip.

Two problems involving contamination of the test with X factor may arise when using these methods: (1) carry-over contamination—that is, contamination of the test by X factor carried over from the primary culture media to test inoculum, and (2) contamination of the basal agar medium used in the test with X factor. A rapid tube method and a commercially available quadrant plate method have been developed using the above principle.

The second paper disc method of demonstrating factor requirements is the delta (δ)-aminolevulinic acid (ALA) porphyrin test which determines the hemin or X factor requirement by assessing the ability of an isolate to synthesize compounds in the biosynthetic pathway to hemin from ALA. This test separates the strains of *Haemophilus* capable of synthesizing hemin from ALA from those strains that cannot. Strains that do not require X factor are capable of converting ALA to hemin and give a positive test result. Thus, a positive porphyrin test indicates an organism is X factor independent, while a negative test indicates that an organism is X factor dependent or requires exogenous X factor to grow. Isolates that are X factor independent synthesize and excrete porphobilinogens and porphyrins that fluoresce red to orange under ultraviolet light and are read as positive tests. Test plates plated with organisms that require exogenous X factor do not fluoresce and are recorded as negative. A tube method for this procedure is also available.

Other tests

As mentioned earlier, the infections caused by *H. influenzae* can be very serious and rapidly progress to life-threatening conditions. Therefore, methods for the rapid presumptive identification of *H. influenzae* have been developed. In a suspected case of meningitis or other life-threatening infection thought to be due to *H. influenzae*, the laboratory investigation typically would include the following efforts to identify the organism responsible for the patient's condition.

Initial processing would include careful Gram-stain examination of CSF for the typical short, slender, gram-negative rods of *H. influenzae*. Exercise great caution in your examination of such a specimen since these organisms often stain poorly and can be easily overlooked or mistaken for debris. Remember, your findings can be instrumental to the initiation of life-saving antimicrobial therapy. Secondly, the detection of the soluble, capsular polysaccharide of *H. influenzae* type *b* should be attempted. Several rapid immunologic procedures are available to accomplish this, using a variety of immunologic reactions ranging from coagglutination, latex agglutination, counter immunoelectrophoresis, or enzyme-linked immunosorbent assay. These immunologic procedures have two advantages over the Gram-stain procedure. They are more sensitive and can detect the soluble antigen even after the initiation of antimicrobial therapy. A DNA probe for the identification of *H. influenzae* is also available from Gen-Probe, San Diego, California.

Biochemical tests are also suggested for the species identification of the genus *Haemophilus*. Carbohydrate fermentation tests using glucose, sucrose, lactose, and mannose may be used for species identification. Reactions are read from phenol red broth supplemented with both the X and V factors and containing a 1% solution of the appropriate carbohydrate. Still other tests that may be used to separate the various strains of *H. influenzae* and *H. parainfluenzae* into biotypes include indole, urease, and ornithine decarboxylase. Biotyping is important since it can reveal meaningful epidemiological information. For example, it has been shown that certain biotypes of *H. influenzae* are associated with the source of isolation, antimicrobial susceptibility, and invasive disease. Also, keep in mind that *H. haemolyticus* and *H. parahaemolyticus* produce a zone of hemolysis on horse blood plates. See the table below for a review of different characteristics of *Haemophilus* species.

Species	X-Factor ^a Required	V-Factor Required	Catalase	Glucose	Sucrose	Lactose	Mannose
<i>H. influenzae</i>	+	+	+	+	—	—	—
<i>H. haemolyticus</i>	+	+	+	+	—	—	—
<i>H. ducreyi</i>	+	—	—	—	—	—	—
<i>H. parainfluenzae</i>	—	+	+/-	+	+	—	+
<i>H. parahaemolytica</i>	—	+	+	+	+	—	—
<i>H. segnis</i>	—	+	+/-	w	w	—	—
<i>H. paraphrophilus</i>	—	+	—	+	+	+	+
<i>H. aphrophilus</i>	—	—	—	+	+	+	+

a = as required by porphyrin test, +/- = different reactions found, w = weak fermentation reaction

209. *Bordetella* species

The genus *Bordetella* contains four species, and it has been proposed that this genus be included in a new family called *Alcaligenaceae* with the genus *Alcaligenes*. The four species of *Bordetella*—*B. pertussis*, *B. parapertussis*, *B. bronchiseptica* and *B. avium*—were formerly listed under the genera *Haemophilus*, *Bacillus*, and *Brucella*, respectively. The most nutritionally fastidious species, *B. pertussis*, is also the primary pathogen of the genus and the causative agent of whooping cough.

General characteristics

Microscopically, bordetellae are small, gram-negative coccobacilli or short rods, occurring singly, in pairs, or in small clumps. Characteristically, they stain very faintly, since they are slow in taking up safranin. It is suggested that the safranin counterstain be allowed to remain on the slide a full two

minutes instead of the usual 30 seconds. Although bordetellae do not require a CO₂ enriched atmosphere to grow, they do benefit from a moist environment, and it is suggested that they be incubated in a candle jar or CO₂ incubator at 35 to 37°C for this reason. *B. pertussis* and *B. parapertussis* are nonmotile, but *B. bronchiseptica* and *B. avium* are motile by peritrichous flagella. Bordetellae do not produce acid from carbohydrates and are inactive metabolically.

Clinical significance

All of the species of *Bordetella*, with the exception of *B. avium*, are known to cause respiratory infections in humans. *B. avium* is strictly an animal pathogen causing respiratory infections in turkeys and will not be discussed in this text. *B. pertussis* causes the most serious respiratory infection of all, which is commonly referred to as whooping cough. The infection may cause nothing more than a troublesome cough or may lead to the obstruction of the airway and death. Three factors seem to determine the seriousness of the infection: the patient's age, immunocompetence, and the level of infection—that is, how heavily the patient is colonized by the organism.

Whooping cough is spread when droplets containing *B. pertussis* are expelled into the atmosphere in aerosol form when an infected patient coughs. Inhalation of these droplets by a susceptible person leads to infection. Within seven to 10 days of infection, mild respiratory symptoms resembling those of the common cold appear. As increased irritation of nasotracheal passages develops so does the characteristic sporadic coughing. The coughing grows progressively worse with the coughs coming in such close sequence that the patient is unable to catch his or her breath. When the urge to cough stops, the patient gasps for air over a partially closed glottis resulting in the typical whooping sound so characteristic of *B. pertussis* infection.

The frequency of *B. pertussis* infection has been underestimated in recent years, since health care providers are not generally familiar with its signs and symptoms. Also, there is a lack of convenient and reliable procedures for the laboratory diagnosis of this condition. The cases seen in clinical practice today are often milder since they occur in immunized individuals who may only experience inflammation of nasotracheal passages and mild bronchitis. However, a *B. pertussis* infection should be suspected in any young child with a severe respiratory infection, and the diagnosis should be considered in older children and adults who have been exposed to pertussis and have the hallmark respiratory symptoms. *B. parapertussis*, as the name implies, is capable of producing a whooping cough like infection. In humans, *B. bronchiseptica* has been recovered from both respiratory and nonrespiratory infections, such as of wounds, endocarditis, peritonitis, bacteremia, and meningitis. It is, however, usually considered a more serious pathogen in animals, and especially in dogs.

Laboratory identification

The specimen of choice for isolation of members of the genus is from secretions of nasotracheal passages collected with a flexible thin wire swab tipped with either Dacron or calcium alginate. This swab should be passed up the patient's nose to the posterior nasopharynx and allowed to remain in place for 30 seconds while the patient is encouraged to cough. Once removed, it should ideally be plated immediately on plain Bordet-Gengou (BG) potato-glycerol-blood agar and BG supplemented with methicillin or cephalexin to inhibit normal nasopharyngeal flora. This media must be fresh; therefore, the laboratory should be notified ahead of time, so this medium can be prepared or obtained through a commercial supplier. Three smears should also be prepared at this time—two for direct fluorescent antibody (DFA) staining and one for Gram-staining. If the swab cannot be immediately plated, Regan-Lowe (RL) or casein hydrolysate transport media must be used for the transfer of the specimen to the laboratory. In any event, the specimen should ideally be plated within two hours.

Colony morphology

Within three days, the colonies of *B. pertussis* develop distinct mercury droplet morphology on BG plates. They are smooth, transparent, convex colonies with a pearly sheen, surrounded by a narrow

zone of hemolysis. *B. parapertussis* will produce larger, duller, slightly brown colonies on BG. *B. bronchiseptica* is the most rapid growing of all, producing well developed colonies on BG in 48 hours that initially look like *B. pertussis* colonies. In time, however, they display their own unique, pitted, metallic morphology and grow larger than the colonies of the other species. *B. parapertussis* and *B. bronchiseptica* will grow on ordinary blood agar unlike *B. pertussis*. On blood agar plates, *B. bronchiseptica* sometimes displays a type of hazy hemolysis, whereas *B. parapertussis* is usually noticeably hemolytic.

Other tests

A presumptive identification of bordetellae can be made based on the symptoms of the patient in conjunction with the colony and microscopic morphology observed. DFA testing is very valuable for diagnosing whooping cough in children since rapid results can be obtained. DFA is the most frequently used laboratory test for diagnosing whooping cough. However, this test requires that the technicians be skilled in the interpretation of DFA stained smears, and most clinical laboratories simply do not have the opportunity to maintain DFA testing proficiency. It is recommended that once smears have been prepared they be shipped to a reference laboratory for DFA examination. Confirmatory testing can be accomplished using slide agglutination tests. For the most part the bordetellae are biochemically inert, and biochemical tests for the identification of *B. pertussis* are generally not performed.

Self-Test Questions

After you complete these questions, you may check your answers at the end of the unit.

208. Haemophilus species

1. How would you describe the microscopic morphology of *Haemophilus*.
2. What physical characteristic is displayed by strains of *Haemophilus* isolated from cases of meningitis or bacteremia?
3. For growth on laboratory media, *Haemophilus* species must have one or the other, or both of what two compounds?
4. Of the X and V factors, which is heat stable and which is heat labile?
5. What is the preferred medium for the isolation of *Haemophilus*?
6. If enrichment supplements are not available, how can blood agar plates be used to cultivate *Haemophilus* species?

7. What is meant by the term “satellitism”?
8. What species of *Haemophilus* is the most commonly isolated pathogen?
9. How are the serotypes of *H. influenzae* designated?
10. What serotype of *H. influenzae* is most commonly isolated from serious, invasive infections?
11. What patients are most likely to experience infections due to *H. influenzae* type *b*?
12. What species of *Haemophilus* is associated with a highly communicable form of conjunctivitis?
13. What species of *Haemophilus* is the causative agent of chancroid?
14. What characteristics of *Haemophilus* are used for identification purposes?
15. What species of *Haemophilus* only require the V factor?
16. What is the principle of the delta-aminolevulinic acid porphyrin test?
17. What does a positive ALA porphyrin test indicate?
18. What substances are produced in a positive ALA porphyrin test, and how are they detected?
19. List four rapid immunologic procedures that can be used to identify *H. influenzae* type *b*?

209. *Bordetella* species

1. What is the causative agent of whooping cough?
2. Describe the microscopic morphology of the bordetellae, and how do you explain why they Gram-stain faintly?
3. All species of *Bordetella*, with what exception, are capable of causing respiratory infections in man?
4. What three factors determine the seriousness of a *B. pertussis* infection?
5. How do individuals become infected with *B. pertussis*?
6. What feature of *B. pertussis* infection of the respiratory tract has earned its common name of whooping cough?
7. What is the specimen of choice for the isolation of bordetellae?
8. What medium is required for the recovery of *B. pertussis*?
9. What is the medium of choice for shipping bordetellae specimens to a reference laboratory?
10. How would you describe the colonies of *B. pertussis* after three days of incubation on Bordet-Gengou agar?
11. On what findings can a presumptive identification of bordetellae be based?
12. What is the most frequently used confirmatory test for diagnosing whooping cough?

210. *Francisella* and *Brucella*

Francisella tularensis causes tularemia, a major zoonotic disease indigenous to many areas of the United States. The organism is transmitted by insect vectors or by the handling or ingestion of infected animals. The reservoir of infective agent is maintained in rodents by biting flies, ticks, and rabbit louse. All of the vectors are capable of spreading the disease from animal to animal. Tularemia is primarily a disease of rodents secondarily acquired by humans. The *Brucellae* are obligate parasites of animals and humans and are characteristically located intracellularly. *Brucella melitensis* usually infects goats while *Brucella suis* and *Brucella abortus* are associated with swine and cattle respectively. In humans, the *Brucellae* cause undulant fever that is characterized by an acute phase and followed by a chronic stage that may extend over many years and involve many tissues.

Francisella species

There are only three known species in this genus: *F. tularensis*, *F. novicida* and *F. philomiragia*. Only *F. tularensis*, the causative agent of tularemia (also known as rabbit or deerfly fever) is pathogenic to humans. *F. novicida* and *F. philomiragia* are animal pathogens, are extremely rare human isolates and, therefore, are not discussed in this text. The genus is named after Edward Francis who studied the organism extensively in Tulare County, California. Two varieties, or biovars, of *F. tularensis* exist and are designated as types A and B. Type A, the most virulent pathogen in humans, is only found in North America where it causes severe disease in humans, domestic rabbits, sheep, and ticks. Type B has been isolated throughout the Northern Hemisphere and is usually found in aquatic mammals, water, or wherever a favorable habitat for *Francisella* exists.

General characteristics

This organism is a strict aerobe that Gram-stains poorly, if at all, since it does not retain the stain well. When Gram-staining is successful, it reveals highly pleomorphic, minute, gram-negative, bipolar, faintly staining coccobacilli. They are nonmotile and nonspore forming. *F. tularensis* is extremely infectious; accordingly, a biosafety level 2 is recommended in the clinical laboratory and a biosafety level 3 is required when culturing large quantities of organisms.

Clinical significance

Humans contract tularemia either by handling the flesh of infected animals or through the bite of an arthropod vector. The main source of infection is the wild rabbit. The bacilli enter through cutaneous abrasions, or possibly through the intact skin of the hunter as he processes the animal for food. An aerosol of body fluids from infected animals may cause infection of the conjunctiva or lungs. Tularemic infections can be acquired in a number of different ways. Individuals of both sexes and all ages can contract tularemia. However, most reported cases are seen in hunters, the majority of whom are men who acquire the infection while skinning wild rabbits. Other means of acquiring tularemia include contact with infected rodents, biting insect vectors (deerflies, ticks, or mosquitoes), or ingesting contaminated food or water. In the United States, tularemia is the third most commonly reported tick-borne disease.

Again, it should be especially noteworthy to you as a laboratory technician that *F. tularensis* is classified as a biosafety level-2 pathogen (extremely invasive) that can be effectively transmitted by aerosolization. It is mandatory that you wear lab coats and gloves and work in a biological safety cabinet when working with material suspected of harboring this organism. Tularemia is one of the most frequently acquired laboratory infections and may be one of the few organisms capable of penetrating intact skin, although this is debatable. Some current literature sources suggest that the average clinical laboratory not work with materials from suspected cases of tularemia, but rather should ship such material to reference facilities for identification.

Tularemia is a zoonotic infection the severity of which depends upon two factors: (1) the virulence of the strain encountered, and (2) the route of infection. Route of infection determines the clinical

symptoms manifested by the patient. For example, there are seven types of tularemic infections: ulceroglandular (the most common), oculoglandular, oropharyngeal, glandular, typhoidal or systemic, pleuropulmonary, and gastrointestinal.

As the name implies, ulceroglandular tularemia involves the formation of a slightly tender erythematous papule that develops on the skin at the point of entry of the organism and progresses to become an ulcer. The ulcer is a result of contact with infected animals or from tick, deerfly, or mosquito bites. Lymph nodes surrounding this ulcer become enlarged and may drain. In oculoglandular tularemia, the organism gains entrance to the body by way of the conjunctiva of the eye. The eye becomes contaminated by way of an aerosol or when a contaminated hand touches the eye. Oropharyngeal tularemia's port of entry is the pharyngeal area and is manifested by a painful sore throat with enlarged tonsils. Glandular is the term used for patients with unexplained regional lymphadenopathy, positive for presence of organisms, but no ulcer can be found.

The typhoidal form of the disease has the highest mortality rate of approximately 30 to 60%, while the other forms result in a mortality rate of about 5% in untreated patients. This form occurs as an acute septicemia without the external ulcer and lymphadenopathy. Patient symptoms include severe headache, high fever, toxemia, and possible delirium; then shock usually occurs. When typhoidal tularemia is suspected, antibiotic therapy must be started immediately or the patient can die before the diagnostic workup is completed. The pleuropulmonary type tularemia is acquired when the organism is inhaled or the pleural cavity is colonized via the bloodstream. In both typhoidal and pulmonary forms of the disease, lung involvement is seen. However, lung involvement is not unique to these forms of tularemia, since some lung involvement may occur with all forms of tularemia and is attributed to a transient bacteremia.

The gastrointestinal form is acquired through ingestion of uncooked or improperly cooked food or contaminated water. Abdominal discomfort, low-back pain, and persistent diarrhea are associated with this form of tularemia. If left untreated, this form can progress to a rapidly fulminating fatal disease.

Laboratory identification

The preferred specimen includes material aspirated from the lesions, tissue from inflamed lymph nodes, respiratory secretions, and conjunctival scrapings. To optimize recovery of *F. tularensis*, place at least 0.5 ml of specimen on primary culture plates. The inoculum is placed in the center of the plate and then evenly distributed. Conjunctival scrapings can be obtained by passing a cotton swab over the conjunctiva while applying gentle pressure. *F. tularensis* is a fastidious obligate aerobe that will not grow on ordinary laboratory media. Its growth requirements have been used to differentiate it from *Pasteurella* because of its inability to grow without enrichments, such as cysteine, animal serum, or blood, and its inability to produce catalase.

Selective media and colony morphology

Two media that will support the growth of *Francisella* are (1) blood cysteine glucose (BCG) agar and (2) blood cysteine heart agar. BCG is the agar of choice for culturing this organism; however, success is not guaranteed even with its use, and, for this reason, freeze a portion of the specimen between -30 to -70°C for direct fluorescent microscopic examination. Colonies of *F. tularensis* are minute, blue-gray, transparent, round, smooth, and mucoid or drop-like. This organism is slow growing, requiring four days to reach a size of 2 to 3 mm. A greening of the agar under the colony occurs after prolonged incubation on BCG. Increased CO₂ does not enhance colony growth.

Serological test

Laboratory identification by observation of growth requirements, microscopic morphology, and biochemical characteristics is possible but considered dangerous. Serological examination is the most reliable diagnostic test when culture attempts fail. A four-fold rise in a patient's antibody titer over

the course of the disease or a convalescent titer greater than 1:160 is diagnostic. The fastest, most specific means for the definitive identification of *F. tularensis* in body exudates or tissue is the direct fluorescent antibody technique. Serological slide agglutination tests are routinely used to confirm the presence of *F. tularensis*. All strains of *F. tularensis* will agglutinate with the antisera that are available at reference laboratories.

***Brucella* species**

Six species of *Brucella* exist, *B. abortus*, *B. melitensis*, *B. suis*, *B. canis*, *B. neotomae* and *B. ovis*. The most common natural habitat for *B. abortus* is cattle, *B. melitensis*—goats and sheep, *B. suis*—swine, *B. canis*—dogs, *B. neotomae*—desert wood rat, and *B. ovis*—sheep. *B. ovis* and *B. neotomae* do not normally cause disease in humans.

General characteristics

Microscopic examination of *Brucella* reveals small (0.5 by 0.6 to 1.5 μm) gram-negative coccobacilli or short rods, singly, in pairs, or in short chains. The brucellae are nonmotile, nonspore forming, and nonencapsulated. It is important to note that you may experience difficulty Gram-staining brucellae since they resist counterstaining with safranin. This difficulty can be overcome in one of two ways: either allow the counterstain to remain on the slide for two minutes instead of the usual 30 seconds, or substitute carbol-fuchsin for the counterstain in the Gram-stain procedure. Of the two solutions, substituting carbol-fuchsin for safranin is the better since it stains the bacteria a more intense red color. Biochemically, the brucellae are oxidase positive (except *B. neotomae* and *B. ovis*), catalase positive, nonhemolytic, do not ferment carbohydrates, and are strict aerobes. Growth is often enhanced by CO_2 . They are relatively inactive metabolically.

Clinical significance

Brucellosis is a worldwide zoonosis that has considerable economic and public health impact. This is especially true in third world countries where the resources to deal with this disease are limited to non-existent. In different parts of the world, brucellosis is referred to by a variety of names, such as undulant fever, Bang's disease, Gibraltar fever, and Mediterranean fever. Its clinical manifestations are also just as varied and include local, systemic, and chronic forms of the disease. The disease may be subclinical (especially in animals) or acute.

In humans, it is characterized by an irregular temperature curve during the course of the illness. For several weeks, the patient may experience high fever followed by a few days of normal temperature readings. Brucellosis has a variable incubation period (7- to 10-month incubations have been reported) and either an abrupt or insidious onset. Localized lesions can occur in any tissue, organ, or bone. Pulmonary, cardiovascular, skeletal, and genitourinary complications can arise. Neurological and visceral complications arise in an estimated 10 to 15% of patients, with the most common complication of brucellosis being osteomyelitis. Brucellosis does not have a characteristic pattern of symptoms or signs, but shares symptoms such as weight loss, malaise, chills, anorexia, arthritis, splenomegaly, hepatomegaly, and night sweats with a number of other more common diseases. The primary virulence factor for *Brucella* seems to be the organism's ability to survive intracellularly since it is an intracellular parasite. This characteristic may also be responsible for the relapsing nature of this disease.

Human infections with *B. abortus* and *B. melitensis* are generally attributable to the consumption of unpasteurized dairy products from cattle and goats respectively, while infections due to *B. suis* and *B. canis* are usually related to close association with pigs and dogs. In developed countries where pasteurization is the norm, brucellosis is generally considered an occupation-related disease, with most cases occurring in the dairy and meatpacking industries.

Laboratory identification

A note of caution is warranted at this point. This is a biohazard type 3 organism and many laboratory-acquired cases of brucellosis have been documented. This organism is easily transmitted via direct skin contact and aerosols that may be created if specimens are not handled properly. Use caution when handling material suspected of harboring *Brucella*—wear lab coats, gloves, masks, and work within the confines of a biological safety cabinet. Make sure other members of the professional staff are aware of the fact that all clinical materials submitted to the laboratory for the isolation of brucellosis need to have cautionary labels attached. This will serve a dual purpose by alerting the laboratory of both the special handling required and special culturing techniques needed. Members of the genus *Brucella* require media that contains tryptose or trypticase.

Body fluids

A wide variety of specimens may be submitted for the recovery of *Brucella*, since culturing is the method of choice for demonstrating the organism in patient specimens. Blood, bone marrow, CSF, joint fluid, and various other body fluids are all possibilities. However, in the acute stage of the disease, blood and bone marrow cultures are most likely to be positive. This organism grows slowly in blood culture bottles, and even if growth takes place, it may not be apparent when culture bottles are visually or radiometrically checked. Therefore, retain blood culture bottles for 30 days, and perform blind subcultures weekly. Subcultures can be made to blood and chocolate agar plates that are retained for seven days because of the organism's slow growth. Regular blood culture bottles can be used and will support the growth of *Brucella*; however, it is recommended that the Castaneda technique be used. This involves using a biphasic blood culture bottle, one containing both liquid and a solid agar. This arrangement has the advantage of increasing your chances of seeing colonies develop directly from the broth or liquid portion of the culture. All blood culture bottles, regardless of type, must be continuously vented to a CO₂ enriched atmosphere. BACTEC and BacT/Alert bottles must also be kept for 30 days to recover *Brucella* species; the reported recovery from BACTEC bottles is five to 29 days and two to eight days with BacT/Alert.

Colony morphology

As mentioned earlier, the brucellae are somewhat fastidious and require agar containing tryptose or trypticase with 5% bovine serum supplement. Most strains will grow on blood and chocolate agar. However, the use of *Brucella* agar or infusions agar is recommended to enhance the recovery.

There are two types of *Brucella* agar: nonselective and selective. The nonselective type contains pancreatic digest of casein, peptic digest of animal tissue, yeast autolysate, and sodium bisulfite. To this list of ingredients, all that is needed to make the selective type of agar is the addition of crystal violet and antibiotics, such as bacitracin. The selective type is especially helpful if culturing material from sites that are normally contaminated and if the specimen must be mailed to a reference laboratory. The addition of 5% heated horse or rabbit serum has been shown to enhance the growth of *Brucella* on all media. Plates are incubated at 35 to 37°C in 5 to 10% CO₂ for at least 10 days.

Visible colonies take two to seven days to appear as convex, pinpoint, and glistening, producing neither pigment nor hemolysis on blood enriched media. These colonies turn gray or brown with age. Different species of *Brucella* require differing lengths of time before they produce visible colonies. *B. suis*, *B. melitensis*, and *B. canis* usually require 48 hours, while 72 hours is required by *B. abortus*. *B. abortus* also distinguishes itself in that it is the only brucellae that usually require CO₂. The brucellae also produce two types of colonies—smooth and rough. *B. abortus*, *B. melitensis*, *B. neotomae*, and *B. suis* usually produce smooth colonies, and *B. canis* and *B. ovis* usually produce rough colonies.

Other tests

The most rapid test for presumptive identification of *Brucella* is the particle test with antismooth *Brucella* serum. This test can only be performed on colonies suspected of being *Brucella* that have a smooth colony morphology. Antismooth agglutination sera will agglutinate *Brucella* organism from smooth colonies. The test is not performed on rough colonies. Other tests include urease and H₂S production, slide and tube agglutination test, growth in the presence of Thionin and basic fuchsin, phage typing, oxidative metabolic test, and DNA probes. All of these require growth of the organism, and since this is difficult, routine diagnosis of brucellosis is accomplished by serological test. These test procedures include agglutination, radioimmune assay, complement fixation and enzyme-linked immunoassay. They are performed on patient serum and detect a rise in antibody titers of IgM and IgG immunoglobulins.

Self-Test Questions

After you complete these questions, you may check your answers at the end of the unit.

210. *Francisella* and *Brucella*

1. What species of *Francisella* is pathogenic to man?
2. What biovars or types of *F. tularensis* exist, and which one is more virulent?
3. What biosafety classification has been given to *F. tularensis*?
4. Most reported cases of tularemia arise in patients who have engaged in what activity?
5. What mandatory safety precautions should be taken when working with *Francisella*?
6. What two factors determine the severity of a tularemic infection?
7. List the seven types of tularemic infections.
8. Passage of *F. tularensis* through what portal of entry results in oculoglandular tularemia?
9. Which type of *F. tularensis* has the highest mortality rate?

10. What is the agar of choice for culturing *F. tularensis*?
11. When culture attempts fail, what is the most reliable diagnostic test for tularemia?
12. What is the fastest most specific means for the definitive identification of *F. tularensis*?
13. Of the six species of *Brucella*, which are human pathogens?
14. Why is *Brucella* difficult to Gram-stain?
15. What other names is Brucellosis commonly referred to as?
16. What is the characteristic symptom of brucellosis?
17. What is thought to be the primary virulence factor of *Brucella*?
18. What is the usual source of infection of *B. melitensis*?
19. What is the biosafety level of the *Brucella*, and why should specimens from suspected cases of brucellosis be labeled as such?
20. What two substances must *Brucella* contain for media growth?
21. What specimens are most likely to yield positive results in the acute stages of brucellosis?
22. What blood culture technique is recommended for the isolation of *Brucella*?

23. What must be added to *Brucella* agar to make it selective?
24. What species of *Brucella* pathogenic to humans require the longest incubation time to produce visible colonies?
25. What species of *Brucella* usually produce rough colonies?

211. Other gram-negative coccobacilli

Organisms of the genus *Pasteurella* have a wide range and are responsible for epidemic and systemic diseases of domestic animals and birds. Many of these zoonotic species may also be transmissible to humans. *Pasteurella* is included with genera whose taxonomic relationship is unclear and includes *Brucella*, *Bordetella*, and *Francisella*. The relationship between these genera is open for further adjustment, and reorganization is likely in the future. Certain *Pasteurella* species and the species of *Actinobacillus* are thought to be very similar and for this reason they are grouped together in this text. Members of the genus *Moraxella* are generally nonpathogenic organisms that colonize the mucous membranes of humans and other warmblooded animals. *Kingella* species are rarely encountered in the clinical laboratory. Isolates that are recovered are most commonly from the blood or from bone or joint-associated sites.

Pasteurella species

Even among the select group of coccobacilliary organisms, *Pasteurella* is a standout since it grows more readily than the others and is fermentative. There are now at least 17 recognized species in the genus *Pasteurella*. *P. multocida*, *P. pneumotropica*, *P. canis*, *P. ureae*, *P. gallinarum*, *P. haemolytica*, and *P. aerogenes* have been reported to cause disease in humans. However, *P. multocida* is the primary pathogen of the genus and will be highlighted in this text.

