LESSON ASSIGNMENT

LESSON 6
Other Pathogenic Gram-Negative Bacilli and Antibiotic Sensitivity Tests.

LESSON ASSIGNMENT
Paragraph 6-1 through 6-23.

LESSON OBJECTIVES
Upon completion of this lesson, you should be able to:

6-1. Select descriptive characteristics of the procedures for identifying oxidative and nonreactive bacteria, with emphasis on Pseudomonas.

6-2. Identify descriptive features of Brucella, Haemophilus Bordetella, Francisella tularensis, Bacteroides fragilis, and Streptobacillus moniliformis and the procedures for identifying the species.

6-3. Identify the purpose and principles of antibiotic sensitivity tests and characteristics of the paper disk method and tube dilution method.

6-4. Given a Kirby-Bauer zone size, the name of an antibiotic, and type of organism, indicate whether the organism is resistant, sensitive, or intermediate in sensitivity.

SUGGESTION
After reading and studying the assignment, complete the exercises at the end of this lesson. These exercises will help you to achieve the lesson objectives.
LESSEN 6
OTHER PATHOGENIC GRAM-NEGATIVE BACILLI
AND ANTIBIOTIC SENSITIVITY TESTS

Section I. **PSEUDOMONAS**

6-1. **INTRODUCTION**

a. General information.

(1) Figure 6-1 provides an identification schema for oxidative and nonreactive gram-negative bacteria.

<table>
<thead>
<tr>
<th>ORGANISM</th>
<th>OXIDASE</th>
<th>OF (Glucose)</th>
<th>NO$_3$ to NO$_2$</th>
<th>MacC</th>
<th>Motility</th>
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</thead>
<tbody>
<tr>
<td>Pseudomonas</td>
<td>+</td>
<td>O</td>
<td>-</td>
<td>+</td>
<td>(+)</td>
</tr>
<tr>
<td>Achromobacter</td>
<td>-</td>
<td>O</td>
<td>+, +</td>
<td>+</td>
<td>(+)</td>
</tr>
<tr>
<td>Herellea</td>
<td>-</td>
<td>O</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Flavobacterium</td>
<td>+, -</td>
<td>O, N</td>
<td>-</td>
<td>+, -</td>
<td>(+)</td>
</tr>
<tr>
<td>Alcaligenes</td>
<td>+</td>
<td>N</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Hima</td>
<td>-</td>
<td>N</td>
<td>-</td>
<td>+, -</td>
<td>-</td>
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<tr>
<td>Moraxella</td>
<td>+</td>
<td>N</td>
<td>-</td>
<td>+, -</td>
<td>-</td>
</tr>
<tr>
<td>Bordetella bronchiseptica</td>
<td>+</td>
<td>N</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

O = Oxidate  
N = Nonreactive  
+ (-) = Positive, rear negative  
MAC = MacConkey's agar (+ = growth, = no growth)

Figure 6-1. Identification schema for oxidative and nonreactive gram-negative bacteria.
(2) Figure 6-2 provides an identification schema for *Pseudomonas* species in particular.

Figure 6-2. Identification schema for Pseudomonas species (TSI): no change)
Table 6-1 indicates the reactions of the different Pseudomonas species to a selected group of tests.

Table 6-1. General characters of diagnostic value for the differentiation of the genus Pseudomonas.

<table>
<thead>
<tr>
<th>Character</th>
<th>P. aeruginosa</th>
<th>P. fluorescens</th>
<th>P. putida</th>
<th>P. cepacia</th>
<th>P. pseudomallei</th>
<th>P. mallei</th>
<th>P. acidovorans</th>
<th>P. testosteroni</th>
<th>P. alcaligenes</th>
<th>P. pseudoalcaligenes</th>
<th>P. stutzeri</th>
<th>P. maltophilia</th>
<th>P. putrefaciens*</th>
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<tr>
<td>Growth at 41°C</td>
<td>+ + + 0</td>
<td>+ + + +</td>
<td>+ + + +</td>
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<td>+ + + +</td>
<td>+ + + +</td>
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<td>Extracellular poly-β-H butyrate dehydrogenase</td>
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<td>+ + + +</td>
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<td>+ + + +</td>
<td>+ + + +</td>
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<td>+ + + +</td>
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<tr>
<td>Oxidase reaction</td>
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<td>-</td>
<td>-</td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>Cleavage mechanism for diphosphopentose</td>
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<td>+ + + +</td>
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<tr>
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<td>Denitrification</td>
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<td>+</td>
<td>+</td>
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<tr>
<td>Arginine deiminase</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Ornithine deaminase</td>
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<td>+</td>
<td>+</td>
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<td>+</td>
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<td>+</td>
</tr>
<tr>
<td>Poly-p-β-butyrate as cellular reserve</td>
<td>+ + + +</td>
<td>+ + + +</td>
<td>+ + + +</td>
<td>+ + + +</td>
<td>+ + + +</td>
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<td>+ + + +</td>
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<td>m = ortho</td>
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</tr>
<tr>
<td>m = variable TSI</td>
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<td>+ + + +</td>
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<tr>
<td>0 = ortho</td>
<td>+ + + +</td>
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<td>m = meta</td>
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</table>
b. Pseudomonads are gram-negative, asporogenous, catalase-producing rods commonly found in soil and water. Indole, methyl red, and Voges-Proskauer tests are negative. Most species are motile, with one polar flagellum or several polar flagella; some are nonmotile and atrichous. Unlike the Enterobacteriaceae, which only ferment carbohydrates, the pseudomonads oxidize carbohydrates and do not obtain energy by fermentative or photosynthetic metabolism.

c. A useful medium called oxidative-fermentative (OF) basal medium has been especially designed to help differentiate between carbohydrate-oxidizing organisms and those that only ferment carbohydrates.

