

**CDC 4T0510**

**Medical Laboratory  
Journeyman: Microbiology**

**Volume 3. Other Medically Important  
Microorganisms**



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THIS VOLUME of CDC 4T051O, *Medical Laboratory Journeyman: Microbiology*, begins in unit 1 with a study of miscellaneous microorganisms to include the spirochetes, mollicutes, chlamydiae, rickettsiae, and viruses. Next in unit 2, we will examine the acid-fast bacilli and some of the unique features of these organisms. The volume will conclude in unit 3 with an introduction to medical mycology and general information about mycology. General characteristics, clinical significance, and laboratory identification for these microorganisms will be discussed. Wherever needed, safety practices and quality control will be reviewed.

A glossary of terms, abbreviations, and acronyms used in this course is included at the end of the volume.

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This volume is valued at 12 hours and 4 points.

**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 unit review exercises.

|  | <i>Page</i>    |
|--|----------------|
| <b>Unit 1. Miscellaneous Microorganisms .....</b>  | <b>1-1</b>     |
| 1-1. The Families <i>Spirochaetaceae</i> and <i>Leptospiraceae</i> .....                               | 1-1            |
| 1-2. The Genera <i>Mycoplasma</i> , <i>Ureaplasma</i> , <i>Chlamydia</i> , and <i>Rickettsia</i> ..... | 1-11           |
| 1-3. Introduction to Virology .....  | 1-17           |
| <b>Unit 2. The Mycobacteria.....</b>   | <b>2-1</b>     |
| 2-1. Processing and Staining Specimens for Mycobacteria .....  | 2-1            |
| 2-2. The <i>Mycobacteriaceae</i> Family.....   | 2-10           |
| <b>Unit 3. Introduction to Medical Mycology .....</b>  | <b>3-1</b>     |
| 3-1. General Characteristics of Fungi and Specimen Collection.....                                     | 3-1            |
| 3-2. Laboratory Identification .....   | 3-17           |
| <br><i>Glossary</i> .....  | <br><i>G-1</i> |
| <i>Bibliography</i> .....  | <i>B-1</i>     |



## Unit 1. Miscellaneous Microorganisms

|  |             |
|--|-------------|
| <b>1–1. The Families <i>Spirochaetaceae</i> and <i>Leptospiraceae</i>.....</b>                             | <b>1–1</b>  |
| 401. <i>Borrelia</i> species .....   | 1–1         |
| 402. <i>Treponema</i> species .....  | 1–3         |
| 403. <i>Leptospira</i> species .....   | 1–6         |
| <b>1–2. The Genera <i>Mycoplasma</i>, <i>Ureaplasma</i>, <i>Chlamydia</i>, and <i>Rickettsia</i> .....</b> | <b>1–11</b> |
| 404. <i>Mycoplasma</i> and <i>Ureaplasma</i> .....   | 1–11        |
| 405. <i>Chlamydia</i> and <i>Rickettsia</i> .....  | 1–12        |
| <b>1–3. Introduction to Virology .....</b>   | <b>1–17</b> |
| 406. Classification and general characteristics of viruses.....  | 1–18        |
| 407. Laboratory identification, collecting, and shipping of viral specimens .....                          | 1–24        |

IN THIS unit you'll learn about the different microorganisms that may be related to bacteria, yet, are quite different. In addition, you'll study organisms that are not related to bacteria, but cause disease in humans. The clinical significance of laboratory identification of the members of the order *Spirochaetales*, organisms of the class *Mollicutes*, obligate intracellular parasites, and viruses will also be discussed. This order contains both commonly encountered pathogens such as the causative agent of syphilis as well as members of the genus *Leptospira*, which are rarely isolated.

### 1–1. The Families *Spirochaetaceae* and *Leptospiraceae*

The order *Spirochaetales* consists of the families *Spirochaetaceae* and *Leptospiraceae*. *Spirochaetaceae* contains five genera: *Spirochaeta*, *Serpulina*, *Cristispira*, *Treponema*, and *Borrelia*. *Borrelia* and *Treponema* are the only two of clinical significance that will be discussed here. The other family in the order *Spirochaetales* is *Leptospiraceae*, which consists of two genera *Leptospira* and *Leptonema*. Differentiation among the genera of the families *Spirochaetaceae* and *Leptospiraceae* is based primarily on morphology. They are only one of the few major bacterial groups for which the classical morphology taxonomy did not change because of molecular biological studies. Other differentiation properties are staining characteristics, oxygen requirements, and cellular motility.

#### 401. *Borrelia* species

Currently, some members of the genus *Borrelia* are given the species names of the arthropod vectors that carry them. For example, *Borrelia hermsii* gets its species name for its arthropod vector *Ornithodoros hermsi*. Two noteworthy exceptions to this practice involve *B. recurrentis* and *B. burgdorferi*. *B. burgdorferi* was named after Dr. W. Burgdorfer who first observed this spirochete. There are 23 different species in the genus *Borrelia*.

#### General characteristics

The louse- and tick-borne spirochetes of the genus *Borrelia* cause relapsing fever and Lyme disease in humans. *B. recurrentis* is the species responsible for louse-borne epidemic relapsing fever, while several species of ticks are responsible for the less severe tick-borne variety of the disease. The arthropod vector for louse-borne *B. recurrentis* is *Pediculus humanus* subspecies *humanus*. Animal reservoirs are mostly rodents and occasionally chipmunks, tree squirrels, cattle, horses, sheep, deer, and fowl. Humans are the reservoirs for only two species, *B. recurrentis* and *B. duttonii*. In relapsing fevers, *Borrelia* species are present in the blood during febrile periods and can be observed microscopically in a diluted drop of blood. The drop of blood must be mixed with a drop of sodium citrate and covered with a cover slip. Morphologically, this spirochete is a spiral thread measuring 0.2 to 0.5  $\mu\text{m}$  in width and 5 to 25  $\mu\text{m}$  in length. It may possess from 4 to 10 spirals, with most cells

showing around 5 spirals. Spirals are large and wavy with a distance of approximately  $2.5\ \mu\text{m}$  between them. Amplitude is about  $1.5\ \mu\text{m}$ , as illustrated in figure 1–1. Occasionally, axial terminal filaments are seen, and these may give the appearance of flagella.

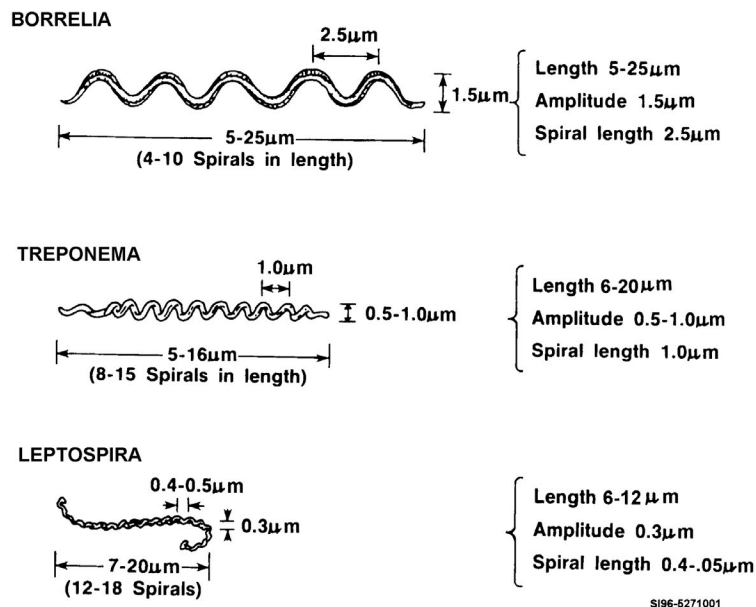


Figure 1–1. Major shapes of spirochetes.

### Clinical significance

Spirochetes of *Borrelia* are the causative agents of an acute febrile illness in humans—relapsing fever—which is transmitted by an infected arthropod. Humans become infected not from the bite of the infected louse vector but rather because of the bite becoming contaminated when scratching crushes infected lice. Louse-borne disease occurs mostly in Eastern Africa, although it is distributed worldwide. Distribution of the disease is dependent on the natural habitat of the vector. Tick-borne relapsing fever is transmitted through the bite via infected saliva. Epidemic relapsing fever is a severe febrile systemic illness with a rapid onset after an incubation period of two to 15 days. During the febrile period, borreliae are found in the blood, spinal fluid, and urine. During this period, the patient experiences shaking chills, fever, severe headache, myalgia, arthralgias, nausea, vomiting, and splenomegaly. After three to seven days of fever, an afebrile (no fever) interval of several days to several weeks follows. A relapse then occurs because of antigenic variant appearing and multiplying. In untreated cases, as many as 13 relapses can occur.

Chronic borreliae infections may involve the central nervous, respiratory, and cardiovascular systems. Pregnant women who contract relapsing fever pass the infection to the infant or may spontaneously abort their fetus. The causative agent of Lyme disease is *B. burgdorferi*, which is transmitted to humans by the bite of its arthropod vectors *Ixodes scapularis*, *Ixodes pacificus*, *Ixodes ricinus*, *Ixodes persulcatus*, and *Ixodes ovatus* ticks. It is transmitted to humans by way of the tick's saliva entering the bite wound directly as the tick takes its blood meal, or indirectly when the wound becomes infected by coxal secretions given off by the tick as it feeds. The reservoir hosts of *B. burgdorferi* are rodents and deer. The disease is named after Lyme, Connecticut, where it was first recognized (in 1975) as a new form of inflammatory arthritis. Other *Borrelia* species involved in Lyme disease are *B. garinii*, *B. afzelii*, and *B. japonica*. A skin lesion referred to as erythema chronicum migrans (ECM) characterizes this spirochetal zoonotic disease. ECM begins as a single or multiple area of a red rash that expands in a circular manner, sometimes reaching diameters of 12 inches or more. The patient experiences rather vague symptoms including headaches, fever, chills, fatigue, arthralgias,



malaise, and stiff neck, due to systemic, neurological, and cardiac involvement. These symptoms may arise before or after the ECM skin lesion. The ECM lesions fade within three weeks.

Within weeks to months after the onset of ECM neurological abnormalities, such symptoms as aseptic meningitis, encephalitis, myocarditis, or facial palsy may develop. Cardiac abnormalities usually arise within weeks of the onset of ECM and include atrioventricular block, cardiomegaly, etc. The hallmark of Lyme disease is the development of a chronic form of arthritis, which may occur weeks or years after the patient experiences ECM. The original treatment for borrelial infections was the use of arsenic compounds, but antibiotics are now preferred. *Borreliae* are susceptible to many antibiotics, however, the choice of antibiotic, dosage, and duration and route of treatment are controversial.

### Laboratory identification

The spirochetes are not gram-positive or gram-negative; therefore, Romanowsky modification, Leishman, May-Grünwald, Wright, or Giemsa stains are used to stain thin- and thick-smears. The mechanics of staining cause some distortion and straightening of the spirals, and experience must be gained if this manner of generic identification is to be relied upon. For best results in viewing morphology, a direct wet preparation is used. Under these conditions, the actively motile spirochetes have a corkscrew movement along with lateral oscillation (side-to-side). The motion is not necessarily directional, and the organism may be seen to move back and forth. Some strains of *Borrelia* have been successfully grown in vitro on Kelly medium, but culture and isolation of this organism is not the preferred procedure for its identification. Attempts to develop serological tests for the diagnosis of relapsing fever met with many technical obstacles due to the spirochetes' ability to change its antigenic composition spontaneously. Therefore, direct microscopic observation is still the preferred method in the diagnosis of relapsing fever. The preferred methods for the diagnosis of Lyme disease, on the other hand, are indirect immunofluorescence assay (IFA) and enzyme-linked immunosorbent assay (ELISA). ELISA procedures are the most widely used serological test for confirming Lyme disease. This is possible since the antigenic composition of *B. burgdorferi* is stable. Deoxyribonucleic acid (DNA) techniques are not very successful because approximately 10,000 spirochetes are needed for detection.

### 402. *Treponema* species

The genus *Treponema* consists of four human pathogens and at least six human nonpathogenic species. Some species are considered strict anaerobic organisms; however others, including *T. pallidum* and *T. pertenue*, are now known to be microaerophilic. The nonpathogenic species are found as part of the normal flora of the mouth, intestinal tract, and genital tract of humans. The status of some of these organisms as nonpathogens is being questioned. Their association with skin ulcers, diarrheal disease, and periodontal disease is being explored; however, their exact role in these diseases has not been clearly defined.

### General characteristics

The spirochetes of *T. pallidum* occur as very fine, delicate spiral forms having a flexible cylindrical body measuring approximately 0.20  $\mu\text{m}$  in diameter and 6 to 20  $\mu\text{m}$  in length. The spiral height (amplitude) is approximately 1.0  $\mu\text{m}$ . The organism possesses from 8 to 15 regular spirals. Motility is generally slow, although the cell rotates rapidly on its axis. During the rotational process, the organism bends to form circular as well as S-shapes. In the material aspirated from the lesion of early syphilis, the cell tends to be elongated (like an over-stretched spring) and movement is more snake-like. This is due to the greater density of the fluid caused by mucus present in the lesion.