General characteristics

As a group, pasteurellae are characterized as being small gram-negative, fermentative, nonmotile, indole, and oxidase-positive coccobacilli or rods (1 to 2 μm in length), with the majority of the species being catalase positive. Microscopically, this organism demonstrates bipolar staining when stained with Giemsa or methylene blue stain giving the appearance of closed safety pins. However, even under the best conditions bipolar staining does not always occur, so it is of limited use as an identifying characteristic. In Gram-stained smears, the other species of *Pasteurella* are larger than *P. multocida* which appears as tiny 0.3 to 0.5 μm by 1 μm coccobacilli when smears from tissue are stained. However, on solid agar the organism can be pleomorphic with rods up to 5 μm . Both *P. multocida* and *P. haemolyticum* can produce capsules. In the case of *P. multocida*, capsule production has been shown to be a virulence factor, since it interferes with phagocytosis of the bacterial cells by neutrophils.

Clinical significance

The pasteurellae are present as normal flora in the respiratory tracts of many domestic animals but, under the right conditions, can cause a variety of infections. The most pathogenic species is *P. multocida* whose Latin name can be translated as “killing many animals”. *P. multocida*, as you might expect from its name, is a zoonosis with important economic and veterinary impact. This organism is responsible for outbreaks of cholera in wild and domestic fowl, as well as hemorrhagic

septicemia in cattle and buffalo. The exact pathogenesis of these diseases is unknown, but outbreaks seem to follow periods of stress such as changes in the environment or viral infections. In domestic livestock, such stress occurs when animals are shipped to market. The resulting hemorrhagic septicemia or pneumonia is referred to as shipping fever.

P. multocida occurs as perhaps the most common organism in human wounds inflicted from cat and dog bites. It is one of the common causes of hemorrhagic septicemia in a variety of animals, including rabbits, rats, horses, and sheep. It can also produce human infections in many systems and at times may be a part of normal human flora. *P. multocida* infections in humans fall primarily into three categories—local, respiratory, or systemic. The vast majority of infections in humans are local infections due to being scratched or bitten by a dog or cat. In fact, 50% of all infections resulting from dog or cat bites yield *P. multocida* when cultured. On occasion, these infections can progress to osteomyelitis or arthritis. Even less commonly, *P. multocida* has been known to cause respiratory infections such as chronic pulmonary infections, and systemic infections such as bacteremia.

Laboratory identification

Clinical specimens include sputum, pus, blood, spinal fluid, and tissues. Members of this genus do not require specialized media, and they grow well at 35°C on blood or chocolate agar. Most strains, except *P. aerogenes* and *P. haemolytica*, do not grow on MAC agar. Only *P. haemolytica* produces beta-hemolysis on blood agar although many strains of pasteurellae produce a brownish discoloration. *P. multocida* produces a characteristic musty odor when grown on blood agar, and mucoid, smooth, or rough colonies may be observed. The smooth colony morphology is the most common. *P. multocida* should be suspected when an organism isolated from an animal bite grows on blood agar (but not MAC agar), ferments glucose and sucrose, and is oxidase positive. Other biochemical characteristics that help the identification of *P. multocida*, as well as other members of the genus, are production of indole, hydrolysis of urea, decarboxylation of ornithine, gas production from glucose, and acidification of maltose and xylose.

Actinobacillus species

The genus *Actinobacillus* is part of the family *Pasteurellaceae* and consists of seven species. Three of these species—*A. lignieresii*, *A. equuli*, and *A. suis*—are primarily animal pathogens, but on occasion have been recovered from bite wounds, blood, sputum, and CSF. *A. capsulatus* has never been recovered from humans but is known to cause arthritis in rabbits. It is considered a veterinary pathogen only and is not discussed in this text. *A. ureae*, *A. hominis*, and *A. actinomycetemcomitans* have been rare isolates from humans.

General characteristics

Actinobacillus are slow growing gram-negative microaerophilic coccobacilli or small rods that are best grown on blood or chocolate agar in a CO₂ atmosphere of 5 to 10% and elevated moisture. *Actinobacillus* are fermentative, nonmotile, and oxidase positive (except *A. actinomycetemcomitans* is sometimes negative).

Clinical significance

Actinobacillus species were first isolated from cattle and have since been isolated from other animals and humans. They have been associated with bacteremia, endocarditis, and periodontitis and recovered from blood, CSF, respiratory tract specimens, and animal bite wounds. *A. actinomycetemcomitans* is the most frequently isolated species of the genus and its primary pathogen. *A. actinomycetemcomitans* is part of the normal oral flora of humans, and human infections are endogenous. In the past, it was usually isolated in conjunction with actinomycotic infections, and its pathogenicity was questionable. More recently, it was recognized as a copathogen with *Actinomyces* and *Arachnia* species, perhaps enhancing the invasiveness of these agents by providing reduced conditions in tissue or by supporting the bacteria through production of extracellular enzymes and

toxins. *A. actinomycetemcomitans* is also capable of causing serious infection alone. It has been frequently isolated in cases of subacute endocarditis, vertebral osteomyelitis, and brain, oral, and thyroid abscesses.

Laboratory identification

After prolonged incubation, *Actinobacillus* has unique star-shaped colony morphology. The colonies enlarge, thicken in the center, and grow into the agar. When viewed from above, these colonies appear as four- to six-point stars that develop after prolonged incubation. These colonies adhere to the agar and are not easily removed. When scraped from the agar surface, they leave a star-shaped impression. In a tube of liquid media, this organism forms delicate granular colonies that cling to the sides of the tube just below the surface of the liquid. In blood cultures, these same granular colonies can be seen on the surface of the undisturbed blood cells or clinging to the sides of the culture bottle.

It is important to carefully examine such broth and blood cultures. In most cases, the surrounding liquid media will appear clear as if no growth is occurring. Important biochemical and key characteristics used to identify *A. actinomycetemcomitans* are no growth (or on rare occasions poor growth) on MAC agar, positive catalase reaction, negative urease and indole, no motility, and fermentation of glucose (but not of lactose and sucrose).

Kingella species

The genus *Kingella* is the latest addition to the family *Neisseriaceae*. Organisms formerly included in the *Moraxella* and classified as *Moraxella kingii* have now been relegated to their own genus *Kingella*. The four species of the genus *Kingella*—*K. kingae*, *K. denitrificans*, *K. oralis*, and *K. indologenes* (now *Suttonella indologenes*)—inhabit the upper respiratory tract, oral cavity, and occasionally, the urogenital tract. Formerly, these species were classified under the genus *Moraxella*, but due to their greater biochemical activity and differing genetic traits, they were placed in their own genus.

General characteristics

Kingella are gram-negative, aerobic coccobacilli or straight rods (2 to 3 μm in length) that occur in pairs or short chains. The fact that they are sometimes seen in pairs and are oxidase-positive can cause them to be initially identified as *Neisseria*; however, unlike *Neisseria* or *Moraxella*, they are usually catalase-negative. They are further distinguished from *Moraxella* in that they ferment glucose.

Clinical significance

K. kingae is considered an opportunistic pathogen that is occasionally a normal inhabitant of the upper respiratory tract. However, it is isolated more commonly from blood, corneal ulcer, nasopharynx, and bone- or joint-associated sites and, on rare occasions, is found in urine, wounds, and pustules. It has also been associated with endocarditis, arthritis, osteomyelitis, and septicemia. *K. kingae* has been isolated from significant infections in children under five years of age suffering from bacteremia, osteomyelitis, or septic arthritis. The occurrence of each of these genera in clinical specimens can complicate the diagnostic problems that you face. *K. denitrificans* on extremely rare occasions has been confirmed as the causative agent of endocarditis and has been described as a cause of granulomatous disease in AIDS patients. *K. indologenes* is infrequently isolated from ocular lesions.

Laboratory identification

After 24 hours, colonies are minute and convex with a flat periphery. Members of the genus *Kingella* differ from the *Moraxella* in that they are catalase negative and ferment glucose. They are similar to *Moraxellae* in being nonmotile, being cytochrome-oxidase positive, and obligating aerobes. They differ from *Moraxellae* in producing beta hemolysis. CO_2 enhances the growth of *K. denitrificans* which is somewhat fastidious requiring blood or blood products, such as serum, to grow and often

exhibits pitting of blood agar. *K. kingae* and *K. indologenes* do not require blood for growth, but they both can pit agar.

Self-Test Questions

After you complete these questions, you may check your answers at the end of the unit.

211. Other gram-negative coccobacilli

1. What two characteristics make the genus *Pasteurella* stand out from other unusual gram-negative bacilli?
2. As a group, how are *Pasteurella* organisms characterized?
3. What two species of *Pasteurella* produce capsules?
4. Why is encapsulation considered to be a virulence factor for *P. multocida*?
5. What are the three categories of *P. multocida* infections in humans?
6. What is the most common type of *P. multocida* infection in humans?
7. Which strains of *Pasteurella* do not grow on MAC agar?
8. Which species of *Pasteurella* is beta-hemolytic?
9. What characteristic odor does *P. multocida* give off?
10. What colony types are produced by *P. multocida*?
11. What are the three *Actinobacillus* that have been isolated from humans?

12. What species of *Actinobacillus* is most frequently isolated and is the primary pathogen of the genus?
13. What is the normal habitat of *A. actinomycetemcomitans*?
14. What other genera has *Actinobacillus* been closely associated with as a copathogen?
15. Upon prolonged incubation, what unusual shape do the colonies of *Actinobacillus* assume?
16. How do colonies of *Actinobacillus* appear in liquid culture media?
17. Why are *Kingella* often initially identified as *Neisseria*; how are they different?
18. From what specimens and sites is *K. kingae* most likely to be isolated?
19. *K. denitrificans* is associated with what type of infections?

Answers to Self-Test Questions

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1. *N. elongata*.
2. Gonorrhea.
3. Estimates range from 25 to 80%.
4. One to seven days.
5. *N. gonorrhoeae*.
6. Aerosols.
7. Overcrowding and fatigue.
8. Less than 10%.
9. The colonies usually appear as tiny to small (0.5 to 1 mm in diameter), gray, convex, shiny, translucent colonies with entire or undulate margins, although other larger, flatter colony types may be present.
10. Every 18 to 24 hours.
11. To accomplish an even distribution and prevent destruction of white cells or tissue cells.
12. *N. gonorrhoeae* can not be differentiated from *N. meningitidis* based on microscopic morphology and both may inhabit common sites. Recent evidence suggests an increase of gonorrhea-like infections in genital

specimens is actually caused by *N. meningitidis*, indicating that the reporting of gonococcal disease based on presumptive criteria is no longer valid.

13. Only 40 to 70%.
14. The cervix and anal canal; to TM or MTM culture plates and Transgrow bottles.
15. Platinum, because nichrome loops will cause a false positive reaction in the oxidase test.
16. (1) Immunologic.
(2) Carbohydrate utilization.
(3) Chromogenic enzymatic substrate.
(4) Nucleic acid probes.
17. Cystine trypticase agar.
18. *N. meningitidis* uses both glucose and maltose; whereas *N. gonorrhoeae* uses only glucose.
19. (1) Is designed to detect acid production from fermentation and is relatively insensitive to the detection of acid production by oxidation.
(2) Requires the suspected organism to grow.
20. (1) Higher concentration of carbohydrate is incorporated into the media.
(2) Heavier suspension of organism is used to inoculate the CTA media.
21. *S. aureus* cells, coated with anti-gonococcal antibodies, agglutinate in the presence of gonococci.
22. Enzyme hydrolysis of chromogenic substrates.
23. Agglutination reactions with commercially available typing sera.

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1. *M. nonliquefaciens*.
2. It is comprised of eight species of nonfermentative, strictly aerobic, catalase positive, nonmotile, oxidase-positive, plump coccobacilli, appearing in pairs, and sometimes short chains; they have a tendency to resist decolorization.
3. *M. lacunata*.
4. Unknown.
5. They are gram-negative diplococci with the characteristic kidney-bean appearance. They can grow on blood agar, and some strains can tolerate lower temperatures and grow well at 28°C.
6. They are smooth, opaque, raised, have an entire edge, and can produce a grayish pink pigment or be gray to white in color. They usually stay intact when moved across the plate or when picked up by a loop.
7. Employing the hydrolysis of tributyrin which is positive for *B. catarrhalis* but negative for the other human *Neisseria* species.

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1. Minute gram-negative rods, often coccobacillary, threadlike filaments, bipolar staining, and encapsulated are common.
2. Encapsulation.
3. X factor-hemin, associated with hemoglobin and V factor, a coenzyme I, nicotinamide-adenine-dinucleotide.
4. X factor is heat stable and V factor is heat labile.
5. Supplemented chocolate.
6. Streak the specimen presumed to hold *Haemophilus* species, and then apply one to two streaks of *Staphylococcus aureus* culture at right angles to the primary inoculum.
7. The phenomenon of small colonies growing in close proximity to another colony which provides the smaller colonies with the required growth factors is referred to as "satellitism".
8. *H. influenzae*.
9. a through f.

10. *H. influenzae* type b.
11. Children from three months to five years of age, immunocompromised patients, and those with chronic pulmonary disease.
12. *H. influenzae* biogroup aegyptius.
13. *H. ducreyi*.
14. Factor requirements, hemolytic properties, CO₂ requirements, carbohydrate fermentation, and biochemical test.
15. *H. parainfluenzae*.
16. Assesses the ability of an isolate to synthesize compounds in the biosynthetic pathway to hemin from ALA.
17. That an organism is X factor independent.
18. Porphobilinogens and porphyrins that fluoresce red to orange under ultraviolet light.
19. Coagglutination, latex agglutination, counter immunoelectrophoresis, or enzyme-linked immunosorbent assay.

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1. *B. pertussis*.
2. The bordetellae are small, gram-negative coccobacilli or short rods, occurring singly, in pairs, or in small clumps and they take up safranin slowly.
3. *B. avium*.
4. Age, immunocompetence, and how heavily the patient is colonized.
5. By inhaling aerosols containing *B. pertussis*.
6. Sporadic coughing that grows progressively worse until the patient is gasping for breath over a partially closed glottis resulting in the typical whooping sound.
7. Secretions from the nasotracheal collected on a flexible wire swab tipped with Dacron or calcium alginate.
8. Bordet-Gengou.
9. Regan Lowe or casein hydrolysate transport media.
10. Mercury droplets.
11. Patient symptoms, colony and microscopic morphology.
12. Direct fluorescent antibody test.

210

1. *F. tularensis*.
2. Biovars, or types A and B; A.
3. A biosafety level 2 is recommended in the clinical laboratory and a biosafety level 3 is required when culturing large quantities of organisms.
4. Hunting.
5. You must wear lab coats and gloves, and work in a biological safety cabinet.
6. Virulence of strain encountered, and route of infection.
7. Ulceroglandular (the most common), oculoglandular, oropharyngeal, glandular, gastrointestinal, typhoidal or systemic, and pleuropulmonary.
8. The eye.
9. Typhoidal.
10. Blood cysteine glucose.
11. Serological examination of the patient's blood.
12. Direct fluorescent antibody techniques.
13. *B. abortus*, *B. melitensis*, *B. suis*, and *B. canis*.
14. It resists counterstaining.

15. Undulant fever, Bang's disease, Gibraltar fever, and Mediterranean fever.
16. An irregular temperature curve.
17. The organism's ability to survive intracellularly.
18. Dairy products from goats.
19. Biosafety level 3 and so that special handling and culturing techniques will be utilized.
20. Tryptose or trypticase.
21. Blood and bone marrow.
22. Castaneda technique.
23. Crystal violet and antibiotics such as bacitracin.
24. *B. abortus*.
25. *B. canis* and *B. ovis*.

211

1. Grows well on ordinary agar and is fermentative.
2. Small gram-negative, fermentative, nonmotile, indole, and oxidase-positive coccobacilli or rods (1 to 2 μm in length), with the majority of the species being catalase positive.
3. *P. multocida* and *P. haemolytica*.
4. It interferes with phagocytosis of the bacterial cells by neutrophils.
5. Local, respiratory, and systemic.
6. Local, due to the bite or scratch of a dog or cat.
7. *P. aerogenes* and *P. haemolytica*.
8. *P. haemolytica*.
9. Musty odor.
10. Mucoid, smooth, and rough.
11. *A. ureae*, *A. hominis*, and *A. actinomycetemcomitans*.
12. *A. actinomycetemcomitans*.
13. Normal oral flora of humans.
14. *Actinomyces* and *Arachnia*.
15. Star shape.
16. Delicate granules that cling to the sides of the tube or culture bottle.
17. *Kingella* are coccobacilli that sometimes appear in pairs and are oxidase-positive causing them to be initially identified as *Neisseria*; however, unlike *Neisseria*, they are usually catalase-negative.
18. Blood, corneal ulcer, nasopharynx, and bone- or joint-associated sites, and, on rare occasions, is found in urine, wounds, and pustules.
19. On extremely rare occasions has been confirmed as the causative agent of endocarditis and has been described as a cause of granulomatous disease in AIDS patients.

Do the Unit Review Exercises (URE) before going to the next unit.

Unit Review Exercises

Note to Student: Consider all choices carefully, select the *best* answer to each question, and *circle* the corresponding letter.

27. (206) Ophthalmia neonatorum, an eye infection of the newborn, is usually caused by *Neisseria*
 - a. *sicca*.
 - b. *elongata*.
 - c. *meningitidis*.
 - d. *gonorrhoeae*.
28. (206) What is the *usual* route of transmission of *Neisseria meningitidis*?
 - a. Direct skin contact.
 - b. Aerosols.
 - c. Sexual.
 - d. Blood.
29. (206) The production of what respiratory enzyme is detected by the oxidase test?
 - a. Peroxidase.
 - b. Hydrogenase.
 - c. Cytochrome oxidase.
 - d. Indole phenol oxidase.
30. (207) What has *generally* been associated with *Moraxella lacunata*?
 - a. Meningitis.
 - b. Otitis media.
 - c. Conjunctivitis.
 - d. Nonspecific urethritis.
31. (207) Which rapid method is used for confirming *Moraxella catarrhalis*?
 - a. Oxidase.
 - b. Catalase.
 - c. Tributyrin hydrolysis.
 - d. Acid production from carbohydrates.
32. (208) What is the *preferred* agar for the isolation of *Haemophilus* spp.?
 - a. Blood.
 - b. Egg based.
 - c. Trypticase.
 - d. Supplemented chocolate.
33. (208) The *Haemophilus influenzae* serotype that is recovered from meningitis and pneumonia most often and can develop rather quickly into a life-threatening infection is serotype
 - a. a.
 - b. b.
 - c. d.
 - d. f.

34. (208) Which organism is associated with the highly communicable form of purulent bacterial conjunctivitis?
- H. ducreyi*.
 - H. aphrophilus*.
 - H. haemolyticus*.
 - H. influenzae* biotype aegyptius.
35. (208) Which organism is the cause of a sexually transmitted genital ulceration known as chancroid?
- H. ducreyi*.
 - H. aphrophilus*.
 - H. haemolyticus*.
 - H. parainfluenzae*.
36. (209) Which organism is the causative agent of whooping cough and is the primary pathogen of the genus *Bordetella*?
- B. avium*.
 - B. pertussis*.
 - B. parapertussis*.
 - B. bronchiseptica*.
37. (209) What is the specimen of choice for isolation of members of the genus *Bordetella*?
- CSF.
 - Urine.
 - Blood.
 - Nasotracheal secretions.
38. (209) What is the medium of choice for shipping specimens suspected of containing *B. pertussis*?
- .85% saline.
 - Regan Lowe.
 - Bordet-Gengou.
 - Buffered phosphate.
39. (210) The biosafety level recommended for the clinical laboratory when culturing *F. tularensis* is classified as
- 1.
 - 2.
 - 3.
 - 4.
40. (210) Which tularemic infection is the *most* common?
- Ulceroglandular.
 - Oculoglandular.
 - Oropharyngeal.
 - Glandular.
41. (210) What is the agar of choice for culturing *F. tularensis*?
- Blood.
 - Chocolate.
 - MacConkey (MAC).
 - Blood cysteine glucose (BCG).

42. (210) What is the *most* common complication of brucellosis?
- Osteomyelitis.
 - Pericarditis.
 - Pneumonia.
 - Arthritis.
43. (210) The biosafety level recommended for *Brucella* is classified as
- 1.
 - 2.
 - 3.
 - 4.
44. (211) The most pathogenic species of *Pasteurella* is
- P. ureae*.
 - P. multocida*.
 - P. aerogenes*.
 - P. haemolytica*.
45. (211) *P. multocida* occurs as perhaps the most common organism in human wounds inflicted by bites from
- cats and dogs.
 - goats and sheep.
 - cattle and horses.
 - ticks and deerflies.
46. (211) What species of *Pasteurella* produces beta-hemolysis on blood agar?
- P. ureae*.
 - P. aerogenes*.
 - P. multocida*.
 - P. haemolytica*.
47. (211) What characteristic odor is given off by *P. multocida*?
- Fecal.
 - Musty.
 - Moldy.
 - Mousy.
48. (211) What species of *Actinobacillus* is *considered* the primary pathogen of the genus?
- A. actinomycetemcomitans*.
 - A. lignieresii*.
 - A. equuli*.
 - A. suis*.
49. (211) After prolonged incubation, *Actinobacillus* has a unique colony morphology. How do the colonies appear?
- Star shaped.
 - Granular.
 - Mucoid.
 - Stringy.

50. (211) Organisms formerly classified as *Moraxella kingii* are now classified in the genus
- a. *Flavobacterium*.
 - b. *Acinetobacter*.
 - c. *Pseudomonas*.
 - d. *Kingella*.

Please read the unit menu for unit 3 and continue ➡

Unit 3. Aerobic Gram-Negative Bacilli

3–1. <i>Enterobacteriaceae</i> Family	3–1
212. <i>Enterobacteriaceae</i> classification and general characteristics	3–1
213. <i>Escherichia</i> , <i>Salmonella</i> , <i>Shigella</i> , and <i>Yersinia</i>	3–7
214. Other <i>Enterobacteriaceae</i> genera	3–12
3–2. Other Gram-Negative Bacilli.....	3–22
215. <i>Pseudomonas</i> and <i>Burkholderia</i>	3–22
216. <i>Acinetobacter</i> , <i>Eikenella</i> , and <i>Xanthomonas</i>	3–23
217. <i>Vibrio</i> , <i>Aeromonas</i> , <i>Plesiomonas</i> , <i>Campylobacter</i> , <i>Arcobacter</i> , and <i>Helicobacter</i>	3–25
218. <i>Legionella</i> and <i>Streptobacillus</i>	3–29

OF ALL the clinical specimens submitted to the laboratory for bacteriological examination, feces or stool specimens contain the greatest number of organisms, both in quantity and the variety of genera represented. The feces normally contain millions of bacteria per gram. Most of the bacteria are harmless normal flora, but others cause some of the world's most prevalent diseases. The pathogenic and nonpathogenic gram-negative bacilli that you isolate from stool specimens are often referred to as the enterics because of their natural habitation in the intestinal tract. Aside from these forms, there are enteric-like organisms that closely resemble the enterics and occur often enough in feces or other clinical specimens to complicate the process of identification.

3–1. *Enterobacteriaceae* Family

The *Enterobacteriaceae* family contains over 50 species of microorganisms that are isolated from human clinical specimens. Four species cause intestinal infections, including diarrhea, seven species are frequently responsible for nosocomial infections, and most of the other species are associated with human or animal infections. These species can be divided into two groups, (1) those that have been well known for many years and are isolated most often in the clinical microbiology laboratory and (2) those that are newly described and are rarely seen.

212. *Enterobacteriaceae* classification and general characteristics

The prompt and accurate identification of enteric organisms serves several useful purposes. It can help locate and isolate carriers and give advance warning of such epidemics as the plague and typhoid fever.

Enterobacteriaceae classification

The bacteria that make up the family *Enterobacteriaceae* are closely related in the physiological sense. Similarities in their fundamental metabolic processes, biochemical reactions, and antigenic analysis usually make generic distinction difficult. To complicate the task of laboratory identification, we often find marked variability from culture to culture of a given species with respect to fermentation patterns and other traits useful in characterizing an organism. Consequently, there is still a great deal of uncertainty regarding the exact taxonomic position of many of the gram-negative enteric forms. Techniques in nucleic acid sequencing and hybridization better define the relationships of this family.

Taxonomy

With the application of highly technical tests to determine the relatedness of one species of bacteria to another, has come a great amount of new information concerning members of the family *Enterobacteriaceae*. Such procedures as deoxyribonucleic acid (DNA) homology studies resulted in

the number of recognized enteric species growing from 26 to over 50 in the last few years. Unfortunately, the assimilation of this glut of information and its practical application has created confusion for the clinical microbiologist. From a clinical point of view, we strive to communicate in a meaningful way the identity of the causative agent of a disease process along with its antibiotic susceptibility pattern to the health care provider. Increasingly complex taxonomic classification schemes, although of importance to epidemiologists and taxonomists, are of limited usefulness to the clinical microbiologist. This text concentrates on the genera of *Enterobacteriaceae* that are most likely to be encountered in the clinical laboratory, and every effort is made to use the most current nomenclature to identify them by their correct genus and species.

General characteristics

Members of the *Enterobacteriaceae* family are truly universal, having as their natural habitat soil, water, plants, insects, fish, and, of course, as their name implies, the intestinal tract of both humans and animals. Their name is derived from two Greek words “enteron” that is translated as “intestine” and “bacter” meaning “small rod”. They are so widely distributed in nature that they are sometimes used as a standard for determining water purity. The number of *E. coli* present in a given amount of water is used to determine if a body of water is a safe source for drinking water or for recreational activities.

Physically, the enterics can be described as gram-negative, straight-sided rods averaging 1 μm wide and up to 6 μm in length. They do not form spores and can survive with or without oxygen and, therefore, are considered facultative anaerobes. If the enteric members are motile, they possess peritrichous flagella. All *Enterobacteriaceae* are able to use glucose fermentatively, reduce nitrates to nitrites, and are oxidase negative. Another biochemical characteristic common to many members of the family is the production of the enzyme catalase.

Cultural characteristics

The growth of members of the family *Enterobacteriaceae* on a variety of agars is not difficult, but what may prove difficult at times is separating the pathogens from the nonpathogens. Media for the cultivation of the *Enterobacteriaceae* fall into four categories: (1) supportive, (2) selective, or (3) differential, or (4) some combination of the three. The supportive agars, as the name implies, support the growth of most types of bacteria; an example is blood agar. The selective agars inhibit the growth of some organisms that may be present in the sample; examples EMB and MacConkey (MAC) inhibit the growth of most gram-positive organisms. Various selective agars that allow only members of *Enterobacteriaceae*, likely to cause disease to grow, are available. These agars are selective because various inhibitory dyes are incorporated into them. The differential agars demonstrate visible morphological differences in growth characteristics for preliminary identification of organisms.

For the most part, the *Enterobacteriaceae* lack distinctive colony morphology with all members producing rather nondescript, large, gray colonies on blood agar. A few members do distinguish themselves by their colony characteristics. *Klebsiella*, for example, has rather distinctive raised mucoid colonies that adhere to inoculating loops when touched and string out when the loop is withdrawn. When cultured on blood agar some members of the genus *Proteus* display unique swarming colony morphology. Swarming limits the identity of the organisms to just three possibilities: *P. mirabilis*, *P. vulgaris*, and *P. penneri*. Likewise, the *Enterobacteriaceae* for the most part do not have a distinctive appearance when grown in broth media. In general, growth is marked by the development of overall turbidity; however, one exception is *Yersinia pestis*, which exhibits a long, filamentous formation that hangs down from the surface of the broth. This unusual morphology is referred to as a stalactite pattern of growth.

As mentioned earlier, one of the greatest challenges in working with enteric organisms is separating the pathogens from the nonpathogens. In no other specimen is this more apparent than stool samples.

The processing of stool specimens for enteric isolation follows a well-established pattern. Let us review in brief the stepwise procedure for isolation of *Enterobacteriaceae* in stool samples as well as in other specimens.

Isolation from stools

First, inoculate an enrichment broth such as gram-negative (GN) broth, selenite F, or tetrathionate broth. These media are inhibitory for the normal intestinal inhabitants and must be used. Concurrently with inoculation of the enrichment broth, streak plates of assorted selective and differential media with the stool specimen.

There are many plating media in use. Some are selective while others are inhibitory. Among those widely used are desoxycholate citrate agar, *Salmonella-Shigella* (SS) agar, Hektoen enteric agar (HEA), bismuth sulfite agar, brilliant green (BG) agar, eosin-methylene blue (EMB) agar, xylose-lysine-desoxycholate (XLD) agar, and MAC agar.

NOTE: To inhibit the spreading of *Proteus* strains on MAC or EMB media, you may increase the agar concentration to 5%. Remember that selective media suppress the growth of contaminating forms, usually nonpathogens, and allow the infectious species to survive and proliferate.

Differential agars, such as eosin-methylene blue and MAC, employ the principle of color distinction between lactose-positive and lactose-negative colonies (nonpathogens and pathogens). Colors are based on changes in pH of an indicator dye when acids are produced from lactose.

Tetrathionate and selenite F enrichment broths are *not* used to isolate enteropathogenic *Escherichia coli*, *Klebsiella*, *Enterobacter*, or *Citrobacter* from stool samples because they are inhibitory to most strains of these genera. MAC or EMB agar is used for primary isolation.