(1) Unlike most fermentation media, basal medium contains about 0.2 percent peptone rather than 1 percent peptone. The higher concentration tends to result in enough alkalinity, as the peptone is attacked, to mask the acidity produced by oxidative organisms. OF basal medium, which contains 1 percent carbohydrate, changes color from blue-green to yellow as acid is produced.

(2) For each isolate, each of two tubes is inoculated by stabbing once with a straight needle, almost to the bottom of the agar. One of the two tubes is layered with sterile mineral oil or melted petroleum.

(3) During incubation, oxidizers produce acidity in the open tube but not the covered tube. Fermentative organisms generally produce acidity in both tubes. Nonreactive organisms fail to produce acidity in either tube. If the organism has grown only along the stab line, it is nonmotile. If it has grown out from the stab line, it is motile.

6-2. PSEUDOMONAS AERUGINOSA

a. Pathogenicity. Pseudomonas aeruginosa may infect surgical wounds, severe burns, and other injuries, where it tends to produce a characteristic blue-green pus. Since it is resistant to antibiotic therapy, it tends to produce dangerous infections at the sites of previous infections eradicated by antibiotics. Infections of the eye, the ear, and the urinary tract are frequently reported. Systemic infections, which may occur in individuals with lowered resistance, tend to be fatal.

b. Identification. P. aeruginosa is most easily identified by its characteristic blue-green pigment, but 4 percent of its strains do not produce this coloration. On agar the colonies are large, flat, with a ground glass appearance, and they tend to spread, especially on nutrient agar or Trypticase soy agar. They have a characteristic grapelike odor. If an organism meets the following criteria, it may be identified as P. aeruginosa:

(1) Polar monotrichous (generally having a single polar flagellum).

(2) Gram-negative, asporogenous, rod-like.

(3) Oxidation of glucose.
(4) Failure to oxidize maltose.
(5) Production of indophenol oxidase.
(6) Reduction of nitrate to gas.
(7) Oxidation of gluconate to ketogluconate.
(8) Growth at 42º C. (Most strains will grow form 25º to 42º C; some will grow from 5º to 10º C.)

6-3. **PSEUDOMONAS PSEUDOMALLEI**

a. **Introduction.** Melioidosis is an infectious disease caused by a gram-negative, motile bacillus, *Pseudomonas pseudomallei*, which is found in soil and water in Southeast Asia. The disease is manifested in a number of ways ranging from an inapparent infection to a fatal septicemia. However, the most common manifestation is an acute pneumonia or pneumonitis. The mode of transmission has not been definitely established; however, there are several possible routes. *Pseudomonas pseudomallei* has been isolated from soil, local fruits and vegetables, well water, and variety of surface waters. Attempts to identify a human or animal reservoir have proven unsuccessful to date. Cultures have been performed on the urine and feces of humans, rats, goats, cattle and chickens. Thus far, on the basis of these cultures, no human or animal reservoir has been identified and it is assumed that the organism leads a saprophytic existence in nature. Likewise, there has been no proof of man-to-man transmission. The disease is frequently associated with traumatic injuries.

b. **Identification.** Early diagnosis of melioidosis should be considered in all cases of febrile disease in persons in an endemic area and in those recently returned from such an area. The only positive laboratory identification of melioidosis is the isolation and identification of *Pseudomonas pseudomallei*. It is isolated from the sputum in the pneumonic form. In other cases blood, urine, feces, spinal fluid, and surgically removed tissue have yielded positive cultures. Culturally the organism grows well in 2 to 3 days on ordinary media such as Trypticase soy agar and blood agar. It is aerobic in its oxygen requirements. Colonies on agar medium appear circular, raised, opaque, creamy, and yellow to brown in color with irregular edges. The colonies tend to become wrinkled after several days (four to five), and this is one of the diagnostic characteristics of the organism. Due to this phenomenon it is important that cultures in all suspected cases be held so that this may be observed. Cultures of the organism give off an earthy (ammoniacal) odor. Gelatin slabs show moderate, crateriform liquefaction. Litmus milk is curdled with slow acid production. The following commonly employed biochemicals are oxidized with the production of acid but no gas: glucose, maltose, lactose, mannitol, and cellobiose. Due to the unfamiliarity of many bacteriologists with the organism, it is sometimes confused with other gram-negative bacilli and has probably been reported as *Escherichia coli*, *Pseudomonas aeruginosa*, or one of the *Klebsiella* or *Enterobacter* species in a number of cases.
Section II. BRUCELLA

6-4. GENERAL TYPES

The genus Brucella includes several species known to infect man. However, man is not the primary host of any of these. Goats are infected by B. melitensis, cattle by B. abortus, swine by B. suis, and dogs by B. canis. Man can be infected with any of these organisms by direct contact or by consumption of milk or milk products. The brucellae exhibit predominantly small coccobacillary forms ranging from 0.4 to 3.0 microns in length and 0.4 to 0.8 microns in breadth. Fresh isolates from disease are encapsulated and form smooth mucoid colonies on agar media. The cells occur singly, in pairs, or in short chains. Brucella species do not possess flagella, nor do they form spores.

6-5. CULTURE

a. The brucellae require complex media for growth. Although many special media have been devised and recommended for cultivation of Brucella species, Trypticase soy broth and agar are used with considerable success.

b. Brucella abortus can be cultivated from clinical specimens only under increased CO₂ tension, preferably 10 percent. Brucella suis and Brucella melitensis will grow with or without increased carbon dioxide but their growth is enhanced by increased CO₂ tension.

c. Prolonged incubation (several weeks) at 37º C is often necessary for initial isolation of Brucella species; however, upon subculture, growth usually occurs within 3 or 4 days. Colonies of Brucella species are small, translucent, smooth, glistening, and blue-gray in color, and they possess entire (even) margins.

d. It is necessary to keep the culture 21 days before reporting a negative result.