### Clinical significance

Three species of the genus that are known pathogens and have been linked with specific disorders are *T. pallidum* subspecies *pallidum*, *T. pallidum* subspecies *pertenue*, *T. pallidum* subspecies *endemicum* and *T. carateum*. *Treponema pallidum* subspecies *pallidum* (pale thread) is the etiologic agent of venereal syphilis, a highly contagious disease transmitted from person-to-person through intimate

contact, usually sexual. Infections may be transmitted occasionally through indirect means such as fomites (drinking cups, towels), but only when the time interval between contamination of the article and its subsequent contact with an individual is very short. For this reason, fomites used by persons with open, infective lesions should be considered highly contagious. *T. pallidum* subspecies *pallidum* can cross the placenta and infect the unborn child causing congenital syphilis.

### ***T. pallidum* subspecies *pallidum***

*Treponema pallidum* subspecies *pallidum* enters the skin or mucous membranes through minute breaks. The spirochetes remain for a time at the site of infection and multiply. The characteristic inflammatory response known as the chancre follows. This stage is referred to as primary syphilis, and the chancre forms within 10 to 90 days after infection. The chancre lasts from one to three weeks and may heal spontaneously. Primary syphilis is generally diagnosed by darkfield microscopic detection of *Treponema pallidum* subspecies *pallidum*, although a positive serologic test for syphilis will occasionally be obtained at this stage of the disease. Within six weeks to six months after the primary chancre heals, a generalized skin rash usually appears. This stage of the disease is referred to as secondary (disseminated) syphilis. Symptoms usually disappear within about three weeks and, in untreated cases, may reappear one or more times as relapses. In secondary syphilis, the characteristic skin lesions contain *Treponema pallidum* subspecies *pallidum*. An increasingly positive reaction in serologic tests is the rule. The subsequent latent stage of syphilis occurs six months to one year post-infection and is usually asymptomatic. It is shown by a persistently positive reaction in serologic tests. Tertiary (late or chronic) syphilis, which can be expected to follow the latent period in untreated cases, can also be detected by reactive serologic tests. Tissue destruction of any organ, aortic aneurysms, neurological disorders, and monocytic infiltrates are some of the symptoms seen in tertiary syphilis.

### ***T. pallidum* subspecies *pertenue***

*Treponema pallidum* subspecies *pertenue* is the causative agent of yaws (frambesia or pian). Yaws is a nonvenereal, systemic infectious disease, most commonly occurring in children in tropical regions, such as Equatorial Africa, West Indies, and tropical areas of the Americas and the Far East. It is divided into three stages:

1. Primary stage, in which a granulomatous initial lesion, the mother yaws, appears at the site of implantation of the spirochete, usually the lower leg or foot, and increases in size, becomes crusted, and spontaneously disappears, leaving a scar.
2. Secondary stage, which occurs weeks or months after the appearance of the primary lesions, and is marked by successive crops of granulomatous papules distributed over the body, especially the face, extremities, and anogenital area; on rare occasions.
3. Tertiary stage may occur in which gummatous lesions of the skin similar to those of syphilis, and destructive lesions of the bones occur after an interval of several years.

### ***T. pallidum* subspecies *endemicum* and *T. carateum***

*Treponema pallidum* subspecies *endemicum* is the causative agent of nonvenereal endemic syphilis. This disease is less serious than venereal syphilis, and is found mostly in children in developing tropical and subtropical countries of the world. Congenital transmission is rare. *Treponema carateum* is the causative agent of pinta. Pinta is a form of treponematoses, in which chronic dyschromic dermatosis occurs. It is prevalent in parts of tropical America and characterized by the presence of spots on the skin, which may be white, coffee colored, blue, red, or violet. The disease is transmitted by direct person-to-person contact.

The table summarizes the characteristics of the treponematoses.

| Organism  | Disease           | Predominant age         | Route of Infection | Incubation Period | Congenital Infection |
|---|-------------------|-------------------------|--------------------|-------------------|----------------------|
| <i>T. pallidum</i> sub-species <i>pallidum</i>  | Venereal Syphilis | Adolescents, Adults     | Sexual Contact     | 10–90 days        | Yes                  |
| <i>T. pallidum</i> sub-species <i>pertenue</i>  | Yaws              | Children                | Skin Contact       | 9–90 days         | No                   |
| <i>T. pallidum</i> sub-species <i>endemicum</i> | Endemic Syphilis  | Children to Adults      | Mucous Membrane    | ?                 | Rarely               |
| <i>T. carateum</i>                              | Pinta             | Children to Adolescents | Skin Contact       | 2–6 months        | No                   |

The Centers for Disease Control and Prevention recommends syphilis treatments based on the stage of syphilis. Penicillin and its derivatives are the preferred antibiotic for syphilis and the other treponematoses.

### Laboratory identification

The four treponemes pathogenic to humans (three subspecies of *T. pallidum* plus *T. carateum*), are indistinguishable morphologically and serologically. Yaws, pinta, and nonvenereal or endemic syphilis must be differentiated from venereal syphilis based on clinical and epidemiologic characteristics. Since *T. pallidum* ssp. *pallidum* is the primary pathogen of the genus and encountered in the laboratory on a day-to-day basis, we shall place emphasis on its identification.

### Staining *T. pallidum*

Staining of *Treponema pallidum* by the Gram-stain technique is usually unsuccessful. Although the organism may take the dye, the amount of protoplasm available by staining is so small that the necessary visual contrast is lacking. Staining can be accomplished satisfactorily, however, by using special techniques such as the silver impregnation method of Levaditi, or one of its several modifications. This technique is based on the coating of the surface of the treponeme with metallic silver to increase contrast. In the Fontana-Tribondeau method, the cell surface is coated with reduced silver nitrate.

### Pathogenic treponemes

The pathogenic treponemes have not been cultivated in the laboratory. Only saprophytic, nonpathogenic treponemes are cultivable. They are anaerobic, or possibly microaerophilic, ferment glucose or amino acids, and require serum bovine albumin or volatile fatty acids for growth.

### Diagnosis of treponemes

Laboratory diagnosis of treponemes is generally based on darkfield examinations and serological procedures (which will be discussed at length in the 4T051C CDC, *Immunology* volume). Let's look at the darkfield technique employed during the first examination of the syphilitic lesion. Most of the microscopy you have done has been with "brightfield" illumination. This form of microscopy was covered in CDC 4T051A, *Chemistry and Urinalysis*. In the darkfield setup, we replace the standard microscope condenser with a special darkfield condenser to provide oblique rather than direct lighting of the specimen.

### Darkfield examination

In brightfield microscopy, the substage condenser provides a solid cone of light that is concentrated on the specimen. In "darkfield illumination" the cone of light is hollow, leaving a dark central area. It is this darkened center that we see when we look through the microscope. Thus, the darkfield condenser provides scattered light that reflects off any object in the darkened field, giving us bright objects against a black background. In most laboratories that perform the darkfield examination, one

microscope adapted for this procedure with special condenser and objective lenses, as shown in figure 1-2, is set-aside for the purpose.

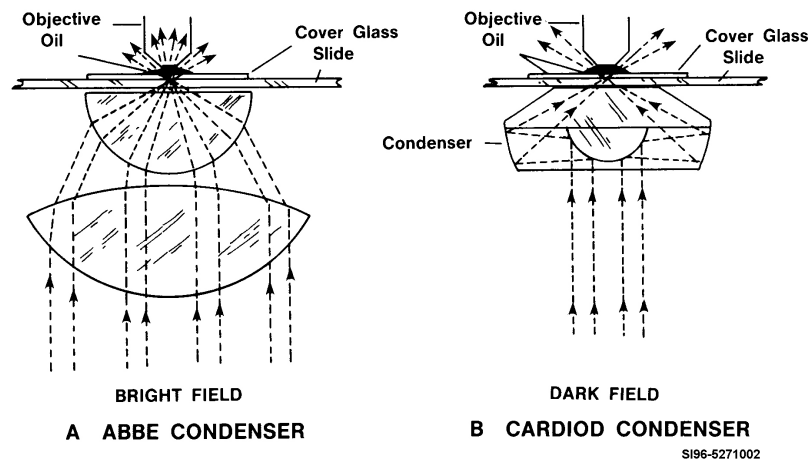


Figure 1-2. Brightfield versus darkfield condenser systems.

### ***Collecting from a syphilitic lesion***

Collecting material from a syphilitic lesion for darkfield study must be done carefully. First, you must clean the lesion of surface crust, detritus, and pus, with gauze or a cotton applicator. If the patient has treated his lesion with a germicidal agent, examination is deferred until all germicide has been removed by washing and several hours have elapsed. Primary lesions are then mildly traumatized to provoke a clear serous exudate. We do this by gently rolling the lesions between the gloved finger and thumb, or by rubbing its surface with a dry cotton applicator. Hemorrhage should be avoided, although a few erythrocytes or pus cells are desirable to aid in obtaining a proper focus. Secondary lesions are merely cleansed with saline saturated gauze and abraded.

### ***Fresh preparations***

Fresh preparations for microscopic examination are made from accessible lesions by touching the slide to tissue juice and immediately placing the cover glass over the drop of fluid. Petroleum jelly placed around the edge of the cover slip will prevent drying. If the lesion is less accessible, the fluid may be collected in a capillary pipette and placed on the slide. Examine immediately with the darkfield microscope for the characteristic morphology and motility of *T. pallidum*.

## **403. *Leptospira* species**

This genus consists of two species, *Leptospira interrogans* and *Leptospira biflexa*. *L. interrogans* includes all pathogenic serovars of leptospire, whereas *L. biflexa* includes the saprophytic or water *Leptospira* that commonly occur in fresh, surface waters. A proposed phylogenetic system of leptospire taxonomy contains 11 species of *Leptospira*. *Leptonema* is new genus in the family *Leptospiraceae* and consist of a single species, *Leptonema illini*.

### **General characteristics**

The species of *Leptospira* are thin, flexible, tightly coiled organisms. The spirochetes measure approximately 0.15  $\mu\text{m}$  in diameter, with a length ranging from 6 to 12  $\mu\text{m}$ . There are generally 12 to 18 spirals in each cell, with each spiral having a length of about 0.4 to 0.5  $\mu\text{m}$ . One characteristic peculiar to most of the *Leptospira* is that the terminal third of the organism is quite flexible and is often seen in the form of a hook. The hook may be on one end or both ends. If on both ends, the hooks may be bent in the same direction or in the opposite directions.

### Clinical significance

Leptospirosis is a zoonotic disease and in humans is primarily associated with occupational exposure. Its reservoirs are wild and domestic animals. Infection usually results from direct or indirect exposure to the urine of infected animals. Work with animals or in rat-infested surroundings poses direct infection hazards. These may include such workers as veterinarians, dairymen, swineherds, miners, fish and poultry processors, and pet owners. Indirect exposure is through contaminated water. Rice farmers, sugarcane workers, sewer workers, military personnel, and swimmers are at risk for indirect sporadic cases. All pathogenic species of *Leptospira* are placed in one species, *L. interrogans*, containing many serovars. Those serovars encountered in the United States include *icterohaemorrhagiae*, *canicola*, *ballum*, *grippotyphosa*, *bataviae*, *autumnalis*, and *pomona*. Certain serotypes are routinely associated with rodents, *canicola* with dogs, *pomona* with cattle and pigs, and *autumnalis* and *grippotyphosa* with mice; however, cross infections do occur. Leptospirosis is an acute illness associated with febrile jaundice and nephritis. Weil first recognized it in 1886 as a clinical entity distinct from other icteric fevers. Referred to as “Weil’s disease”, the infection is caused by a *Leptospira* transmitted to humans from infected rodents. The principal sources of *Leptospira* infecting humans are urine and tissues of infected animals. The usual sequence of events begins when a person becomes infected through the mucosa of the mouth and upper respiratory tract, conjunctiva, or skin contact with urine from an infected animal or by exposure to urine-contaminated water or soil. Incubation period is from two to 20 days and symptoms are fever, chills, rigors, intense headache, myalgia, nausea, and vomiting. After four to seven days, the organisms enter the blood and then invade various tissues and organs, particularly the kidney, liver, meninges, and conjunctiva. One serotype, *icterohaemorrhagiae*, causes a more severe illness referred to as Weil’s disease, icteric leptospirosis, or infectious jaundice. The fatality rate from Weil’s disease may run as high as 10 percent. At this time, there is no standardized in vitro procedure for antibiotic susceptibilities. Physicians can consult different publications for the treatment of microorganisms, where in vitro susceptibility testing is not possible. Examples of these publications are *The Sanford Guide to Antimicrobial Therapy*, *The Merck Manual of Diagnosis and Therapy*, and *Current Medical Diagnosis and Treatment*.

### Laboratory identification

In darkfield microscopy, the organism is actively motile with a progressive undulating movement, and it displays rapid spinning around its long axis. *L. icterohaemorrhagiae* cannot be easily stained by routine procedures. One of the silver impregnation methods works well, however, in trained hands. These methods may be of value when cultural and serological procedures are not possible.