Isolated colonies on these media furnish the inoculum for one of the more complex formulations such as triple sugar iron (TSI) or Kligler's agar (KIA) slants. Reactions on TSI or KIA provide presumptive information upon which we can base our choice of fermentation and other metabolic tests to establish genus and species identity. The three color patterns possible with TSI or KIA agar are:

1. Acid (yellow) reaction throughout—lactose fermentation.
2. Acid (yellow) butt and alkaline (red) slant—glucose fermentation.
3. Alkaline (red) butt and slant—no sugars fermented.

In addition, bubbles or large breaks/cracks in the medium indicate gas formation, and H₂S production is evidenced by a blackening of the agar in the butt of the tube. TSI contains a third sugar, sucrose, which, if fermented, points to one of the slow-lactose fermenters. A positive sucrose gives an all-yellow reaction identical to that of lactose fermentation.

Isolation from urine

Members of *Enterobacteriaceae* isolated from the urinary tract include species of *Escherichia* and certain members of *Salmonella*, *Klebsiella*, *Enterobacter*, *M. morganii*, *Providencia*, *Serratia* and *Proteus* genera. Inoculate urine specimens directly onto plating media. Blood agar and a differential plating media are recommended.

Biochemical characteristics

The enterics can be tentatively identified based on their colony morphology on both supportive and selective/differential media, microscopic morphology, rapid biochemical test, and reaction patterns displayed on TSI or KIA. However, species identification requires demonstrating the phenotypic features of individual organisms. Traditionally, this was accomplished by running a biochemical battery of tests in a series of 13×100 mm test tubes. With the advent of miniaturized or automated

commercial identification systems, the older more cumbersome and labor-intensive biochemical tube tests have fallen out of favor. However, the reactions observed in these tests are still valid and serve as the basis of the more modern test kits. We'll, therefore, review the principles of the older tube tests and briefly discuss some of the new test systems.

Oxidase test

This test can be critical in separating true enterics that are all oxidase negative from other gram-negative rods, such as *Pseudomonas*, that are oxidase positive. The test is easily performed using filter paper and a solution of freshly prepared tetramethyl-p-phenylenediamine dihydrochloride (Kovac's reagent). The presence of the oxidase enzyme reacts with the Kovac's reagent to form a blue to black color. The oxidase test must be performed with colonies growing on agar that does not contain dyes that might obscure the color reaction. Read the test reaction within 10 to 20 seconds since many organisms, including a few enterics, may produce delayed false-positive reactions.

Urease

This test determines if an organism is capable of hydrolyzing urea to ammonia and water. A urea slant should only be inoculated on its surface to determine aerobic reactions. For best results, heavily inoculate urea broths and thoroughly disperse the organism in the broth. The heavier the inoculum the faster a positive result is obtained. The phenol red indicator changes to pink or red as the pH of the media rises due to the production of the ammonia end product. Incubate the media at 37°C, and check it at 10 minutes, 30 minutes, 1 hour, and several hours after inoculation.

Orthonitrophenyl galactosidase (ONPG) activity

The ONPG test is a rapid means of determining if an organism can utilize lactose, by detecting the presence of β -galactosidase. This can be especially helpful when you are dealing with certain strains of *E. coli*, *Shigella sonnei*, *Hafnia alvei*, *Serratia marcescens*, and some *Yersinia* that possess β -galactosidase but do not possess permease. Permease is responsible for actively transporting lactose across the cell membrane (fig. 3-1), making it available to intercellular β -galactosidase. These organisms ferment lactose slowly, since they must wait for it to slowly diffuse across the cell membrane before it can be acted upon. True nonlactose fermenters do not possess β -galactosidase.

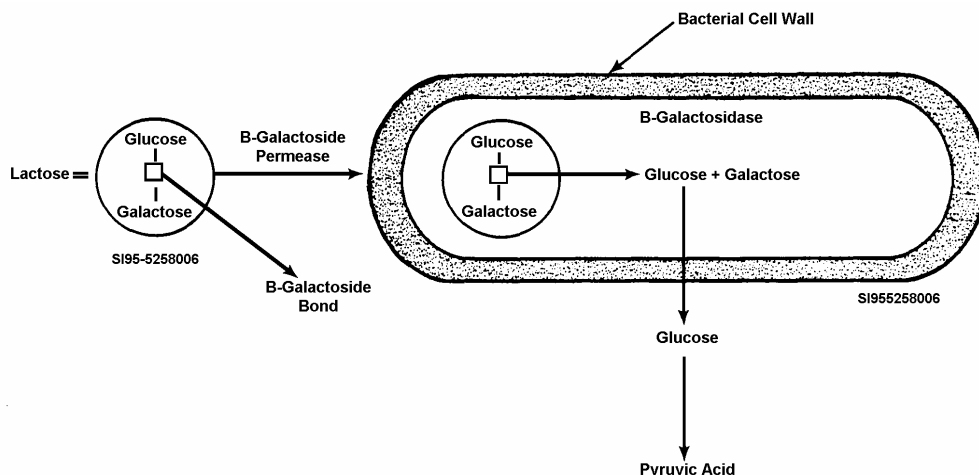


Figure 3-1. Permease transporting lactose through the cell membrane.

ONPG test detects the presence of intercellular β -galactosidase (fig. 3-2). β -galactosidase acts on the substrate orthonitrophenyl-beta-D-galactopyranoside in the same way as the enzyme hydrolyzes lactose to form galactose and glucose. The end product of the ONPG reaction is orthonitrophenol and

is yellow. Perform this test by mixing a heavy inoculum of the suspected organism in a small volume of alkaline buffer solution and then adding a paper disc impregnated with the reagents. The tube is then incubated and examined for the development of a yellow color that indicates a positive test. Negative tests remain colorless after incubation. This is an important determination since enteric pathogens such as *Salmonella* and *Shigella* are usually considered nonlactose fermenters.

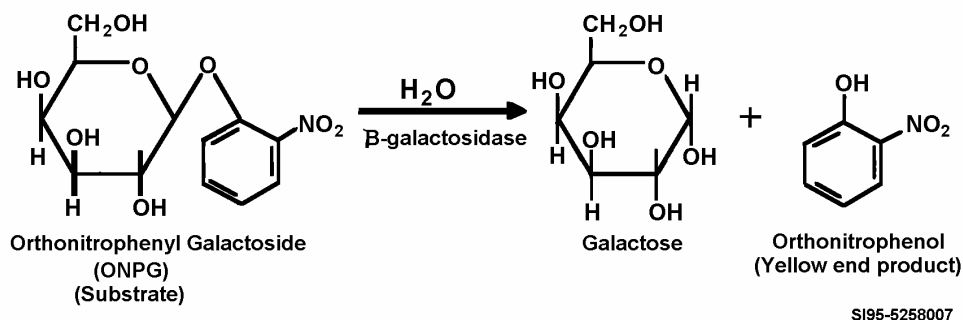


Figure 3-2. ONPG test detecting the presence of intracellular β -galactosidase

Indole test

This test determines if an organism possesses the enzyme tryptophanase. If so, the organism is able to deaminate the amino acid tryptophan, resulting in the production of indole, pyruvic acid, and ammonia (fig. 3-3). The indole can then be detected by the addition of either Kovac's or Ehrlich's reagent, that cause a positive red color reaction in the presence of indole. Remember that Ehrlich's reagent is more sensitive and should be used when working with nonenteric organisms.

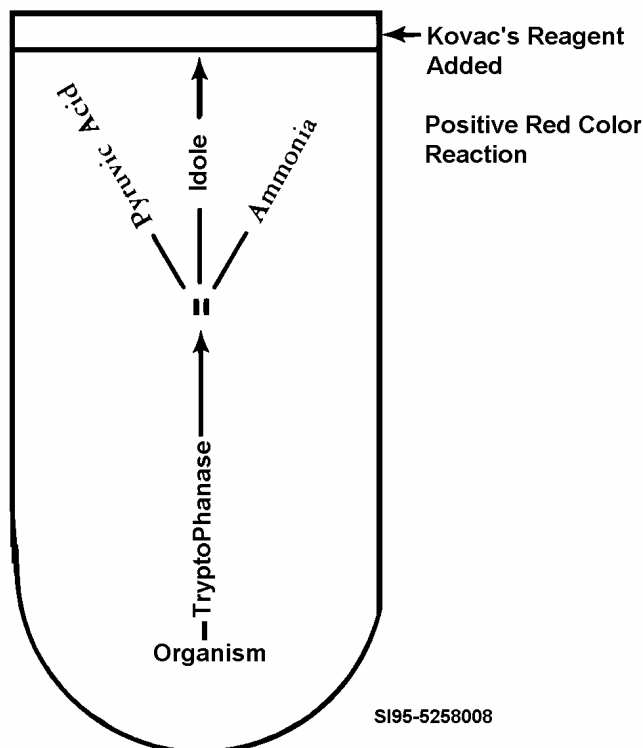


Figure 3-3. Tryptophan test.

Methyl red (MR) and Voges-Proskauer (VP) test

Both of these tests detect the formation of acid end products of metabolism of glucose. Some organisms use glucose and produce strong acid end products such as formic and acetic acid. Other organisms produce less acidic end products such as acetoin and butanediol. The MR and VP tests detect end products of two divergent metabolic pathways in the utilization of glucose by bacteria. The MR test detects the formation of formic acid and acetic acid, while the VP test detects acetoin and butanediol (fig. 3-4). The same 5 ml substrate broth is inoculated and incubated and then used in both tests. After the substrate broth is incubated for 48 hours, it is divided equally and one aliquot is tested for the MR reaction while the other is tested for the VP reaction. Perform by adding 0.5 ml of methyl red to the MR tube and observing to see if the tube remains red after the addition of methyl red (positive reaction), or changes to yellow (negative reaction). Two reagents must be added to the VP tube before it can be read, 0.6 ml of alpha naphthol followed by 0.2 ml of 40% KOH. The tube is then mixed and allowed to stand for 15 minutes. The development of a pink to red color is recorded as a positive test, while a colorless to yellow reaction is considered a negative.

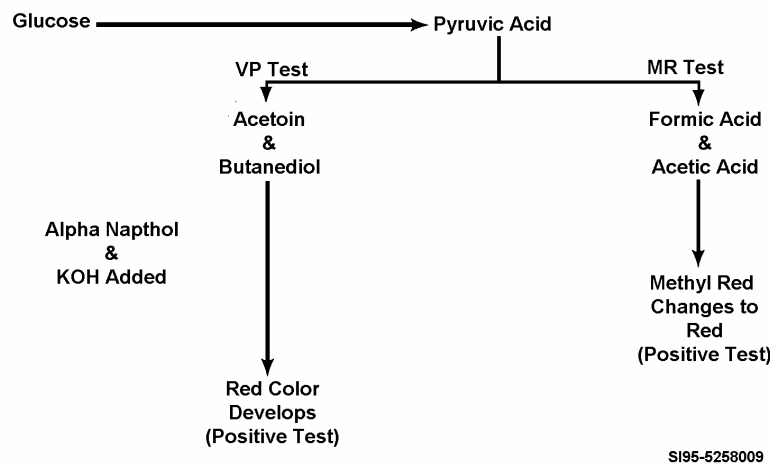


Figure 3-4. MR and VP test.

Phenylalanine test

Most members of the family *Enterobacteriaceae* cannot deaminate phenylalanine; however, the genera *Proteus*, *Providencia*, *Morganella*, and occasionally certain strains of *Enterobacter agglomerans* can. Phenylpyruvic acid is the end product of the deamination of phenylalanine and can be detected with the addition of 10% ferric chloride. Perform by inoculating phenylalanine agar slants heavily and incubating them overnight. A 10% solution of ferric chloride is then added; it reacts with any phenylpyruvic acid that may be present to form a green color.

Citrate utilization test

This test determines if an organism can use citrate as its sole source of carbon. Simmon's citrate agar, containing buffers, salts, cations, and bromthymol blue as a pH indicator, is used to perform this test. The production of alkaline end products from the metabolism of citrate causes the indicator to turn from green to blue. The formation of this blue color is considered a positive test reaction. The reaction is an aerobic one, so the agar slant is lightly inoculated on its surface and incubated at 35 to 37°C for 24 hours with its screw cap slightly ajar.

Motility test

This test determines if an organism is capable of moving through a semisolid agar. This characteristic can be helpful in differentiating nonpathogens like *E. coli* that is usually motile from pathogens like *Shigella* that is almost never motile. Motility agars are prepared as butt tubes to a depth of 5 cm. They

are inoculated with a single straight stab into the center of the tube to a depth of 2 cm. Motility is determined by hazy growth occurring around the stab line after overnight incubation. The preferred incubation temperature is dependent upon the suspected identity of the isolate you are working with. Not all organisms are motile at a given temperature; for example, *Yersinia enterocolitica* is not motile at 37°C, but is motile at room temperature. Some motility agars such as sulfide-indole-motility (SIM) agar not only demonstrate motility but also hydrogen-sulfide and indole production.

Decarboxylation and dihydrolation tests

These tests determine the ability of an organism to break down the amino acids lysine, arginine, and ornithine. The amine end product from the decarboxylation of lysine and arginine, and the dihydrolation of ornithine causes an alkaline pH shift to occur in the media. This shift is detected when the indicator bromocresol purple, that originally is pale purple, turns a deeper purple. If an organism is incapable of degrading the amino acid present, but does ferment glucose, the media turns yellow. Moeller's decarboxylase medium is used to perform this test. This medium is formulated to contain the appropriate amino acid, glucose, cresol red indicator, and the enzyme activator pyridoxal. This media is inoculated by placing a large loopful of the isolate beneath the surface of the liquid media, making sure it is well mixed with the broth. The broth is then overlaid with sterile mineral oil since the reaction must take place in an anaerobic environment, and the tube is incubated at 37°C for up to 14 days. A base tube containing glucose without an added amino acid is included as a negative control with each set.

Lysine iron agar (LIA) slants may also be used to detect the decarboxylation of lysine as well as the production of H₂S and the fermentation of glucose. Inoculate this agar by stabbing the butt and streaking the slant. The decarboxylation reaction takes place in the anaerobic environment present in the butt of the tube. A positive LIA tube for the decarboxylation of the amino acid lysine has a purple butt. If the organism cannot utilize the amino acid lysine but does ferment glucose, the butt turns yellow. H₂S production is seen as a black precipitate in the medium.

Rapid identification systems

The enterics are very commonly encountered and are very labor intensive to be identified by traditional methods. These two characteristics have led to the development of numerous commercially prepared rapid identification systems. These systems may perform standard-type biochemical tests in a reduced volume, or they may test for preformed enzymes for more rapid results. Some of these systems use computer-assisted instrumentation to read test results and interpret their meaning. Many of these systems are numerically coded with an identification number generated based on the test results read by the instrument. These systems have as their primary advantages cost effectiveness, rapidity, and standardization, since they can evaluate a large number of biochemical parameters in a uniform manner. These parameters are captured by the computer then compared to an existing database, and identification achieved within a relatively short time. One computer-assisted system is the Vitec AutoMicrobic System that makes use of biochemical-impregnated wells within the plastic matrix of a card. Once inoculated and incubated, growth and color change in the wells are detected and read by a computer-integrated instrument. The results are available visibly on the CRT screen or printed out for hard copy. Refer to volume 1 for a review of these systems.

213. *Escherichia*, *Salmonella*, *Shigella*, and *Yersinia*

Escherichia, *Salmonella*, *Shigella*, and *Yersinia* are the four genera that are associated with, but not limited to, gastrointestinal infections. Species of *Salmonella*, *Shigella*, *Yersinia*, and specific strains of *E. coli* are capable of causing severe gastroenteritis and life-threatening systemic illness. General characteristics for these organisms were included in the previous lesson. Additional characteristics are given as needed.

***Escherichia* species**

This genus consists of five species: *E. coli*, *E. hermannii*, *E. vulneris*, *E. fergusonii*, and *E. blattae*. The first three of the species listed are known to be pathogenic to humans.

Clinical significance

E. coli is the most common aerobic organism in the large bowel and accounts for 99% of the *Escherichia* isolates. When compared to the prevalence of *E. coli*, the other species of *Escherichia* are uncommon. *E. coli* is the primary pathogen of the genus and is usually associated with urinary tract infections; although it causes a number of other syndromes. It is estimated that 90% of all acute urinary tract infections and 30% of all nosocomial urinary tract infections are due to *E. coli*. Other syndromes attributable to *E. coli* include diarrhea, gastroenteritis, pyelitis, pyelonephritis, appendicitis, peritonitis, and endocarditis. The inclusion of diarrhea and gastroenteritis in the list of pathogenic conditions associated with *E. coli* may first appear strange since we have already noted that *E. coli* is part of the normal flora of the bowel. However, it may produce four types of enteric disease: enteropathogenic, enterotoxigenic, enteroinvasive, and hemorrhagic or enterohemorrhagic.

Enteropathogenic E. coli (EPEC)

EPEC produces an enteric disease and diarrhea, generally in small children. Serotypes O55, O111, and others correlate with worldwide infantile diarrhea. This disease has become increasingly more prevalent in the past 20 years, and strains of EPEC are frequently encountered in third world countries. The stool produced by the patient is watery and usually contains mucus but not blood. Also, the patient has a fever accompanied by vomiting and fever. The loss of fluid can result in dehydration and death. Detection of EPEC includes growth on routine enteric media, polyvalent antisera for detecting the O-antigen groups, enzyme-linked immunosorbent assays (ELISA), and cell culture assays.

Enterotoxigenic E. coli (ETEC)

ETEC, as the name implies, produces toxins and is responsible for dehydrating infantile diarrhea in developing countries (rare in the United States) and traveler's diarrhea. It accounts for 40 to 70% of all cases in certain geographical locations. ETEC is usually transmitted via contaminated food and water. Traveler's diarrhea or secretory diarrhea is due to the production of heat-stable enterotoxins (ST) and heat-labile enterotoxins (LT) that cause a profuse, watery diarrhea. In developing countries, enteropathogenic *E. coli* may produce a mild cholera-like condition in adults. This cholera-like condition is the most serious form of ETEC gastroenteritis that is severe but rarely fatal. The other forms of gastroenteritis caused by ETEC are milder and are characterized by diarrhea, nausea, vomiting, abdominal cramps, and low fever following an incubation period of 24 to 48 hours. Detection of ETEC enterotoxins is accomplished by cell culture in reference or research laboratories or commercial products just recently available.

Enteroinvasive E. coli (EIEC)

EIEC causes a gastroenteritis that is clinically similar to shigellosis and is accompanied by fever, abdominal cramps, and watery diarrhea. Strains of EIEC enter the mucosal cells of the bowel causing destruction of these cells and sloughing of the mucosal lining. The stool specimens passed by patients suffering from this type of gastroenteritis contain blood, mucus, and leukocytes. Growth on routine enteric media, serogrouping, and ELISA procedures is used for identification. The most common serotypes of EIEC are O152 and O124.

Enterohemorrhagic E. coli (EHEC)

EHEC is due to specific strains of *E. coli* designated as serotype O157:H7. Although there are over 50 strains, O157:H7 is usually the only one that causes diarrhea. Patients are usually infected with this serotype by consuming undercooked hamburger. The beef is frequently contaminated at the

slaughterhouses where it contacts bovine feces. After a period of one to eight days incubation, symptoms start with a mild diarrhea with nonbloody stools progressing to severe abdominal pain and grossly bloody stools *not* accompanied by fever. These strains produce two distinct toxins. One that inhibits protein synthesis and another that damages vascular endothelial cells, causing hemorrhaging into the bowel. In most patients, the illness is self-limited and lasts five to eight days without further problems. About 2% to 7% of patients develop serious complications including hemolytic anemia; thrombocytopenia; acute renal failure; chronic renal, cardiac, neurological complications and death. The Centers for Disease Control and Prevention recommend that all microbiology laboratories routinely culture for *E. coli* O157:H7, at least for a period to determine its prevalence in that geographical area. It has been isolated in up to 40% of all bloody stools in certain areas. *E. coli* O157:H7 does not ferment (or very slowly) D-sorbitol; however, approximately 80% of most healthy bowel flora *E. coli* does ferment D-sorbitol within 24 hours. Bloody stool specimens are plated to a MacConkey-sorbitol (SMAC) agar plate. Those colonies that are colorless do not ferment sorbitol and can be screened directly from the SMAC (or subcultured) with latex agglutination tests for *E. coli* O157 antigen. Before the results can be sent out, the isolate must be confirmed by standard biochemical tests.

Laboratory identification

When gram-negative lactose fermenting colonies are isolated from extraintestinal sites such as wounds and urine, they should be speciated. This can be most easily accomplished by observing the following biochemical reactions of the isolate in question. *E. coli* is also typically positive for gas production from glucose, lysine, arabinose, mannitol, trehalose, and xylose. It is usually negative for DNase, phenylalanine deaminase, inositol, and KCN. *Escherichia* and *Shigella* are so closely related that the characteristics of each must be considered during the laboratory identification of either genus from stool specimens. Many of the nonmotile, anaerogenic strains of *E. coli* may be confused with *Shigellae*. They may easily be differentiated by agglutination with a polyvalent antiserum.

Salmonella species

The genus *Salmonella* is composed of a more complex and diverse group of organisms than the genus *Shigella*. These organisms infect almost all animals besides humans and are capable of invading extraintestinal tissues causing enteric fevers—the most severe of which is typhoid fever. Some salmonellae are species specific; for example, humans are the only known reservoir for *S. typhi*. *Salmonella* is more biochemically reactive and contains over 2,000 antigenic types or serotypes. *Salmonella* species include *S. typhi*, *S. choleraesuis*, *S. paratyphi*, *S. gallinarum*, and *S. pullorum*. This genera also contains subgroups or DNA groups 1, 2, 3a, 3b, 4, 5, and 6 strains. The above species and 99% of all salmonellae isolated are in DNA group 1. The classification of the salmonellae has been the source of a great deal of debate over the years. From time to time, the Centers for Disease Control and Prevention have changed their classification schemes and at one time included only three biochemically distinct species—*S. typhi*, *S. choleraesuis* and *S. enteritidis*. Under this scheme, *Arizona* was viewed as a separate genus, but more recently a new classification based on DNA-DNA hybridization studies has been suggested. Under this new system, *Salmonella* and *Arizona* are classified under the same genus and all strains are closely related. To avoid confusion, serotype names are still being used instead of trying to apply new species names. However, reporting specific serotype names is often beyond the ability of many clinical laboratories, since it would require testing an organism with an extensive battery of antisera. Such antisera would not be available in most clinical laboratories, and, therefore, serogroup reporting is more common. This approach is much more practical for the average clinical laboratory and is made possible through the use of commercially available polyvalent antisera designated A, B, C₁, C₂, D, E, F, G, H, and Vi. With these antisera, it's possible to perform a very abbreviated serologic grouping of *Salmonella* isolates, since some of the more common agents of enteric disease can be classified by their agglutination with one or more of these polyvalent antisera. Examples of various species of *Salmonella* that react with

particular antisera include *S. paratyphi* A being agglutinated by antisera A, *S. typhimurium* by antisera B, *S. paratyphi* B by antiserum B, *S. choleraesuis* by antiserum C, and *S. typhi* by antiserum D.

Clinical significance

Virulent *Salmonella* penetrate the epithelial lining of the small bowel like invasive *Shigella*. However, unlike *Shigella*, the *Salmonella* do not merely reside in the epithelial lining, but pass directly through the epithelial cells into the subepithelial tissue. The biochemical mechanism of penetration is not known, but the process appears to be similar to phagocytosis. The ability of the *Salmonella* to survive intracellularly may be due to the surface O antigens or, in the case of *S. typhi*, the presence of the Vi antigen. The actual disease process may be present as any of three distinct clinical entities. They are self-limiting gastroenteritis (the most common), a septicemia with focal lesions, or an enteric fever such as typhoid fever. *Salmonella* species are also associated with bacteremia, meningitis, respiratory disease, cardiac disease, osteomyelitis, and other local infections. Contaminated food and water are the mechanisms of transmission for all *Salmonella*, including *S. typhi*. The only difference is the source of infection. In *S. typhi* infection (typhoid fever), the human carrier is the source, whereas in the other salmonellosis, animals are most important.

Laboratory identification

Routine differential (MAC, EMB) and selective (SS, XLD, Hektoen) enteric agar media are used for isolating *Salmonella* species. The salmonellas, with the exception of a rare isolate, do not ferment lactose. They are usually motile, but nonmotile forms do occur. With the exception of *S. gallinarum*, most produce gas from glucose. In addition, the H₂S production by *S. typhi* may be very slight, sometimes being described as resembling a button at the point of inoculation. Suspected colonies of salmonellae on isolation media are inoculated to slants of TSI (or KIA) agar. Isolates that produce acid, gas, and hydrogen sulfide in the butt and an alkaline slant in this medium and are urease negative should be tested with *Salmonella* polyvalent antisera. The three most clinically important salmonellae, *S. choleraesuis*, *S. typhi*, and *S. paratyphi* A, should be identified both biochemically and serologically, since infections due to these organisms are associated with high mortality. These organisms may be separated on the basis of their biochemical reactions. You only have to identify other salmonellae to the genus and serogroup level and then ship them to reference laboratories for serotyping when necessary.

Shigella species

The genus *Shigella* is composed of *S. sonnei*, *S. flexneri*, *S. boydii*, and *S. dysenteriae*. The serogroup of *S. sonnei* is D, *S. flexneri* is B, *S. boydii* is C, and *S. dysenteriae* is A. These species or serogroups designated A through D, can be separated based on both their biochemical and antigenic characteristics. The species or serogroups can be further subdivided into serotypes, usually at reference laboratories, on the basis of the O-antigens that they possess.

Clinical significance

The four species of *Shigella* genus are carried primarily by humans and are not disseminated in nature. All of the *Shigellae* are capable of causing bacillary dysentery or shigellosis. The organisms are ingested by the patient in contaminated food or water, or by person-to-person via the fecal-oral route. The species ingested determines the severity of the disease experienced by the patient. Most victims are children between the ages of one and five years old. This is especially true in third world countries. The disease caused by *S. sonnei* is relatively mild and self-limited, but that due to *S. dysenteriae* is severe with reported fatality rates up to 20%. *S. boydii* and *S. flexneri* are intermediate in severity, with *S. flexneri* causing a more severe disease than *S. boydii*. In the United States, 64% of the *Shigella* isolates are *S. sonnei*, 23% are *S. flexneri*, and the other 13% are the other serogroups or are not completely typed. *Shigellae* are resistant to gastric acidity and only a small number need be

ingested in order to cause dysentery. The incubation period ranges from one to seven days. Initially, the patient experiences fever, cramping, abdominal pain, and watery diarrhea. The second phase of the disease occurs as the bacilli continue to invade the epithelial cells of the large bowel, producing shallow ulcers. Frequent but scant stools that contain blood, mucus, and white cells mark this second phase. Microscopically, these specimens display large sheets of polymorphonuclear leukocytes when stained with new methylene blue. This stage of the disease is referred to as dysentery. The *Shigellae* rarely enter the circulation, but dysentery is characterized by fever and severe abdominal cramps. Children may experience convulsions at this point in the disease process.

Laboratory identification

Differential and moderately selective enteric agar media are used for the isolation of *Shigella*. Members of the genus *Shigella* are very closely related to the genus *Escherichia*, but because they cause such a clinically important disease as dysentery, they retain their identity as a separate genus. The *Shigellae* are among the most biochemically least reactive of all the *Enterobacteriaceae*. They are rated as no more than variably positive for any of the commonly used biochemical tests other than glucose fermentation and nitrate reduction, and all isolates are always nonmotile and lysine negative. Because *S. dysenteriae* and *S. boydii* are so rare in the United States, these isolates must be re-tested and confirmed before a final report is sent out.

***Yersinia* species**

Eleven clinically isolated species of *Yersinia* are now recognized: *Y. enterocolitica*, *Y. pestis*, *Y. pseudotuberculosis*, *Y. frederiksenii*, *Y. kristensenii*, *Y. intermedia*, *Y. rohdei*, *Y. aldovae*, *Y. bercovieri*, *Y. mollaretii* and "*Yersinia*" *ruckeri*. *Y. pestis* is a gram-negative nonmotile coccobacillus. Cells appear short, plump, sometimes elongated and pleomorphic, usually singly or in pairs, and occasionally in short chains.

Clinical significance

Y. enterocolitica has been implicated in human disease with a variety of clinical syndromes. They include a severe form of gastroenteritis that mimics appendicitis, as well as bacteremia, peritonitis, cholecystitis, visceral abscesses, and mesenteric lymphadenitis. Infections with *Y. enterocolitica* are often sporadic, or outbreaks are associated with the consumption of contaminated food or water. *Y. pestis* is the causative agent of both urban and sylvatic plague, a disease with a high mortality in humans, rats, and infectious mice, guinea pigs, and rabbits. The natural reservoirs for the urban ("city") plague are rats, and for the sylvatic ("country") plague, the natural reservoirs are ground squirrels, field and wood rats, rabbits, and domestic cats. When plague is acquired directly or indirectly from animals, it is referred to as *bubonic*. If the infection is acquired via aerosols from infected individuals, it is termed *pneumonic*. Most cases in the United States are due to accidental exposure to infected animals. The rat flea is the main vector for transmission. *Y. pseudotuberculosis* causes an illness similar to that of *Y. enterocolitica* usually seen in children ages five to 15, and is usually isolated from blood. Rodents, wild animals, and game birds throughout the world are the natural reservoirs.