6-6. PATHOGENICITY

Brucella abortus, Br. melitensis, Br. suis, and Br. canis are all pathogenic for man. Following the ingestion of raw milk from infected animals, the brucellae may invade the oral mucous membranes or the lining of the alimentary tract. Infections also result from direct contact with infected tissue. Occasional cases of pulmonary brucellosis suggest that infections may be acquired by inhalation. The incidence of brucellosis is much higher among slaughterhouse attendants, veterinarians, sausage-makers, butchers, dairymen, or similar occupational groups exposed to infected animals.

a. After entry into the human host, the organisms progress by way of lymphatic channels to the thoracic duct. They enter the blood stream and are widely disseminated
to various tissues including the liver, spleen, bone marrow, and other areas of the reticuloendothelial system. The organisms form multiple intracellular abscesses in the particular tissue affected. Osteomyelitis or meningitis may occur.

b. Characteristic symptoms begin insidiously (usually 10 to 14 days following infection), with slight periodic fever, weakness, and malaise. The lymph nodes and spleen gradually become enlarged; deep pain and symptoms of in coordination may also occur. At the height of infection, acute febrile episodes occur as a result of organisms being periodically released into the blood stream. Frank symptoms usually subside within three months and the bacilli remain dormant in deep tissues (chronic brucellosis) and long as the general physiological well being of the individual is maintained. Relapses of acute symptoms may occur when the resistance of the infected individual is lowered.

6-7. LABORATORY IDENTIFICATION

a. Isolation. The specimens usually examined for Brucella species are blood samples taken during the febrile stage. Lymph aspirations, biopsy materials, spinal fluid, or swab specimens of deep lesions may also be examined. All specimens from suspected brucellosis should be inoculated on Trypticase soy agar and broth. Blood specimens should be collected in a Castaneda bottle if they are available. All specimens must be cultivated at 37º C under 10 percent carbon dioxide. Cultures should be held at least 30 days before being discarded as negative. Although the gram-negative, coccobacillary cells of Brucella species may be observed in sputum or deep tissue fluids, such smears are rarely of diagnostic value since other bacteria may exhibit similar morphology.

CAUTION: The brucellae are dangerous pathogens and strict aseptic technique must be maintained at all times. A bacteriological hood should be employed.

b. Differentiation. To differentiate the species many procedures are available. Agglutinin absorption will separate Br. melitensis from Br. suis and Br. abortus (see table 6-2). Br. suis and Br. abortus cannot be separated by this method. The brucellae show differential sensitivity to a number of aniline dyes, for example, thionine, basic fuchsin, and crystal violet. Carbohydrates are fermented by the three species in the following manner. Brucella melitensis ferments only glucose while Br. abortus ferments glucose, inositol, mannose, and rhamnose. Brucella suis ferments glucose, mannose, maltose and trehalose. Brucella suis hydrolyzes urea rapidly while the other two are very slow or do not hydrolyze at all. Nitrates are reduced by all three species and all have catalase. Brucella suis is very active and Br. abortus is least active for catalase. Hydrogen sulfide is produced abundantly by Br. suis, to a lesser extent by Br. abortus and only in trace amounts by Br. melitensis. Indole, methyl red, and Voges-Proskauer tests are all negative for all three species. Ammonia is produced in varying amounts. Brucella suis produces the greatest amount of urease, catalase, and nitrate reduction. The best test is to use aniline dyes and observe the growth or inhibition in them.
Table 6-2. Differential characteristics of Brucella species.

Section III. HAEMOPHILUS

6-8. MORPHOLOGY

The genus Haemophilus is a heterogeneous group of small, gram-negative bacilli that are nonmotile and nonspore-forming. They are minute gram-negative rods that may be pleomorphic (occurring in various distinct forms), sometimes almost coccal, and sometimes threadlike. Although in many instances the various stains of Haemophilus species are microscopically indistinguishable, the better known forms have been shown to exhibit some cellular features of differential value.
a. **Haemophilus influenzae** is usually observed as very small bacilli, rarely exceeding 1.5 microns in length and 0.3 microns in breadth. The cells occur predominantly as coccobacillary forms, although longer, slender bacilli may be observed. There is a marked tendency for the organism to produce long thin filaments, or other aberrant forms in culture. The organisms are more difficult to stain than most bacteria. In smears of young smooth (S) colonies, definite capsules are exhibited. These capsules are rapidly dissolved by autolytic enzymes as the culture becomes older.

b. **Haemophilus haemolyticus** strains vary from small rods resembling **H. influenzae**, to larger bacillary forms occurring as irregularly shaped filaments. In addition, many pleomorphic forms are often observed which may be spheroid, tenpin, or dumbbell-shaped, or even club-shaped in appearance.

c. **Haemophilus ducreyi** (Ducrey's bacillus) appears in gram-stained smears of tissue exudates as short ovoid rods in end-to-end pairs (diplobacilli) or short chains. Individual cells are about 1 to 1.5 microns in length and 0.6 microns in breadth.

### 6-9. CULTURAL CHARACTERISTICS

a. **Growth Requirements.** **Haemophilus** species grow best at 37° C in 10 percent CO₂.

   (1) The genus is composed of hemophilic or hemoglobinophilic species. **Haemophilus influenzae** and closely related species require two factors in blood for growth. One factor, designated as X, is a heat-stable derivative of hemoglobin. The other factor, designated as V, is a heat-labile fraction of blood consisting of coenzyme I. The V factor is also formed by yeasts, staphylococci, and certain other microorganisms. Blood or commercially available additives should be incorporated in isolation media for the **Haemophilus** species requiring X and/or V factors.