### Cultural growth

Culturally, the leptospiral forms are aerobic and grow best at an optimal temperature of 30°C. Serotype *icterohaemorrhagiae* and some of the other species can be easily cultivated in a liquid medium containing 10% rabbit serum or serum albumin plus fatty acids at pH 6.8 to 7.8. The incubation time for optimal growth ranges from a few days to four weeks or longer, but usually six to 14 days. Fletcher’s semisolid and Stuart’s liquid media containing rabbit serum will grow most strains of leptospires. Colonies on the surface of agar plates are rarely seen. Fluid media inoculated with leptospires become faintly turbid.

### Observation

The observation of *Leptospira* in a darkfield examination of blood would provide strong support; however, unequivocal diagnosis requires that the organisms be grown and identified as *Leptospira* by serological methods. The bacteria can be isolated early in the disease by culturing the patient’s blood or urine and by animal inoculation (guinea pigs or hamsters). Antibodies may be detected in the patient’s serum within one week after the onset of the disease. Various serological techniques have been used, including the microscopic agglutination-lysis test, the genus-specific hemagglutination test, and direct fluorescent-antibody test. Specialized sera, not normally available in the diagnostic

laboratory, are required for identification of specific serotypes. The most useful strain-specific serologic test is the microscopic agglutination test (MAT). This test was the original method for determining antibody response to leptospirosis and remains the reference method.

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### Self-Test Questions

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

#### 401. *Borrelia* species

1. What vectors are responsible for transmitting relapsing fever?
2. How does man become infected with the louse-borne spirochetes that cause relapsing fever?
3. What specimens will contain the spirochetes of *Borrelia* during the febrile period of relapsing fever?
4. What causes the relapses that characterize relapsing fever?
5. What is the causative agent of Lyme disease?
6. How is Lyme disease transmitted to man?
7. What are the reservoir hosts of *B. burgdorferi*?
8. What is the hallmark symptom of Lyme disease?
9. What are the stains used to stain thin- and thick-smears for identification of the *Borrelia*?
10. What are the preferred methods for the diagnosis of Lyme disease?

**402. *Treponema* species**

1. How do you describe the morphological appearance of *Treponema pallidum*?
2. In early syphilis, the spirochete tends to be elongated like an over-stretched spring and movement is more snakelike. What factor is probably responsible for this condition?
3. What disease is caused by *Treponema pallidum* ssp. *pallidum*?
4. How is syphilis transmitted?
5. How does *Treponema pallidum* ssp. *pallidum* enter the skin or mucous membranes?
6. During which stage of syphilis does the chancre usually first appear?
7. During the secondary stage of syphilis, where may the spirochetes of *T. pallidum* ssp. *pallidum* be isolated?
8. What organisms are the causative agents of yaws, nonvenereal syphilis, and pinta, respectively?
9. Why is the spirochete of *Treponema pallidum* difficult to stain with Gram-stain?
10. Staining may be accomplished satisfactorily by what other technique using what principle?
11. Laboratory diagnosis of treponemes is generally based on what two procedures?

**403. *Leptospira* species**

1. How do you describe the characteristics of the species *Leptospira*?
2. What is the major morphological characteristic peculiar to the spirochete of *Leptospira*?

3. How is the disease transmitted to man?
4. What condition is associated with Leptospirosis?
5. All pathogenic species of *Leptospira* are classified as what species?
6. After the organisms enter the blood, what tissues and organs are affected?
7. What serotype causes a more severe illness referred to as Weil's disease?
8. What type of environmental condition is required for growth of *Leptospira*, and at what optimal temperature?
9. Serotype *icterohaemorrhagiae* and some other species can be easily cultivated in a liquid medium containing what substances?
10. What is the incubation time required for optimal growth?
11. Fluid media inoculated with leptospires show what appearance?
12. Unequivocal diagnosis requires that leptospiral organisms are grown and identified by which methods?
13. How soon after the onset of the disease is the antibody detected in the patient's serum?
14. What serological techniques have been used for detection of leptospiral antibodies?
15. Which of the serological techniques is considered the most useful strain specific?



## 1-2. The Genera *Mycoplasma*, *Ureaplasma*, *Chlamydia*, and *Rickettsia*

Members of the class *Mollicutes* (soft skin) have been derived from ancestral anaerobic bacteria (clostridia) because of gene deletion. There are four orders, five families, and eight genera in this class. The genera encountered in humans are *Mycoplasma*, *Ureaplasma*, and *Acholeplasma*. *Chlamydia trachomatis* is now considered one of the most common sexually transmitted diseases. It is estimated that between 2 and 3 million cases occur annually in the United States. *Chlamydia trachomatis* causes a wide spectrum of disease and results in 1 billion dollars in direct and indirect costs. The order *Rickettsiales* includes a very diverse group of microorganisms that primarily infect various arthropods (lice, fleas, ticks, or mites), but may frequently infect humans and other vertebrates as accidental hosts.

### 404. *Mycoplasma* and *Ureaplasma*

The species of the genera *Mycoplasma* and *Ureaplasma* are found throughout the animal and plant kingdom as saprophytes and parasites.

#### *Mycoplasma* species

There are over 100 species of *Mycoplasma* but only 13 have been detected in humans:

*M. pneumoniae*, *M. hominis*, *M. salivarium*, *M. orale*, *M. buccale*, *M. faucium*, *M. lipophilum*, *M. genitalium*, *M. fermentans*, *M. primatum*, *M. spermatophilum*, *M. pirum*, and *M. penetrans*.

#### General characteristics

Mollicutes are prokaryotes that differ from most other bacteria because of their very small size. They are the smallest free-living (extracellular) microorganisms, and are contained only by a cell membrane. Since the mollicutes do not have a cell wall, they are resistant to  $\beta$ -lactam and other cell wall-active antibiotics, are highly pleomorphic, and do not take up Gram-stain. Mollicutes vary in size from 0.2 to 0.3  $\mu\text{m}$ ; hence they are about the size of poxviruses. By darkfield and phase-contrast microscopy, they appear as round or filamentous depending on the mycoplasmal species.

#### Clinical significance

The mycoplasmas of human origin colonize the oropharynx and genitourinary tracts. *M. pneumoniae* is the major causative agent of primary atypical pneumonia, also known as “walking pneumonia”. *M. pneumoniae* infections tend not to be seasonal and are usually mild. Transmission is via respiratory tract secretions and requires close person-to-person contact. Children, young adults, and immunocompromised patients are the most susceptible. *M. salivarium* is commonly found in the gingival crevice, especially in patients with periodontal disease. Other inhabitants of the oral cavity are *M. orale*, *M. buccale*, *M. faucium*, *M. lipophilum*, and *M. fermentans*. *M. fermentans* has been associated with adult respiratory distress syndrome with or without systemic disease. *M. hominis* and *M. genitalium* are found in the genitourinary tract of both males and females. *M. genitalium* detection has increased in urethral specimens from men with acute nonchlamydial, nongonococcal urethritis (NGU). *M. hominis* has been isolated from blood specimens from patients with postabortal and postpartum fever, solid organ transplants, trauma, and genitourinary manipulations. It has also been found in wound infections, brain abscesses, and osteomyelitis lesions. Sternal wound infections caused by *M. hominis* in heart-lung transplant patients is becoming quite common. Immunocompromised hosts, including AIDS patients, are at risk for extrapulmonary and extragenital mycoplasmal infections.

#### Laboratory identification

Swab specimens are suitable for mycoplasmal cultures, but swabs should not be exposed to antiseptics, analgesics, or lubricants due to their inhibitor effect. Swabs from any site should be transported in mycoplasmal transport medium and not be allowed to dry. Because of their size and genetic make-up, the mollicutes are very fastidious. They need cholesterol from serum supplements and preformed nucleic acid precursors from yeast extract in both broth and agar mediums. Growth

from broth is, at most, a faint haze after six hours of incubation and the organism yield is very low. On blood agar *M. hominis* produces nonhemolytic pinpoint colonies after two days incubation at 35°C in an atmosphere of 95% N<sub>2</sub> plus 5% CO<sub>2</sub>, and on A7 agar they have a characteristic “fried-egg” appearance. However, *M. pneumoniae* colonies require 20 days or more for growth on specialized (Edward-Hayflick agar) media at 35°C in air and 5% CO<sub>2</sub>, and they usually do not appear as fried-eggs. In view of their growth characteristics and requirements, most laboratories do not perform mycoplasma cultures. Laboratory diagnosis is usually made serologically using complement fixation (CF), ELISA, and IFA tests. Polymerase chain reaction (PCR) testing is more widely available in reference laboratories for detection. Nonspecific cold agglutinins occur in approximately 50% of the patients with primary atypical pneumonia.

### ***Ureaplasma species***

*Ureaplasma urealyticum* is the only species in the genus *Ureaplasma*. General characteristics of *Ureaplasma* are identical to the other species in the class *Mollicutes*.

### ***Clinical significance***

*U. urealyticum* has been isolated from internal organs of infants, spontaneously aborted fetuses, and stillborn and premature infants at a higher rate than from normal full-term infants. Recent studies have shown a correlation between *U. urealyticum* colonization and fetal morbidity. *U. urealyticum* as well as *M. hominis* have been acquired in utero or during birth. In the very low birth weight newborn, they are associated with respiratory disease, chronic lung disease, and even death. Both organisms can invade the cerebrospinal fluid of the premature infant within the first few days of life. They produce a mild subclinical meningitis to neurologic damage with permanent handicaps.

### ***Laboratory identification***

Commercially prepared Shepard’s A7–B agar is usually satisfactory for the cultivation of *Ureaplasma*. After 48 hours in 5% CO<sub>2</sub>, or under anaerobic conditions, the colonies appear dark-brown in color and must be visualized under 100 X magnification stereoscope. Unfortunately, at this time, most serological tests are not ready for routine clinical use in the laboratory for the diagnosis of *Ureaplasma*.

## **405. *Chlamydia* and *Rickettsia***

*Chlamydia*, *Rickettsia*, and other related bacteria are grouped together because of their small size, unusual cell wall structure, and obligate intracellular parasitism. The application of molecular methods continues to change and add to their species classification and identification.

### ***Chlamydia species***

The organisms that are today known as *Chlamydia* have had a variety of names and classifications in the past. In the early 1950s, the agents of trachoma and inclusion conjunctivitis were accepted as members in the psittacosis-lymphogranuloma venereum (LGV) group of viruses. This group became known as the PLT group of atypical viruses. This nomenclature persisted until the 1960s when the name “*Bedsoniae*” came into use. The *Bedsonia* organisms were named after Sir Samuel Bedson, who isolated and characterized the agent of psittacosis. Shortly afterwards, microbiologists recognized that these organisms were not viruses, but bacteria, and assigned them to a separate genus, *Chlamydia*. The genus currently contains four species, *C. trachomatis*, *C. pneumoniae*, *C. psittaci*, and *C. pecorum*.

### ***General characteristics***

The chlamydiae are a group of parasitic organisms that infect vertebrates and may occasionally be associated with arthropods. They may elicit a variety of well-defined disease patterns, but exist more commonly in the natural host as subclinical infections. In the past, chlamydiae were thought to be viruses because, like viruses, they will not grow on artificial media. They differ from viruses, however, in that chlamydiae contain both ribonucleic acid (RNA) and DNA, possess ribosomes,

multiply by binary fission, are susceptible to certain antibiotics, and have a cell wall similar to bacteria. The chlamydiae are nonmotile, gram-negative organisms that have a similar morphology, share a common group antigen, and possess some metabolically active enzymes. Chlamydiae attaches to the heparan sulfate-like molecule on the surface of the host cell through a receptor on the elementary body (EB). Elementary bodies are rigid structures that envelop the infectious particles of the chlamydiae and are approximately 0.3  $\mu\text{m}$  in diameter, as shown in figure 1–3. The susceptible host cells then ingest the chlamydiae by a mechanism similar to receptor-mediated endocytosis. They go through a unique developmental cycle within the cytoplasm of their host's cells. These obligate intracellular parasites are now regarded as bacteria that lack some important mechanism for producing metabolic energy. More specifically, they have been called energy parasites, since they use the adenosine triphosphate (ATP) produced by the host cell for their own requirements.

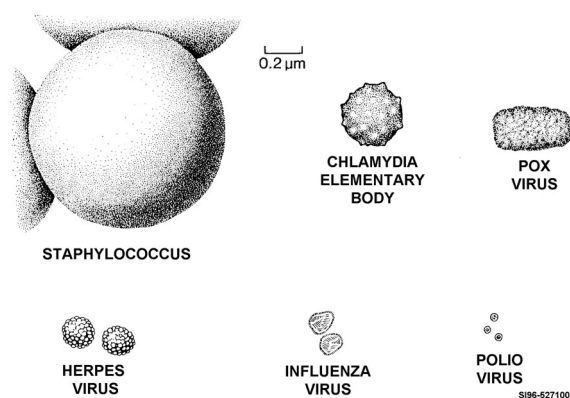


Figure 1–3. Size comparison of *Staphylococcus*, *Chlamydia*, and viruses.