Laboratory identification

Exercise caution when handling suspected cultures or pathological materials. Perform your work under a bacteriological safety hood. Clinical materials containing *Y. pestis* are extremely hazardous. Specimens suspected for *Yersinia* species should be inoculated to cefsulodin-irgasan-novobiocin (CIN) agar. Carefully collect and place aspirates from buboes, pus from the area of the flea bite, sputum, throat swabs, or blood in Cary-Blair transport media for transfer to the laboratory. Cells show marked bipolar staining, especially in tissue impressions, aspirates of buboes, and pus stained with polychrome stains, such as the Giemsa and Wayson stains, but not Gram-stain. The cells have a safety-pin appearance with the polar bodies staining blue and the remainder light blue to reddish.

Coccoid, round, filamentous, elongated, and other forms commonly occur, and especially in old cultures. A capsule can be demonstrated in animal tissue and in young cultures that yields a positive fluorescent antibody reaction by which a presumptive diagnosis may be made.

Y. pestis are facultative anaerobes. They are anaerogenic and usually do not ferment lactose. They are catalase and oxidase negative, urea positive, and motile at 25°C but not at 37°C. The organism grows slowly on nutrient agar, producing small, nonhemolytic, round, transparent, glistening, colorless colonies with an undulate margin. Opaque colonies with yellowish centers and whitish edges, that develop a soft mucoid consistency due to capsular material, are noted in older enlarged types. After 24 hours on MAC, colonies are small and colorless and become much larger following an additional 24 hours incubation at room temperature. On TSI agar *Yersinia* species produce an acid/acid reaction with no gas or H₂S.

Y. pestis is a dangerous organism to work with so exercise caution when an isolate is suspected of being *Y. pestis*. *Y. pestis* is not included in the data banks of most commercial identification systems. The definitive identification of this organism usually depends upon close observation of its macroscopic and microscopic appearance, bacteriophage typing, serological assays, and *in vivo* pathogenicity tests using rats and guinea pigs. Such testing is beyond the abilities of most clinical laboratories and is best performed in a reference laboratory.

The typical biochemical reactions displayed by *E. coli*, *Salmonella*, *Shigella*, and *Yersinia* species are shown in the following table.

Test	<i>E. coli</i>	<i>Salmonella</i>	<i>Shigella</i>	<i>Yersinia</i>
Indole	+	–	– ^b	+/- ^a
Methyl Red	+	+	+	+
Voges-Proskauer	–	–	–	–
Citrate	–	+/- ^a	–	–
H ₂ S	–	+	–	–
Urease	–	–	–	+
Motility	+	+	–	–
ONPG	+	+/- ^a	+	+
+/- ^a = The reaction is different for each species.				
– ^b = <i>S. sonnei</i> species are negative, and 50% of the other <i>Shigella</i> species or strains are indole positive.				

214. Other *Enterobacteriaceae* genera

In this lesson, we'll continue our look at the family *Enterobacteriaceae*. The general characteristics and laboratory identification were briefed in the first lesson. A more complete description of the following genera is presented below.

Citrobacter species

At the time of this writing, the genus *Citrobacter* contains three species: *C. diversus* (synonym *C. koseri*), *C. amalonaticus*, and *C. freundii*. Proposals have been made to include eight new species.

Clinical significance

C. diversus is a causative agent of neonatal sepsis and meningitis and is associated with nursery outbreaks. *C. amalonaticus* has been isolated from stool specimens but very seldom from extraintestinal specimens. *C. freundii* causes sepsis, infects a number of tissues, and has been implicated in gastrointestinal infections.

Some strains of *C. freundii* resemble *Salmonella* species biochemically and cross-react with *Salmonella* polyvalent O antiserum. Review colony morphology and biochemical reactions before finalizing results.

***Edwardsiella* species**

The genus *Edwardsiella* consists of three species and a biogroup: *E. tarda*, *E. hoshinae*, *E. ictaluri*, and *E. tarda* biogroup 1.

Clinical significance

Edwardsiella species are found in the intestines of cold-blooded animals and in water. They are pathogens for eels and catfish. In humans, *E. tarda* is associated with soft tissue infections, meningitis, osteomyelitis, sepsis, hepatic abscesses, and a mild diarrheal disease. The other *Edwardsiella* species are rare clinical isolates.

***Enterobacter* species**

The genus *Enterobacter* contains 11 currently recognized species: *E. cloacae*, *E. aerogenes*, *E. agglomerans*, *E. gergoviae*, *E. sakazakii*, *E. amnigenus*, *E. taylorae*, *E. intermedium*, *E. cancerogenus*, *E. hormaechei*, and *E. asburiae*.

Clinical significance

The organisms contained in this genus are commonly found in soil, water, and the intestinal tracts of humans and animals. Only the first six species listed are considered clinically significant.

Enterobacter, like most *Enterobacteriaceae*, are capable of producing disease in any body tissue but have been most frequently isolated from urinary tract infections. *E. aerogenes* and *E. cloacae* are the members of the genus most frequently isolated in clinical laboratories and are causes of nosocomial infections. These species are closely related to one another and share the traits of being nonencapsulated and motile. *E. agglomerans* is especially noteworthy since it is often associated with life-threatening neonatal meningitis and sepsis. The others are rare clinical isolates.

Several physical and biochemical characteristics can be used to separate the genus *Enterobacter* from *Klebsiella*. Unlike *Klebsiella*, *Enterobacter* is nonencapsulated and motile. Biochemically the ornithine decarboxylase test is helpful in separating the genera since *Klebsiella* is negative and most strains of *Enterobacter* are positive. *E. agglomerans* is highly variable biochemically since it actually consists of several genetically distinct groups and may cause identification problems. Most isolates of *E. agglomerans* produce a yellow pigment that can help in their identification.

***Klebsiella* species**

Klebsiella is the second most populous facultative enteric genus found in the bowel of humans. The genus consists of eight species, but only the following five are considered of clinical significance: *K. pneumoniae*, *K. ozaenae*, *K. rhinoscleromatis*, *K. oxytoca*, and *K. planticola*. *K. pneumoniae* has the greatest clinical significance. All members of the genus are nonmotile, encapsulated short rods. Both *K. ozaenae*, and *K. rhinoscleromatis* are thought to be variants of *K. pneumoniae*, but since they are responsible for specific human diseases, they have been given species names.

Clinical significance

K. oxytoca and *K. pneumoniae* are the most frequently isolated members of the genus. *K. pneumoniae* is also the primary pathogen of the genus and has been isolated from the upper respiratory and intestinal tract of about 5% of normal individuals. It is thought to be responsible for about 2% of the bacterial pneumonias. This organism has also been found to be a secondary invader in such diseases as bronchiectasis, tuberculosis, influenza, pleurisy, and pyelonephritis and is a significant cause of nosocomial infections. *K. rhinoscleromatis* is associated with granulomatous infections of the mucous

membranes of the external nares, mouth, and pharynx, and *K. ozaenae* has been recovered from patients with atrophic rhinitis. These two species are rarely encountered in the United States.

The members of the genus *Klebsiella* are gram-negative, nonmotile, and encapsulated short rods. On blood agar, EMB, MAC, trypticase soy agar, and other routine media, they normally give rise to large mucoid colonies that have a tendency to coalesce. When touched with an inoculating loop the colonies usually string out. Also, the growth in broth can be very stringy and sometimes difficult to break when making transfers. In contrast with *K. pneumoniae*, *E. aerogenes* is usually considered motile, may liquefy gelatin, and is not as distinctly capsulated. *E. aerogenes* does not react with *Klebsiella* antiserum and is ornithine decarboxylase positive. The capsules that surround *Klebsiella* organisms can be used to type members of the genus using the quellung reaction. Over 70 different heat labile polysaccharide K antigens have been described. All species of *Klebsiella* share common antigens and thus are able to be typed with the same set of antisera.

***Morganella* species**

This genus consists of a single species *M. morganii* originally classified in the genus *Proteus*.

Clinical significance

M. morganii is a well known cause of urinary tract infections and has been associated with sepsis. Organisms in this genus are closely related to those in the genera *Proteus* and *Providencia*. The genus is differentiated from the genus *Proteus* since it is H₂S negative, gelatin negative, and D-mannose positive. It can be separated from the genus *Providencia* by its ability to utilize decarboxylate ornithine and its inability to utilize citrate.

***Proteus* species**

This genus consists of four species: *P. mirabilis*, *P. vulgaris*, *P. penneri*, and *P. myxofaciens*.

Clinical significance

Organisms of the genus *Proteus* are found in soil, water, sewage, and decaying animal matter, as well as the human intestinal tract. *Proteus* species cause human infections when introduced into tissues other than the normal intestinal tract. *Proteus* ranks next to *E. coli* as the etiological agent of cystitis. *P. mirabilis* is the most frequently recovered species of *Proteus*, particularly as the causative agent of urinary tract and wound infections. *P. vulgaris* is also a cause of urinary tract infections. *P. penneri* has been associated with nosocomial infections. *P. myxofaciens* has not yet been implicated in human infections. The organisms are encountered frequently in eye and ear infections, and occasionally in pleurisy, peritonitis, and suppurative abscesses in various areas of the body. *Proteus* species are commonly associated with other bacteria in purulent wounds and may contribute to the severity of these infections.

The *Proteus* species are phenylalanine deaminase positive and urea positive. They rapidly decompose urea to ammonia in one to four hours, yielding a highly alkaline environment that can be detected with a pH indicator dye. Lactose is not fermented, although sucrose-positive strains give an acid slant and butt in TSI agar. *P. mirabilis*, *P. penneri*, and *P. vulgaris* swarm on blood agar, producing wavelike or thin, veil-like confluent growth over the surface. Swarming may be inhibited on MAC and on EMB plates if the agar concentration is increased to 5%. Addition of sodium azide, chloral hydrate, and phenylethyl alcohol is also preventive. Members of the genus *Proteus* produce nonpigmented colonies on EMB and MAC and may be confused with nonlactose fermenting pathogens such as the salmonellae or *Shigellae*. *P. mirabilis*, *P. penneri*, and *P. vulgaris* produce H₂S and can be even more easily confused with the salmonellae.

The species identification of members of the genus *Proteus* is important because of the varying susceptibility of various members of the genus. For example, *P. mirabilis* is, as a rule, sensitive to penicillin and ampicillin, but *P. vulgaris* is resistant. Therefore, a patient diagnosed as having an

infection due to *P. mirabilis* can be immediately treated effectively with a suitable form of penicillin. *P. mirabilis* and *P. penneri* are indole negative, whereas *P. vulgaris* is indole positive. *P. penneri* characteristically produces a small zone of inhibition around the chloramphenicol disk.

***Providencia* species**

The genus *Providencia* currently consists of five species: *P. alcalifaciens*, *P. stuartii*, *P. rettgeri*, *P. heimbachae* and *P. rustigianii*. Only the first three species listed are known to be pathogenic to humans.

Clinical significance

The genus *Providencia* has been associated with a number of nosocomial infections involving urinary tract infections, septicemia, wound infections, and pneumonia. *P. stuartii* and *P. rettgeri*, in particular, have been implicated in urinary infections, especially with underlying urologic disorders, and various infections of patients in burn units. *P. alcalifaciens* is usually a rare human isolate. These organisms grow well on moist, enteric isolation media. They are gram-negative motile rods that are lactose negative. Since they are hydrogen-sulfide negative and may or may not produce gas in glucose, they often resemble *Shigellae* on TSI or KIA agar. When sucrose is fermented, the reaction is delayed, and thus fermentation of this carbohydrate will not be detected in 48 hours in the TSI slant. *Providencia* may be distinguished from the *Shigellae* by their motility and utilization of citrate.

***Serratia* species**

The genus *Serratia* is gram-negative motile rods. Only a small percentage of strains are chromogenic. The eight species that currently exist are *S. marcescens*, *S. liquefaciens*, *S. ficaria*, *S. fonticola*, *S. odorifera*, *S. plymuthica*, *S. proteomaculans*, subspecies *quinovora*, and *S. entomophila*. The strains of *S. marcescens* that are chromogenic produce a red, nonwater-soluble pigment at room temperature, but rarely at 35°C or above. Organisms of the genus *Serratia* can be differentiated from other enterics by the production of an extracellular DNase.

Clinical significance

S. marcescens occurs widely in nature, water, soil, milk, and foods. It is associated with pulmonary infections, urinary tract infections, and septicemia. In addition, *Serratia* has been implicated in nosocomial outbreaks associated with blood transfusions, in urinary tracts, and with surgeries. Burn cases are particularly susceptible to the organism. *Serratia* can also be a secondary invader in certain types of lung disease. *S. entomophila* is the only *Serratia* that does not occur in human specimens. Unlike the other enteric forms, colonies on agar are generally circular, thin, smooth, or rough; occasionally mucoid; and often pigmented red, pink, or magenta. In broth cultures growth may form a red ring at the surface or a pigmented pellicle. Infections with pigmented *Serratia* may cause sputum to be tinged with red, thus giving the false impression of hemoptysis.

Self-Test Questions

After you complete these questions, you may check your answers at the end of the unit.

212. *Enterobacteriaceae* classification and general characteristics

1. What three general biochemical characteristics do all the enterics have in common?
2. Media for the cultivation of the *Enterobacteriaceae* fall into what four categories?

3. Why are EMB and MAC considered selective agars?
4. What distinctive colony morphology is displayed by most of the enterics?
5. What unusual colony morphology does *Yersinia pestis* display when grown in broth media?
6. What are three examples of enrichment broths that must first be inoculated for isolation of *Enterobacteriaceae* from stool specimens?
7. List eight plating media used for the isolation of enterics.
8. What can be done in the preparation of MAC and EMB media to inhibit the spreading of *Proteus* strains?
9. What is the purpose of selective media?
10. How do EMB and MAC agars provide for the differentiation of lactose and nonlactose fermenting organisms?
11. In attempting to isolate enteropathogenic *E. coli*, *Klebsiella*, *Enterobacter*, and *Citrobacter* from stool samples, why is the use of tetrathionate and selenite F enrichment broths *not* recommended?
12. On TSI or Kligler's agar when the reaction gives an acid (yellow) butt and alkaline (red) slant, what carbohydrate is most likely fermented?
13. What carbohydrate is found in TSI and not Kligler's iron agar?
14. A positive sucrose gives what type of reaction in the tube?

15. What are some members of *Enterobacteriaceae* isolated from the urinary tract?
16. On the basis of what observations can the enterics be tentatively identified?
17. Why must the oxidase test be read within 10 to 20 seconds?
18. What does the urease test determine?
19. What substance is detected in the ONPG test?
20. What function does permease play in the fermentation of lactose?
21. The production of what color indicates a positive ONPG test?
22. What do both the methyl red and Voges-Proskauer tests detect?
23. What acid end products of the metabolism of glucose does the MR test detect? The VP test?
24. What genera of the family *Enterobacteriaceae* can deaminate phenylalanine?
25. What is the end product of the deamination of phenylalanine?
26. What color is a positive citrate test?
27. How do you describe a positive LIA tube test?

213. *Escherichia*, *Salmonella*, *Shigella*, and *Yersinia*

1. What percentage of the *Escherichia* isolated are *E. coli*?
2. With what disorder is *E. coli* usually associated?
3. What four types of enteric disease may be produced by *E. coli*?
4. Match each type of *E. coli* in column B with a statement in column A by placing the letter of the column B item beside the number of the column A item that most nearly describes it. Each element in column B may be used once, more than once, or not at all.

Column A

- ___ (1) Traveler's diarrhea.
- ___ (2) Infant diarrhea.
- ___ (3) May produce a cholera-like condition in adults.
- ___ (4) Causes a gastroenteritis similar to shigellosis.
- ___ (5) Hemorrhagic colitis.
- ___ (6) Stool does not contain blood but usually contains mucus.
- ___ (7) Stool contains blood, mucus, and leukocytes.

Column B

- a. *Enteropathogenic E. coli*.
- b. *Enterotoxigenic E. coli*.
- c. *Enteroinvasive E. coli*.
- d. *Enterohemorrhagic E. coli*.

5. Why is reporting *Salmonella* isolates by specific serotypes beyond the ability of most clinical laboratories?
6. How may some of the more commonly encountered salmonellae that are agents of enteric disease be identified?
7. What three distinct clinical entities may result as a result of infection with salmonellae?
8. What is the source of infection for typhoid fever?
9. What type of H₂S production is sometimes exhibited by *S. typhi*?

10. What are the three most clinically significant salmonellae?
11. In what two ways can the species of *Shigella* be differentiated?
12. On what basis can the species of *Shigella* be subdivided into serotypes?
13. What species of *Shigella* are capable of causing dysentery?
14. What age group is usually affected by shigellosis?
15. What species of *Shigella* produces the most severe form of dysentery?
16. What stain should be used to visualize polymorphonuclear cells in stools from a suspected case of dysentery?
17. Which of the species of *Yersinia* is the causative agent of plague?
18. What are the two clinical forms of plague?
19. What insect is the main vector for transmission of plague?
20. What clinical specimens suspected for *Yersinia* may be obtained for culture?
21. For these specimens, what transport medium has been recommended?
22. How do the cells appear in tissue impressions, aspirates of buboes and pus, when stained with polychrome stains, such as the Giemsa and Wayson stains?

23. What morphological forms commonly occur in older cultures?
24. A presumptive diagnosis of *Y. pestis* may be made from a positive fluorescent test from animal tissue and young cultures when what morphological form is demonstrated?
25. How does *Y. pestis* appear on nutrient agar?

214. Other *Enterobacteriaceae* genera

1. Which *Citrobacter* species is associated with nursery outbreaks?
2. *Edwardsiella tarda* causes what kind of infections in humans?
3. Where are organisms normally found in the genus *Enterobacter*?
4. Which two species of *Enterobacter* are the most frequently isolated?
5. Which species of *Enterobacter* is associated with neonatal meningitis and sepsis?
6. What biochemical test is helpful in separating *Klebsiella* from *Enterobacter*?
7. What are the five clinically significant species of the genus *Klebsiella*?
8. What are some diseases caused by *K. pneumoniae*?
9. How do colonies of *Klebsiella* generally appear on blood agar, EMB, MAC, and other routine agars?

10. What characteristic is noted when a colony of *Klebsiella* on routine media is touched with an inoculating loop?
11. In contrast to *K. pneumoniae*, by what three characteristics does *E. aerogenes* differ?
12. In general, what biochemical traits characterize the genera *Proteus*, *Providencia*, and *Morganella*?
13. What is the most frequently recovered species of *Proteus*?
14. What type of reaction do sucrose-positive strains of *Proteus* give on TSI?
15. How may swarming be inhibited on MAC and EMB plates?
16. Which species of *Proteus* produces H₂S and can be easily confused with the salmonellae?
17. Why is the species identification of *Proteus* important?
18. *P. stuartii* and *P. rettgeri* are, in particular, incriminated in what types of infections?
19. What distinguishes *Providencia* from the *Shigellae*?
20. How do you describe the chromogen produced by *S. marcescens*?
21. What type of injury is particularly susceptible to infection with *Serratia*?

3-2. Other Gram-Negative Bacilli

In this section we'll discuss gram-negative bacilli that neither use carbohydrates as a source of energy nor degrade them through metabolic pathways other than fermentation. They are also oxidase positive, or are known as fastidious gram-negative bacilli. These organisms cause a wide array of infections, such as diarrhea, septicemia, meningitis, cholera, pneumonia, and rat-bite fever.

215. *Pseudomonas* and *Burkholderia*

In addition to the organisms that make up the enterics, there is a large group of gram-negative aerobic, facultatively anaerobic, and usually saprophytic bacilli. Members of the genera *Pseudomonas* and the new genus *Burkholderia* are nonfermenters and discussed in this lesson.

Pseudomonas species

The genus *Pseudomonas* was divided into five groups (I, II, III, IV, V) based on molecular studies. Subsequently, three of the five groups have been reclassified or a new genus was designated. Group II organisms belong to the genus *Burkholderia*; group III organisms are now in the genera *Comamonas* and *Acidovorax*, and the organisms of group V are now in the genera *Xanthomonas*. As these studies of *Pseudomonas* continue, many pseudomonads are reclassified into other genera and the entire genus seems to be in a constant state of flux. The clinically significant *Pseudomonas* includes *P. aeruginosa*, *P. fluorescens*, *P. stutzeri*, *P. alcaligenes*, *P. putida*, and *P. vesicularis*.

General characteristics

The genus *Pseudomonas* is composed of a large number of nonfermentative, aerobic, nonspore-forming, gram-negative rods that are straight or slightly curved. Rods may occur singly, in pairs, or in short chains. These organisms are motile due to single or multiple polar flagella. They are catalase positive and most are oxidase positive. Some species can grow at 4°C, although most are mesophilic, with optimal growth at temperatures between 30 and 37°C.

Clinical significance

They inhabit the soil and water, and are found on plants including fruits and vegetables. These organisms play an important role in the decomposition of organic matter in their normal habitat. While most *Pseudomonas* species do not infect humans, some are important opportunistic pathogens that infect immunocompromised individuals. The *Pseudomonas* species most frequently associated with human disease is *P. aeruginosa*. Infections with *P. aeruginosa* range from superficial skin infections to fulminant sepsis that has a high mortality rate. It may infect burn sites, wounds, ears, eyes, tissue, joints and bones, the urinary tract, and the lower respiratory tract. These infections occur especially in patients whose defenses have been impaired. In some hospitals, this organism causes 10 to 20% of the nosocomial infections. *P. aeruginosa* has been isolated from numerous sources, such as swimming pools; hot tubs; baby, whirlpool, and hydrotherapy baths; sink traps; showerheads and respiratory therapy and dialysis equipment. It has also been isolated from various solutions, such as disinfectants, ointments, soaps, irrigation fluids, eye drops, dialysis fluids, contact lens solutions, and illicit injectable drugs. Other sources include distilled water, injectable medicines, cosmetics, and the innersoles of sneakers. It is rarely found as normal flora in healthy individuals.

Laboratory identification

The organism produces a spreading, flat colony with an erose margin and a metallic sheen on blood agar. Mucoid strains are frequently isolated from the sputum of patients with cystic fibrosis. Colonies tend to spread and give off a characteristic grape-like odor. Most strains excrete pyocyanin and fluorescein (pyoverdine), giving the colony a characteristic blue-green or yellow-green color. Perhaps the most striking feature of *P. aeruginosa* is its ability to produce a blue-green pigment on Mueller-Hinton agar. This pigment does not color the colony but readily diffuses throughout the surrounding medium. However, approximately 4% do not produce pyocyanin. *P. aeruginosa* is oxidase-positive

by Kovac's method and utilizes glucose oxidatively in O-F medium; gluconate is oxidized to ketogluconate. *P. aeruginosa* is lysine and ornithine decarboxylase-negative and arginine dihydrolase-positive. Most strains grow at 42°C on trypticase agar slants. The organism grows on EMB on MAC as a nonlactose fermenter. You may mistakenly place this organism on TSI or KIA agar because of its appearance on EMB or MAC. Suspicious colonies from stool specimens may be incorrectly identified because of the alkaline slant and butt reaction characteristic of this organism.

***Burkholderia* species**

The genus *Burkholderia* contains five species: *B. cepacia*, *B. pseudomallei*, *B. gladioli*, *B. mallei*, and *B. pickettii*. *B. cepacia* and *B. pseudomallei* (formerly *P. cepacia* and *P. pseudomallei*) are well-known human pathogens.

General characteristics

Burkholderia species are also aerobic, nonspore-forming, gram-negative straight or slightly curved rods. They are also motile due to one or more polar flagella.

Clinical significance

B. cepacia is a cause of nosocomial infections associated with contaminated disinfectants, equipment, and medications. Unlike *P. aeruginosa*, *B. cepacia* is of low virulence; therefore, morbidity and mortality associated with *B. cepacia* infections is low. The other *Burkholderia* species generally considered pathogenic for humans is *B. pseudomallei*. This organism is a common inhabitant of the soil in northern Australia and Southeast Asia. It causes melioidosis, a glanders-like disease in humans. Abscesses produced by this organism occur in the lungs, liver, spleen, and lymph nodes. The abscesses can also occur in the skin, soft tissue, joints and bones. The symptoms of this organism can mimic those of *Mycobacterium tuberculosis*. It is acquired by either inhalation or through cuts of skin in contact with contaminated soil or water. Although apparently rare in natives, the disease was an important and sometimes fatal infection in the U.S. Armed Forces in Vietnam.

Laboratory identification

B. pseudomallei bacteria have three or more flagella per pole and are morphologically similar to *P. aeruginosa*. The colonies frequently are wrinkled and on prolonged incubation become umbonate in character, particularly on blood agar. These organisms may be cultivated on most laboratory media, growing well on trypticase soy agar, on blood agar, and on MAC, but not on *Salmonella-Shigella* agar or cetrimide agar. The oxidase reaction is positive; an oxidative acidity is produced in glucose O-F medium and growth occurs at 42°C. The agglutination and fluorescent-antibody reactions are useful for identification of *B. pseudomallei*. Commercial kits and automated systems can also be used to identify most *Pseudomonas* and *Burkholderia* species.

216. *Acinetobacter*, *Eikenella*, and *Xanthomonas*

Acinetobacter, *Eikenella*, and *Xanthomonas* are nonfermenters and are found in nature or as part of the normal flora of the mucous membranes.

***Acinetobacter* species**

The *Acinetobacter* genus consists of seven species and 12 genospecies that have replaced the old biovars: *A. calcoaceticus* biotype: *anitratus*, *lwoffii*, *haemolyticus*, and *alcaligenes*. All the species are oxidase negative (a trait that distinguishes them from all other members of the family *Neisseriaceae*), grow well on MAC, and resemble members of the family *Enterobacteriaceae* when grown on blood agar. This organism is everywhere in the environment and often colonizes hospital patients. This is usually without pathological effect. However, immunocompromised patients and those undergoing invasive techniques, such as tracheostomy, urinary catheterization, or neurosurgical procedures are at risk to developing nosocomial infections with this agent.

General characteristics

The genus *Acinetobacter* is another member of the family *Neisseriaceae*. They are gram-negative (although at times they resist decolorization with alcohol), aerobic, nonmotile bacilli organisms that do not ferment glucose. They are somewhat pleomorphic appearing coccoid, coccobacillary or as filamentous rods. At times they occur in pairs from cervical and urethral smears and may be easily mistaken for gonococci.

Clinical significance

Acinetobacter species are very common inhabitants of soil and water. They are also frequently encountered as normal flora of the skin, pharynx, and genitourinary tract. However, *Acinetobacter* species are infrequently seen as the cause of nosocomial infections. The biotypes of *Acinetobacter* are ubiquitous, growing in soil and water throughout the natural environment. In hospital settings they colonize sink traps, respiratory equipment, humidifiers, and other moist habitats. A lower percentage of healthy adults have been found to have this same organism colonizing the pharynx and genitourinary tract. Although members of this genus commonly colonize hospital patients, the organism is rarely pathogenic. However, infections of surgical sutures and burns do occur, as well as cases of pneumonia and tracheobronchitis. When infection does occur, it can generally be traced to the performance of some invasive procedure such as endotracheal intubation, intravenous therapy or urinary catheterization. Pneumonia and tracheobronchitis have been the most frequently documented nosocomial infections caused by this organism, and most cases can be traced to transmission of the organism via the hands of hospital staff or from contaminated equipment. Patients who succumb to these types of infections generally have some underlying predisposing medical condition. Outside the clinical setting, individuals occasionally suffer what is termed community-acquired pneumonia caused by *Acinetobacter* contaminated dust particles of free silica or metal dust. This condition is an occupational hazard of foundry workers who are exposed to such material or may be acquired by the very elderly who have debilitating illnesses such as chronic renal failure, lung disease, alcoholism, and cirrhosis. *A. baumannii* is the species most frequently isolated, followed by *A. lwoffii*, *A. haemolyticus*, and *A. johnsonii*.

Laboratory identification

Acinetobacter species are oxidase negative, nitrate negative, and the second most commonly isolated nonfermenter. Hemolysis, carbohydrate utilization, proteolytic activity and antibiotic susceptibility are all characteristics that may be used to identify the species of *Acinetobacter*.

***Eikenella* species**

Eikenella corrodens is the only species in the genus *Eikenella*.

General characteristics

E. corrodens is nonmotile, oxidase positive, usually catalase negative, capnophilic, facultatively anaerobic, and with straight slender rods (1.5 to 4 μm in length). In order to grow aerobically, hemin is required.

Clinical significance

Part of the normal flora of mucous membranes, *E. corrodens* is important when isolated from single or mixed infections in human bite wounds, dental infections, postsurgical infections, soft tissue abscesses (including brain), and pleuropulmonary infections. It has been the causative agent in endocarditis, meningitis, pneumonia, appendicitis, and arthritis.

Laboratory identification

Minute colonies may appear after 24 hours of incubation on 5% blood agar plates, but further incubation is required. After further incubation, flat, spreading colonies with moist, clear centers are

seen. Slight yellow pigmentation may occur with additional incubation. *E. corrodens* is urease and gelatin negative and lysin decarboxylase positive, reduces nitrate, and usually does not produce acid from carbohydrates.