   (2) Some variations exist in the ability of certain haemophilus species to grow on human blood. The use of human blood as the enrichment is not recommended; it should not be used since it may contain antibodies against this organism. Rabbit blood is the blood of choice.

   (3) Sheep blood contains substances that inhibit the growth of **Haemophilus** species, and can only be used for the preparation of chocolate agar. The inhibitory substances in chocolate agar are destroyed by the necessary temperature (70° C to 75° C) required of the sterilized agar base for lysis of red blood cells. Since heat-labile V factor is also destroyed in preparing chocolate agar, it must be restored to the medium. This is accomplished by adding enrichment supplements A or B to chocolate agar at a temperature of 50° C.
(4) If staphylococci are able to grow on a blood agar medium, it may be heavily inoculated with the specimen presumed to contain a Haemophilus species. Immediately thereafter, one or two streaks of staphylococcal organisms are applied at right angles to the primary streaks. After 24 to 48 hours of incubation, Haemophilus influenzae or similar species requiring V factor will appear as small colonies growing in close proximity to the colonies of staphylococci. This is referred to as "satellitism" and results from the production of V factor by the Staphylococcus species. Diffusion of V factor into the surrounding medium provides a readily available enrichment source for the Haemophilus species, and little or no growth of the organism will occur except along the staphylococcal growth line.

b. Observable Colony Characteristics. Haemophilus influenzae strains isolated from most pathologic sources occur in the so-called S (smooth) phase. The S colonies are raised, slimy, mucoid confluent, and nonhemolytic. Microscopic examination will reveal the presence of capsules. The R (rough) colonies are small, nonhemolytic, transparent to translucent, and present a discrete dewdrop appearance. The cells of the R phase are nonencapsulated. The S phase colonies are readily differentiated from R phase colonies in that S colonies are larger (2 to 4 mm) and more slimy and irregular than the well-defined colonies of the R phase. After 2 to 3 transfers on culture media, the encapsulated S colonies revert to the nonencapsulated R phase. The colonies of H. haemolyticus may be confused with the colonies of beta streptococci because of similarity of gross morphology and hemolysis. All colonies appearing to be beta streptococci must be verified by gram staining.

6-10. PATHOGENICITY AND IDENTIFICATION

Characteristics useful in differentiating the Haemophilus species are given in table 6-3.

<table>
<thead>
<tr>
<th>ORGANISM</th>
<th>NEED FOR X FACTOR</th>
<th>NEED FOR V FACTOR</th>
<th>HEMOLYSIS</th>
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<tr>
<td>H. Influenzae</td>
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<td>+</td>
<td>-</td>
</tr>
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<td>H. duereyi</td>
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<td>+</td>
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<td>H. parahaemolyticus</td>
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</tr>
<tr>
<td>H. aprophilus</td>
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</tr>
</tbody>
</table>

Table 6-3. Hemolytic activity and growth requirements of Haemophilus species.
a. **Haemophilus influenzae.** The encapsulated strain of *H. influenza* may cause pharyngitis, conjunctivitis, otitis, sinusitis, *pneumonia*, or meningitis. Meningitis is rare, occurring primarily in children under three years of age. The nonencapsulated variety of *H. influenzae* is considered to be normal flora in the upper respiratory tract of adults. *Haemophilus influenzae* is a fastidious organism and requires a medium enriched with blood or hemoglobin to supply the X factor and also a supplement for the V factor. All strains of *H. influenzae* reduce nitrates and are soluble in sodium deoxycholate; indole is produced by the encapsulated strains and fermentation reactions are variable. In cases of suspected meningitis caused by *H. influenzae*, spinal fluid is submitted. The specimen should be centrifuged and the supernatant disposed of in accordance with local laboratory procedures. The sediment is inoculated on a blood agar and a chocolate agar plate to which supplement has been added, or on which a staphylococcal streak is made. A smear is also made, gram-stained, and thoroughly examined. If gram-negative coccobacilli are seen, the requesting physician should be immediately notified. Throat swabs may also be submitted, and if *H. influenzae* is suspected, a chocolate agar plate should be inoculated along with a blood agar plate. Satellitism will occur if bacteria producing the V factor are present. R phase colonies of *H. influenzae* are usually seen in throat swabs of carriers and adults, where the organism is normally present in small numbers. When *H. influenzae* is recovered from throat swabs, its presence should be confirmed by subculturing to chocolate agar with added supplement or with a "staph" streak. Final identification of *H. influenzae* is accomplished by serotyping.

b. **Haemophilus ducreyi.** *Haemophilus ducreyi* is the etiologic agent of chancroid, a venereal disease characterized by the formation of ragged, soft ulcers in the genital region. The regional lymph nodes of the groin may become secondarily infected and are referred to as buboes. The ulcers differ from typical hard chancre of primary syphilitic lesions in that their edges are soft. Such localized infections are called soft chancres or chancroids. Chancroid ulcers often become infected with other bacteria. In gram-stained exudates from chancroids, these gram-negative organisms, about 1.3 microns by 0.5 microns in size, may appear singly, in small clusters, or in parallel rows suggestive of a school of fish. *H. ducreyi* is extremely hard to culture. When infections are considered to originate from *H. ducreyi*, material collected from beneath the craterlike edges of soft chancres (chancroids) are examined. Specimens may also be obtained by aspiration of any intact buboes that may be present.

c. **Haemophilus aegyptius.** *Haemophilus aegyptius* (Koch-Weeks bacillus) causes a highly communicable form of conjunctivitis ("pink eye") and morphologically resembles *H. influenzae*. It requires both the X factor and the V factor for growth and will produce satellite colonies when grown with adjoining *Staphylococcus* colonies.
Section IV. BORDETELLA