### ***Clinical significance***

*Chlamydia* organisms have a wide host range within the vertebrates. Numerous species of birds and mammals, including man, may become infected. The organisms of *C. psittaci* are primarily animal pathogens; those of *C. trachomatis* and *C. pneumoniae*, primarily human pathogens. Human infections with the *C. psittaci* organisms of psittacosis or ornithosis are usually accidental infections resulting from contact with infected birds, most notably parakeets, parrots, and turkeys. As human pathogens, the organisms of *C. trachomatis* cause a number of diseases. Primarily, these are diseases of the eye (trachoma and inclusion conjunctivitis) and urogenital tract (lymphogranuloma venereum, urethritis, and cervicitis). In men, *C. trachomatis* is associated with nongonococcal urethritis, epididymitis, and Reiter's syndrome. In women, cervicitis, urethritis, endometritis, and salpingitis—which can produce tubal scarring, infertility, and ectopic pregnancy. Other diseases in which *Chlamydia* are implicated are infant pneumonia and pelvic inflammatory disease (PID).

*C. pneumoniae* is the causative agent of a variety of respiratory tract diseases. Chlamydial infections range in severity from blinding trachoma and complicated LGV to asymptomatic cases that serve as reservoirs of infection. Chlamydia organisms can coexist with other infectious agents, such as *Neisseria gonorrhoeae*, and be transmitted simultaneously with them. Chlamydial infections can be treated with certain antibiotics such as tetracycline, erythromycin, chloramphenicol, and sulfonamides (*C. trachomatis* infections only). Of these, tetracycline is usually the drug of choice unless contraindicated by conditions such as pregnancy. Penicillin therapy, the routine treatment for syphilis and gonorrhea, is ineffective in treating chlamydial infections. Although infection with chlamydial organisms does stimulate antibody production, the development of protective immunity is faulty. Relapses and re-infections are not uncommon. In some of the diseases (trachoma, for example), the symptomatology becomes more severe with repeated infections. Treatment also becomes more difficult. Chlamydial infections can be subdivided into interrelated groups that are based on the clinical disease, the route of transmission, the distribution of the diseases, and the prevalent immunotypes (see following table). *C. trachomatis* organisms inhabit primarily the eye or the genital

tract and can be transmitted from these sites in three ways: (1) eye to eye; (2) genital tract to genital tract; and (3) genital tract to other susceptible tissue, including the eye. Chlamydial diseases are distributed worldwide.

| Transmission                       | Diseases                    | Infected Sites             | Distribution   | Predominate Immunotypes                          |
|------------------------------------|-----------------------------|----------------------------|--|--|
| Eye to Eye                         | Trachoma                    | Eyes                       | Endemic:<br>Southeast Asia,<br>United States-<br>Cultural Groups | A, B, Ba, C                                      |
| Genital to Genital                 | LGV                         | Lymph Nodes                | Tropics, United<br>States Select<br>populations                  | L <sub>1</sub> , L <sub>2</sub> , L <sub>3</sub> |
| Genital to Genital                 | Urethritis                  | Urethra                    | Worldwide  | D-K  |
| Genital to Genital                 | Female Infections           | Cervix, Fallopian<br>Tubes | Worldwide  | D-K  |
| Genital to Other<br>Tissue—Adults  | Inclusion<br>Conjunctivitis | Eyes                       | Worldwide  | D-K  |
|                                    | Nonendemic<br>Trachoma      | Eyes                       | Sporadic,<br>Worldwide   |  |
| Genital to Other<br>Tissue—Infants | Inclusion<br>Conjunctivitis | Eyes                       | Worldwide  | D-K  |
|                                    | Pneumonia                   | Lungs                      | Probably<br>Worldwide  |  |

### **Laboratory identification**

Three general approaches are used in the laboratory diagnosis of chlamydial infections: (1) direct examination of clinical specimens, (2) isolation of the organisms, and (3) detection of the antibodies. Swabs, scrapings, and small tissue samples are suitable for chlamydiae isolation. Specimens should be placed in a special transport medium and should only be handled in appropriate containment facilities.

#### *Direct examination*

Direct examination of conjunctival scrapings for the presence of *Chlamydia* inclusions is an established method for diagnosing active eye infections. Properly performed, direct examination is as sensitive as isolation for diagnosing ocular infection in the newborn. In adult infection, the sensitivity is somewhat reduced but still of value, particularly in patients who have been treated with antibiotics which may interfere with the recovery of the organism. Direct examination of genital material for diagnosis of *Chlamydial* infections is of limited value. For direct examination, a slide is prepared from the clinical specimen, stained, and microscopically examined for the presence of *Chlamydial* organisms. Giemsa and immunofluorescent staining are the two most widely used procedures.

#### *Isolation*

Isolation of the *Chlamydial* organism is the most sensitive method for the laboratory diagnosis of both male and female infections of the genital tract. Since *Chlamydia* species are obligate intracellular parasites, they require a living host in which to multiply. All types of *Chlamydia* may be grown in egg yolk sacs and cell cultures, but the cell culture is the most sensitive, simplest, and safest method for the isolation of *C. trachomatis*. *Chlamydia* organisms grow in a number of cell types, but mouse L cells (McCoy cells) and HeLa are most commonly used.

#### *Detecting antibodies*

Because of a number of problems, serological testing for the diagnosis of chlamydial infections is limited. No single test is totally satisfactory for all the chlamydiae, but complement fixation is the

most widely used serological test. Antibody response may be inadequate for measurement by certain tests. Paired sera may be difficult to obtain because the infection may not be apparent or may have a long incubation, which thwarts the collection of acute serum. High-risk populations have high rates of background antibodies. In sexually transmitted diseases clinics, approximately 25% of the men and over 60% of the women from whom the organism is not isolated have *Chlamydia* antibodies. Serum antibodies persist for extended periods. Detection of antibodies in a single serum indicates only previous exposure to the organism. A fourfold rise in antibody titer between an acute and a convalescent serum when accompanied by clinical symptomology supports the diagnosis of a chlamydial infection. A commercially available DNA probe test (GenProbe) has been developed for identification of *Chlamydia* species.

### ***Rickettsia* species and other related organisms**

The order of *Rickettsia* includes the genera, *Rickettsia*, *Coxiella*, *Ehrlichia*, *Cowdria*, *Neorickettsia*, *Rochalimaea*, *Afipia*, and *Bartonella* (some species were formerly known as *Rochalimaea* species).

### ***General characteristics***

The *Rickettsia* and other related bacteria are morphologically and biochemically similar to other gram-negative bacteria. The *Rickettsia* species are gram-negative, short, rod-shaped or coccobacillary organisms. The other related bacteria vary from short, rod-shaped microorganisms to extremely pleomorphic rods.

### ***Clinical significance***

This group of microorganisms produces diseases with many common features, usually a fever that can be acute or chronic and mild to severe. Untreated cases can result in death. The following table is a review of the most common and clinically significant microorganisms in this group that affect humans.

| Organism   | Diseases   | Natural Host                   | Mode of Transmission                    | Distribution                                |
|--|--|--------------------------------|---|---|
| Spotted fever rickettsiae group                  | Rocky Mountain spotted fever (RMSF)  | Ticks, mites                   | Tick or mite bite                       | Western Hemisphere                          |
| Typhus rickettsiae group                         | Epidemic, recrudescent (Brill-Zinsser), sporadic, and murine typhus                      | Fleas, lice, mammals           | Flea or louse feces                     | Worldwide—area is species dependent         |
| <i>Rickettsia tsutsugamushi</i>                  | Scrub typhus   | Chiggers                       | Chigger bite                            | Asia, northern Australia, Pacific Islands   |
| <i>Coxiella burnetii</i>                         | Q-fever  | Mammals, ticks                 | Infectious aerosol                      | Worldwide                                   |
| <i>Ehrlichia chaffeensis</i> group               | Human ehrlichiosis   | Human, dogs, ticks             | Tick bite                               | United States                               |
| <i>Ehrlichia sennetsu</i> group                  | Sennetsu ehrlichiosis  | Human, other unknown           | Unknown, infected salmon fluke          | Japan                                       |
| <i>Bartonella</i> species ( <i>Rochalimaea</i> ) | Oroya fever, bacteremia and bacillary angiomatosis in AIDS patients, cat scratch disease | Sand flies, humans, cats, lice | Sand fly bite, cat scratch, louse feces | Oroya fever-South American Andes, Worldwide |
| <i>Afipia</i> species                            | Cat scratch disease  | Unknown (soil?)                | Wound contamination                     | Worldwide                                   |

**Laboratory identification**

There are three basic approaches to the laboratory diagnosis of rickettsial diseases: (1) isolation of rickettsiae from tissues, (2) direct detection of rickettsiae in patient tissues, and (3) serological test for rickettsial antibodies. Isolation of living rickettsiae is hazardous and requires biosafety level 3 containment facilities. Therefore, cultivation of rickettsiae is not recommended for routine diagnosis. Immunofluorescence, CF, microagglutination, ELISA, latex agglutination, indirect hemagglutination (IHA), radioimmunoassay (RIA), and the Weil-Felix have been used or studied for the diagnosis of rickettsial infections. The basis of the Weil-Felix test is the fact that several *Rickettsia* and *Proteus* OX (OX-2, OX-19, and OX-K) strains share common antigens and can be tested serologically. This method can be used for the diagnosis of typhus, scrub typhus, and RMSF. Although no technique constantly provides a diagnosis early enough to affect the outcome of the disease, several new rapid diagnostic techniques have shown promise in research laboratories and these tests may soon be available to the clinical laboratory. These procedures include PCR technology, detection of the antigen in the peripheral blood cells, and immunoglobulin M (IgM) antibody capture immunoassays. Most *Ehrlichia* species can be identified by direct microscopic examination of Giemsa- or Diff-Quik-stained peripheral blood buffy coat smears. A cluster of round, dark-purple-stained, small dots or clusters of dots (morulae) in the cytoplasm of leukocytes indicates *Ehrlichia* organisms are present. The specimen source for most isolates of *Bartonella* and *Afipia* species is blood or tissue. Culture protocols designed to yield other slow-growing organism can also result in the recovery of *Bartonella* species since most of these organisms require more than seven days of incubation before they can be detected. Cell culture techniques (HeLa cell line) are the most successful for the isolation of *Afipia* species. Serological test for the diagnosis of *Bartonella* species are being developed at this time.

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**Self-Test Questions**

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

**404. *Mycoplasma* and *Ureaplasma***

1. Match each 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, more than once, or not at all.

*Column A*

- \_\_\_ (1) Major causative agent of primary atypical pneumonia.
- \_\_\_ (2) Has characteristic “fried-egg” appearance on A7 agar.
- \_\_\_ (3) Associated with sternal wound infections in heart-lung transplant patients.
- \_\_\_ (4) Found in urethral specimens from men with NGU.
- \_\_\_ (5) Associated with colonization and fetal morbidity.

*Column B*

- a. *M. genitalium*.
- b. *M. pneumoniae*.
- c. *M. hominis*.
- d. *U. urealyticum*.

**405. *Chlamydia* and *Rickettsia***

1. How many species are contained in the genus *Chlamydia*?
2. How do Chlamydiae differ from viruses?

3. Why are Chlamydiae called energy parasites?
4. How are human infections with *C. psittaci* acquired?
5. What are the primary diseases of man caused by *C. trachomatis*?
6. What are the three modes of transmission for *C. trachomatis*?
7. What three approaches are used in the laboratory diagnosis of chlamydial infections?
8. What is the most sensitive method for the laboratory diagnosis of chlamydial infections of the genital tract?
9. Match each 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, more than once, or not at all.

*Column A*

- \_\_\_\_ (1) Transmission by infected salmon fluke.
- \_\_\_\_ (2) Causative agent(s) of Q-fever.
- \_\_\_\_ (3) Transmission by chigger bite.
- \_\_\_\_ (4) Causative agent(s) of cat scratch disease.
- \_\_\_\_ (5) Transmission by tick bite.
- \_\_\_\_ (6) Causative agent(s) of human ehrlichiosis.
- \_\_\_\_ (7) Transmission by sand fly bite and louse feces.
- \_\_\_\_ (8) Causative agent(s) of bacillary angiomatosis in AIDS patients.

*Column B*

- a. Spotted fever rickettsiae group.
- b. Typhus rickettsiae group.
- c. *Rickettsia tsutsugamushi*.
- d. *Coxiella burnetii*.
- e. *Ehrlichia chaffeensis* group.
- f. *Ehrlichia sennetsu*.
- g. *Bartonella (Rochalimaea)* species.
- h. *Afipia* species.

### 1-3. Introduction to Virology

Virology is a rapidly expanding field that for years was ignored by many microbiologists. However, with the advent of acquired immunodeficiency syndrome (AIDS) and other devastating diseases caused by viruses, virology has moved to the forefront of microbiology and into the public spotlight. An extensive review of virology would be impossible in the short space allotted. Therefore, we will concentrate on the following areas:

- Viral structure and replication.
- Serologic diagnosis of viral infections.
- Collection and shipping procedures.