***Xanthomonas* species**

Xanthomonas maltophilia (formerly *Pseudomonas maltophilia*) is the only species in the genus *Xanthomonas*. In the future, *X. maltophilia* may be moved to the new genus *Stenotrophomonas*.

General characteristics

X. maltophilia is a gram-negative, short to medium-sized straight rod with a polar cluster of flagella. The organism is oxidase negative, positive for esculin and gelatin hydrolysis, and ONPG and DNase positive; it produces acid from glucose and maltose.

Clinical significance

X. maltophilia is found everywhere in nature and has been isolated in the hospital environment. In the clinical laboratory, it is the third most frequently isolated nonfermentative, gram-negative rod. It is associated with hospital acquired septicemia, pneumonia, wound infections, endocarditis, and meningitis.

Laboratory identification

X. maltophilia grows on MAC and on blood agar produces large, smooth, glistening colonies with undulated margin, and lavender-green to light purple pigmentation. Traditional and commercial test systems can be used to identify this organism.

217. *Vibrio*, *Aeromonas*, *Plesiomonas*, *Campylobacter*, *Arcobacter*, and *Helicobacter*

These organisms are gram-negative bacilli that are related to diarrheal disease or chronic gastritis and are not considered normal flora of the gastrointestinal tract.

***Vibrio* species**

The genus *Vibrio* is one of four genera belonging to the family *Vibrionaceae*. The other genera are *Aeromonas*, *Plesiomonas*, and *Photobacterium*. The species of the genus *Vibrio* share features with the members of the families *Enterobacteriaceae* and *Pseudomonadaceae*. The genus contains over 30 species, 12 of which are known to be pathogenic in humans. These 12 species are: *V. cholerae*, *V. parahaemolyticus*, *V. vulnificus*, *V. mimicus*, *V. fluvialis*, *V. furnissii*, *V. alginolyticus*, *V. damsela*, *V. carchariae*, *V. cincinnatiensis*, *V. hollisae* and *V. metschnikovii*.

General characteristics

The typical vibrio appears as a gram-negative, slightly curved, or comma-shaped rod upon initial isolation. In older cultures the organisms may demonstrate a great degree of pleomorphism—appearing coccoid or straight-sided bacilli. This latter appearance may cause them to be mistaken for enteric organisms. All species are motile, displaying a darting motility, usually due to polar flagellum, although some species do have lateral flagella—a characteristic much more likely to be observed if the organism is cultured on solid media. These organisms have a tendency to produce spheroplasts, but they do not form spores and do not have capsules. The salt requirements of members of the genus *Vibrio* can be used to divide members of the genus into two classes: (1) nonsalt-requiring species, such as *V. cholera* and *V. mimicus*, and (2) the rest of the pathogenic *Vibrio* members that do require salt.

Clinical significance

Vibrios are among the most common bacteria in surface and marine water worldwide. They are associated with the ingestion of contaminated water or consumption of contaminated shellfish or seafood. However, they are rarely seen in Western industrialized nations or in laboratories that serve

inland populations. On the other hand, Japan is exceptional in that *Vibrio parahaemolyticus* is the most common cause of gastroenteritis. This is due to the dietary preference of many Japanese for eating raw fish.

Vibrio cholerae is the causative agent of Asiatic or epidemic cholera and is the species in this genus most readily associated with human disease. In its most severe form, Asiatic cholera is an acute diarrheal disease characterized by massive loss of fluid and electrolytes that, if untreated, may result in cardiovascular collapse and death in a single day. Even though such cases are the exception, epidemiological studies indicate that for each severe case there are 25 to 100 mild to asymptomatic infections. The disease is produced by a heat-labile enterotoxin produced by *V. cholerae* multiplying in the small bowel. This causes vomiting and profuse diarrhea (rice-water stools), resulting in severe dehydration, anuria, hypochloremia, acidosis, and circulatory failure. The mortality rate ranges between 30 to 50% of untreated cases. Cholera is transmitted by the fecal-oral route or from infected individuals or convalescing carriers through contaminated food and water. *Vibrio cholerae* can be subdivided by serological methods into over 60 serovars, with those associated with epidemics being designated as O1 or Non-O1 serovars. The O1 serovar may be further subdivided into subtypes based on the specific antigenic determinants they possess. These subtypes are designated Ogawa, Inaba, and Hikojima. It is possible to separate the O1 *V. cholerae* stains into two biovars—Classical and El Tor—based on their phenotypic characteristics. Most authorities feel that El Tor biotype is less susceptible to environmental changes than the other *Vibrio cholerae* types and, for this reason, is more readily recovered from specimens submitted to the laboratory.

The pathogenic *Vibrio* species are all capable of causing three clinical syndromes: gastroenteritis; soft tissue infections subsequent to traumatic injury; and systemic infections, of which septicemia is the most common. *V. parahaemolyticus* is a marine organism that inhabits estuaries throughout the world and is perhaps more important in the Gulf Coast area of the United States. *V. parahaemolyticus* can cause gastroenteritis or food poisoning related to the consumption of contaminated seafood.

Extraintestinal infections by *V. parahaemolyticus* such as septicemia with shock, hemolytic anemia, and disseminated intravascular coagulation (DIC) also occur. Wound infections are the most common extraintestinal infection caused by *V. parahaemolyticus*. The strains of *V. parahaemolyticus* most likely to infect wounds distinguish themselves from the more common environmental strains by producing beta hemolysis on Wagatsuma agar that contains human red cells. This reaction is referred to as the Kanagawa phenomenon. More than 95% of the isolates from wounds are Kanagawa positive, while only about 1% of the environmental marine strains display this characteristic.

V. parahaemolyticus is not the only species of the genus *Vibrio* that has been isolated from wounds that have been exposed to a marine environment. *V. alginolyticus* has been isolated from eye and ear infections, intracranial infections, and wound infections. *V. alginolyticus* is the most common isolate recovered from traumatic injuries. *V. damsela* almost exclusively causes wound infections and can cause a rapidly progressive necrotizing infection. *V. vulnificus* is the most virulent strain of vibrio mentioned above and causes the most serious wound infections and primary septicemia due to the consumption of seafood, especially raw oysters. *V. vulnificus* has been recovered from wounds due to lacerations by shells of marine animals or direct exposure to seawater. Wound infections due to *V. vulnificus* rapidly develop swelling and erythema, often extending to involve adjacent areas where bullae or vesicles appear and necrosis sometimes develops. Primary septicemia due to the consumption of seafood contaminated with this organism is often life threatening, having a mortality rate of nearly 50%.

Laboratory identification

The isolation of vibrios from stools and extraintestinal sites is not considered difficult. These organisms have no special handling or media requirements. Likewise, identification of these organisms should be within the capabilities of most clinical laboratories. They are usually identified by performing standard biochemical tests, latex agglutination test, and molecular methods. However,

many of the automated or kit tests may be unsuitable for their isolation since the media used in these packaged identification kits may not contain enough NaCl to support the growth of halophilic vibrios. Collect and process extraintestinal specimens from infected wounds or suspected cases of septicemia by routine procedures. *Vibrio* species can be recovered on nonselective media that contain at least 0.5% NaCl; MAC is a suitable selective medium for *Vibrio* species. Liquid stool is best collected by rectal catheter, but rectal swabs inserted beyond the anal sphincter are acceptable. Obtain specimens as early on in the acute phase of the disease as possible since this increases your chances of recovering any vibrios that may be present. As always, when possible, make every attempt to acquire specimens before antibiotics are administered. Plate specimens as soon after collection as possible since vibrios are sensitive to dessication. Transport formed or liquid (rice-water) stool or rectal swabs in Cary-Blair transport medium; buffered glycerol saline is unsuitable for transport of this organism. If Cary-Blair is not available, filter paper can be soaked in liquid stool, placed in a zip-lock bag, and transported to the laboratory as soon as possible.

Direct microscopic examination of stool specimens using a wet-prep technique is *not* recommended since *Vibrio* members are often pleomorphic and would be difficult to distinguish from other motile, straight, or curved, rod-shaped bacteria. However, stools may be examined by dark-field microscopy for characteristic size, shape, and darting motility, especially after a brief incubation in broth.

Routine stool culture procedures are usually sufficient for the recovery of *Vibrio* members. However, in situations where they are specifically suspected, the use of specialized media is warranted. Thiosulfate-citrate-bile salts-sucrose (TCBS) agar is selective for *Vibrio* species and differentiates between sucrose fermenting strains that are yellow and sucrose negative strains that appear as blue or green colonies is suggested. To further enhance your chance of recovering *Vibrio* members, it is suggested that alkaline peptone broth (1% NaCl, pH 8.5) be used as an enrichment medium. After inoculation, incubate alkaline peptone broth six to eight hours at 35°C and then subcultured to MAC or TCBS agar, and a nonselective medium such as blood.

Test isolates suspected of belonging to the genus *Vibrio* for oxidase cytochrome production. All members of the genus are oxidase positive with the exception of *V. metschnikovii*. All other species produce a strong oxidase-positive test reaction. Colonies taken from acidic medium may give false negative reactions; therefore, it is suggested that only colonies from nonselective medium be used to perform the oxidase test. The production of oxidase by all with the exception of *V. metschnikovii* separates the genus from the family *Enterobacteriaceae*. Two other characteristics that are useful in identifying members of the genus are sensitivity to the vibriostatic compound O/129 and their halo-tolerance. *Vibrio* members are considered halophilic organisms; that is, they prefer to live in a saline environment. The presence of NaCl actually stimulates growth, but the concentration of the NaCl required for stimulation varies from species to species.

Inoculate suspected colonies into KIA since all the pathogenic varieties produce alkaline slant over acid butt (K/A) reactions. Colonies that are oxidase positive and give a K/A, KIA reaction can be tentatively identified as *Vibrio* species.

***Aeromonas* and *Plesiomonas* species**

The genus *Aeromonas* has expanded from *A. hydrophila* (a mesophilic species) and *A. salmonicida* (a psychrophilic species) to 14 genomospecies or DNA groups. *Plesiomonas* includes only one species, *P. shigelloides*.

General characteristics

The *Aeromonas* are straight gram-negative rods that can appear as coccobacilli or filamentous rods. Except for *A. salmonicida* and *A. media*, they are motile, catalase and oxidase positive, and reduce nitrate to nitrite. They grow over a wide temperature range of 0 to 45°C. *Plesiomonas* are straight gram-negative rods that can occur singly, in pairs, or in short chains. They are motile due to two to

five polar flagella and are also catalase and oxidase positive, and reduce nitrate to nitrite. *Plesiomonas* grow in a temperature range of 8 to 45°C.

Clinical significance

Aeromonas and *Plesiomonas* species are found in aquatic environments worldwide. Plesiomonads are usually found in tropical aquatic climates; whereas, the Aeromonads have been isolated from freshwater, chlorinated water, and polluted water, although they prefer brackish or estuarium water. *Aeromonas* species are associated with gastroenteritis, endocarditis, meningitis, pneumonia, osteomyelitis, peritonitis, and conjunctivitis. *P. shigelloides* is usually linked to a diarrheal disease or septicemia often accompanied by meningitis.

Laboratory identification

Blood agar and CIN are useful in isolating *Aeromonas* species from stool specimens. A semi-specific medium for *Aeromonas* or a nonselective agar is best. *P. shigelloides* can be isolated from selective and differential agars. Growth in the presence of 6% NaCl, growth on TCBS agar, and susceptibility to the vibriostatic agent O/129 can be used to differentiate *Aeromonas*, *Plesiomonas*, and *Vibrio* isolates. Serological procedures can also be used.

Campylobacter and Arcobacter species

The genera *Campylobacter* and *Arcobacter* are in the family *Campylocacteraceae*. The genus *Campylobacter* contains 18 species and subspecies, and the genus *Arcobacter* consists of four species.

General characteristics

The *Campylobacter* species are gram-negative, nonspore-forming, curved, S-shaped, or spiral rods. They are motile by the means of a single polar flagellum at one or both ends. Some strains grow aerobically or anaerobically and may require increased hydrogen for growth. Most *Campylobacter* species require an atmosphere containing approximately 5% O₂, 10% CO₂, and 85% N₂ that can be provided by commercial gas generator packs. *Arcobacter* species also consist of gram-negative, slightly curved, curved, or S-shaped nonspore-forming rods. These organisms are motile due to a single polar, microaerophilic flagellum and do not require hydrogen for growth.

Clinical significance

Campylobacter infections are associated with the ingestion of contaminated milk and water, and ingestion of improperly cooked food (usually poultry products). The *Campylobacter* species, *C. jejuni* and *C. coli*, are most commonly associated with a diarrheal illness that can range from asymptomatic to severe. Symptoms include fever, abdominal cramping, and diarrhea. Stool specimens may or may not contain blood and leukocytes. *C. fetus* is associated with bacteremia and extraintestinal infections. The other species have been isolated from a variety of clinical specimens. *Arcobacter* species are linked to bacteremia, endocarditis, peritonitis, and diarrhea.

Laboratory identification

For optimum recovery of *Campylobacter*, selective plating media and incubation methods are required. The selective media include Campylobacter-cefoperazone-vancomycin-amphotericin (CVA), blood-containing Skirrow medium, blood-free charcoal cefoperazone deoxycholate agar (CCDA), and charcoal-based selective medium (CSM). Once inoculated, plates are incubated under microaerophilic conditions at 42°C. Depending on the selective media used colonies usually appear gray, flat, irregular, and spreading on fresh media. In older cultures where less moisture is available, colonies may become convex, circular, and glistening with little spreading. *Arcobacter* species grow well on Skirrow medium at a 37°C. They are morphologically similar to *Campylobacter*. Nonculture methods for identifying *Campylobacter* are continually being developed and improved.

***Helicobacter* species**

Established in 1989, the genus *Helicobacter* is a result of taxonomic restructuring of the genus *Campylobacter*. There are at least nine species of *Helicobacter*, and only three are significant human pathogens: *H. pylori*, *H. fennelliae*, and *H. cinaedi*.

General characteristics

Helicobacter organisms are gram-negative, helical, curved or straight, unbranched rods. They are catalase and oxidase positive, and are motile by means of a single polar flagellum (*H. fennelliae* and *H. cinaedi*) or multiple unipolar or bipolar, lateral flagella (*H. pylori*). *H. pylori* is rapidly urease positive; whereas, *H. fennelliae* and *H. cinaedi* are rapid urease negative. They are microaerophilic with optimum growth at 37°C.

Clinical significance

The human gastric mucosa is the major habitat for *H. pylori* that is associated with chronic gastritis. The natural habitat of *H. cinaedi* is the intestinal tract of rodents, but it has also been isolated from rectal cultures and feces. *H. cinaedi* and *H. fennelliae* were first isolated from homosexual men with proctitis, enteritis, and bacteremia.

Laboratory identification

Multiple gastric biopsy specimens are needed for the identification of *H. pylori*. Selective media are required for the isolation of *Helicobacter* species. Plates must be incubated in a microaerobion environment with high relative humidity. After three to four days, colonies appear small, pinpoint, translucent and nonhemolytic. Nongrowth-detection methods are commercially available for *H. pylori*.

218. *Legionella* and *Streptobacillus*

The average clinical laboratory encounters most of these organisms so infrequently that diseases caused by these organisms may prove difficult to diagnose. It is important that the laboratory be informed if the health care provider suspects one of these unusual organisms since special selective media and processing techniques may be required to isolate and identify many of these organisms.

***Legionella* species**

The family *Legionellaceae* contains one genus *Legionella*. To date, over 35 species of *Legionella* have been isolated and expansion of the genus is likely as additional studies are performed. *L. pneumophila*, *L. micdadei*, *L. gormanii*, *L. anisa*, *L. bozemanii*, *L. dumoffii*, *L. feelei*, *L. hackeliae*, *L. israelensis*, *L. jordanis*, *L. sainthelensi*, *L. longbeachae*, *L. maceachernii*, *L. oakridgensis*, *L. wadsworthii*, *L. birminghamensis*, *L. cincinnatiensis*, *L. tucsonensis*, and *L. lansigensis* have been isolated from humans.

General characteristics

The bacilli belonging to the genus *Legionella* are mesophilic, gram-negative, motile, nonsporulating, and fastidious, obligate aerobes. Legionellae are unique among gram-negative bacilli in three ways: (1) their requirement of L-cysteine for growth, (2) the structure of their cellular fatty acids, and (3) DNA composition. This genus is fastidious, and its members will not grow on ordinary laboratory media. Initial work with *Legionella* dates back to 1947 when it was successfully grown using animal inoculation techniques. At present, the nutritionally fastidious organism can be successfully grown on buffered charcoal yeast extract (BCYE) agar that does contain the required L-cysteine. The majority (≥80%) of the cellular fatty acids of the members of this genus are branched acids. This is indeed unique among gram-negative bacilli being more akin to gram-positive organisms, such as corynebacteria and mycobacterium. DNA hybridization studies alone are the sole criterion used to

distinguish species of *Legionella*, and new species are being rapidly added to the genus, that some estimate may have the complexity of *Salmonella*.

Clinical significance

Aquatic environmental sources, such as lakes, air conditioning system cooling towers, showerheads, public fountains, water storage tanks, and ground water are the organism's natural habitat. To date, no animal reservoir has been discovered. The organism now known as *Legionella pneumophila* was once thought to be an aquatic saprophyte. Now it is known to be the causative agent of Legionnaires' disease, the most commonly isolated species of the genus, and its primary pathogen. Legionnaires' disease is described as a multisystem disease having pneumonia as its primary clinical manifestation. It may occur as a nosocomial or community-acquired, pneumonic disease either sporadically or in epidemic proportions. Inhalation of aerosols is usually the route of infection, and to date no person-to-person transmission has been documented. Only one case of laboratory-acquired infection has been documented, but you are cautioned to perform any laboratory procedure likely to create an aerosol in a biological safety cabinet. The fact that other serious pathogens, such as *F. tularensis* and *Coccidioides immitis* can grow on the same media as legionellae makes taking precautions all the more prudent.

Identification important

Two characteristics of Legionnaires' disease make its prompt and accurate identification imperative: (1) antimicrobial agents usually used to treat pneumonia are ineffective against *L. pneumophila*, and (2) this organism is capable of appearing in epidemic form with a high mortality rate. Your efforts in promptly identifying this agent may result in both a lower mortality rate and prevention of a possible epidemic. An estimated 3 to 25% of all adult pneumonias are due to legionellae. This is not a rare disease with 25,000 to 50,000 cases occurring annually in the United States. Most of these cases occur during the summer and early fall seasons that coincide with an increased prevalence of the organism in temperate climates.

Other diseases

Legionnaires' disease is not the only illness caused by the legionellae; a wide spectrum of other possibilities exists. *Legionella* infections can be divided into four categories: (1) subclinical infection, (2) nonpneumonic disease, (3) pneumonia, and (4) extrapulmonary inflammatory disease. Legionellae have, on occasion, been isolated from cases of peritonitis, pericarditis, endocarditis, lymphadenitis, pyelonephritis, and wounds. However, the second most common illness due to legionellae is Pontiac fever—a self-limiting, nonpneumonic, febrile disorder.

Laboratory identification

It is agreed that respiratory secretions or lung tissue are required for the isolation of legionellae. However, what is not agreed upon is how the respiratory secretions are to be collected. One school of thought places little value on expectorated sputum samples urging the use of invasive procedures such as transtracheal aspiration or bronchoalveolar lavage to acquire secretions. Others think that these invasive techniques are not warranted, that they expose the patient to unnecessary risk, and that expectorated sputum is acceptable. Both agree that if these expectorated specimens are utilized, they should be placed on both nonselective and semi-selective media and undergo acid decontamination techniques since they contain oropharyngeal flora. These techniques reduce the possibility of normal oropharyngeal flora from interfering with the isolation of pathogens.

Transportation

Legionellae are hardy organisms, and special transport media are not required. They are, however, sensitive to desiccation, so a small amount of sterile water may be added to the specimen. Do not use sterile saline to prevent desiccation, since it has been found to be inhibitory to some legionellae. If the

specimen is to be transported or stored for up to three days, maintain it at 5°C. If it is to be held any longer than three days, lower its temperature to -70°C.

Media

It is essential that appropriate media of the highest quality be used for the isolation of legionellae. BCYE agar containing yeast extract, L-cysteine, iron, activated charcoal is the preferred medium when it is supplemented with α -ketoglutaric acid and is termed BCYE α . BCYE α supplemented with cefamandole, polymyxin B, and anisomycin is selective and referred to as BMPA α .

Growth

All the *Legionella*, with the exception of *L. gormanii* grow in room air. However, increasing both the humidity and CO₂ content of the incubator enhances growth. The legionellae have the capacity to grow in a rather wide temperature range; ideally, they should be incubated at 35°C. They do not grow anaerobically and even under the best environmental conditions require three to five days to produce visible colonies. For this reason, you must retain culture plates for seven days before discarding them as negative. This prolonged incubation requirement, coupled with the organisms' need for a moist environment, may present a problem. The media must not be allowed to dry out during prolonged incubation. A solution to both problems is to place the culture plates in a CO₂ permeable plastic. Thus, CO₂ is available for growth, and moisture loss is prevented.

Appearance

After three to five days on BCYE α , the colonies are described as pinpoint, convex, circular, gray, glistening, and having an entire margin. When viewed from the side, under magnification, they are said to have a ground-glass appearance. To speed identification of this organism, it is suggested that the plates be examined under magnification either with a hand-held lens or preferably a dissecting microscope.

Identification

Speciation of the genus *Legionella* is of epidemiologic importance, but not of importance at the clinical level. The legionellae are biologically inert and there are few biochemical reactions on which speciation may be determined. There is also little benefit in identifying the organism as to species, since all legionella infections are treated identically. Therefore, an isolate suspected of being legionellae may be presumptively identified based on colony morphology on BCYE α agar, and microscopic morphology. To help substantiate this presumptive identification, subculture colonies suspected of being legionellae to BCYE α and an agar that does not contain L-cysteine, such as blood agar. Growth on BCYE α , but not on the blood agar, would further confirm your suspicion that the organism in question is a species of *Legionella*.

Other methods

We will now discuss other methods used to confirm a diagnosis of legionellosis. Confirmation tests include direct fluorescent antibody (DFA) test, serological test, and radioimmunoassay (RIA) test. The quickest most specific means of diagnosing legionellosis is the performance of the DFA procedure. This can be accomplished on respiratory secretions and tissues collected in a variety of ways. Studies have indicated that DFA procedures have a specificity approaching 95%, but a sensitivity of only 50 to 75%. This means a positive test is highly indicative of legionellosis; since there are very few organisms known to cross-react with the DFA conjugates used to detect *Legionella*. However, a 50 to 75% sensitivity rate means that the potential exists for 25 to 50% of the tests to be false negatives. The time needed to become proficient in performing DFA testing coupled with the specialized reagents required are generally thought to prohibit the average laboratory from offering this diagnostic technique. Serum indirect immunofluorescent antibody (IFA) test can be used to demonstrate seroconversion by a patient with legionellosis. This test has epidemiological

value, but little clinical significance since it offers only a retrospective diagnosis. It has the disadvantage of requiring a specimen to have been collected during the acute stage of the disease, as well as an additional specimen 10 to 14 days after the onset of the disease. This collection regimen is necessary in order to demonstrate a diagnostic four-fold rise in antibody titer. Lastly, an RIA procedure that detects an antigenic polysaccharide substance excreted in the urine of patients with legionellosis has been developed. This procedure may offer a quick and specific means of detecting legionellosis, but it must first undergo additional clinical trials.

***Streptobacillus* species**

The genus *Streptobacillus* contains only one species, *S. moniliformis*, whose species name is Latin for “necklace shaped”. This is an apt description of the organism’s microscopic morphology displayed only when the organism is grown under less than ideal conditions.

General characteristics

S. moniliformis is a facultatively anaerobic gram-negative rod. Under ideal conditions, the cells are highly pleomorphic, long, curved, looped filaments. These filaments may have irregular swellings that can be two to five times the width of the filament giving it the appearance of a string of beads. *S. moniliformis* is catalase and oxidase negative, and it may produce acid without gas from some sugars, arginine dihydrolase, and H₂S.

Clinical significance

S. moniliformis is one of two agents capable of causing rat-bite fever; the other organism is *Spirillum minus*. *S. moniliformis* is a normal inhabitant of the oropharynx of rats, mice, weasels, and squirrels. It is transmitted to humans in one of three ways:

1. Directly by way of a rodent bite.
2. Indirectly due to the consumption of dairy products contaminated with rat feces.
3. In an unknown manner to people living in rat infested buildings.

When the disease is acquired indirectly, it is referred to as “Haverhill fever”. The onset of rat-bite fever due to *S. moniliformis* is abrupt, sometimes beginning within 12 hours, but always within a 10-day incubation period. Clinical symptoms include fever, chills, severe headache, vomiting, and irregular or wavering temperature curve. A rash covering the palms and soles of the feet is often seen. However, the hallmark of this infection in over 50% of the patients is the development of polyarthrititis.

Laboratory identification

Two forms of this bacteria exist: (1) bacterial-phase organisms and (2) L-phase or L-form organisms that are cell-wall-defective variants that arise spontaneously. The bacterial-phase organisms have a normal cell wall and can be grown on a number of basal media enriched with 15% sterile, defibrinated rabbit blood, or 20% horse serum. The L-form organisms or cell-wall-defective form is nutritionally more fastidious and requires a more enriched medium. Rogosa medium was especially developed for the growth of L-form organisms. It is a clear, heart-infusion medium enriched with horse serum. Since natural body fluids in the form of blood, serum, or ascitic fluid are required for growth of both forms of *S. moniliformis*, it will not grow on ordinary media such as KIA, MAC, or TSI. When 10 to 30% ascitic fluid or serum is added to thioglycollate medium, the organism grows within two to six days in the form of breadcrumb or fluff-ball colonies.

Blood and joint fluids, as well as pus and other body exudates, may be submitted for the isolation of *S. moniliformis*. The initial processing of blood and body fluids is identical. Both are mixed with equal volumes of sterile 2.5% sodium citrate to prevent clotting, and three smears are prepared. One of each of these smears should be stained with Gram, Wayson, and Giemsa stains.

After this point in processing, blood and joint fluids are handled differently. In the case of blood, the specimen is centrifuged for 30 to 40 minutes and then 0.1 ml of cells are mixed with an equal amount of Rogosa broth and inoculated onto a plate of Rogosa agar. No loop or other inoculating device is used in this process. The mixture is simply placed in the center of the plate and distributed over the surface by gently tilting. Two additional tubes of Rogosa broth should also be inoculated with 0.1 ml of sedimented cells.

Joint fluid or other body fluids, on the other hand, need not be centrifuged after being diluted with sodium citrate. All that is required is that 1 ml amounts of the anticoagulated mixture be placed in each of two tubes of Rogosa broth medium. Inoculate swab specimens taken from the wound site into one tube of broth and one plate of solid agar, and then prepare and stain three smears.

Bacterial-phase colonies grown under increased CO₂ and moisture levels appear as small, discrete colonies with a glistening surface and irregular edges within two to three days. Spontaneously arising L-phase colonies may develop beneath or on the surface of the agar and adjacent to bacterial-phase colonies and have a “fried egg” appearance.

Several factors influence the microscopic morphology displayed by this organism. The age, media used, and manner in which the culture was incubated all have an impact on what phase of *S. moniliformis* is seen and the microscopic morphology displayed. Uniform gram-negative bacilli with rounded or pointed ends usually predominate under ideal conditions. Under less than optimum conditions, the organism is extremely pleomorphic; long filaments and unevenly staining “necklace forms” may be seen. If L-phase organisms are present, they’ll exhibit bipolar staining and appear as tiny, gram-negative coccoid or coccobacillary forms.

Self-Test Questions

After you complete these questions, you may check your answers at the end of the unit.

215. *Pseudomonas* and *Burkholderia*

1. What is the range of infections from *Pseudomonas aeruginosa*?
2. What are eight solutions from which *P. aeruginosa* has been isolated?
3. What type colony appearance does *P. aeruginosa* produce on blood agar?
4. Mucoid strains are isolated from the sputum of patients with what disease?
5. What other significant characteristics are noted about the colony appearance *P. aeruginosa*?
6. What causes the colony to have a characteristic blue-green color?

7. What percent do not produce this characteristic color?
8. What reactions do *P. aeruginosa* produce on lysine and ornithine decarboxylase? Arginine dihydrolase?
9. If *Pseudomonas* is mistaken for an enteric organism and placed on TSI or KIA, it may be incorrectly identified because of what characteristic reaction?
10. What disease is caused by *Burkholderia pseudomallei*?
11. How do the colonies of *B. pseudomallei* appear on blood agar?
12. *B. pseudomallei* do *not* grow well on what two media?
13. What reaction is obtained on the oxidase test? Glucose O-F medium?
14. What techniques are useful for identification of *B. pseudomallei*?

216. *Acinetobacter*, *Eikenella*, and *Xanthomonas*

1. Match the organism in column B with the statement in column A by placing the letter of the column B item beside the number of the column A item that most nearly describes it. Each element in column B may be used once or more than once.