6-11. INTRODUCTION

The genus Bordetella consists of three species, Bordetella pertussis, Bordetella parapertussis, and Bordetella bronchiseptica, which are all minute, gram-negative coccobacilli and very closely resemble Haemophilus species. Bordetella pertussis is the causative agent of whooping cough. The Bordetella species do not require the X factor or the V factor for growth; however, blood is definitely a stimulus to members of this group and should be incorporated in isolation media. The glycerin-potato-blood agar of Bordet and Gengou is most often used for cultivation of these organisms. The growth of Bordetella pertussis on this medium is slow, usually requiring from 3 to 5 days' incubation. The colonies are small and dome-shaped, possessing a gray metallic luster, resembling mercury droplets. Although the colonies of pertussis bacilli are beta hemolytic, the zone of hemolysis is difficult to observe since Bordet-Gengou agar contains 15 to 20 percent blood.

6-12. IDENTIFICATION

Species identity is confirmed by the use of FA (fluorescent antibody) staining, slide agglutination tests with absorbed sera, or biochemical tests.

a. Biochemical Reactions. The relative inertness of Bordetella cells, is indicated by their failure to ferment sugars, liquefy gelatin, produce indole, produce acetyl methylcarbinol, or produce H₂S. However, most strains are catalase-positive, litmus milk is alkalized, and most media are alkalized. B. bronchiseptica rapidly produces urease. Other differential traits are illustrated in table 6-4.

b. Slide Agglutination Tests. Specific antisera may be used to identify Bordetella organisms as soon as growth is apparent on Bordet-Gengou plates.

<table>
<thead>
<tr>
<th>TEST</th>
<th>B. pertussis</th>
<th>B. parapertussis</th>
<th>B. bronchiseptica</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urease</td>
<td>-</td>
<td>+</td>
<td>+4h</td>
</tr>
<tr>
<td>Nitrate</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Motility</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Growth on blood-free peptone agar</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Browning of peptone agar</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Table 6-4. Differential characteristics of Bordetella species.
Section V. FRANCISELLA TULARENSIS

6-13. MORPHOLOGY

Francisella tularensis (formerly known as Pasteurella tularensis), the gram-negative coccobacillus that causes tularemia, is much smaller than the plague bacillus; it is 0.3 to 0.5 by 0.2 microns upon initial isolations but it becomes rodlike upon transfer. It may be quite pleomorphic and filamentous in old cultures. It occurs singly, is nonencapsulated in vitro and nonmotile; stains faintly with aniline dyes, and may show bipolar staining. Capsulated forms occur in vivo.

6-14. CULTURAL CHARACTERISTICS

F. tularensis will not grow on ordinary media and may fail to grow even on highly enriched blood agar unless cystine or cysteine is added. Cultivation is most successful when using blood-cystine-glucose agar slants. Primary growth of F. tularensis from specimens usually requires 4 to 7 days' incubation when blood-cystine-glucose agar slants are used. In young cultures the colonies are very thin, although gram-stained smears of fluid from the base of the slant will reveal numerous cells. Later, relatively heavy growth of small, gray, transparent to translucent, mucoid colonies develop. Subcultures to blood-cystine-glucose agar usually yield abundant growth within 2 to 3 days.

6-15. LABORATORY IDENTIFICATION

F. tularensis is an extremely dangerous pathogen and strict aseptic technique must be maintained at all times. When tularemia is suspected, special media must be inoculated in addition to the routine media--blood agar and thioglycollate broth. This is necessary since other bacterial agents may cause systemic disease similar to tularemia. Direct microscopic examination of F. tularensis is seldom productive. The final identification of colonies suspected of being F. tularensis is usually made by testing for agglutination with a standard, specific antiserum and the demonstration of virulence by intraperitoneal inoculation of guinea pigs. Inoculation of animals is a highly dangerous procedure that requires special facilities.
6-16. PATHOGENICITY

*F. tularensis* is the causative agent of tularemia; which is primarily a disease of rodents, rabbits, hares, and birds. Humans are only accidental, terminal hosts. The reservoir of infective agents is maintained among wild animals by biting flies (*chrysops*), ticks, and the rabbit louse; all of which are capable of spreading the disease from animal to animal. Humans can contact tularemia either directly through handling the flesh of infected animals or indirectly by an insect vector. The primary source of human infection is the rabbit. In the process of preparing the animal for food, the bacilli may enter through cutaneous abrasions or possibly through the intact skin. Aerosols of body fluids from infected animals may result in infections of the conjunctivae or lungs. Following invasion of the skin and mucous membranes, an ulcerating papule usually develops at the site of entry. The bacilli spread rapidly to the regional lymph nodes that become enlarged and suppurative. A transient bacteremia during the first week of illness serves to distribute the organisms to various internal organs where various foci of infection develop. As the disease progresses, pneumonia and fulminating septicemia may develop, resulting in death in untreated cases. Frequently the clinical signs are suggestive of the portal of entry. This is evidenced in that infections may be oculoglandular, following infection by way of the conjunctivae; ulceroglandular, following entry through the skin, or pneumonic, resulting from primary inhalation of infectious droplets. In some cases, there are no signs of localized involvement, but only the picture of a febrile systemic illness.