Careful study of this section will provide a greater understanding of virology and better equip you to deal with requests for viral diagnosis.

#### 406. Classification and general characteristics of viruses

Early classification schemes for viruses were based on host range, type of tissue affected, host response, and type of lesion produced. These criteria were frequently subject to change. The International Committee on Taxonomy of Viruses (ICTV) has recently approved 73 families and groups of viruses.

##### Classification

Refer to figures 1–4, 5, 6, and 7. Currently, viruses are separated into families based on the type and form of their nucleic acid genome, size, shape, substructure, and mode of replication of the virus particle. The three key properties upon which the 73 families and groups are classified are the kind of nucleic acid (DNA or RNA), type of strandedness (single- or double-stranded), and the presence or absence of a lipoprotein envelope. Enveloped viruses are susceptible to chemical agents such as chloroform and ether.

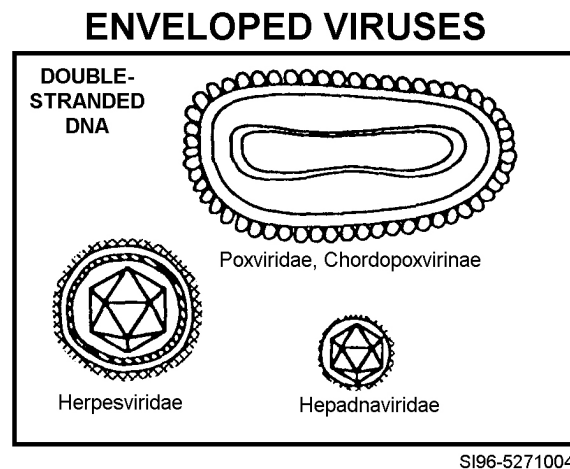


Figure 1–4. Enveloped DNA viruses.

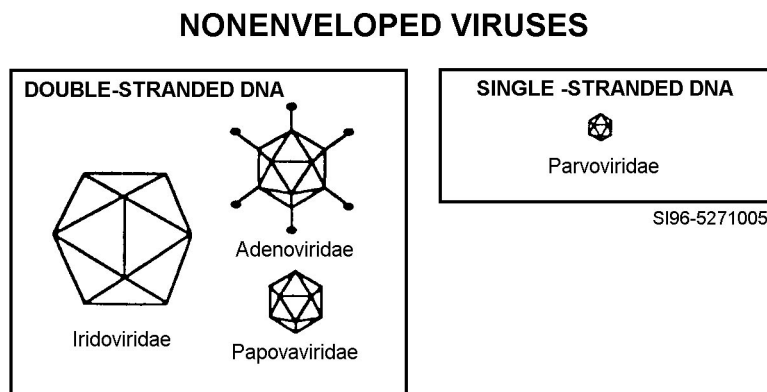
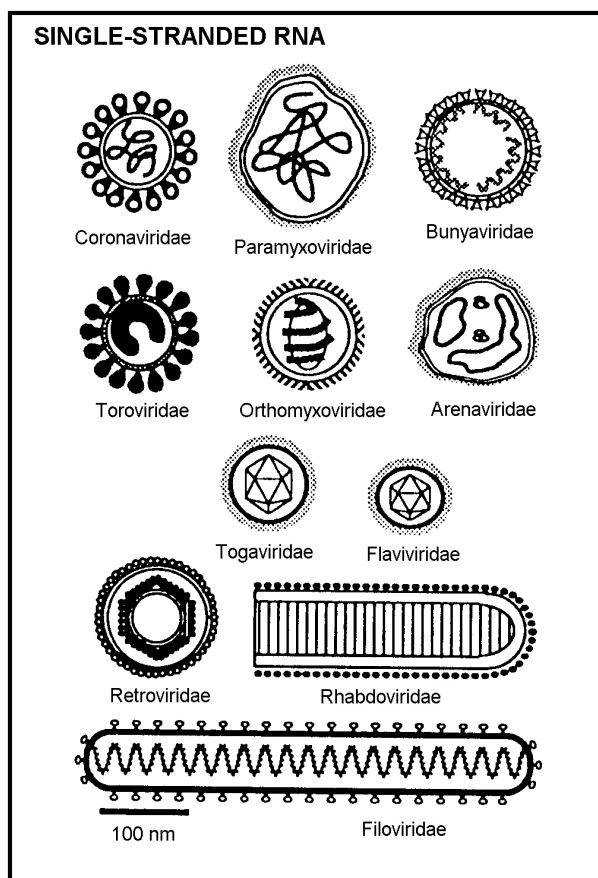


Figure 1–5. Non-enveloped DNA viruses.



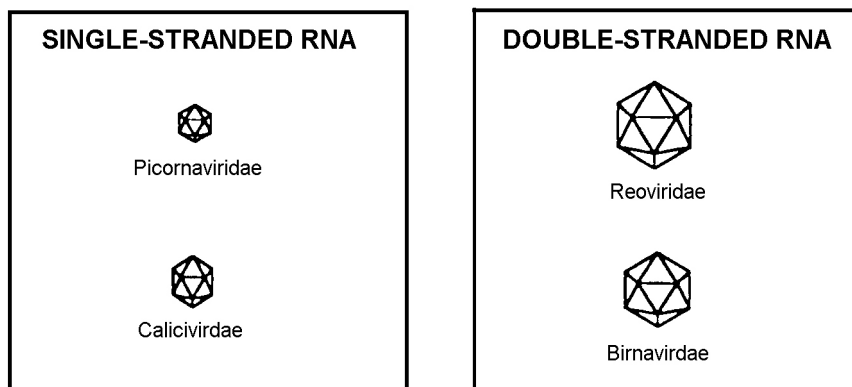
## ENVELOPED VIRUSES



SI96-5271006

Figure 1-6. Enveloped RNA viruses.

## NONENVELOPED VIRUSES



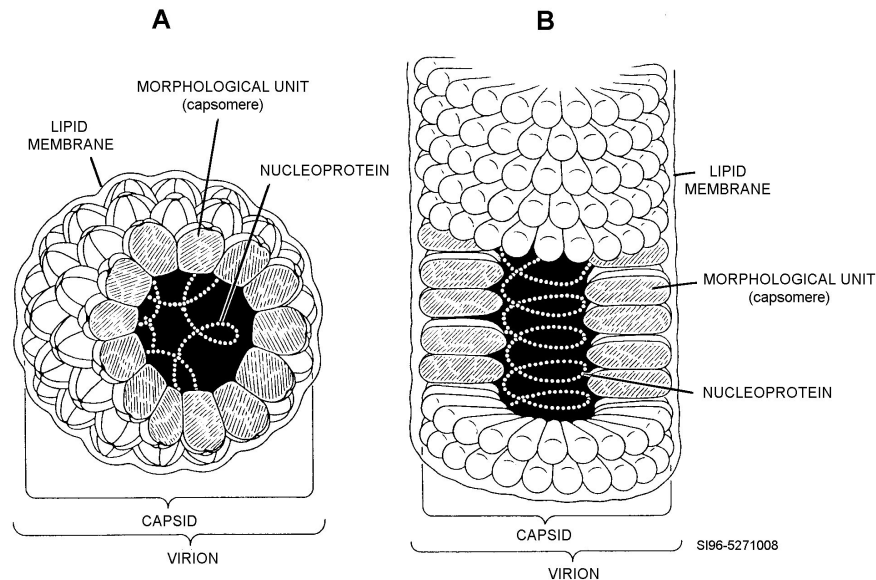
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Figure 1-7. Non-enveloped RNA viruses.

### Structure

Viruses are a unique group of organisms, originally distinguished by their ability to pass through bacterial filters. However, size is only one distinguishing characteristic. Viruses consist of an inner

core of nucleic acid, either DNA or RNA (never both), and an outer protein coat. Certain viruses are surrounded by an envelope containing cellular lipids and viral proteins (glycoprotein spikes). The entire virus is called the virion or viral particle. A protein coat called a capsid surrounds the nucleic acid genome. The capsid is composed of repeating units of protein called capsomers or morphological units. The two most common shapes (symmetry) of the protein coat may be either icosahedral or helical (fig. 1-8).



**Figure 1-8. Viral construction—(A) icosahedral shape and (B) helical shape.**

### **Replication**

Viruses lack enzymes and are unable to replicate outside a host cell. For this reason, they are referred to as obligate intracellular parasites. The steps, or phases in virus replication are also known as the infectious cycle. This cycle can be divided into the following phases:

1. Attachment and penetration.
2. Uncoating and host cell synthesis of viral components.
3. Assembly of components into complete virus particles.
4. Release of virus from host cell.

A virus attaches to a host cell and is either engulfed into the cell through the process of phagocytosis, the viral envelope fuses with the cell membrane, or it injects its nucleic acid core into the cell. Once inside the cell and free of its protein coat (uncoating), the nucleic acid (DNA or RNA) directs the host cell to manufacture the necessary enzymes and proteins required for viral replication and structural components. The host cell, according to the genetic code of the invading virus, assembles protein components and newly manufactured nucleic acids into complete infectious virus particles. The completed virus particles are then released from the cell by budding through the cell (the lipoprotein envelope is acquired in this manner) or through cell lysis. A host cell can respond to a viral infection in three ways.

1. Abortive infection or failed infection.
2. Lytic infection or cell death.
3. Persistent infection or virus production without cell death.

There is no effect on the fate of the cell with abortive infections. Lytic infections result in the death of the cell because viral replication is often incompatible with essential cell functions and viability. This

may be brought about through the prevention of the synthesis of cellular macromolecules, or production of degradative enzymes and toxic proteins. A persistent infection occurs in an infected cell that is not killed by the virus, because the viruses are gently released from the cell or by budding from the plasma membrane. Some viruses establish persistent infections that can also stimulate uncontrolled cell growth, causing transformation or immortalization of the cell. These viruses are called “oncogenic viruses” which can rapidly cause tumors. Clinical significance

The following tables identify the members of the virus families, genera, common species, mode of transmission, and diseases caused by the DNA and RNA viruses that most commonly infect humans.

| DNA VIRUS CHART                                      |                          |   |  |  |
|--|--------------------------|---|--|--|
| Family/Subfamily of DNA Viruses                      | Genus                    | Common Species                                    | Mode of Transmission   | Disease(s)   |
| <i>Adenoviridae</i>                                  | <i>Mastadenovirus</i>    | Human adenovirus                                  | Fecal oral, aerosol droplet, direct contact (eye)                                | Conjunctivitis, keratitis, pharyngitis, pneumonia, hemorrhagic cystitis, gastroenteritis           |
| <i>Hepadnaviridae</i>                                | <i>Hepadnavirus</i>      | Hepatitis B virus-HBV                             | Infected secretions including sexual contact and blood products                  | Hepatitis, cirrhosis, hepatocellular carcinoma   |
| <i>Herpesviridae</i><br>- <i>Alphaherpesvirinae</i>  | <i>Simplexvirus</i>      | Human herpes simplex virus 1 & 2—<br>HSV-1, HSV-2 | Infected oral or<br>Infected secretions (sexual), blood transplacentally         | Local skin lesions, neonatal sepsis, conjunctivitis, keratitis, pharyngitis, cervicitis, pneumonia |
|  | <i>Varicellovirus</i>    | Varicella-zoster virus-VZV (herpes virus 3)       | Infected oral secretions   | Chickenpox (varicella), shingles (zoster)  |
| <i>Herpesviridae</i><br>- <i>Betaherpesvirinae</i>   | <i>Cytomegalovirus</i>   | Human cytomegalovirus-CMV (herpes-virus 5)        | Infected secretions, blood transfusions, organ transplants, and transplacentally | Asymptomatic infection, congenital infection, hepatitis, mononucleosis pneumonia                   |
| <i>Herpesviridae</i><br>- <i>Gammapherpesvirinae</i> | <i>Lymphocryptovirus</i> | Epstein-Barr virus-EBV (herpes-virus 4)           | Infected oral secretions   | Infectious mononucleosis hepatitis, pharyngitis, Burkitt's lymphoma, nasopharyngeal carcinoma      |
| <i>Papovaviridae</i>                                 | <i>Papillomavirus</i>    | Human papilloma-viruses-HPV (warts)               | Contact with infected secretions (sexual)  | Cutaneous, genital and laryngeal warts, head and neck tumors                                       |
|  | <i>Polyomavirus</i>      | JC and BK viruses of humans                       | Contact with infected secretions   | Progressive multifocal leuko-encephalopathy (JCV), hemorrhagic cystitis-kidney (BKV)               |
| <i>Parvoviridae</i>                                  | <i>Parvovirus</i>        | Human parvovirus B19                              | Close contact, probably respiratory  | Gastroenteritis, erythema infectiosum of childhood, hemolytic                                      |