Column A	Column B
___ 1. In order to grow aerobically, hemin is required.	a. <i>Acinetobacter</i> .
___ 2. Community-acquired pneumonia caused by contaminated dust particles of free silica or metal dust.	b. <i>Eikenella</i> .
___ 3. Found everywhere in nature and has been isolated in the hospital environment.	c. <i>Xanthomonas</i> .
___ 4. Important when isolated from single or mixed infections in human bite wounds, postsurgical infections, soft tissue abscesses (including brain), and pleuropulmonary infections.	
___ 5. A member of the family Neisseriaceae.	
___ 6. Normal flora of the skin, pharynx, and genitourinary tract.	
___ 7. Formerly <i>Pseudomonas</i> .	

17. *Vibrio*, *Aeromonas*, *Plesiomonas*, *Campylobacter*, *Arcobacter* and *Helicobacter*

1. How do you describe the typical microscopic appearance of the vibrios?
2. What two species of pathogenic vibrios do *not* require salt?
3. What is the causative agent of Asiatic or epidemic cholera?
4. What symptoms characterize Asiatic cholera?
5. The pathogenic *Vibrio* species are all capable of causing what three clinical syndromes?
6. What is the most common extraintestinal infection caused by *V. parahaemolyticus*?
7. What species of *Vibrio* is the most commonly isolated from traumatic injuries due to lacerations by shells of marine animals or exposure to seawater?
8. Why are many automated kit test products for the isolation of pathogenic bacteria unsuitable for the identification of vibrios?
9. What medium should be used to transport specimens suspected of containing vibrios?
10. What selective agar is suggested for recovering vibrios?
11. How can the recovery of vibrios be enhanced?
12. What biochemical characteristic separates all the vibrios, with one exception, from the family *Enterobacteriaceae*? Which *Vibrio* species is the exception?

13. What tests can be used to differentiate *Aeromonas*, *Plesiomonas*, and *Vibrio* isolates?
14. What are the two *Campylobacter* species most commonly associated with diarrheal illness?
15. What is the optimal method for recovering *Campylobacter*?
16. Which *Helicobacter* species is associated with chronic gastritis?

218. *Legionella* and *Streptobacillus*

1. How many genera are included in the family *Legionellaceae*?
2. In what three ways are legionellae unique among gram-negative bacilli?
3. What is the natural habitat of *Legionella*?
4. What is the causative agent of Legionnaires' disease?
5. In what forms can Legionnaires' disease occur?
6. What is the usual route of infection with Legionnaires' disease?
7. Why is it prudent to take laboratory precautionary measures when working with *Legionella*?
8. What two characteristics make the prompt identification of Legionnaires' disease imperative?
9. During what time of the year do most cases of Legionnaires' disease occur?

10. What is the second most common type of legionellosis?
11. What types of specimens are usually required for the isolation of legionellae?
12. What is the preferred medium for the isolation of *Legionella*?
13. Increasing what two incubation conditions enhances the growth of legionellae?
14. Why is speciation of legionellae clinically unimportant?
15. What is considered the quickest most specific confirmatory test for legionellae?
16. How do you describe the microscopic appearance of *Streptobacillus moniliformis* grown under ideal conditions?
17. What two agents can cause rat-bite fever?
18. What is the normal habitat of *S. moniliformis*?
19. In what three ways are humans infected with *S. moniliformis*?
20. What is the hallmark symptom of infection with *S. moniliformis*?
21. What two forms of *S. moniliformis* exist?
22. What medium was especially developed for the growth of *S. moniliformis* L-phase organisms?

23. How do colonies of *S. moniliformis* appear when grown in thioglycolate enriched with 10 to 30% ascitic fluid or serum?
24. What is the first step in processing both blood and joint fluid for the isolation of *S. moniliformis*?
25. How do you describe the appearance of L-phase colonies on a plate of Rogosa medium?

Answers to Self-Test Questions

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1. Use glucose fermentatively, reduce nitrates to nitrites, and are oxidase negative.
2. Supportive, selective, differential, or some combination of the three.
3. They inhibit the growth of most gram-positive organisms.
4. For the most part enterics *lack* distinctive colony morphology.
5. Stalactite pattern of growth.
6. GN, selenite F, and tetrathionate.
7. (1) Desoxycholate citrate agar.
(2) SS agar.
(3) HEA.
(4) Bismuth sulfite agar.
(5) BG.
(6) EMB agar.
(7) XLD agar.
(8) MAC agar.
8. Increase the agar concentration to 5%.
9. Suppress growth of contaminating forms, usually nonpathogens, and allow the infectious species to survive and proliferate.
10. Employ the principle of color distinction since colors are based on a change of pH when acids are produced because of lactose fermentation. This change in pH affects the color of the indicator dye present in the agar.
11. These broths are inhibitory to most strains of these genera.
12. Glucose.
13. Sucrose.
14. All-yellow reaction.
15. Species of *Escherichia* and certain members of *Salmonella*, *Klebsiella*, *Enterobacter*, *M. morganii*, *Providencia*, *Serratia* and *Proteus*.
16. Colony morphology, microscopic morphology, rapid biochemical test, and TSI or KIA reaction patterns.
17. Many organisms, including a few enterics, may produce delayed false-positive reactions.
18. If an organism is capable of hydrolyzing urea to ammonia and water.
19. β -galactosidase.
20. Actively transporting lactose across the cell membrane.
21. Yellow.

22. Formation of acid end products from the metabolism of glucose.
23. The MR test detects the formation of formic acid and acetic acid. The VP test detects the formation of acetoin and butanediol.
24. *Proteus*, *Providencia*, *Morganella*, and some strains of *Enterobacter agglomerans*.
25. Phenylpyruvic acid.
26. Blue.
27. Purple butt.

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1. 99%.
2. Urinary tract infections.
3. (1) EPEC.
(2) ETEC.
(3) EIEC.
(4) Hemorrhagic or EHEC.
4. (1) b, (2) a, (3) b, (4) c, (5) d, (6) a, (7) c.
5. The extensive battery of antisera required is not available in most laboratories.
6. Serogrouping with polyvalent antisera.
7. Gastroenteritis, septicemia with focal lesions, and enteric fever.
8. Human carriers.
9. Slight, described as button-like at the point of inoculation in the butt of a TSI or KIA tube.
10. (1) *S. choleraesuis*.
(2) *S. typhi*.
(3) *S. paratyphi*.
11. Biochemically and antigenically.
12. On the basis of the O-antigens they possess.
13. All four.
14. One to five years of age.
15. *S. dysenteriae*.
16. New methylene blue.
17. *Yersinia pestis*.
18. Bubonic and pneumonic.
19. The rat flea.
20. Aspirates from buboes, pus from the area of the fleabite, sputum, throat swabs, or blood.
21. Cary-Blair transport media.
22. Cells show marked bipolar staining. They have a safety pin appearance with polar bodies staining blue and the remainder light blue to reddish.
23. Coccoid, round, filamentous, elongated forms.
24. A capsule.
25. Small, nonhemolytic, round, transparent, glistening colorless colonies with an undulate margin.

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1. *C. diversus*.
2. Soft tissue infections, meningitis, osteomyelitis, sepsis, hepatic abscesses, and a mild diarrheal disease.
3. Soil, water, and the intestinal tract of man and animals.
4. *E. aerogenes* and *E. cloacae*.

5. *E. agglomerans*.
6. Ornithine decarboxylase test.
7. *K. pneumoniae*, *K. ozaenae*, *K. rhinoscleromatis*, *K. oxytoca*, and *K. planticola*.
8. Bacterial pneumonia and this organism have also been found as a secondary invader in such diseases as bronchiectasis, tuberculosis, influenza, pleurisy, pyelonephritis and as a significant cause of nosocomial infections.
9. As large mucoid colonies that have a tendency to coalesce.
10. The colonies usually string out.
11. *E. aerogenes* is usually motile, may liquefy gelatin, and is not distinctly capsulated.
12. *Morganella* is differentiated from the genus *Proteus* since it is H₂S negative, gelatin negative, and D-mannose positive. It can be separated from the genus *Providencia* by its ability to use decarboxylate ornithine and its inability to use citrate.
13. *P. mirabilis*.
14. Acid slant and butt.
15. Increase the agar concentration to 5%. Addition of sodium azide, chloral hydrate, and phenylethyl alcohol is also preventive.
16. *P. mirabilis*, *P. penneri*, and *P. vulgaris*.
17. Because of the varying susceptibility to antibiotics of various members of the genus.
18. Urinary infections and infections of burn patients.
19. *Providencia* spp. are motile and utilize citrate.
20. Red, nonwater-soluble pigment usually formed at room temperature.
21. Burns.

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1. Infections range from superficial skin infections to fulminant sepsis that has a high mortality rate.
2. Any eight of the following:
 - (1) Disinfectants.
 - (2) Ointments.
 - (3) Soaps.
 - (4) Irrigation fluids.
 - (5) Eye drops.
 - (6) Dialysis fluids.
 - (7) Contact lens solutions.
 - (8) Illicit injectable drugs.
 - (9) Distilled water.
 - (10) Injectable medicines.
 - (11) Cosmetics.
3. Spreading and flat colony with an erose margin and a metallic sheen on blood agar.
4. Cystic fibrosis.
5. They tend to spread and give off a characteristic grape-like odor.
6. The secretion of pyocyanin and fluorescein (pyoverdine).
7. Approximately 4%.
8. Negative; positive.
9. Alkaline slant and butt.
10. Melioidosis, a glanders-like disease in man.
11. They are wrinkled and on prolonged incubation become umbonate.

12. *Salmonella-Shigella* agar and cetrimide agar.
13. Positive; oxidative acidity.
14. The agglutination and fluorescent-antibody reactions.

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1. (1) b.
(2) a.
(3) c.
(4) b.
(5) a.
(6) b.
(7) c.

217

1. Gram-negative, slightly curved or comma-shaped rod upon initial isolation; in older cultures, a great degree of pleomorphism-appearing coccoid or straight-sided bacilli.
2. *V. cholera* and *V. mimicus*.
3. *Vibrio cholerae*.
4. An acute diarrheal disease characterized by massive loss of fluid and electrolytes that, if untreated, may result in cardiovascular collapse and death in a single day.
5. (1) Gastroenteritis.
(2) Soft tissue infections.
(3) Systemic infections.
6. Wound infections.
7. *V. vulnificus*.
8. The media used in these packaged test kits may not contain enough NaCl to support the growth of halophilic vibrios.
9. Cary-Blair transport medium.
10. Thiosulfate-citrate-bile salt-sucrose agar.
11. By using alkaline peptone broth as an enrichment medium.
12. Production of oxidase, *V. metschnikovii*.
13. Growth in the presence of 6% NaCl, growth on TCBS agar, and the susceptibility to the vibriostatic agent O/129 can be used to differentiate *Aeromonas*, *Plesiomonas*, and *Vibrio* isolates. Serological procedures can also be used.
14. *C. jejuni* and *C. coli*.
15. Primary plating on selective media incubated under microaerophilic conditions at 42°C.
16. *H. pylori*.

218

1. One.
2. (1) Their requirement for L-cysteine for growth.
(2) Cellular, fatty acid composition.
(3) DNA composition.
3. Aquatic environmental sources.
4. *L. pneumophila*.
5. As nosocomial or community acquired in sporadic or epidemic proportions.
6. Inhalation of aerosols.

7. Laboratory-acquired infection is possible and other serious pathogens may grow on the same media as legionellae.
8. (1) It does not respond to the antibiotics normally used to treat pneumonia.
(2) It is capable of causing epidemics.
9. Summer and early fall.
10. Pontiac fever.
11. Respiratory secretions or lung tissue.
12. BCYE α .
13. Humidity and CO₂.
14. All legionellosis infections are treated the same.
15. Direct fluorescent antibody test.
16. Under ideal conditions, the cells are gram-negative, highly pleomorphic, long, curved, looped filaments.
17. *S. moniliformis* and *Spirillum minus*.
18. Oropharynx of rats, mice, weasels, and squirrels.
19. (1) Direct by rodent bites.
(2) Indirectly by consumption of contaminated dairy products.
(3) In an unknown manner from living in rat infested buildings.
20. Polyarthrititis.
21. (1) Bacterial-phase.
(2) L-phase or L-form.
22. Rogosa agar.
23. As bread -crumb or fluff-ball colonies.
24. Dilute with an equal volume of sterile 2.5% sodium citrate.
25. "Fried egg" colonies.

Do the Unit Review Exercises (URE) before going to the next unit.

Unit Review Exercises

Note to Student: Consider all choices carefully, select the *best* answer to each question, and *circle* the corresponding letter.

51. (212) What genus of the family *Enterobacteriaceae* is sometimes used as a standard for determining water purity and if it is safe for drinking?
 - a. *E. coli*.
 - b. *S. typhi*.
 - c. *P. vulgaris*.
 - d. *K. pneumoniae*.
52. (212) Which biochemical test determines if an organism possesses the enzyme tryptophanase?
 - a. Indole.
 - b. Urease.
 - c. Oxidase.
 - d. Voges-Proskauer.
53. (213) Which of the four types of *E. coli* enteric disease is associated with serotype O157:H7?
 - a. Enterohemorrhagic.
 - b. Enteropathogenic.
 - c. Enterotoxigenic.
 - d. Enteroinvasive.
54. (213) In *S. typhi* infection (typhoid fever), the sources of infection are
 - a. fish.
 - b. cattle.
 - c. humans.
 - d. snails and eels.
55. (213) The salmonellae, with the exception of a rare isolate, do *not* ferment
 - a. trehalose.
 - b. arabinose.
 - c. glucose.
 - d. lactose.
56. (213) Which genera is *usually* considered the most biochemically inactive?
 - a. *Proteus*.
 - b. *Shigella*.
 - c. *Salmonella*.
 - d. *Escherichia*.
57. (213) What is the *main* vector for the transmission of *Y. pestis*?
 - a. Lice.
 - b. Ticks.
 - c. Rat fleas.
 - d. Mosquitoes.
58. (214) Which two species of *Enterobacter* are *most* frequently isolated?
 - a. *E. agglomerans* and *E. gergoviae*.
 - b. *E. sakazakii* and *E. amnigenus*.
 - c. *E. aerogenes* and *E. cloacae*.
 - d. *E. asburiae* and *E. taylora*.

59. (214) Which is the *second* most populous facultative enteric genus found in the bowel of humans?
- Proteus*.
 - Klebsiella*.
 - Citrobacter*.
 - Enterobacter*.
60. (214) Which two characteristics may be used to differentiate *Morganella* from *Providencia*?
- Positive motility and ability to utilize citrate.
 - Positive Voges-Proskauer and ability to liquefy gelatin.
 - Positive Voges-Proskauer and ability to hydrolyze urea.
 - Positive ornithine decarboxylase and negative citrate utilization.
61. (214) Which two characteristics may be used to distinguish *Providencia* from *Shigellae*?
- Positive motility and ability to utilize citrate.
 - Positive motility and ability to hydrolyze urea.
 - Positive Voges-Proskauer and ability to liquefy gelatin.
 - Positive Voges-Proskauer and ability to hydrolyze urea.
62. (214) The chromogenic strains of *Serratia marcescens* show
- red, water-soluble pigment at 35°C or above.
 - brown, water-soluble pigment at room temperature.
 - brown, non-water-soluble pigment at 35°C or above.
 - red, non-water-soluble pigment at room temperature.
63. (215) The disease melioidosis, and endemic glanders-like disease in humans, is caused by
- Burkholderia mallei*.
 - Burkholderia pseudomallei*.
 - Pseudomonas aeruginosa*.
 - Pseudomonas fluorescens*.
64. (215) You may culture *Burkholderia pseudomallei* on all of the following *except*
- trypticase soy agar.
 - MacConkey (MAC) agar.
 - blood agar.
 - Salmonella-Shigella (SS)* agar.
65. (216) Which miscellaneous gram-negative bacilli are in the family *Neisseriaceae*?
- Eikenella*.
 - Legionella*.
 - Acinetobacter*.
 - Xanthomonas*.
66. (216) Which miscellaneous gram-negative bacilli were formally in the *Pseudomonas* genus?
- Eikenella*.
 - Legionella*.
 - Acinetobacter*.
 - Xanthomonas*.
67. (217) Which is the *most* virulent strain of vibrio mentioned that causes the most serious wound infections and primary septicemia due to the consumption of seafood, especially raw oysters?
- V. damsela*.
 - V. vulnificus*.
 - V. alginolyticus*.
 - V. parahaemolyticus*.

68. (217) Many automated kit tests for the identification of pathogenic bacteria are unsuitable for the genus *Vibrio* because the
- media used may contain too much NaCl.
 - media used may not contain enough NaCl.
 - pH of the media used may be too acidic.
 - pH of the media used may be too alkaline.
69. (217) Which transport medium has been *recommended* for transporting specimens which may contain members of the genus *Vibrio*?
- Stuart.
 - Charcoal.
 - Selenite F.
 - Cary-Blair.
70. (217) What is the specimen of choice for the recovery and identification of *H. pylori*?
- Stool.
 - Blood.
 - Joint fluid.
 - Gastric biopsy.
71. (218) What solution *may* be added to specimens for the isolation of *Legionella* to prevent desiccation during transport?
- Sterile water.
 - 10% Formalin.
 - Physiological saline.
 - 5% buffered phosphate.
72. (218) In which genera do L-phase organisms arise spontaneously?
- Pasteurella*.
 - Francisella*.
 - Legionella*.
 - Streptobacillus*.
73. (218) Which media was especially developed for the growth of L-phase organisms?
- Rogosa.
 - Brucella.
 - Fletchers.
 - Thayer-Martin.

Please read the unit menu for unit 4 and continue ➔

Student Notes

Unit 4. Anaerobic Gram-Positive and Gram-Negative Organisms

4-1. General Characteristics and the Gram-Negative Anaerobes	4-1
219. General characteristics of anaerobes	4-1
220. Anaerobic gram-negative cocci and bacilli	4-1
4-2. Anaerobic Gram-Positive Cocci and Bacilli.....	4-7
221. Anaerobic gram-positive cocci.....	4-7
222. Anaerobic gram-positive bacilli	4-8

IN VOLUME 1 we discussed the collection and processing of specimens for the recovery of anaerobic bacteria. In this unit, we will consider the more common and clinically significant anaerobes. If you recall, an obligate anaerobe does not grow in culture when exposed to atmospheric oxygen. Free oxygen is toxic to the anaerobe's respiratory mechanism, and hydrogen atoms are usually transferred only between certain organic compounds fabricated by the cell from constituents of the culture medium. Obligate anaerobic bacteria can be divided into two basic groups: (1) strict anaerobes that cannot survive even minute amounts of oxygen, and (2) moderate anaerobes which can tolerate small amounts of oxygen. If exposed to atmospheric oxygen for just 10 minutes on an open laboratory bench, some of the strictest anaerobes will rapidly die. The reasons for the difference in oxygen sensitivity are multiple and vary among each genera and species. Let's begin with a review of general characteristics of anaerobes and then move to the anaerobic gram-negative bacteria.

4-1. General Characteristics and the Gram-Negative Anaerobes

Along with aerobic bacteria, molecular biological studies have changed and will change the taxonomy of anaerobic bacteria. However, at this time, there are over 70 different genera and 250 described species of anaerobic bacteria. Some species are facultative anaerobes; in other words, some species of aerobic bacteria grow anaerobically. Other species are obligate anaerobes. Nearly all of the anaerobic bacteria can be found in humans, animals, or nature. In nature, they have been isolated from soil, water, food, and animals. In humans, they are normal flora in the oral cavity, in genitourinary and gastrointestinal tracts, and on the skin. We'll discuss only those of greatest concern to the clinical microbiologist.

219. General characteristics of anaerobes

It is crucial to isolate and identify anaerobic bacteria because these infections are associated with high morbidity and mortality. Also, the antibiotic therapy of the anaerobic infection varies with the bacterial species involved. The most common sites or disease processes of the human body for anaerobic infections are:

- brain abscess
- chronic sinusitis
- periodontal infections
- lung abscess
- necrotizing pneumonia
- empyema
- secondary bacteremia
- subdiaphragmatic
- hepatic and subhepatic abscess
- abdominal infections (post-surgical, post-traumatic, following ruptured viscus, malignancy)
- vagina and uterus in females (endometritis, postabortal infections, pelvic and tubo-ovarian abscess)

- endocarditis
- breast abscess
- perirectal abscess
- necrotizing cellulitis (frequently with gas) infected vascular gas gangrene

Anaerobic infections are usually a mixture of aerobic, facultatively anaerobic, and other anaerobic bacteria. Because of this mixture, enriched and selective media are required for optimal recovery of anaerobes. Once a suspected anaerobe is recovered, there are three levels of identification for all anaerobic bacteria.

Level I—Presumptive identification

Presumptive identification can be made on atmospheric requirements (aerotolerance testing), Gram-stain results, and colony morphology. Specimen source and growth or no growth on the selective media can also aid presumptive identification.

Level II—Preliminary grouping

Preliminary grouping includes Gram-stain reaction; cellular and colony morphology; spot indole and catalase test (using 15% hydrogen peroxide instead of 3% hydrogen peroxide); nitrate reduction and antibiotic susceptibility disk test; and simple procedures for glutamic acid decarboxylase production and L-analyl-L-analylaminopeptidase production. Additional tests include colony hemolysis, pigment, or pitting of agar; long-wavelength (366 nm) fluorescence using a UV light; 20% bile-resistance test; formate and fumarate (F/F) growth-stimulation test; urease, motility, and sodium polyanetholsulfonate (SPS) susceptibility test; Ethanol spore test; lecithinase test, lipase reaction, and production of fatty acids from peptone-yeast-glucose broth; Nagler and reverse CAMP test; and arginine growth-stimulation test. These tests may incorporate disk, tubes, or agar methods. Exact procedures depend on the commercial source and reagents used.

Level III—Species identification

Species identification encompasses biochemical testing using convectional tube test or microtubule methods (example: API); enzyme substrate test systems; gas-liquid chromatography (GLC) for determining metabolic products; and deoxyribonucleic acid (DNA) probes. GLC techniques are the most sensitive and specific, but the average clinical laboratory does not have the ability to perform these procedures.

NOTE: Many laboratories have adopted various commercially prepared micromethod test kits for the identification of anaerobic bacteria. Interpretation of results from commercial microtubule methods may require additional confirmatory testing before reporting species identification. Most rapid identification methods can only be considered presumptive.

Methods for Antimicrobial Susceptibility Testing of Anaerobic Bacteria—Second Edition, NCCLS Document M11-A2, 1991, is published by the Clinical and Laboratory Standards Institute, formally known as the National Committee for Clinical Laboratory Standards, and should be referenced for anaerobic antimicrobial procedures.

220. Anaerobic gram-negative cocci and bacilli

Acidaminococcus, *Megasphaera*, and *Veillonella* are considered the only anaerobic gram-negative cocci described at this time. *Acidaminococcus* and *Megasphaera* are part of the normal, human fecal flora and are very rare isolates. There are over 45 genera in the group of anaerobic gram-negative bacilli. It is beyond the scope of these CDCs to name them all. The anaerobic gram-negative bacilli isolated most often in the clinical laboratory are; *Bacteroides*, *Biophilia*, *Campylobacter*, *Fusobacterium*, *Mobiluncus*, *Porphyromonas*, and *Prevotella*. The genus *Campylobacter* was covered in unit 3; however, two species are obligate anaerobes. *C. curvus* and *C. rectus* (formerly *Wolinella* species) are found in patients with periodontal disease.

***Veillonella* species**

The genus *Veillonella* is the most frequently isolated anaerobic gram-negative cocci in clinical specimens. There are only three *Veillonella* species out of seven that are human isolates—*V. parvula*, *V. atypica*, and *V. dispar*.

General characteristics

Veillonella species are tiny, gram-negative cocci that appear in pairs and packets.

Clinical significance

Veillonella species are part of the normal flora in the oral cavity. They are associated with oral, head, neck, and miscellaneous soft-tissue infections, as well as, human bite wounds.

Laboratory identification

Veillonella colonies are small, convex, and translucent to transparent, with an entire margin on blood agar. They may show red fluorescence under long-wave ultraviolet light; however, the fluorescence is rapidly lost after the colonies are exposed to air. *Veillonella* species grow slowly in broth and produce a very fine, granular turbidity.

***Bacteroides* species**

The genus *Bacteroides* has been undergoing a lot of taxonomic changes in recent years. It has been suggested to restrict the genus *Bacteroides* to the *Bacteroides fragilis* group. The *B. fragilis* group contains: *B. fragilis*, *B. vulgatus*, *B. distasonis*, *B. caccae*, *B. merdae*, *B. thetaiotaomicron*, *B. uniformis*, *B. ovatus*, *B. stercoris*, and *B. eggerthii*.

General characteristics

The *B. fragilis* group of organisms is gram-negative, nonmotile, straight rods with rounded ends. This group of anaerobic bacteria is the most commonly recovered anaerobes in clinical specimens and are more resistant to antimicrobial drugs than the other anaerobes.

Clinical significance

The *B. fragilis* group is the predominant normal flora in the gastrointestinal tract. They are normally present in the female genital tract and, although rare, in the oral cavity. *Bacteroides* species are frequently recovered from intra-abdominal and soft tissue infections, liver abscesses, decubitus ulcers, and blood cultures. They are rarely, but can be, associated with pleuropulmonary infections and brain abscesses. Of this group, the two that are of greatest clinical significance are *B. fragilis* and *B. thetaiotaomicron*. *B. fragilis* produces an endotoxin that is biologically weaker than the toxins produced by the aerobic gram-negative bacilli.

Laboratory identification

Colonies of the *B. fragilis* group are circular with an entire margin, smooth, white to gray, and are nonhemolytic on blood agar. Preliminary grouping of this organism includes growth on *Bacteroides* bile esculin (BBE) agar. BBE agar contains 20% bile that inhibits most anaerobes and 100 µg/ml of gentamicin, which inhibits most aerobic bacteria. Colonies on BBE agar are also circular, entire, and raised with varying degrees of esculin hydrolysis. Three distinct morphotypes exist:

1. A low convex, dark gray colony surrounded by a dark gray zone.
2. A glistening, convex, medium gray colony surrounded by a gray zone.
3. A glistening, convex, medium gray colony with no gray zone.

NOTE: *Bilophila wadsworthia* also grows on BBE agar but only after three to five days, and the colonies are usually gray with black centers. *Bilophila wadsworthia* is a rare, fastidious, gram-negative bacillus that has been isolated from patients with acute appendicitis, perforated and

gangrenous appendicitis, and other related abscesses. It has also been recovered from blood, joint fluid, pleural fluid, oral secretions, vaginal secretions, and feces.

***Fusobacterium* species**

The *Fusobacterium* genus consists of *F. nucleatum* (*F. nucleatum* subsp. *animalis*, *fusiforme*, *nucleatum*, *polymorphum*), *F. necrophorum* (*F. necrophorum* subsp. *funduliforme* and *necrophorum*), *F. mortiferum*, *F. alocis*, *F. sulci*, *F. periodonticum*, *F. russii*, *F. ulcerans*, and *F. varium*. *F. nucleatum* is the most commonly isolated species.

General characteristics

Fusobacterium species are thin, straight or slightly curved, nonmotile, anaerobic gram-negative bacilli with round ends. *F. nucleatum* is the exception with pointed ends.

Clinical significance

The *Fusobacterium* are found as normal flora in the oral, upper respiratory, genital, and gastrointestinal tracts. They have been associated with infections throughout the body including peritonsillar abscesses, metastatic abscesses, jugular-vein septic thrombophlebitis, gingivitis and periodontitis, intra-abdominal, head, neck, and upper respiratory infections, and bacteremia.

Laboratory identification

Fusobacterium species are slightly hemolytic and produce a greening of the blood agar. The colonies of *F. nucleatum* look like breadcrumbs or appear convex, glistening, and grainy on the inside. *F. necrophorum* produces an umbonate colony. *F. varium* colonies are translucent and opaque in the center, have an undulate margin, and resemble fried eggs.

***Mobiluncus* species**

This group of microorganisms was not recognized until 1984. To date, there are only three species named—*M. mulieris*, *M. curtisii*, and *M. curtisii* SLH-29 group.

General characteristics

Mobiluncus species are curved, gram-variable or gram-negative, nonspore-forming bacilli with tapered ends. They occur singly or in pairs and may resemble “gull-wings”. They are motile by multiple subpolar flagella. Although they stain gram-negative, they possess a multilayered gram-positive-type cell wall and may be classified as gram-positive bacilli.

Clinical significance

Mobiluncus are found in the reproductive tracts and recta of humans and other primates. They are associated with bacterial vaginosis (BV) and other pelvic infections in females. *Mobiluncus* species have been isolated from rectal and urethral specimens from male sex partners of women with BV and from rectal swabs of women with BV. They have also been isolated from breast abscesses, blood cultures, and the placenta after preterm births. This genus is very fastidious and is a rare isolate because of inadequate laboratory recovery procedures or because it has little pathogenic potential.

Laboratory identification

After 48 hours on anaerobic blood agars, *Mobiluncus* colonies are minute and may be overlooked in mixed cultures. The colonies are low convex, translucent, and only 1 to 2 mm after three to five days of incubation.