Section VI. MISCELLANEOUS ORGANISMS

6-17. BACTEROIDES FRAGILIS

*Bacteroides fragilis* is a nonsporogenous, nonencapsulated, nonmotile, gram-negative bacillus. It is a strict anaerobe requiring the complete absence of atmospheric oxygen. The optimum temperature for growth and isolation is 37º C. Initial isolation of this organism is accomplished by using Brewer's thioglycollate broth or deep tubes of meat media overlayed with paraffin. This will provide the anaerobic conditions necessary for growth of this bacterium according to the anaerobic techniques in Lesson 2, Section II. The organisms are transferred from this media to blood agar plates or ascitic agar plates to obtain pure colonies of *Bacteroides fragilis*. The colonies when grown in pure culture are smooth, convex, with an entire (even) edge, and they may exhibit pigmentation. The pigment may be white, gray, or yellow. The organism may be found in specimens taken from the mouth, vagina, or the intestinal tract. In fact, it is the anaerobe most frequently isolated from clinical infections, and it is the most common organism in the normal intestinal tract.
6-18. STREPTOBACILLUS MONILIFORMIS

Streptobacillus moniliformis is a gram-negative bacillus. The bacilli may be small and slender or may be looped or have curved filaments. The loops resemble a necklace or a string of beads (moniliform). Initial isolation is best obtained by using thioglycollate broth with the addition of ascitic fluid. Further culture may be obtained in solid media such as blood agar or serum agar with blood. The colonies growing on these media are small, smooth, and glistening, with irregular edges, and are colorless to gray. This organism is a normal inhabitant of the throat and nasopharynx of the rat. The infection in man is also known as rat-bite fever and is contracted by the bite of this animal.

Section VII. ANTIBIOTIC SENSITIVITY TESTS

6-19. GENERAL COMMENTS

a. Chemotherapy involves the systemic use of chemical agents for the treatment of infectious disease, and antibiotics are substances, having antimicrobial properties. The basis of chemotherapy, then is selective toxicity; the drug will destroy the disease-producing microbe nonmotile having relatively little effect on the host. This selective toxicity represents the main functional difference between antibiotics and disinfectants. Disinfectants and antiseptics lack cellular specificity.

b. An antibiotic must be effective in vivo in concentrations not harmful to the host. It is possible for an antibiotic to be effective in vitro ("in a test tube") but not in vivo. In vitro the antibiotic is in contact with only one biological system, namely that of the bacteria. In vivo an entirely different situation exists. In vivo the antibiotic is in contact with at least two biological systems, the bacteria and the host. The response of the host may be such that it alters the antibiotic to a form not effective against the pathogenic bacteria. The in vivo activity of the antibiotic is extremely complex and the mechanism of antibiotic activity in vivo is not generally known.

c. The laboratory specialist should take great care in performing antibiotic sensitivity tests and reporting their results. He should try to attain a high lever of accuracy and dependability. The laboratory report is in effect a recommendation for the usefulness or withdrawal of an antibiotic. In either case, the consequences for the patient can be tremendous.

6-20. ANTIBIOTIC RESISTANCE

Of extreme importance in the area of chemotherapy is the subject of bacterial resistance to antimicrobial agents. In the bacteriology section it is often necessary to determine an effective agent for an organism that has become resistant to another more commonly used agent.
a. **Resistance by Mutation.** An organism becomes resistant by mutation. Within a given bacterial population, some bacteria will develop that are more resistant to an antibiotic than the rest of the population due to the natural occurrence of mutations. The administration of an appropriate antibiotic kills all of the population except the resistant mutants. These mutants are called first-generation mutants. Each succeeding generation of the first generation mutants exposed to the antibiotic will demonstrate a greater degree of resistance to the agent. Most drug resistant bacteria are "selected" in this manner. An example of this method of resistance having great clinical significance is the case of penicillin-resistant *Staphylococcus aureus*. When penicillin was first used, it was employed indiscriminately. In effect, first-generation mutants were selected out from the general bacterial population. Through the years, resistance to penicillin increased through all succeeding generations of this bacterium. Today, the penicillin-resistant "staph" often presents a grave problem.

b. **Cross-Resistance.** Second, bacteria may become resistant to an antibiotic by a process of cross-resistance. The organism develops resistance to another antibiotic that is similar chemically, even though the bacteria had not been exposed to the second agent. For example, bacteria resistant to streptomycin may also be resistant to dihydrostreptomycin, which is similar chemically.

### 6-21. PREVENTING EMERGENCE OF RESISTANT STRAINS OF BACTERIA

Once the problem of drug-resistant strains of bacteria was recognized, several ways of handling the situation were developed. First, high blood levels of the antibiotic must be maintained. This will help prevent resistance by holding back the emergence of first-step mutants. Second, combined therapy is sometimes very effective. Combined therapy involves the concomitant use of two or more antimicrobial agents to combat infectious disease. Under combined therapy, one drug may be effective against those mutants resistant to the other drug. Naturally, drugs for which the bacteria may be cross-resistant must not be used.

### 6-22. LABORATORY ANTIBIOTICS METHODS FOR DETERMINING SENSITIVITY OF AN ORGANISM TO ANTIBIOTICS

a. **Paper Disk Method.** A paper disk containing a predetermined concentration of antibiotic is placed on the surface of an agar plate previously inoculated with the organism to be tested. The size of a zone of inhibited growth surrounding the disk is used as an index of sensitivity. The size of a zone surrounding a disk is dependent upon such factors as the amount of inoculum, thickness of the medium, and diffusion and solubility of the antibiotics. The detection of isolated colonies growing within a zone of inhibition indicates the presence of resistant organisms from the culture. The disk method of antibiotic sensitivity is a convenient and rapid technique for use in the clinical laboratory. This method is discussed further later in this lesson.
b. **Tube Dilution Method.** Employing the serial dilution technique, decreasing concentrations of antibiotics are prepared in broth for inoculation with a culture of the organism to be tested. After inoculation and incubation of the dilutions with the organism, its sensitivity is determined by the presence or absence of growth in the varying concentrations of therapeutic agents. If there is no growth in the control tube, which contains no antibiotic, the test should be considered invalid. If there is excellent growth in the control tube, look for the tube containing the lowest concentration of antibiotic in which growth is inhibited. If there is a clear-cut distinction between two adjacent tubes in increasing antibiotic concentration, report the result as the lowest concentration of antibiotic in which bacterial growth is inhibited. If there is a gradual decrease in the amount of growth as the concentration of antibiotic increases, report the results as the lowest concentration of antibiotic to which the bacterial growth is sensitive and the lowest concentration of antibiotic to which the growth is completely resistant. The tube dilution method is applicable to research procedures and other situations requiring quantitative determinations.