| DNA VIRUS CHART                                |                      |                                  |                                 |   |
|--|----------------------|----------------------------------|---------------------------------|---|
| Family/Subfamily of DNA Viruses                | Genus                | Common Species                   | Mode of Transmission            | Disease(s)  |
|  |                      |                                  |                                 | disease, fetal infection and still birth              |
| <i>Poxviridae</i><br>- <i>Chordopoxvirinae</i> | <i>Orthopoxvirus</i> | Variola-smallpox virus, vaccinia | From infected humans or animals | Cutaneous ulcerative lesions<br>smallpox (eradicated) |

| RNA VIRUS CHART         |                       |  |  |  |
|-------------------------|-----------------------|--|--|--|
|                         |                       |  |  |  |
| <i>Arenaviridae</i>     | <i>Arenavirus</i>     | Lymphocytic chorio-meningitis virus  | Secretions from infected rodents or patients | Febrile illness, meningitis  |
|                         |                       | Lassa fever virus, South American hemorrhagic fevers   | Secretions from infected rodents or patients | Hemorrhagic fevers   |
| <i>Bunyaviridae</i>     | <i>Bunyavirus</i>     | California encephalitis viruses, LaCrosse virus  | Bite of infected mosquitoes                  | Febrile illness, aseptic meningitis, encephalitis  |
|                         | <i>Hantavirus</i>     | Hantaan virus  | Secretions from infected rodents             | Hemorrhagic fever with renal syndrome, kidney disease, acute respiratory failure (approximately 50% fatality rate) |
| <i>Caliciviridae</i>    | <i>Calicivirus</i>    | Norwalk gastro-enteritis virus   | Fecal-oral, contaminated water               | Gastroenteritis  |
| <i>Coronaviridae</i>    | <i>Coronavirus</i>    | Human coronavirus  | Person to person, presumably by aerosol      | Common cold, rarely lower respiratory disease  |
| <i>Filoviridae</i>      | <i>Filovirus</i>      | Marburg virus, Ebola virus   | Unknown but close contact required           | Hemorrhagic fever with fatality rates as high as 90%   |
| <i>Flaviviridae</i>     | <i>Flavivirus</i>     | Yellow fever virus, St. Louis encephalitis, dengue, Mosquito-borne viruses, Tick-borne viruses | Bites from infected mosquito and ticks       | Febrile illnesses, encephalitis, hemorrhagic fever   |
|                         |                       | Hepatitis C virus  | Parenteral or sexual                         | Acute and chronic hepatitis  |
| <i>Orthomyxoviridae</i> | <i>Influenzavirus</i> | Influenza virus type A, B, and C   | Aerosol droplet spread from person to person | Common cold, respiratory infections, pneumonia, in children-croup, bronchiolitis, otitis media                     |
| <i>Paramyxoviridae</i>  | <i>Paramyxovirus</i>  | Human parainfluenza viruses  | Contact with respiratory secretions          | Common cold, tracheo-bronchitis, rarely pneumonia  |

| RNA VIRUS CHART                   |                      |   |  |  |
|-----------------------------------|----------------------|---|--|--|
|                                   |                      |   |  |  |
|                                   | <i>Morbillivirus</i> | Mumps virus   | Droplet aerosol, person-to-person                            | Parotitis, pancreatitis, orchitis, meningitis  |
|                                   |                      | Measles virus   | Droplet aerosol or direct inoculation                        | Rash, pneumonia, encephalitis, subacute sclerosing pan-encephalitis  |
|                                   | <i>Pneumovirus</i>   | Human respiratory syncytial virus-RSV                                     | Person-to-person by hand and respiratory secretions          | Tracheo-bronchitis and pneumonia especially in young children  |
| <i>Picornaviridae</i>             | <i>Enterovirus</i>   | Polioviruses, Coxsackie-viruses group A and B, Echoviruses, Enteroviruses | Fecal-oral from human to human or contaminated water         | Polio, febrile illness, meningitis, encephalitis myocarditis, pericarditis, acute hemorrhagic conjunctivitis |
|                                   | <i>Heparnavirus</i>  | Hepatitis A virus   | Fecal-oral from human to human or contaminated water         | Hepatitis  |
|                                   | <i>Rhinovirus</i>    | Rhinovirus  | Human respiratory tract by aerosol or on hands               | Common cold  |
| <i>Reoviridae</i>                 | <i>Reovirus</i>      | Reoviruses  | Fecal-oral by direct contact                                 | Gastroenteritis  |
|                                   | <i>Orbivirus</i>     | Colorado tick fever and Kemerovo viruses                                  | Tick or animal bites   | Febrile illnesses  |
|                                   | <i>Rotavirus</i>     | Human rotaviruses   | Animal to human  | Gastroenteritis  |
| <i>Retroviridae -Oncovirinae</i>  | HTLV-BLV group       | Human T-cell lymphotropic virus types 1 and 2                             | Unknown  | T-cell leukemia and lymphoma, tropical spastic paraparesis   |
| <i>Retroviridae -Spumavirinae</i> | <i>Supmavirus</i>    | Syncytial and foamy viruses   | Unknown  | Asymptomatic infections  |
| <i>Retroviridae -Lentivirinae</i> | <i>Lentivirus</i>    | Human immuno-deficiency virus-HIV   | Transfusions, contaminated needles, body secretions (sexual) | Acquired immuno-deficiency syndrome (AIDS) 100% fatal after many years                                       |
| <i>Rhabdoviridae</i>              | <i>Lyssavirus</i>    | Rabies virus  | Bite from infected animal                                    | Encephalitis   |
|                                   | <i>Vesiculovirus</i> | Vesicular stomatitis virus-VSV  | Infected animal secretions                                   | Veterinary disease (VSV)   |
| <i>Togaviridae</i>                | <i>Rubivirus</i>     | Rubella virus   | Aerosols or transplacental                                   | Rash illness, congenital infection   |
|                                   | <i>Alphavirus</i>    | Sindbis virus and other mosquito-borne viruses                            | Mosquito or tick bites                                       | Hepatitis, meningitis, encephalitis, arthritis, febrile illness  |

The following list presents the incubation periods of the most common viral infections.

| Disease                   | Days | Disease                  | Days       |
|---------------------------|------|--------------------------|------------|
| Influenza                 | 1–2  | Smallpox                 | 12–14      |
| Common cold               | 1–3  | Chickenpox               | 13–17      |
| Bronchiolitis, croup      | 3–5  | Mumps                    | 16–20      |
| Acute respiratory disease | 5–7  | Rubella                  | 17–20      |
| Dengue                    | 5–8  | Hepatitis A              | 15–40      |
| Herpes simplex            | 5–8  | Infectious mononucleosis | 30–50      |
| Enteroviruses             | 6–12 | Rabies                   | 30–100     |
| Poliomyelitis             | 5–20 | Hepatitis B              | 50–150     |
| Measles                   | 9–12 | Warts                    | 50–150     |
|                           |      | AIDS                     | 1–10 years |

### Treatment

Viral diseases are not susceptible to antibiotics, but antibiotics are used to prevent complications in patients who are vulnerable to bacterial infections. New antiviral drugs, except for topical antiseptics, are always being developed and many have been released in the last 10 years. This diverse group of drugs is divided into two basic categories: antiviral agents and antiretroviral agents. Unlike bacterial antibiotics, there are only a few antiviral drugs. Most of the antiviral agents interfere with viral DNA polymerase, which inhibits viral replication. Other agents interfere with viral entry into the host cell. Acyclovir, Amantadine, Famciclovir, Foscarnet, Ganciclovir, Interferon Alfa, Ribavirin, Rimantadine, and Vidarabine are antiviral agents. They are used in the treatment of herpes simplex, herpes zoster, EBV, CMV, influenza A, chronic hepatitis B and C, and varicella. The antiretroviral agents are Didanosine-ddI, Stavudine-d4T, Zalcitabine-ddC, and Zidovudine-AZT, which are used almost exclusively for the treatment of HIV infection. All of the agents work by inhibiting the actions of HIV reverse transcriptase once they enter the host cell. Keep in mind that these drugs do not necessarily kill the virus or viruses, but they slow down the disease processes and/or treat the symptoms of infection.

### 407. Laboratory identification, collecting, and shipping of viral specimens

Laboratory identification from a microbiologist in a clinical lab is limited; nonetheless, it is appropriate to review the basic procedures in this CDC. In virology, as in other areas of microbiology, it is imperative that the proper specimen is collected in the proper manner and, if necessary, shipped under appropriate conditions. Techniques for collecting specimens for viral analysis are available in many references and will briefly be discussed here.

#### Laboratory identification

The standard by which all diagnostic virology procedures are measured is the isolation and identification of a virus in cell or tissue cultures, or by direct detection of viral components in infected tissues or fluids using an electron microscope. However, relatively few hospital laboratories are equipped to perform virus isolation or electron microscopy. Therefore, they must rely on serodiagnostic procedures. Viral infections elicit immune responses directed against one or more antigens (for example; core and envelope proteins and/or glycoprotein spikes). Antibodies of the IgM class appear initially and are followed by IgG antibodies. IgM antibodies disappear within several weeks, while IgG antibodies may persist at detectable levels for years or a lifetime. By demonstrating the presence of IgM antibodies or a rise in the titer of IgG antibodies, a viral infection may be diagnosed.

There are many serodiagnostic tests used in diagnostic virology. This section will briefly concentrate on the following: ELISA, immunofluorescence, hemagglutination inhibition (HI), and CF. These

procedures can be used to measure both antigen and antibody. A more in-depth study is presented in the *Immunology* volume of the CDCs.

### ***ELISA***

ELISA stands for enzyme-linked immunosorbent assay. In order to detect the presence of an antibody, a known antigen is attached to a solid-phase, generally a plastic microtiter plate. The patient's serum is added, allowed to incubate, washed, and an enzyme labeled antihuman antibody added. After incubation and washing, a substrate is added and a colored end product is formed. The amount of color present is in direct proportion to the concentration of the antibody in the patient's sample. Different enzymes and substrates yield different colors.

### ***Immunofluorescence***

These procedures also involve the use of a solid-phase microscope slide. A known antigen is attached to a slide. Patient serum is added to the slide and incubated. After incubation and washing the slide, an antihuman antibody labeled with a fluorescent dye is added. After incubation and washing, the slide is examined under an ultraviolet microscope. The intensity of the fluorescence is in direct proportion to the concentration of the antibody in the patient's sample.

### ***HI***

This procedure is dependent upon the ability of various viruses to agglutinate erythrocytes of various species. To measure antibody using the HI procedure, a standardized suspension of viral particles is added to the wells of a microtiter plate. Serial dilutions of a patient's serum are then added and the mixtures are allowed to incubate. A standardized erythrocyte suspension is added. If the patient serum contains antibodies against the virus, the virus will be neutralized and unable to agglutinate the erythrocytes. As a result, the erythrocytes settle to the bottom of the well in a compact red button. A lack of antibodies in the patient's serum results in agglutination, which is characterized by a red diffused lining on the bottom of the well.

### ***CF***

To measure an antibody using the CF procedure, a known antigen is mixed with a patient's sample and a standard amount of complement. The mixture is incubated. If the proper antibody is present in the patient's serum, then the antibody and antigen will form a complex and the complex will "fix" the complement. A standardized suspension of erythrocytes is added and if the complement has been fixed, it will be unable to lyse the erythrocytes. If the antigen and antibody did not form a complex, or the sample did not contain any antibody, the complement will not have been fixed and is thereby free to lyse the erythrocytes.

### ***Collection***

The proper collection of specimens is highly important to the success of any subsequent laboratory examination. The type of specimen collected depends on the nature of the illness, and often several types of specimens are required. Common specimens include nasal, pharyngeal, oral, eye, and cervical swabs; nasal washings; vesicle fluids; skin scrapings; stool and rectal swabs; urine; semen; CSF; serum; blood; bone marrow; and autopsy and biopsy tissue. Specimens from non-sterile sites should be treated with antimicrobial agents, which substantially reduce overgrowth of microorganisms in the host cell culture during virus isolation attempts. A brief clinical history should be included with each specimen. This history should include:

- Name of patient, age and sex.
- Summary of patient clinical history to include date onset, physical findings, and clinical tests.
- Viral disease suspected.
- Specimen source and collection date.
- Viral vaccines administered.

- Similar cases in family or vicinity (if applicable).
- Exposure to animal or insect vectors.

When collecting serum samples for serodiagnostic procedures, it is important to remember that circulating antibodies are not usually formed in the early stages of an infection. Therefore, paired serum samples are drawn: one shortly after the symptoms appear, and the other two to three weeks later. An elevated antibody titer (usually four-fold) in the second or convalescent sample may indicate a recent or current infection.