***Porphyromonas* species**

P. asaccharolytica, *P. endodontalis*, *P. gingivalis*, *P. canoris*, *P. circumdentaria*, and *P. salivosa* are in the genus *Porphyromonas*. The first three are of human origin, and the last three are of animal origin.

General characteristics

Porphyromonas species are nonmotile, straight bacilli with rounded ends.

Clinical significance

Porphyromonas species are normal flora in the oral, female genital, and gastrointestinal tracts. *P. asaccharolytica* can be isolated from any type of infection, and *P. gingivalis* is important in periodontal disease. They are also found in human or animal bite infections.

Laboratory identification

On blood agar, the colonies of *Porphyromonas* are mucoid, dark brown to black, and smooth with an entire margin. Before the pigment is produced, they fluoresce brick red under ultraviolet light, except for *P. gingivalis*. They are difficult to grow in broth or on some selective agars that contain vancomycin.

Prevotella species

The genus *Prevotella* is divided into three categories based on pigmentation. *P. intermedia*, *P. nigrescens*, *P. corporis*, *P. melaninogenica*, *P. loescheii*, and *P. denticola* are pigmented. *P. oralis*, *P. veroralis*, *P. buccalis*, *P. oulora*, *P. oris*, *P. buccae*, *P. heparinolytica*, and *P. zoogloformans* are nonpigmented. The third category of species consists of *P. bivia* and *P. disiens*.

General characteristics

Besides having specific pigmentation characteristics, *Prevotella* species are also nonmotile, straight bacilli with rounded ends.

Clinical significance

As with *Bacteroides* and *Porphyromonas*, *Prevotella* are found as normal flora in the oral, female genital and gastrointestinal tracts. *P. disiens* and *P. bivia* are found in females with genital tract infections. The other *Prevotella* species are isolated from oral, head and neck, and pleuropulmonary infections. The species of *Prevotella* and *Porphyromonas* are isolated less frequently than those of the *B. fragilis* group.

Laboratory identification

Prevotella species produce varying amounts of pigments that may be helpful with their presumptive identification. *P. melaninogenica* produce smooth, tan to light brown colonies; whereas, *P. intermedia*, *P. nigrescens*, and *P. corporis* produce dry, dark brown to black colonies. Most *Prevotella* species also fluoresce brick red before exposure to air. *P. bivia* and *P. disiens* fluoresce orange to pink (coral).

Self-Test Questions

After you complete these questions, you may check your answers at the end of the unit.

219. General characteristics of anaerobes

1. Why is it crucial to isolate and identify anaerobic bacteria?
2. Anaerobic infections are usually a mixture of what variety of bacteria?

3. What media are used for optimal recovery of anaerobes?
4. Describe Level I identification.
5. Describe Level II identification.
6. Describe Level III identification.
7. Where can you find information on anaerobic antimicrobial procedures?

220. Anaerobic gram-negative cocci and bacilli

1. What are the three human isolates of the *Veillonella* species?
2. Define *Veillonella* species microscopic cellular morphology and colony morphology.
3. Which group of anaerobic bacteria is the most commonly recovered anaerobes in clinical specimens and is more resistant to antimicrobial drugs than the other anaerobes?
4. What organisms in the *B. fragilis* group have the greatest clinical significance?
5. What is the agar used for preliminary grouping of *B. fragilis* group?
6. Describe the colonies of *F. nucleatum*.
7. How do you describe the colonies of *F. varium*?
8. What makes *Mobiluncus* species different from the other gram-negative anaerobic bacilli?

9. Describe colony morphology of *Porphyromonas*.

10. What are the three categories of *Prevotella*?

4-2. Anaerobic Gram-Positive Cocci and Bacilli

The anaerobic gram-positive cocci make up about 30% of the anaerobes isolated from anaerobic infections in the clinical laboratory. These organisms are part of the normal flora in human oral, genital, and gastrointestinal tracts. The anaerobic gram-positive bacilli are also indigenous flora of the oral cavity, genitourinary tract, gastrointestinal tract, and skin.

221. Anaerobic gram-positive cocci

The group of anaerobic gram-positive cocci found in humans consists of the genera *Coprococcus*, *Peptococcus*, *Peptostreptococcus*, *Ruminococcus*, and certain strains or species of *Staphylococcus*, *Streptococcus*, and *Gemella*. *Coprococcus* and *Ruminococcus* are part of the normal flora in the human bowels, are rarely isolated and, therefore, will not be discussed further. The obligate anaerobic staphylococci and streptococci are *Staphylococcus saccharolyticus* (formerly *Peptococcus saccharolyticus*), *Streptococcus hansenii*, *Streptococcus phemorphus*, and *Streptococcus parvulus*. They are rare clinical isolates and the general characteristics were discussed in unit 1.

Peptococcus species

The genus *Peptococcus* contains only one species—*P. niger*.

General characteristics

P. niger is an anaerobic gram-positive cocci that occurs in pairs, tetrads, irregular masses or chains. The cellular morphology of *Peptococcus niger* is identical to *Peptostreptococcus* species.

Clinical significance

P. niger can be recovered from the vaginas of 20 to 30% of pregnant women. It has also been associated with endometrial infections although it is rarely encountered in the clinical laboratory.

Laboratory identification

After the initial incubation on the primary media, the colonies of *P. niger* are circular, convex, smooth in appearance, and black to olive green in color. This pigment fades quickly once exposed to oxygen. The pigment may not be visible to the naked eye but is clearly seen under a dissecting microscope or stereoscope. A mustard-yellow pigment may be produced in cultures over a week old or from subculturing. *P. niger* is not in the identification database or analytical profile index of the commercial systems RapID-ANA II or AN-IDENT. The colony morphology of *P. niger* is what distinguishes it from some of the *Peptostreptococcus* species. Otherwise, molecular biology techniques are the only definitive methods for identification.

Peptostreptococcus species

P. anaerobius, *P. asaccharolyticus*, *P. magnus*, *P. micros*, *P. prevotii*, *P. tetradius*, *P. productus*, *P. lactolyticus*, *P. vaginalis*, *P. lacrimalis*, and *P. hydrogenalis* are in the genus *Peptostreptococcus*. The first three species are the most common isolates in this genus.

General characteristics

The cellular morphology is the same as *P. niger*. Some species of *Peptostreptococcus* may resemble staphylococcal cells on a Gram-stain. Consider *P. tetradius*, *P. magnus*, or *P. prevotii* if gram-positive cocci are seen on the direct specimen Gram-stain, but, there are no staphylococci recovered on the aerobic culture.

Clinical significance

The species of *Peptostreptococcus* are found in head and neck infections, pulmonary disease, intra-abdominal infections, and most often in obstetric and gynecologic infections. Bacteremia caused by these organisms usually follows obstetric and gynecologic infections.

Laboratory identification

The anaerobic gram-positive cocci grow on most nonselective media with best results on blood or Brucella agar supplemented with vitamin K and hemin. Satisfactory broth media include chopped meat-glucose, thioglycolate supplemented with vitamin K, hemin, and 5% rabbit serum, and peptone-yeast extract-glucose. Colony morphology for the *Peptostreptococcus* species varies in size and elevation. Most are small, dull, smooth (rough variants may occur), and convex. *P. asaccharolyticus* colonies may have a yellow pigment, and *P. anaerobius* may produce a sweet odor.

222. Anaerobic gram-positive bacilli

Anaerobic gram-positive bacilli are divided into two groups: (1) spore-forming bacilli and (2) nonspore-forming bacilli. The spore-forming bacilli are *Clostridium* and *Desulfotomaculum*; however, *Clostridium* is the only genus of human clinical significance. The nonspore-forming bacilli are *Acetobacterium*, *Actinomyces*, *Arcanobacterium*, *Bifidobacterium*, *Eubacterium*, *Lachnospira*, *Lactobacillus*, *Methanobacterium*, *Rothia*, and *Propionibacterium*. We will only discuss those with the greatest clinical significance. Twenty percent of *Lactobacillus* species are obligate anaerobes. See unit 1 for *Lactobacillus* genera characteristics.

***Clostridium* species**

The genus *Clostridium* consists of over 100 described species. Most species are considered harmless saprophytes found throughout the world in soil, freshwater, sludge, and marine sediments. Other species are found only in the intestinal tracts of humans and animals. Clostridial infections can be exogenous (caused by outside sources) or endogenous (developing or originating from within) in origin. Clostridial diseases generally fall into one of three categories:

1. Noninvasive disease in which toxins are responsible for the symptoms.
2. Invasive disease in which tissue destruction occurs.
3. Purulent disease in which a closed space, such as the peritoneal cavity, is infected with multiple organisms.

General characteristics

Members of the genus *Clostridium* are large, gram-positive rods of variable length and breadth, ranging from long filamentous forms to short, plump bacilli. The bacilli may have round, blunt, or tapered ends and may occur singly, in pairs, or in chains. In an optimal environment, most species produce a single, round, or oval spore that may be located centrally, subterminally, or terminally within the vegetative cell. The spores appear as swollen bodies since they are generally wider than the diameter of the rods in which they develop. The shape and position of the spore, as well as the fact that it does or does not distort the vegetative cell, are characteristics that help in species identification.

Spores of clostridia are not stained by the routine aniline dyes. In methylene blue and Gram-stained smears, spores are seen as unstained areas against the darkly stained cytoplasm or as free hyaline bodies (fig. 4-1). The relatively impervious spores may be effectively stained by the Wirtz-Conklin

technique. Stained smears of culture materials usually reveal spores, except that *C. perfringens* fails to sporulate on most laboratory media. Gram-stained smears of fresh clinical specimens show large gram-positive rods with or without spores. Unfortunately, however, the bacilli of gas gangrene cannot be distinguished morphologically from the saprophytic putrefactive anaerobes that may be associated with gangrene. Frequently, specimens from gangrenous lesions are contaminated with gram-negative rods and gram-positive cocci. For these reasons, direct smears are only of presumptive value, and gangrenous lesions must be cultured. Direct examination of suspected foods from outbreaks of botulism is of little or no value since very few organisms are ordinarily present in such specimens.

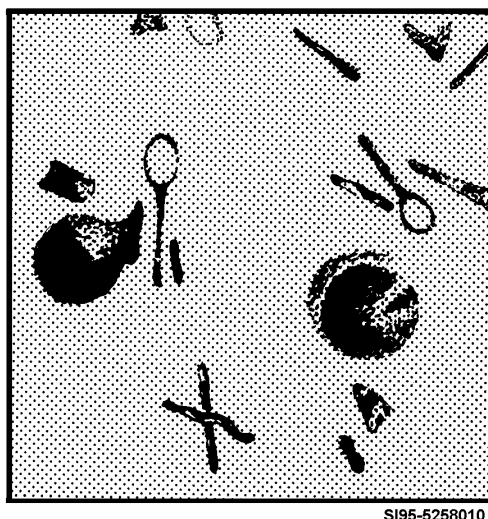


Figure 4-1. Microscopic cellular morphology of *Clostridium tetani*.

The majority of clostridia is obligate anaerobes and is motile, except *C. perfringens*. Growth may be obtained over a wide range of temperature, but 37°C is optimum for pathogenic species. Although the nutritive requirements among the clostridia vary, the organisms can be isolated from specimens using blood agar or thioglycolate medium containing 0.6% glucose. The clostridia grow well in the depths of thioglycolate medium, which provides the necessary anaerobic conditions without the necessity of providing an airtight seal for the mouth of the culture tube.

Clinical significance

Clostridia are widely distributed in soil, dust, and water and are common inhabitants of the intestinal tract of animals and humans. The pathogenic species produce soluble toxins, some of which are extremely potent. True exotoxins are produced by the pathogenic clostridia, several species of which are of medical significance. The following table outlines the four major groups of pathogenic clostridia.

Four major groups of pathogenic clostridia	
Major group	Description
Histotoxic clostridia	Cause of a variety of tissue infections subsequent to wounds or other traumatic injury.
<i>Clostridium tetani</i>	Causative agent of tetanus or "lockjaw", which produces disease through a potent exotoxin.
<i>Clostridium botulinum</i>	Cause of botulism, a food poisoning caused by the ingestion of a powerful exotoxin previously formed by the organisms in contaminated food.
<i>Clostridium difficile</i>	Causative agent of antibiotic-associated diarrhea and pseudomembranous colitis, usually due to chemotherapeutic suppression of the normal flora of the bowel.

Histotoxic clostridia

These organisms cause a severe infection of the muscle called clostridial myonecrosis. The frequently used and older synonyms for this infection are gas gangrene and clostridial myositis. The term *gas gangrene*, however, is misleading since the presence of gas in the infected tissues may be a late or variable manifestation of the disease, and the clostridial myositis suggests muscle inflammation rather than the actual pathological condition of necrosis. The most important histotoxic clostridia are *Clostridia perfringens*, *C. novyi*, and *C. septicum*. Two other organisms of lesser importance are also capable of producing clostridial myonecrosis—*C. histolyticum* and *C. bifermentans*. *C. sporogenes*, *C. tertium*, *C. sordelli*, and *C. fallax* have been isolated from patients with myonecrosis, but their significance is unknown. All of these histotoxic clostridia produce a variety of toxins of different potencies. For each species, toxins are designated by Greek letters in order of importance or discovery. Thus, the alpha toxins of unlike species are not identical. These histotoxic clostridia are not highly invasive pathogens, but they play an opportunistic role that requires a special set of conditions within tissue in order to initiate infection. *C. perfringens* is cultured from 70 to 90% of myonecrosis specimens. *C. perfringens* has five toxin types designated as A through E. Type A produces the classic gas gangrene in humans. *C. novyi* is divided into four toxinogenic types: A, B, C, and D. Type A, again, is implicated in gas gangrene of humans. *C. perfringens* is the most frequent cause of the disease, either singly or in combination with other anaerobes.

Clostridial myonecrosis develops as a complication of severe traumatic injuries, especially lacerated wounds accompanied by a compound fracture. In these injuries the blood circulation to a local tissue area is often impaired or destroyed. The resulting necrotic tissue, void of oxygen and rich in microbial nutrients, affords an ideal anaerobic environment in which clostridial spores germinate and multiply. The organisms actively metabolize tissue carbohydrates to acid and gas. The gangrenous process extends to other tissues primarily because of exotoxins excreted by the pathogenic clostridia. In addition, bacterial enzymes may exhibit hemolytic, necrotizing, and lethal effects on tissue cells. Clostridial myonecrosis consists usually of a mixed infection of toxigenic (toxin-producing) and proteolytic (protein destroying) clostridia and other gram-positive or gram-negative anaerobic organisms. The accessory organisms may contribute to the severity of the infection. The antitoxin employed in treating gangrene usually consists of pooled, concentrated immune globulins against toxins of *C. perfringens*, *C. novyi*, and *C. septicum*.

Clostridium perfringens food poisoning

C. perfringens is the third most common (behind *Salmonella* species and *S. aureus*) cause of food poisoning in the United States and elsewhere. Most of the outbreaks are due to *C. perfringens* type A strains. Symptoms usually occur seven to 15 hours after consumption of improperly cooked meat or meat products and include cramping and abdominal pain with diarrhea. Only 25% of the patients have a fever, nausea, or vomiting. The stool can be foamy and foul smelling. The illness is usually mild and lasts two to three days. The diagnosis is confirmed by culturing the implicated food and by quantitative spore counts from stool specimens. These procedures are usually performed in a reference laboratory.

Clostridium tetani

C. tetani is the etiologic agent of tetanus (lockjaw). This disease follows the introduction of tetanus spores (from soil or feces) into puncture wounds, burns, surgical sutures, or traumatic injuries. If anaerobic conditions prevail in the wound, the spores germinate to form the vegetative bacilli that produce toxin whose action precipitates the disease. The rapidly absorbed toxin acts on the tissue of the spinal cord and peripheral motor nerve endings. The toxemia is evidenced by muscle spasms of the jaw muscles (lockjaw). The nerves of other voluntary muscles are progressively involved causing toxic spasms and convulsions. Tetanus is largely a disease of nonimmunized people, since the vaccine has been available for many years.

Clostridium botulinum

C. botulinum is responsible for an often-fatal type of food poisoning called botulism. Outbreaks occur following the ingestion of food in which the organism has produced a highly potent exotoxin. In the anaerobic environment of food, the spores germinate to form vegetative cells that, in turn, produce the toxin. The spores will withstand a temperature of 100°C for at least three to five hours. Toxin-containing foods may appear spoiled and rancid, and cans may be swollen due to gas formation by the organism. In some cases, however, the food appears entirely innocuous. Heating the food at 100°C for 10 minutes destroys the toxin itself. Outbreaks of botulism are rare in the United States because of rigid quality control in commercial canning and food preservation. Nevertheless, cases of botulism still occur from consumption of home-prepared foods containing *C. botulinum* exotoxin. Infant botulism is the most frequently encountered form of botulism, and the only clearly defined risk factors are breast-feeding and exposure to honey. Honey is a potential source of spores.

There are seven distinct serological types of *C. botulinum*. They are designated A through G on the basis of the type of toxin produced. Humans are susceptible to types A, B, E, and F, birds primarily to A and C, rodents to C and D, and mink to A, B, C, and E. Type G organisms (now called *C. argentinense*) have been isolated from the soil in Argentina and from autopsy material from five individuals who died suddenly. Type A toxin is one of the most poisonous substances known. Typically, within 18 to 36 hours (can range from eight hours to eight days) following the consumption of toxic food, neurotoxic symptoms occur, such as visual disturbances, inability to swallow, and speech difficulty. Progressive paralysis is exhibited, and the disease can terminate fatally from respiratory failure and cardiac arrest.

Clostridium difficile

This organism is the causative agent of pseudomembranous colitis (PMC) and antibiotic-associated diarrhea. PMC usually occurs in association with antibiotic and chemotherapeutic treatment. PMC is characterized by the development of a raised, adherent, whitish-yellow or greenish-yellow pseudomembrane. This membrane is composed of epithelial cells, mucin, granulocytes and fibrin. Complications of PMC include electrolyte imbalance, dehydration, and perforation of the colon. PMC has been linked to treatment with just about every antimicrobial agent and several anticancer chemotherapeutic agents, but the most frequently implicated antibiotics are ampicillin, clindamycin, and the cephalosporins.

Pathogenicity of this organism is expressed when the normal flora of the bowel is suppressed and overgrown by *C. difficile*. This organism is routinely found in soil, water, and the intestinal tract of various lower animals. Studies suggest that 2 to 3% of normal adult population harbors *C. difficile*, and up to 30% of hospitalized patients may be colonized. Such a high percentage of hospitalized patients being colonized by this agent, and the fact that its spores may remain viable for long periods of time in the hospital environment, place it high on the list of agents likely to cause nosocomial infections. Mounting evidence suggests that this organism is being transmitted to patients on the contaminated hands of hospital personnel. Most strains of *C. difficile* isolated from patients are toxigenic. Normally, two toxins designated A (enterotoxin) and B (cytotoxin). It is still unclear as to how these toxins produce disease in the bowel of humans.

The laboratory identification of this organism may be accomplished by culturing patient samples on medium containing cycloserine and cefoxitin, but this is sometimes difficult and always time-consuming. The best way to diagnose PMC is by demonstrating the presence of toxin B in patient stool samples by a tissue culture cytotoxicity assay, which is considered the standard, most definitive method. This procedure demonstrates the presence of toxins in a cell-free extract of the patient's stool on tissue culture cells and then shows that the effects are neutralized by *C. sordelli* or specific *C. difficile* antitoxins.

Laboratory identification

After overnight incubation on blood agar, typical colonies of nearly all *Clostridium* species produce distinct, complete hemolysis. *Clostridium perfringens* produce a distinct characteristic pattern of hemolysis on blood agar plates, precipitation in serum on egg-yolk media, and stormy fermentation in milk media. For example, on blood agar the colonies of most strains demonstrate a characteristic target or double zone of hemolysis (fig. 4-2). This results from a narrow zone of complete hemolysis due to the theta toxin and a much wider zone of incomplete hemolysis due to the alpha toxin. This is seen as a definite, narrow, 1 to 2 mm zone immediately around the colony, surrounded by a wider 4 to 5 mm zone of partial hemolysis. If growth of large gram-positive bacilli is obtained on blood agar in an anaerobic culture, a member of the genus *Clostridium* should be strongly suspected.

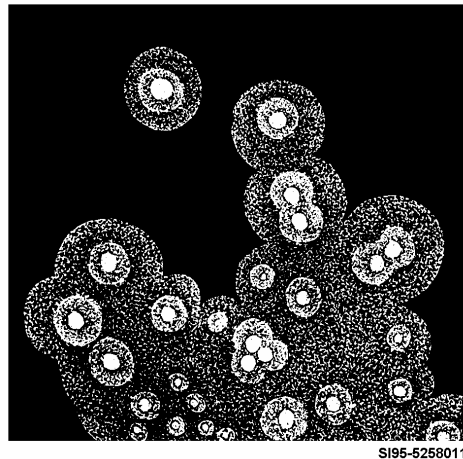


Figure 4-2. Double zone hemolysis exhibited by *Clostridium perfringens*.

In addition to clostridia, gangrenous infections may contain enteric bacteria or *Pseudomonas* species. Under such circumstances, the primary anaerobic blood agar plate may be overgrown with these gram-negative organisms, thereby making the isolation of *Clostridium* species on blood agar difficult or impossible. If overgrowth occurs, incubate the primary thioglycolate medium (inoculated at the time the plate was streaked) for 48 to 72 hours. During this extended period of incubation, the gram-negative bacilli will tend to die out, allowing isolation of clostridia in subculture.

When spores of a possible *Clostridium* species are seen in thioglycolate medium containing a mixed culture, heat resistance of the spore form may be used to an advantage in obtaining isolation. An inoculum consisting of a sample of the mixed bacterial culture is placed in a fresh tube of thioglycolate medium and heated at 80°C for 15 to 30 minutes. This heating will destroy all vegetative cells, but not the spores. The heated medium is then incubated for 24 to 48 hours, and the germinating spores will give rise to a population of clostridia, free of nonspore-forming species. Rarely will *Clostridium perfringens* be isolated by this method because the bacterium generally fails to sporulate in culture. Thus, this method is not recommended for *C. perfringens*. Cooked-meat medium may also be used for cultivation of the spore-forming anaerobes. Tubes of this medium require a petrolatum seal if obligate anaerobes are being cultured. Proteolytic activity is recognized by digestion of the solid material of the medium that is quite readily detected.

The Nagler reaction is recommended for rapid identification of *C. perfringens*. The medium in this reaction, 10% egg yolk in blood agar base, is placed in a Petri dish and one-half the surface is smeared with a few drops of *C. perfringens* type A antitoxin (anti-lecithinase). The culture is then streaked in a single line diagonally across the plate. A dense opalescence (precipitate) will form on the portion of the plate without the type A antitoxin. This reaction is caused by alpha toxin (a lecithinase C). On the other side of the plate the precipitation reaction does not occur since the

antitoxin neutralizes the alpha toxin produced by the organism. After 24 to 48 hours of incubation at 35°C, inhibition of the precipitation on the half of the plate containing the antitoxin constitutes a positive Nagler reaction. *C. bifermentans*, *C. sordelli*, as well as *C. perfringens* will give you this reaction since the antitoxin is not species specific for *C. perfringens*. However, these other species of *Clostridium* are not generally isolated in the clinical laboratory, so most clostridia isolated in the clinical laboratory that give a positive Nagler reaction are *C. perfringens*. Nonetheless, perform additional testing to make a definitive identification of the isolates you encounter.

The reverse CAMP test is one such test. It is more specific for *C. perfringens* than the Nagler test and is based on the enhanced production of hemolysis when colonies of group B streptococci and *C. perfringens* are grown in close proximity to one another. A known culture of group B streptococci is streaked at a right angle to a suspected *C. perfringens* colony on a blood agar, leaving a gap of about 5 mm at their closest point. A positive result is reported if bullet or crescent-shaped areas of enhanced hemolysis are seen in this 5 mm gap.

Bacteria may be tentatively identified as members of the genus *Clostridium* on the basis of strict anaerobiosis, and characteristic microscopic and colony morphology. Final identification is dependent upon the results of biochemical studies and the demonstration of exotoxin production.

***Actinomyces* species**

The human isolated species of *Actinomyces* are: *A. israelii* (the most frequently isolated species), *A. naeslundii*, *A. odontolyticus*, *A. viscosus*, *A. meyeri*, *A. pyogenes*, *A. neuii*, *A. hyovaginalis*, *A. georgiae*, and *A. gerencseriae*.

General characteristics

Actinomyces are nonspore-forming, gram-positive bacilli that are usually branching and beaded. A tangled mass of long filamentous forms can be seen in a Gram-stain from actinomycetous pus and “sulfur granule” specimens. They are microaerobic (aerotolerant) but grow better anaerobically. *A. meyeri* is the only obligate anaerobe.

Clinical significance

Actinomyces are normal flora in the female genital tract and oral cavity of humans and animals. They produce infection primarily as endogenous opportunistic pathogens and are often found in mixed infections with anaerobic cocci and anaerobic gram-negative bacilli. *Actinomyces* has been isolated from various sites; for example appendix, liver abscess, scrotal or testicular abscess, vagina, periapical abscess (surrounding the apex of a tooth), and periodontal pockets. They play a significant role in dental caries and periodontal disease.

Laboratory identification

Most species of *Actinomyces* have characteristic colony morphology. On blood agar plates they produce a white, opaque, pulvinate colony that may resemble a molar tooth. Others produce a bread-crumble-like raspberry, or smooth, flat to convex, gray-white translucent colonies with entire margins. Growth in broth media may appear crumbly or granular. *Actinomyces* generally grow slowly and should be incubated for a minimum of five to seven days.

***Bifidobacterium* species**

Bifidobacterium species, which are considered rare human isolates, are *B. dentium*, *B. bifidum*, *B. infantis*, *B. globosum*, *B. catenulatum*, *B. adolescentis*, *B. longum*, and *B. breve*. *B. dentium* appears to be the only species with pathogenic potential.

General characteristics

Bifidobacterium species are gram-positive, pleomorphic bacilli. They may also appear clubbed or branching (but thicker than *Actinomyces*) with bifurcated or forked ends.

Clinical significance

Bifidobacterium species are part of the normal flora in the female cervix and the human gastrointestinal tract, and may help in preventing overgrowth of the enteric pathogens in the intestines. They have been found in sewage and intestines of animals. They are associated with dental caries and pulmonary infections.

Laboratory identification

Most bifidobacteria are anaerobic, but some will grow aerobically in the presence of CO₂. They are acidophilic and grow best on a low pH agar, for example, tomato juice agar. They produce white, convex, shiny colonies with an irregular shape on blood agar.

***Eubacterium* species**

The genus *Eubacterium* consists of over 50 species, but the human isolates are *E. lentum* (most common), *E. nodatum*, *E. timidum*, and *E. brachy*.

General characteristics

Eubacterium species vary greatly on Gram-stain. They are gram-positive, straight, and uniform or curved rods, but may also appear pleomorphic. The rods or coccobacilli can occur in pairs or short chains.

Clinical significance

Eubacterium species are normal inhabitants of soil, various plants, and intestines and oral cavities of humans and other animals. They are also found in specimens from soft tissue infections. *E. lentum* is usually isolated from abscesses and wounds, but rarely from blood cultures. *E. nodatum*, *E. timidum*, and *E. brachy* are associated with periodontal disease, and possibly with head and neck, thorax, bone, skin, and pelvis infections.

Laboratory identification

On blood agar, the colonies are usually not distinctive. They can be circular with an entire margin, raised to convex, and may be transparent to translucent. *E. nodatum* may be raspberry-shaped or molar-tooth-like and be confused with *Actinomyces israelii*.

***Propionibacterium* species**

Propionibacterium species include: *P. acnes*, *P. avidum*, *P. propionicus*, and *P. granulosum*.

General characteristics

Propionibacterium species are sometimes known as the “anaerobic diphtheroids”. The gram-positive bacilli are pleomorphic and often club shaped, and occur as short, branched, or beaded rods.

Clinical significance

Propionibacterium species are predominantly normal skin flora but have been isolated from the upper respiratory, gastrointestinal, and urogenital tracts. Because of their presence on skin, they are often considered contaminants of blood and body fluid specimens collected through skin puncture. These organisms can appear as primary pathogens and should not be instantly dismissed as contaminants. *P. acnes* is associated with acne vulgaris, corneal ulcers, surgical procedures, prosthetic devices, heart valves, and ventricular shunts. These infections can lead to endocarditis, bacteremia, meningitis, and osteomyelitis. *P. propionicus* can cause lacrimal or tear-duct infections and actinomycosis (enlargement of lymph nodes or “lumpy jaw”). Because of its similarity to *Actinomyces* species, it was formerly known as *A. propionica*.

Laboratory identification

Some species of *Propionibacterium* are aerotolerant, but they grow best anaerobically. Young colonies on blood agar are small and white to gray-white; however, older colonies may be yellow. *P. avidum* is beta-hemolytic. A presumptive identification of *P. acnes* can be made if an anaerobic diphtheroid is catalase positive and indole positive.

Self-Test Questions

After you complete these questions, you may check your answers at the end of the unit.