6-23. **KIRBY-BAUER METHOD FOR DETERMINING BACTERIAL SENSITIVITY**

a. A few well-isolated colonies (3 to 8) of the organism to be tested are transferred with a wire loop from the original culture plate to a test tube containing 4 ml of tryptase phosphate or Trypticase soy broth. (Both of these media will support the growth of the great majority of bacteria found in clinical infections.)

b. Incubate tubes two to five hours to produce a bacterial suspension of moderate cloudiness.

c. Dilute suspension, if necessary, with sterile water or saline solution to a turbidity visually comparable to that of a standard prepared by adding 0.5 ml of one percent barium chloride to 99.5 ml of one percent sulfuric acid (0.36N). An alternate procedure is to dilute overnight broth cultures to the density of the opacity standard (10- to 100-fold).

d. For sensitivity plates, large (15 cm) petri dishes are used with Mueller-Hinton agar (5 to 6 nm in depth).

(1) The large petri dishes are spacious enough to accommodate about nine disks in an outer ring and three or four more in the center.

(2) It is advantageous to place antibiotics that diffuse well in the outer circle and disks that produce smaller inhibition zones (such as vancomycin, polymyxin B, and Kanamycin) in the central area of the plate.

e. Plates are dried for about 30 minutes before inoculation and are used within four days of preparation.
f. The bacterial broth suspension is streaked evenly in three planes onto the surface of the medium with a cotton swab (not a wire loop or glass rod). Surplus suspension is removed from the swab by being rotated against the side of the tube before the plates are seeded.

g. After the inoculum has dried (three to five minutes) the disks are placed on the agar with flamed forceps or a disk applicator and gently pressed down to insure contact.

h. Plates are incubated immediately, or within 30 minutes. Incubate overnight (optimum 14 hours) at 37º C.

i. Measure zone diameters (including the 6-mm disk). A reading of 6 mm indicates no zone. Zone diameters may be read after incubation for 6 to 8 hours if they are needed.

j. The end point is taken as complete inhibition of growth as determined by the naked eye. See table 6-5 for interpretation of zone sites.

(1) In the case of sulfonamides, organisms must grow through several generations before inhibition takes effect. Slight growth (80 percent or more inhibition) with sulfonamides is therefore disregarded; the margin of heavy growth is read to determine the zone size.

(2) Swarming (spreading) of Proteus species is not inhibited by all antibiotics; a veil of swarming into an inhibition zone should also be ignored.

(3) If colonies are seen within a zone of inhibition, the strain should be checked for purity and retested.

k. Standard control organisms of known susceptibility should be employed at least once a week as a check on the activity of the disks and on the reproducibility of the test.
<table>
<thead>
<tr>
<th>Antibiotic or Chemotherapeutic Agent</th>
<th>Disk Potency</th>
<th>mm-Inhibition Zone Diameter to nearest mm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Resistant</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>10 ug</td>
<td>11 or less</td>
</tr>
<tr>
<td>Staphylococci and highly penicillin sensitive organisms</td>
<td>10 ug</td>
<td>20 or less</td>
</tr>
<tr>
<td>Haemophilus</td>
<td>10 ug</td>
<td>19 or less</td>
</tr>
<tr>
<td>Carbenicillin²</td>
<td>50 ug</td>
<td>17 or less</td>
</tr>
<tr>
<td>Proteus and E. coli</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>50 ug</td>
<td>12 or less</td>
</tr>
<tr>
<td>Cephalexin³</td>
<td>30 ug</td>
<td>14 or less</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>30 ug</td>
<td>12 or less</td>
</tr>
<tr>
<td>Clindamycin</td>
<td>2 ug</td>
<td>14 or less</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>15 ug</td>
<td>12 or less</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>10 ug</td>
<td>12 or less</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>30 ug</td>
<td>13 or less</td>
</tr>
<tr>
<td>Methicillin⁴</td>
<td>5 ug</td>
<td>9 or less</td>
</tr>
<tr>
<td>Neomycin</td>
<td>30 ug</td>
<td>12 or less</td>
</tr>
<tr>
<td>Nitrofurantoin⁵</td>
<td>300 ug</td>
<td>14 or less</td>
</tr>
<tr>
<td>Staphylococci</td>
<td>10 U</td>
<td>20 or less</td>
</tr>
<tr>
<td>Penicillin-G</td>
<td>10 U</td>
<td>11 or less</td>
</tr>
<tr>
<td>Other organisms</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Polymyxin-B</td>
<td>300 U</td>
<td>8 or less</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>10 ug</td>
<td>11 or less</td>
</tr>
<tr>
<td>Sulfonamide</td>
<td>300 ug</td>
<td>12 or less</td>
</tr>
<tr>
<td>Sulfonamide-trimethoprim (19:1)⁵</td>
<td>25 ug</td>
<td>10 or less</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>30 ug</td>
<td>14 or less</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>30 ug</td>
<td>9 or less</td>
</tr>
</tbody>
</table>

³Class disk for ampicillin and hetacillin.
⁴A change to a 100-μg disk is being considered. This would entail new standards.
⁵Class disk for cephalexin, cephaloridine, cephalexin and cefazolin.
⁶Class disk for penicillinase-resistant penicillins with staphylococci.
⁷Urinary tract infections only.