### **Shipping**

Maintaining specimens at a low temperature is imperative to ensuring the viability of most viruses. Exposure to room temperature for only a few hours may inactivate a virus. Most specimens for viral analysis should be frozen at a temperature of  $-70^{\circ}\text{C}$  and shipped using dry ice. The temperature of a freezer on an ordinary household refrigerator is  $-20^{\circ}\text{C}$ . This can be used to store certain specimens prior to shipping, but not for more than several days. Specimens should be packed to avoid breakage during shipment. Leak-proof containers should be used and sufficient packing material included preventing movement of the container. Styrofoam shipping boxes can be loaded with wet ice in plastic bags, or dry ice, depending on whether or not the specimen is to be shipped chilled or frozen. Specimens for the isolation of Cytomegalovirus and Varicella virus should be rapidly frozen. The Styrofoam box may be placed within a protective outer cardboard container and sealed for shipment. A label for etiologic agents/biomedical material must appear on the outside of the shipping container. Specimens can be shipped to a military or civilian contracted reference laboratory.

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## **Self-Test Questions**

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

### **406. Classification and general characteristics of viruses**

1. Current classification of viruses is based on what key properties?
2. What is the name given to the entire virus?
3. For what reasons are viruses referred to as obligate intracellular parasites?
4. What are the four phases of the life cycle of a typical virus?
5. What are the three ways a host cell can respond to a viral infection?



6. Match each viral infection 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, more than once, or not at all.

| <i>Column A</i>  | <i>Column B</i>              |
|--|------------------------------|
| ____ (1) Associated with cirrhosis and hepatocellular carcinoma.             | a. Common cold.              |
| ____ (2) In the subfamily <i>Alphaherpesvirinae</i> .                        | b. Herpes simplex.           |
| ____ (3) Associated with the genus <i>Lymphocryptovirus</i> .                | c. Measles.                  |
| ____ (4) Common species name—Varicella-zoster virus.                         | d. Chickenpox.               |
| ____ (5) Associated with the genus <i>Coronavirus</i> .                      | e. Rubella.                  |
| ____ (6) Associated with the genus <i>Morbillivirus</i> .                    | f. Hepatitis A.              |
| ____ (7) Mode of transmission is the fecal-oral route or contaminated water. | g. Infectious mononucleosis. |
| ____ (8) In the subfamily <i>Lentivirinae</i> .                              | h. Hepatitis B.              |
| ____ (9) Common species name—Rhinovirus.                                     | i. HIV.                      |
| ____ (10) In the subfamily <i>Togaviridae</i> .                              |                              |

#### 407. Laboratory identification, collecting, and shipping of viral specimens

1. Which class of antibody appears first in response to infection?
2. Which class of antibody is detectable for extended periods after exposure to a virus?
3. What are the four types of serodiagnostic procedures for viruses?
4. Which procedure utilizes a fluorescent dye?
5. Which procedure(s) utilizes a microtiter plate?
6. How is a negative result in CF indicated?
7. What factor determines the type of specimen to be collected?
8. Why is more than one specimen often required for serodiagnostic procedures?
9. What is the significance of a rise in titer in a second or convalescent serum sample?
10. Most specimens for viral analysis should be frozen at what temperature?

## Answers to Self-Test Questions

### 401

1. Lice and ticks.
2. Contamination of the vector's bite when infected lice are crushed as a result of scratching.
3. Blood, spinal fluid, and urine.
4. Antigenic variants appearing and multiplying.
5. *B. burgdorferi*.
6. Directly as the tick takes its blood meal, or indirectly when the wound becomes infected with coxal secretions given off by the tick as it feeds.
7. Rodents and deer.
8. Development of a chronic form of arthritis.
9. Romanowsky modification, Leishman, May-Grünwald, Wright, and Giemsa.
10. Indirect immunofluorescence assay and enzyme-linked immunosorbent assay.

### 402

1. The spirochetes of *T. pallidum* occur as very fine, delicate spiral forms having a flexible cylindrical body measuring approximately 0.20  $\mu\text{m}$  in diameter and 6 to 20  $\mu\text{m}$  in length. The spiral height (amplitude) is approximately 1.0  $\mu\text{m}$ . The organism possesses from eight to 15 regular spirals.
2. This is due to the greater density of the fluid caused by mucus present in the lesion.
3. Syphilis.
4. Through intimate contact, usually sexual. Infections may be transmitted occasionally through indirect means, such as fomites (drinking cups, towels) when the time interval between contact with an individual is very short.
5. Through minute breaks.
6. The primary stage.
7. Skin lesions.
8. *T. pallidum* subspecies *pertenue*, *T. pallidum* subspecies *endemicum*, and *T. carateum*.
9. The amount of protoplasm available for staining is so small that the necessary usual contrast is lacking.
10. Silver impregnation method; this technique is based on the coating of the surface of the treponeme with metallic silver to increase contrast.
11. Laboratory diagnosis of treponemes is generally based on darkfield examinations and serological procedures.

### 403

1. The species of *Leptospira* are thin, flexible, tightly coiled organisms. The spirochetes measure approximately 0.15  $\mu\text{m}$  in diameter, with a length ranging from 6 to 12  $\mu\text{m}$ . There are generally 12 to 18 spirals in each cell, with each spiral having a length of about 0.4 to 0.5  $\mu\text{m}$ .
2. The terminal third of the organism is quite flexible and often forms a hook.
3. Infection usually results from direct or indirect exposure to the urine of infected animals.
4. An acute illness with febrile jaundice and nephritis.
5. *Leptospira interrogans*.
6. The kidney, liver, meninges, and conjunctiva.
7. *Icterohaemorrhagiae*.
8. Aerobic condition; 30°C.
9. Ten percent rabbit serum or serum albumin plus fatty acids.
10. Usually six to 14 days, but can range from a few days to four weeks or longer.
11. Become faintly turbid.
12. Serological methods.

13. Within one week.
14. Microscopic agglutination-lysis test, the genus-specific hemagglutination test, and the fluorescent antibody test.
15. The microscopic agglutination.

**404**

1. (1) b.  
(2) c.  
(3) c.  
(4) a.  
(5) d.

**405**

1. The genus currently contains four species: *C. trachomatis*, *C. pneumoniae*, *C. psittaci*, and *C. pecorum*.
2. Chlamydiae contain both ribonucleic acid and deoxyribonucleic acid, possess ribosomes, multiply by binary fission, are susceptible to certain antibiotics, and have a cell wall similar to bacteria.
3. They lack the mechanism for producing metabolic energy. More specifically, they have been called energy parasites, since they are unable to synthesize adenosine triphosphate.
4. Accidentally, due to contact with infected birds.
5. Primarily, these are diseases of the eye (trachoma and inclusion conjunctivitis) and urogenital tract (lymphogranuloma venereum, urethritis, and cervicitis).
6. *C. trachomatis* organisms inhabit primarily the eye or the genital tract and can be transmitted from these sites in three ways: (1) eye to eye; (2) genital tract to genital tract; and (3) genital tract to other susceptible tissue, including the eye.
7. Direct examination, isolation, and detection of antibodies.
8. Isolation of the Chlamydial organism.
9. (1) f.  
(2) d.  
(3) c.  
(4) h.  
(5) a, e.  
(6) e.  
(7) g.  
(8) g.

**406**

1. They are classified by the kind of nucleic acid (DNA or RNA), type of strandedness (single- or double-stranded), and the presence or absences of a lipoprotein envelope.
2. Virion or virus/viral particule.
3. Viruses lack enzymes and are unable to replicate outside a host cell.
4. Attachment and penetration, uncoating and host cell synthesis of viral components, assembly of components into complete virus particles, and release of virus from host cell.
5. (1) Abortive infection or failed infection; (2) Lytic infection or cell death; (3) Persistent infection or virus production without cell death.
6. (1) h.  
(2) b.  
(3) g.  
(4) d.  
(5) a.  
(6) c.

- (7) f.
- (8) i.
- (9) a.
- (10) e.

**407**

- 1. IgM antibodies.
- 2. IgG antibodies.
- 3. ELISA, immunofluorescence, hemagglutination inhibition, and complement fixation.
- 4. Immunofluorescence procedures.
- 5. Hemagglutination inhibition procedures.
- 6. Hemolysis of the erythrocytes.
- 7. The nature of the illness.
- 8. Circulating antibodies are not usually formed in the early stages of an infection.
- 9. An elevated antibody titer in the second or convalescent sample may indicate a recent or current infection.
- 10.  $-70^{\circ}\text{C}$ .

**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. 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.**

1. (401) What is the causative agent of Lyme disease?
  - a. *Borrelia recurrentis*.
  - b. *Borrelia burgdorferi*.
  - c. *Leptospira biflexa*.
  - d. *Leptospira interrogans*.
2. (401) What is the preferred method for diagnosing relapsing fever?
  - a. DNA probe.
  - b. Agglutination tests.
  - c. Direct microscopic observation.
  - d. Indirect immunofluorescence assay.
3. (401) What is the preferred method for diagnosing Lyme disease?
  - a. DNA probe.
  - b. Agglutination tests.
  - c. Direct microscopic observation.
  - d. Indirect immunofluorescence assay.
4. (402) The spirochetes of *T. pallidum* are
  - a. 4 to 10  $\mu\text{m}$  in length with 3 to 8 spirals.
  - b. 6 to 12  $\mu\text{m}$  in length with 12 to 18 spirals.
  - c. 6 to 20  $\mu\text{m}$  in length with 8 to 15 spirals.
  - d. 5 to 25  $\mu\text{m}$  in length with 4 to 10 spirals.
5. (402) Which one of the following is the etiological agent of venereal syphilis?
  - a. *Treponema carateum*.
  - b. *Treponema pallidum* subspecies *pertenue*.
  - c. *Treponema pallidum* subspecies *pallidum*.
  - d. *Treponema pallidum* subspecies *endemicum*.
6. (402) During secondary syphilis, the most logical place to isolate *Treponema pallidum* would be the
  - a. urethra.
  - b. chancre.
  - c. bloodstream.
  - d. skin lesions that make up the rash.
7. (403) The spirochetes of *Leptospira* are
  - a. 4 to 10  $\mu\text{m}$  in length with 3 to 8 spirals.
  - b. 6 to 12  $\mu\text{m}$  in length with 12 to 18 spirals.
  - c. 6 to 20  $\mu\text{m}$  in length with 8 to 15 spirals.
  - d. 5 to 25  $\mu\text{m}$  in length with 4 to 10 spirals.

8. (403) A peculiar characteristic of *Leptospira* is the fact that terminal one-third of the organism is
  - a. flexible and undulated.
  - b. inflexible and undulated.
  - c. flexible and hook shaped.
  - d. inflexible and hook shaped.
9. (403) Which serotype of *Leptospira* causes a severe illness referred to as Weil's disease, icteric leptospirosis, or infectious jaundice?
  - a. *Leptospira interrogans* serovar ballum.
  - b. *Leptospira interrogans* serovar canicola.
  - c. *Leptospira interrogans* serovar autumnalis.
  - d. *Leptospira interrogans* serovar icterohaemorrhagiae.
10. (403) Which method is considered the most useful strain-specific test and remains the reference method for leptospirosis?
  - a. Direct fluorescent antibody test.
  - b. Indirect fluorescent antibody test.
  - c. Genus-specific hemagglutination test.
  - d. Microscopic agglutination test.
11. (404) Because of their size and genetic make-up, the mollicutes are very particular and for proper growth require which of the following additives to growth media?
  - a. Fatty acids and cholesterol.
  - b. Steroids and carbohydrates.
  - c. Carbohydrates and yeast extracts.
  - d. Cholesterol and yeast extract nucleic acid precursors.
12. (404) Since the mollicutes do not have a cell wall, they are resistant to
  - a.  $\alpha$ -lactam.
  - b.  $\beta$ -lactam.
  - c.  $\gamma$ -lactam.
  - d.  $\Sigma$ -lactam.
13. (404) Which *Mycoplasma* species is associated with NGU?
  - a. *M. hominis*.
  - b. *M. salivarium*.
  - c. *M. genitalium*.
  - d. *M. pneumoniae*.
14. (404) On A7 agar and under ideal atmospheric conditions *Mycoplasma* produces what characteristic colony appearance?
  - a. Fuzzy.
  - b. Fried-egg.
  - c. Swarming.
  - d. Molar tooth.
15. (404) Which microorganism is associated with stillborn infants and shows a correlation between colonization and fetal morbidity?
  - a. *M. fermentans*.
  - b. *M. pneumoniae*.
  - c. *C. pneumoniae*.
  - d. *U. urealyticum*.