221. Anaerobic gram-positive cocci

1. What is the only species in the genus *Peptococcus*?
2. Describe the genus *Peptococcus*' colony morphology.
3. What are the three most common isolates of *Peptostreptococcus* species?
4. If gram-positive cocci are seen on the direct specimen Gram-stain, but there is no staphylococci recovered on the aerobic culture, what *Peptostreptococcus* species should be considered?
5. What media gives the best growth results for anaerobic gram-positive cocci?
6. Describe the colony morphology of *Peptostreptococcus* species?

222. Anaerobic gram-positive bacilli

1. Describe the microscopic morphology of members of the genus *Clostridium*?
2. How do the spores of clostridia appear in methylene blue and Gram-stained preparations?
3. What are the four major groups of pathogenic clostridia?
4. What type of disease is caused by *C. tetani*?

5. What is botulism and what causes it?
6. What disease is caused by the histotoxic clostridia?
7. Why is the term *gas gangrene* considered misleading?
8. What three organisms are considered the most important histotoxic clostridia?
9. Since all the other histotoxic clostridia produce a variety of toxins, how are they designated?
10. What type of role do the histotoxic clostridia play within the tissue in order to initiate infection?
11. How many toxin types does *C. perfringens* have? What are they?
12. What organism is the most frequent cause of clostridial myonecrosis?
13. What species of *Clostridium* is most frequently isolated from clinical material?
14. How does clostridial myonecrosis develop?
15. Most of the food poisoning outbreaks are due to what strain of *C. perfringens*?
16. What are some ways in which *C. tetani* enters the body?
17. Rapidly absorbed toxins of *C. tetani* act upon what tissue?

18. In the anaerobic environment of a foodstuff, the spores of *C. botulinum* germinate to produce what substance?
19. Humans are susceptible to which of the serological types of toxins produced by *C. botulinum*?
20. What type toxin is one of the most poisonous substances known?
21. What are some initial symptoms that follow 18 to 36 hours after the consumption of food contaminated with the toxins of *C. botulinum*?
22. What organism causes pseudomembranous colitis and antibiotic-associated diarrhea?
23. What antibiotics are frequently implicated in the development of PMC?
24. What must usually occur before *C. difficile* expresses its pathogenicity?
25. What toxins are produced by most strains of *C. difficile*?
26. What is the best way of diagnosing PMC?
27. *C. perfringens* produces a characteristic pattern on what three media?
28. On blood agar, what characteristic type of hemolysis is demonstrated by *C. perfringens*?
29. What causes the characteristic type of hemolysis produced on blood agar by *C. perfringens*?
30. If overgrowth occurs in the primary isolation of *Clostridium* species, what should be done to eliminate this condition?

31. When a sample of the mixed bacterial culture is placed in a fresh tube of thioglycolate medium and heated at 80°C for 15 to 30 minutes, the heating will destroy all vegetative cells, but not spores. Why will *Clostridium perfringens* rarely be isolated by this method?
32. In the cooked-meat medium, how is proteolytic activity recognized?
33. The Nagler reaction is recommended for rapid identification of what organism?
34. In the Nagler reaction, what inhibits the reaction caused by the alpha toxin (a lecithinase C)?
35. Members of the genus *Clostridium* may be tentatively identified on the basis of what three characteristics?
36. *Actinomyces* plays a significant role in what type of infections?
37. How do you describe the colony morphology of *Actinomyces* species?
38. Describe the cellular morphology of *Bifidobacterium* species.
39. What is the colony morphology of *E. nodatum*?
40. *Propionibacterium* species are predominantly isolated from what site?

Answers to Self-Test Questions

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1. These infections are associated with high morbidity and mortality; also, the antibiotic therapy of the anaerobic infection varies with the bacterial species involved.
2. Mixture of aerobic, facultatively anaerobic, and other anaerobic bacteria.
3. Enriched and selective.
4. Presumptive identification can be made on atmospheric requirements (aerotolerance testing), Gram-stain results, and colony morphology.

5. Preliminary grouping includes Gram-stain reaction; cellular and colony morphology; spot indole and catalase test (using 15% hydrogen peroxide instead of 3% hydrogen peroxide); nitrate reduction and antibiotic susceptibility disk test; and simple procedures for glutamic acid decarboxylase production and L-analyl-L-analylaminopeptidase production. Additional tests include colony hemolysis, pigment, or pitting of agar; Long wavelength (366 nm) fluorescence using a UV light; 20% bile-resistance test; F/F growth-stimulation test; urease, motility, and SPS susceptibility test; Ethanol spore test; lecithinase test, lipase reaction, and production of fatty acids from peptone-yeast-glucose broth; Nagler and reverse CAMP test; and arginine growth-stimulation test.
6. Species identification encompasses biochemical testing using convectional tube test or microtubule methods (example: API); enzyme substrate test systems; GLC for determining metabolic products; and DNA probes.
7. *Methods for Antimicrobial Susceptibility Testing of Anaerobic Bacteria -- Second Edition*, NCCLS Document M11-A2, 1991, published by the Clinical and Laboratory Standards Institute, formally known as the National Committee for Clinical Laboratory Standards.

220

1. *V. parvula*, *V. atypica*, and *V. dispar*.
2. *Veillonella* species are tiny, gram-negative cocci that appear in pairs and packets. Colonies are small, convex, and translucent to transparent, with an entire margin on blood agar and may show red fluorescence under long-wave ultraviolet light.
3. The *B. fragilis* group of organisms.
4. *B. fragilis* and *B. thetaiotaomicron*.
5. BBE agar.
6. *Fusobacterium* species are slightly hemolytic and produce a green sheen on the blood agar. The colonies of *F. nucleatum* look like breadcrumbs, or appear convex, glistening, and grainy on the inside.
7. They are translucent and opaque in the center, have an undulate margin, and resemble fried eggs.
8. Although they stain gram-negative, they possess a multilayered gram-positive-type cell wall and may be classified as gram-positive bacilli.
9. On blood agar the colonies of *Porphyromonas* are mucoid, dark brown to black, and smooth with an entire margin. Before the pigment is produced, they fluoresce brick red under ultraviolet light, except for *P. gingivalis*.
10. Pigmented, nonpigmented, and other.

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1. *P. niger*.
2. Circular, convex, smooth in appearance, and black to olive green in color.
3. (1) *P. anaerobius*.
(2) *P. asaccharolyticus*.
(3) *P. magnus*.
4. *P. tetradius*, *P. magnus*, or *P. prevotii*.
5. Blood or Brucella agar supplemented with vitamin K and hemin.
6. It varies in size and elevation. Most are small, dull, smooth (rough variants may occur), and convex. *P. asaccharolyticus* colonies may have a yellow pigment and *P. anaerobius* may produce a sweet odor.

222

1. Large, gram-positive rods of variable length and breadth, ranging from long filamentous forms to short, plump bacilli. The bacilli may have round, blunt, or tapered ends and may occur singly, in pairs, or in chains. In an optimal environment, most species produce a single round or oval spore, which may be located centrally, subterminally, or terminally within the vegetative cell.
2. As unstained areas against the darkly stained cell cytoplasm or as free hyaline bodies.
3. (1) Histotoxic clostridia.

- (2) *Clostridium tetani*.
- (3) *Clostridium botulinum*.
- (4) *Clostridium difficile*.
- 4. Tetanus or lockjaw.
- 5. Often fatal type of food poisoning caused by ingestion of a powerful exotoxin previously formed by the organism *C. botulinum* in contaminated food.
- 6. Clostridial myonecrosis.
- 7. Because the presence of gas in the infected tissues may be a late or variable manifestation of the disease.
- 8. (1) *Clostridium perfringens*.
(2) *C. novyi*.
(3) *C. septicum*.
- 9. By Greek letters in order of importance or discovery.
- 10. They play an opportunistic role but are not highly invasive.
- 11. Five; A through E.
- 12. *C. perfringens*.
- 13. *C. perfringens*.
- 14. As a complication of severe traumatic injury, especially lacerated wounds, accompanied by a compound fracture. Blood circulation is impaired or destroyed. The necrotic tissues, void of oxygen, provide a rich medium from which clostridial spores germinate and multiply.
- 15. Type A strains.
- 16. Through puncture wounds, burns, surgical sutures, or traumatic injuries.
- 17. Tissue of the spinal cord and peripheral motor nerve endings.
- 18. A highly potent exotoxin.
- 19. A, B, E, and F.
- 20. Type A.
- 21. Neurotoxic symptoms are evidenced by visual disturbances, inability to swallow, and speech difficulty.
- 22. *C. difficile*.
- 23. Ampicillin, clindamycin, and the cephalosporins.
- 24. The suppression of the normal flora of the bowel.
- 25. A (an enterotoxin) and B (a cytotoxin).
- 26. By demonstrating the presence of toxin B by a tissue culture cytotoxicity assay.
- 27. (1) Distinct characteristic pattern of hemolysis on blood agar plates.
(2) Precipitation in serum or egg yolk media.
(3) Stormy fermentation in milk media.
- 28. A target or double zone of hemolysis.
- 29. A narrow zone of complete hemolysis due to the theta toxin and a much wider zone of incomplete hemolysis due to the alpha toxin.
- 30. Incubate the primary thioglycolate medium for 48 to 72 hours. During the extended period of incubation, the gram-negative bacilli will tend to die out, allowing isolation of clostridia in subculture.
- 31. The bacterium generally fails to sporulate in culture.
- 32. By digestion of the solid material of the medium.
- 33. *C. perfringens*.
- 34. The presence of *C. perfringens* antitoxin.
- 35. Strict anaerobiosis, microscopic morphology, and colony morphology.
- 36. Dental caries and periodontal disease.

37. On blood agar plates they produce a white, opaque, pulvinate colony that may resemble a molar tooth. Others produce a bread-crumble-like raspberry or smooth, flat to convex, gray-white translucent colonies with entire margins. Growth in broth media may appear crumbly or granular.
38. They are gram-positive, pleomorphic bacilli. They may also appear clubbed or branching (but thicker than *Actinomyces*) with bifurcated or forked ends.
39. *E. nodatum* may be raspberry-shaped or molar-tooth-like.
40. Skin.

Unit Review Exercises

Note to Student: Consider all choices carefully, select the *best* answer to each question, and *circle* the corresponding letter. When you have completed all unit review exercises, transfer your answers to ECI Form 34, Field Scoring Answer Sheet.

Do not return your answer sheet to ECI.

74. (219) It is crucial to isolate and identify anaerobic bacteria because
 - a. these infections are associated with high morbidity and mortality.
 - b. there are over 70 different genera and 250 described species.
 - c. they are usually a mixture of other bacteria.
 - d. they are difficult to recover.
75. (219) Which is *not* a common site of the human body for anaerobic infections?
 - a. Endocarditis.
 - b. Urinary tract.
 - c. Periodontal infections.
 - d. Necrotizing pneumonia.
76. (219) Anaerobic infections are usually *not* mixed with
 - a. yeast.
 - b. aerobic bacteria.
 - c. other anaerobic bacteria.
 - d. facultatively anaerobic bacteria.
77. (219) "Species identification" is associated with which identification level?
 - a. I.
 - b. II.
 - c. III.
 - d. IV.
78. (220) Which genus is the most frequent anaerobic gram-negative cocci isolated in clinical specimens?
 - a. *Bilophila*.
 - b. *Veillonella*.
 - c. *Megasphaera*.
 - d. *Acidaminococcus*.
79. (220) The two *Bacteroids* which are of the greatest clinical significance are
 - a. *B. caccae* and *B. merdae*.
 - b. *B. ovatus* and *B. stercoris*.
 - c. *B. vulgatus* and *B. distasonis*.
 - d. *B. fragilis* and *B. thetaiotaomicron*.

80. (220) *Prevotella* species produce varying amounts of pigments. Which is *not* a pigment of *Prevotella*?
- Tan.
 - Black.
 - Green.
 - Dark brown.
81. (221) Which is the only species in the genus *Peptococcus*?
- P. niger*.
 - P. micros*.
 - P. anaerobius*.
 - P. asaccharolyticus*.
82. (221) Which is *not* a common isolates of the genus *Peptostreptococcus*?
- P. magnus*.
 - P. anaerobius*.
 - P. hydrogenalis*.
 - P. asaccharolyticus*.
83. (222) Which species of Clostridia is nonmotile?
- Clostridium tetani*.
 - Clostridium septicum*.
 - Clostridium botulinum*.
 - Clostridium perfringens*.
84. (222) The histotoxic clostridia produce
- lockjaw.
 - botulism.
 - myonecrosis.
 - food poisoning.
85. (222) Members of the genus *Clostridium* may be *tentatively* identified on the basis of all of the following *except*
- microscopic morphology.
 - colony morphology.
 - strict anaerobiosis.
 - capsule formation.

Glossary

Terms

Abscess—A localized collection of pus caused by infection buried in tissues, organs, or confined spaces.

Acidophilic—Growing in highly acid media.

Aerobically—Grown in the presence of molecular oxygen.

Aeromonas—A genus of bacteria of the family *Pseudomonadineae*, occurring as small rod-shaped cells and usually found in water.

Aerophilic—Requiring air for proper growth.

Agranulocytosis—A symptom complex characterized by marked decrease in the number of granulocytes and by lesions of the throat and other mucous membranes.

Anaerobically—Growing in the absence of molecular oxygen.

Anaerobiosis—Life only in the absence of molecular oxygen.

Anorectal—Pertaining to the anus and rectum or to the junction region between the two.

Antigenic—Having the properties of an antigen, capable of eliciting an immune response.

Antimicrobial—Killing microorganisms, or suppressing their multiplication or growth.

Antisera—Serum that contains antibodies; it may be obtained from an animal that has been immunized either by injection of antigen into the body.

Arginine—An amino acid produced by the hydrolysis or digestion of proteins.

Ascitic—Effusion and accumulation of serous fluid in the abdominal cavity.

Asporogenous—Not producing spores.

Asymptomatic—Showing or causing no symptoms.

Autolysis—The spontaneous disintegration of tissues or of cells by the action of their own autogenous enzymes, such as occurs after death.

Bacillary—Pertaining to rod like forms.

Bacteriogenic—Bacterial in origin.

Betalysin—A relatively thermostable lysin for gram-positive bacteria.

Biotype or Biovars—A variant strain of a bacterial species, differing in identifiable physiologic characteristics.

Bronchopneumonia—A name given to an inflammation of the lungs which usually begins in the terminal bronchioles. These become clogged with a mucopurulent exudate forming consolidated patches in adjacent lobules.

Capnophilic—Growing best in the presence of carbon dioxide; said of bacteria.

Carbuncles—A necrotizing infection of skin and subcutaneous tissue composed of a cluster of boils.

Catalase—A crystalline enzyme that specifically catalyzes the decomposition of hydrogen peroxide and that is found in practically all cells except certain anaerobic bacteria.

Cellulitis—Inflammation of cellular tissue; especially purulent inflammation of the loose subcutaneous tissue.

Cerebrospinal—Pertaining to the brain and spinal cord.

Cervical—Pertaining to the neck, or to the neck of any organ or structure.

Cervicitis—Inflammation of the cervix uteri; called also trachelitis.

Chemotherapeutic—Pertaining to chemotherapy.

Chemotherapy—The treatment of disease by chemical agents; first applied to use of chemicals that affect the causative organism unfavorably but do not harm the patient.

Cholera—An acute infectious disease caused by *Vibrio cholerae* and characterized by severe diarrhea with extreme fluid and electrolyte depletion, and by vomiting, muscle cramps, and prostration.

Chromatography—A method of chemical analysis in which the solution to be analyzed is poured into a vertical glass tube containing an adsorbent (or stationary phase), the different solutes moving through the stationary phase at different velocities according to their degree of attraction to it, and producing bands of color at different levels of the adsorption column.

Chromogen—Any substance that may give origin to a coloring matter.

Chromogenic—Producing a pigment or coloring matter.

Chromophore—Any chemical group whose presence gives a decided color to a compound and which unites with certain other groups (auxochromes) to form dyes; called also color radical.

Coagulase—An antigenic substance of bacterial origin, produced chiefly by the staphylococci, that may be causally related to thrombus formation.

Coccobacillus—An oval bacterial cell intermediate between the coccus and bacillus forms.

Colony—A collection or group of bacteria in culture derived from the increase of an isolated single organism or group of organisms.

Colorimetric—A chemical procedure in a reagent reacts with a particular chemical constituent to produce a measurable color.

Colostomy—To provide with an opening, or mouth. The surgical creation of an opening between the colon and the surface of the body; also used to refer to the opening, or stoma, so created.

Conjunctiva—The delicate membrane that lines the eyelids and covers the exposed surface of the sclera.

Counterstain—A stain applied to render the effects of another stain more discernible.

Cystine—An amino acid, produced by the digestion or acid hydrolysis of proteins.

Cystitis—Inflammation of the urinary bladder hypersensitivity, characterized by a large number of mononuclear leukocytes and eosinophils in the bladder mucosa and musculature, and in the urinary sediment.

Cystostomy—The formation of an opening into the bladder .

Diplococcus—A spherical bacterium occurring predominantly in pairs as a consequence of incomplete separation following cell division in a single plane.

Dysuria—Painful or difficult urination.

Endocarditis—Inflammation of the endocardium; a disease generally associated with rheumatic fever, and sometimes with other acute febrile diseases.

Endocervical—Pertaining to the interior of the cervix uteri.

Endocervix—The mucous membrane lining the canal of the cervix uteri.

Endogenous—Growing from within.

Endospore—A spore produced in the hyphae or cell, as in a spherule of *Coccidioides immitis*.

Endotoxin—A heat-stable toxin present in the bacterial cell but not in cell-free filtrates of cultures of intact bacteria.

Enterococcus—Any streptococcus of the human intestine.

Enterocolitis Inflammation involving both the small intestine and the colon.

Epididymitis—Inflammation of the epididymis.

Epiglottitis—Inflammation of the epiglottis.

Erysipelas—An acute superficial form of cellulitis involving the dermal lymphatics.

Etiologic—Pertaining to the cause of a disease.

Exogenous—Growing by additions to the outside; developed or originating outside the organism.

Extracellular—Outside a cell or cells

Extrapulmonary—Not connected with the lungs.

Exudate—Material, such as fluid, cells, or cellular debris, which has escaped from blood vessels and has been deposited in tissues or on tissue surfaces, usually as a result of inflammation.

Facultative—Not obligatory; pertaining to or characterized by the ability to adjust to particular circumstances or to assume a particular role.

Filamentous—Composed of long threadlike structures; said of bacterial colonies.

Fistula—An abnormal passage between two internal organs or leading from an internal organ to the surface of the body.

Flagellum—A mobile, whip-like process or stout cilium, especially a coiled filamentous appendage, originating in the cell wall or outer layers of cytoplasm of some rod-shaped bacteria, and serving as an organ of locomotion.

Fluorochrome—A fluorescent compound, as a dye, used to mark protein with a fluorescent label.

Fluoroscopy—Examination by means of a fluoroscope.

Folliculitis—Inflammation of a follicle or follicles, usually in reference to hair follicles.

Furuncles—A painful nodule formed in the skin by inflammation of the corium and subcutaneous tissue, enclosing a central core. Also called a boil.

Gastrostomy—Surgical creation of an artificial opening into the stomach.

Genitourinary—Pertaining to the genital and urinary organs; urogenital; urinosexual.

Genotypic—Pertaining to or expressive of the genotype.

Gingivae—The gums: the mucous membrane, with the supporting fibrous tissue, which overlies the crowns of unerupted teeth and encircles the necks of those that have erupted.

Glomerulonephritis—Inflammation of the capillary loops in the glomeruli of the kidney.

Glomerulus—A tuft or cluster; used in anatomical nomenclature as a general term to designate such a structure.

Halophilic—Requiring a high concentration of salt for optimal growth.

Hemolysin—A substance which liberates hemoglobin from red blood corpuscles by interrupting their structural integrity.

Hemolysis—The liberation of hemoglobin

Ileostomy—Surgical creation of an opening into the ileum, usually by establishing an ileal stoma on the abdominal wall.

Immunodeficiency—A deficiency in immune response, either in that mediated by humoral antibody or in that mediated by immune lymphoid cells.

Immunoglobulins—A protein of animal origin endowed with known antibody activity.

Immunosuppression—The artificial prevention or diminution of the immune response, as by irradiation or by administration of antimetabolites, antilymphocyte serum, or specific antibody.

Indole—A compound, obtained from coal tar and indigo, and produced by the decomposition of tryptophan in the intestines.

Inoculum—The substance used in inoculation.

Intracellular—Situated or occurring within a cell or cells

Lake—To undergo separation of hemoglobin from the erythrocytes, a phenomenon sometimes occurring in blood.

Lavage—The irrigation or washing out of an organ, such as the stomach or bowel.

Lyse—To cause or produce disintegration of a compound, substance, or cell.

Meatus—A general term for an opening or passage; a general term for an opening in the body.

Meningeal—Of or pertaining to the meninges

Meningitis—Inflammation of the meninges.

Meningoencephalitis—Inflammation of the brain and meninges.

Mesophilic—Fond of moderate temperature; said of bacteria which develop best at temperatures between 20 and 45 degrees C.

Metabolite—Any substance produced by metabolism or by a metabolic process.

Metachromatic—Staining differently with the same dye; said of tissues in which different elements take on different colors when a certain dye is applied.

Microaerophilic—Growing best in only a small amount of atmospheric oxygen.

Microbiota—The microscopic living organisms of a region; the combined microflora and microfauna of a region.

Monoclonal—Derived from a single cell.

Mordant—A substance capable of intensifying or deepening the reaction of a specimen to a stain.

Mucoid—Any one of a group of mucus-like conjugated proteins of animal origin.

Mucosa—A mucus membrane

Nasopharynx—The part of the pharynx which lies above the level of the soft palate.

Necrosis—Death of tissue, usually as individual cells, groups of cells, or in small localized areas.

Necrotizing—Causing necrosis.

Nephropathy—Disease of the kidney.

Nephrostomy—The creation of a permanent fistula leading directly into the pelvis of the kidney.

Neutropenia—A decrease in the number of neutrophilic leukocytes in the blood.

Neutrophil—A granular leukocyte having a nucleus with three to five lobes connected by slender threads of chromatin, and cytoplasm containing fine inconspicuous granules; called also polymorphonuclear, polynuclear, or neutrophilic leukocytes.

Nosocomial—Pertaining to or originating in a hospital, as nosocomial disease.

Nuchal—Pertaining to the nucha, or back of the neck.

Ophthalmia—A severe inflammation of the eye or the conjunctiva, or deeper structures of the eye.

Oropharynx—That division of the pharynx which lies between the soft palate and the upper edge of the epiglottis.

Osteomyelitis—Inflammation of bone caused by a pyogenic organism.

Otitis media—Inflammation of middle ear.

Oxidase—Any of a class of (metalloprotein) enzymes that catalyze the reduction of molecular oxygen independently of hydrogen peroxide.

Papule—A small circumscribed, superficial, solid elevation of the skin.

Paracentesis—Surgical puncture of cavity for the aspiration of fluid.

Pericardium—The fibroserous sac that surrounds the heart and the roots of the great vessels.

Peritonitis—Inflammation of the pericardium.

Pertussis—Whooping cough.

Pharyngitis—Inflammation of the pharynx.

Pleomorphic—Occurring in various distinct forms.

Pneumonia—Inflammation of the lungs with consolidation.

Polymicrobial—Characterized by the presence of several species of microorganisms.

Polymorphonuclear—Having cells of many forms.

Polysaccharide—A carbohydrate which on hydrolysis yields more than 10 monosaccharides.

Prostate—A gland in the male which surrounds the neck of the bladder and the urethra.

Prosthesis—An artificial substitute for a missing body part.

Psychophilic—Fond of the cold.

Purulent—Consisting of or containing pus; associated with the formation of or caused by pus.

Pyocyanin—A blue-green antibiotic pigment derived from alpha-hydroxyphenazine by methylation.

Pyogenous—Caused by pus.

Pyuria—The presence of pus in the urine.

Rehydration—The restoration of water or of fluid content to a body or substance which has become dehydrated.

Salmonellosis —Infection with certain species of the genus *Salmonella*, usually caused by the ingestion of food containing the organisms or their products and marked by violent diarrhea attended by cramps and tenesmus and/or paratyphoid fever.

Saprophytes—Any organism, such as a bacterium, living upon dead or decaying matter.

Septicemia—Systemic disease associated with the presence and persistence of pathogenic microorganisms in the blood.

Serotype—The type of a microorganism as determined by the kinds and combinations of constituent antigens present in the cell.

Serous—Pertaining to or resembling serum.

Subculture—A culture of bacteria derived from another culture.

Subcutaneous—Beneath the skin.

Substrate—A substance upon which an enzyme acts.

Sucrose—A disaccharide, obtained from sugar cane sugar beet, or other sources.

Synovia—A transparent alkaline viscid fluid, resembling the white of an egg secreted by the synovial membrane, and contained in joint cavities, bursae, and tendon sheaths; called also synovial fluid.

Thermophilic—Growing best at or having a fondness for high temperatures

Thrombophlebitis—Inflammation of the veins associated with thrombus formation.

Trachea—The cartilaginous and membranous tube descending from the larynx and branching into the right and left main bronchi.

Tracheostomy—The surgical creation of an opening into the trachea through the neck.

Tryptophan—An amino acid, existing in proteins, from which it is set free by tryptic digestion; essential for optimal growth in infants and for nitrogen equilibrium in human adults.

Turbidimetric—A procedure in which the turbidity of a test solution is measured.

Umbilicate—Shaped like or resembling the umbilicus.

Umbonate—Knob-like; button-like; having a button-like raised center.

Urease—A colorless, crystalline globulin first extracted by Takeuchi from soy bean. It is also found in mucous urine passed during inflammation of the bladder. It is an enzyme elaborated by various microorganisms, and is capable of causing the change of urea into benzoic acid and glycolic acid.

Urethritis—Inflammation of the urethra.

Viable—Capable of living.

Virulence—The degree of pathogenicity of a microorganism as indicated by the severity of the disease produced and its ability to invade the tissues of a host.

Zoonotic—Transmissible from animals to man under natural conditions.

Abbreviations and Acronyms

α	alpha
α'	alpha-prime
β	Beta
γ	Gamma
δ	Delta
AFB	acid fast bacill
AGN	acute glomerulonephritis
ALA	aminolevulinic acid
ATCC	American Type Culture Collection
BBE	<i>Bacteroides</i> bile esculin
BCG	blood cysteine glucose
BCYE	buffered charcoal yeast extract
BG	Bordet-Gengou, brilliant green
BV	bacterial vaginosis
CCDA	charcoal cefoperazone deoxycholate agar
CDC	career development course
CIN	cefsulodin-irgasan-novobiocin
CLSI	Clinical and Laboratory Standards Institute
CNA	Columbia colistin-nalidixic acid
CoNS	coagulase negative <i>Staphylococcus</i>
CRF	coagulase-reacting factor
CSF	cerebrospinal fluid
CSM	charcoal-based selective medium
CTA	cystine trypticase agar

CVA	campylobacter-cefoperazone-vancomycin-amphotericin
DFA	direct fluorescent antibody
DGI	disseminated gonococcal infection
DIC	disseminated intravascular coagulation
DIC	disseminated intravascular coagulation
DNA	deoxyribonucleic acid
EF	edema factor
EHEC	Enterohemorrhagic E. coli
EIEC	Enteroinvasive E. coli
ELISA	enzyme-linked immunosorbent assays
EMB	eosin-methylene blue
EPEC	Enteropathogenic E. coli
ETEC	Enterotoxigenic E. coli
GBS	group B streptococci
GLC	gas-liquid chromatography
GN	gram-negative
GNID	gram-negative intracellular diplococci
GPI	Gram-Positive Identification
HEA	Hektoen enteric agar
IFA	immunofluorescent antibody
KIA	Kligler's iron agar
LAP	leucine aminopeptidase
LF	lethal factor
LIA	lysine iron agar
LPM	lithium chloride-phenylethanol-moxalactam
LT	labile enterotoxins

MAC	MacConkey
MIS	Microbial Identification System
MR	methyl red
MSA	mannitol salt agar
MTM	modified Thayer-Martin
NAD	nicotinamide-adenine-dinucleotide
NYC	New York City
OF	oxidation fermentation test
ONPG	orthonitrophenyl galactosidase
PA	Protective antigen
PEA	phenylethyl alcohol agar
PID	pelvic inflammatory disease
PMC	pseudomembranous colitis
PYR	L-pyrrolidonyl- β -naphthalamide
RF	rheumatic fever
RFT	rapid-fermentation tests
RIA	radioimmunoassay
RL	Regan-Lowe
SBE	subacute bacterial endocarditis
SIM	sulfide-indole-motility
SMAC	MacConkey-sorbitol
SS	<i>Salmonella-Shigella</i>
ST	stable enterotoxins
SXT	trimethoprim-sulfamethoxazole
TCBS	thiosulfate-citrate-bile salts-sucrose
TM	Thayer-Martin

TNase	thermonuclease
TSI	triple sugar iron
TSS	toxic shock syndrome
TSST-1	toxic shock syndrome toxin-1
USDA	United States Department of Agriculture
VP	Voges-Proskauer
WZα	wide zone alpha
XLD	xylose-lysine-desoxycholate

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