Table 6-5. Kirby-Bauer susceptibility test method: zone size interpretative chart.

Continue with Exercises
EXERCISES, LESSON 6

INSTRUCTIONS: Answer the following exercises by marking the lettered responses that best answers the exercise, by completing the incomplete statement, or by writing the answer in the space provided.

After you have completed all of the exercises, turn to "Solutions to Exercises" at the end of the lesson and check your answers. For each exercise answered incorrectly, reread the material referenced with the solution.

1. To determine the pattern of carbohydrate metabolism of oxidative bacteria, we use OF basal medium because it contains:
   a. Uric acid.
   b. Petrolatum.
   c. No peptone.
   d. Less peptone than other fermentation media.

2. When OF basal medium is inoculated with a fermentative organism, acidity generally occurs in:
   a. Only the open tube.
   b. Only the petrolatum-covered tube.
   c. Both the open and petrolatum-covered tube.
   d. Neither the open nor the petrolatum-covered tube.
3. Which of the following gram-negative bacilli causes serious infections in which blue-green pus may be produced?
   a. *Escherichia coli.*
   b. *Bacteroides fragilis.*
   c. *Klebsiella pneumoniae.*
   d. *Pseudomonas aeruginosa.*
   e. *Proteus mirabilis.*

4. Upon primary isolation, which of the following species of *Brucella* will not grow without increased carbon dioxide tension?
   a. *suis.*
   b. *abortus.*
   c. *melitensis.*
   d. *canis.*

5. Which of the following species of *Brucella* is pathogenic for man?
   a. All OF THE BELOW.
   b. *abortus.*
   c. *melitensis.*
   d. *suis.*
6. When examining blood cultures, the technician should be particularly cautious if the organism is suspected to be:
   a. Bacillus.
   b. Bordetella.
   c. Borrelia.
   d. Brucella.

7. Differentiation of the species of Brucella is based primarily on:
   a. The gram stain.
   b. Cellular morphology.
   c. Growth on Castaneda medium.
   d. Growth in presence of thionine and basic fuchsin.

8. The "satellitism" phenomenon is illustrated by:
   a. Staphylococcus, forming colonies in the presence of Haemophilus species.
   b. Streptococcus, species forming colonies in the presence of Haemophilus species.
   c. Haemophilus, species forming colonies in the presence of Staphylococcus.
   d. Haemophilus, species not being able to grow in the presence of Staphylococcus.

9. Haemophilus influenzae requires a medium containing:
   a. X factor only.
   b. V factor only.
   c. X and V factors.
   d. Neither X nor V factor.
10. Differentiation of the species of *Haemophilus* is based primarily on:
   a. Motility.
   b. Satellite growth.
   c. Gram staining reaction.
   d. Requirement for X and V factors for growth.

11. The causative agent of whooping cough belongs to the genus:
   a. *Bordetella*.
   b. *Brucella*.
   c. *Haemophilus*.
   d. *Pasteurella*.

12. Species identity of a *Bordetella* isolate is confirmed by:
   a. ANY OF THE BELOW.
   b. Biochemical tests.
   c. Slide agglutination tests.
   d. Fluorescent antibody staining.

13. Which of the following media is used for the cultivation of suspected *Francisella tularensis*?
   a. OF basal medium.
   b. Bordet-Gengou agar.
   d. Blood agar with added V factor.
14. Animal virulence studies for the presence of *Francisella tularensis* may be performed safely in most laboratories.
   
a. True.
   
b. False.

15. Which of the following is a strict anaerobe?
   
a. *Bordetella pertussis*.
   
b. *Francisella tularensis*.
   
c. *Bacteroides fragilis*.
   
d. *Streptobacillus moniliformis*.

16. The basis of chemotherapy is:
   
a. Selective toxicity.
   
b. General protoplasmic poisoning.
   
c. Dermal application of disinfectants.
   
d. Oral administration of antiseptics.

17. The results of antibiotic sensitivity tests are used primarily for:
   
a. Epidemiology.
   
b. Biological research.
   
c. Pharmaceutical research.
   
d. Determining how to treat a patient.
18. The diameter of a zone of inhibited growth around a paper disk containing an antibiotic is used as an index of the cultured organism's:
   a. Ability to synthesize the antibiotic.
   b. Ability to utilize the antibiotic as a nutrient.
   c. Sensitivity to the antibiotic.
   d. Virulence.

19. Using the tube dilution method for determining antibiotic sensitivity, the absence of bacterial growth in the control tube indicates:
   a. An invalid sensitivity test.
   b. A valid sensitivity test.
   c. A questionable sensitivity test.
   d. An organism extremely sensitive to the antibiotic being tested.

20. When the Kirby-Bauer method is used to test the sensitivity test of a staphylococcal strain to penicillin G, a zone size of 18 mm means that the staphylococci are:
   a. Resistant.
   b. Sensitive.
   c. Intermediate in sensitivity.

Check Your Answers on Next Page
SOLUTIONS TO EXERCISES, LESSON 6

1. d (para 6-1c(1))
2. c (para 6-1c(3))
3. d (para 6-2a)
4. b para 6-5b; Table 6-1)
5. a (para 6-6)
6. d (para 6-7a)
7. d (para 6-7b; Table 6-1)
8. c (para 6-9a(4))
9. c (para 6-10a; Table 6-2)
10. d (para 6-10a; Table 6-2)
11. a (para 6-11)
12. a (para 6-12)
13. c (para 6-14)
14. b (para 6-15)
15. c (para 6-17)
16. a (para 6-19a)
17. d (para 6-19c)
18. c (para 6-22a)
19. a (para 6-22b)
20. a (Table 6-5)

End of Lesson 6