16. (405) Chlamydiae are obligate intracellular organisms referred to as
- a. L-form organisms, because like viruses they lack a cell wall.
  - b. super bugs, because of their ability to resist a wide range of antibiotics.
  - c. mutant forms, because of their unique developmental life cycle within the cytoplasm of the host cell.
  - d. energy parasites, because they use the ATP produced by the host cell for their own requirements.
17. (405) Which one of the following is not a method for laboratory diagnosis of chlamydial infections?
- a. Detection of antibodies in serum.
  - b. Isolation of organisms in cell culture.
  - c. Direct examination of clinical specimens.
  - d. Growth in liquid medium supplemented with 10% rabbit serum.
18. (405) The following procedures are used in the routine diagnosis of rickettsial infections except
- a. cultivation.
  - b. radioimmunoassay.
  - c. complement fixation.
  - d. indirect hemagglutination.
19. (405) The Weil-Felix serologic method is based on the fact that Rickettsia share antigens with
- a. Proteus spp.
  - b. Providencia spp.
  - c. Propionibacterium spp.
  - d. Peptostreptococcus spp.
20. (405) The Weil-Felix test can be used to diagnose which of the following?
- a. Typhus and scrub typhus only.
  - b. Q-fever, typhus, and scrub typhus.
  - c. Q-fever, typhus, and cat scratch disease.
  - d. Typhus, scrub typhus, and Rocky Mountain spotted fever.
21. (406) The nucleic acid genome of a virus is surrounded by a protein coat called
- a. a virion.
  - b. a capsid.
  - c. a capsomer.
  - d. an envelope.
22. (406) Viruses lack enzymes and are unable to replicate outside the host cell, for this reason they are referred to as
- a. intracellular energy parasites.
  - b. extracellular energy parasites.
  - c. obligate intracellular parasites.
  - d. obligate extracellular parasites.
23. (406) Antiviral agents interfere with the virus' ability to
- a. mutate.
  - b. migrate.
  - c. enter the cell.
  - d. leave the host.

24. (407) What is the standard by which all virology procedures are measured?
- a. Hemagglutination.
  - b. Immunofluorescence.
  - c. Isolation and identification in cell culture.
  - d. ELISA.
25. (407) Viral infections elicit immune responses directed against which antigens?
- a. Core proteins, envelope proteins, glycoprotein spikes.
  - b. Core proteins, envelope proteins, carbohydrate spikes.
  - c. Core carbohydrates, envelope carbohydrates, lipid spikes.
  - d. Core carbohydrates, envelope lipids lipid spikes.
26. (407) Why is it necessary to obtain paired serum specimens two or three weeks apart in diagnosing a viral infection?
- a. Viral isolation is easier as the disease progresses.
  - b. Antibody titer drops rapidly after the first week of infection.
  - c. The second specimen serves as a reserve supply of serum if the first becomes contaminated or is lost in transit.
  - d. Antibodies are usually not formed during the early phase of the disease, and the first specimen provides a base line for the second in testing antibody levels.
27. (407) Most specimens for viral analysis should be frozen at a temperature of
- a.  $-10^{\circ}\text{C}$ .
  - b.  $-30^{\circ}\text{C}$ .
  - c.  $-50^{\circ}\text{C}$ .
  - d.  $-70^{\circ}\text{C}$ .
28. (407) When shipping specimens for the isolation of cytomegalovirus and varicella virus, the contents should be
- a. double sealed.
  - b. sealed airtight.
  - c. rapidly frozen.
  - d. kept at room temperature.

**Please read the unit menu for unit 2 and continue ➔**



## Unit 2. The Mycobacteria

|   |             |
|---|-------------|
| <b>2–1. Processing and Staining Specimens for Mycobacteria.....</b> | <b>2–1</b>  |
| 408. Processing specimens for mycobacteria isolation.....           | 2–1         |
| 409. Staining and examining smears for acid-fast bacilli .....      | 2–6         |
| <b>2–2. The <i>Mycobacteriaceae</i> Family .....</b>                | <b>2–10</b> |
| 410. <i>Mycobacterium</i> species.....                              | 2–10        |
| 411. Laboratory identification .....                                | 2–13        |

**D**ISEASE caused by mycobacterial organisms has been reported in the bones of prehistoric humans and animals. *Mycobacterium tuberculosis* was discovered by Robert Koch in 1882 and has proven to be one of the most pathogenic organisms known to man. In the 18th and 19th centuries, it was known as the white plague. It devastated and wiped out certain populations of Europe. Mycobacterial infections have resulted in an enormous amount of human suffering and death, and for this reason we shall discuss processing, staining, isolating, and identifying these agents.

### 2–1. Processing and Staining Specimens for Mycobacteria

As mentioned above, tuberculosis is one of the world’s oldest diseases. The disease is of a persistent and chronic nature that is usually caused by *Mycobacterium tuberculosis*. Untold numbers of people suffered from the effects of tubercular infection throughout history, and these infections are still a great public health concern in the developing countries of the world. After steady declines in the mid–20<sup>th</sup> century mycobacterial diseases are a concern in the western world due to the rise in immunosuppressive diseases. Correct processing and immediate staining are crucial for diagnosis and treatment of all mycobacterial diseases. Mycobacteria are difficult to stain, but once stained, they resist decolorization with acid-alcohol. Organisms possessing the ability to retain a stain in spite of washing with acid-alcohol are referred to as acid-fast.

#### 408. Processing specimens for mycobacteria isolation

Ideally, all specimens for isolation of mycobacteria are delivered to the laboratory within one hour and processed immediately. Delays in processing can result in a decrease in the number of positive cultures and an increase in the contamination rate. Specimens not delivered or processed within one hour of collection must be stored in the refrigerator (except blood cultures) under conditions that avoid drying.

#### Safety precautions

Before discussing specimen processing, it is important to emphasize that many of the techniques employed in the mycobacteriology laboratory have the potential for creating hazardous aerosols of infectious “droplet nuclei” which contain mycobacterial organisms. Tuberculosis ranks high among laboratory acquired infections. It is essential that any laboratory that attempts to isolate mycobacteria must be equipped with a properly operating class I or II biological safety cabinet (BSC). All specimen and culture manipulations must be performed in a BSC. Biosafety level 2 practices are required for laboratories performing acid-fast smears and cultures. Biosafety level 3 practices are required for labs performing identification and susceptibility testing of mycobacterial isolates. Other safety measures include wearing gowns, gloves, and masks, using 0.5% sodium hypochlorite as a disinfectant, and using safety pipetting devices. Also, cleaning contaminated loops in alcohol-sand before incinerating, using aerosol-free safety carriers (buckets with covers and safety domes) in the centrifuge, and appropriate hand washing are a must. Amphyl, phenol-soap mixtures, or 0.5% sodium hypochlorite with UV lighting are acceptable for surface decontamination. Personnel working in the mycobacteriology laboratory must have a tuberculin skin test done at least every six months.

Individuals who convert or have a positive tuberculin test are referred to a physician. The physician will consider isoniazid (INH) prophylaxis and order chest X-rays annually.

### **Collecting and processing acid-fast bacilli specimens**

One of the keys to efficient recovery and detection of mycobacteria in clinical specimens is, obviously, the collection of an adequate specimen. Specimens should be collected in sterile, leakproof, disposable, lab-approved containers. **NOTE:** Do not use waxed containers because they render false-positive smear results.

### ***Pulmonary disease***

Sputum is the specimen most often encountered in the laboratory because 85% of the mycobacterioses in the United States involve the lung. Examination of a single sputum specimen may not be sufficient since the isolation procedure may not be adequate or the specimen may be overgrown with other microorganisms. Some patients do not yield positive smears or cultures until multiple specimens have been submitted to the laboratory. Nonetheless, there seems to be little advantage in collecting more than three or five specimens per patient. In general, 5 to 10 ml of sputum expectorated immediately on arising in the morning, on three to five successive days, is preferred. Patients with positive results collect specimens at weekly intervals beginning three weeks after initiation of therapy. The patient should be instructed that nasopharyngeal discharge and saliva are not adequate specimens and that the desired material is brought up from the lungs after a productive cough. When the patient cannot provide a natural expectoration, having the patient breathe heated sterile hypertonic saline or a nebulized saline solution can induce sputum. This technique is a recommended alternative to gastric lavage (washing) for non-sputum-producing patients. However, gastric lavage is still performed on younger children or on persons unable to provide direct or induced sputum specimens. Gastric washings are collected in a hospital immediately after the patient awakens. Mycobacteria die rapidly in gastric washings. This type of specimen is processed as soon as possible after collection. The gastric fluid is neutralized with buffer tablets or 10% sodium carbonate if a delay is unavoidable. In fact, it is most desirable to routinely neutralize these specimens at the bedside. Other types of specimens include laryngeal swabs, transtracheal aspiration, and samples taken during a bronchoscopy procedure. The recommended collection amount for the various fluid specimens is 5 to 10 ml.

### ***Extrapulmonary disease***

Mycobacteria may not be suspected as the causative agent of an extrapulmonary disease because the chest X-ray is normal or the tuberculin skin test is negative, or both. Because mycobacteria can infect almost any organ in the body, the laboratory should expect to receive a variety of extrapulmonary specimens such as aseptically collected body fluids, surgically excised tissues, aspirated or draining pus, and urine. These specimens are divided into two groups:

1. Aseptically collected specimens, usually free of microorganisms (except the implicated pathogen).
2. Specimens known to contain contaminating normal flora or specimens not collected aseptically.

### ***Aseptically collected specimens—fluid***

The physician collects body fluids (spinal, pleura, pericardial, peritoneal, synovial, ascitic, blood, and bone marrow) aseptically using aspiration techniques or surgical procedures. Acid-fast bacilli (AFB) are difficult to isolate from these specimens because the large fluid volume often dilutes them. Therefore, as much fluid as possible should be collected with a minimum of 10 to 15 ml. Chances of isolating mycobacteria from these specimens are improved if the laboratory personnel and clinician make previous arrangements to inoculate aseptically collected fluid immediately into a liquid medium (ratio of 1 part specimen to 1 part 7H9 broth). When aseptically collected fluid may clot and cannot be inoculated immediately into a liquid medium, add sterile heparin (0.1 mg/ml) or EDTA

(1.0 mg/ml) and transport the specimen to the laboratory as quickly as possible. At least 2 ml of CSF should be collected. However, if the amount is less than 2 ml, place the specimen into a 12B BACTEC bottle (Becton-Dickinson Diagnostic Instrument Systems, Cockeysville, MD) or Septi-Chek AFB System (Becton-Dickinson) broth. Use this procedure for small amounts of any body fluid that is collected aseptically.

#### *Aseptically collected specimens—tissue*

Aseptically collected tissue specimens suspected to contain mycobacteria are placed in sterile containers without fixatives or preservatives. If the specimen is to be shipped by mail, protect the tissue from drying by adding sterile saline and pack the container in wet ice to maintain a temperature of 5 to 10°C. If the specimen were indeed collected aseptically, homogenize in a sterile tissue grinder (aerosol-free grinder preferred) with a small amount of sterile 0.85% saline or sterile 0.2% bovine albumin, and inoculate directly to liquid and solid media as with body fluids. When the tissue is not known to be sterile, homogenize and inoculate half directly to liquid and solid media; the other half is decontaminated (same as sputum). Tissue material inoculated to liquid media is examined regularly by smear and inoculated onto solid media when smears become positive.

#### *Abscess aspirates and swabs*

Abscess aspirate specimens are usually collected in small amounts. As with small amounts of body fluids, place into 12B BACTEC bottles or Septi-Chek AFB system. Swabs are not recommended, but if the abscess cannot be aspirated use a swab. Swabs should be placed in Amies or Stuart's transport medium. Dry swabs are not acceptable.

#### *Culturing blood*

Disseminated mycobacterial infections have been the focus of attention in severely immunocompromised patients. Recovery of mycobacteria from blood, especially in cases of disseminated disease associated with acquired immunodeficiency syndrome (AIDS), presents some unique problems. Recently, the lysis-centrifugation blood culture technique with the Isolator System (Wampole Laboratories, Cranbury NJ) tube has successfully recovered *M. chelonae*, *M. avium* complex, and *M. tuberculosis* bacilli from blood. The lysis-centrifugation technique has provided a more rapid and higher recovery of mycobacteria from blood cultures. Bone marrow aspirates can be injected into Wampole Pediatric Isolator tubes. This method involves inoculating 10 ml of blood to a double-stoppered evacuated tube containing poly-ethanol sulfonate, EDTA, and saponin (which lyses the erythrocytes). The tube is inverted several times to mix the contents. Next, it is allowed to stand for one hour to lyse the blood cells. After lysis, the tube is centrifuged at  $3000 \times g$  ( $g$  = relative centrifugal force), or more, to concentrate the material into 1.5 to 2.0 ml of sediment. The entire sediment is spread over the surface of several tubes or plates of isolation media. By using appropriate dilutions of sediment (or diluting lysed specimen prior to centrifugation), it is also possible to quantify the extent of the bacteremia. Smears of the sediment can also be prepared. Direct inoculation of blood onto the media is not recommended. The radiometric BACTEC 13A blood culture bottle is suggested because it has a lysing agent present in the medium. It is safer than the lysis-centrifugation technique, but it cannot determine CFU counts per volume of blood. Follow the blood culture venipuncture procedure stated in volume 1 and inoculate a BACTEC 13A bottle with 5 ml of blood.

#### *Direct smears on feces*

In addition to culturing blood for mycobacteria, another effective procedure not commonly used is performing direct smears on feces for AFB. This procedure has yielded a large number of positives from severely immunocompromised patients and reflects probable bowel involvement with mycobacteria. It is especially useful for evaluating *M. avium* complex (MAC) infections in AIDS patients. Both procedures allow quantitation of mycobacteria, and it is recommended that weekly acid-fast smears of stool and blood cultures be obtained to follow the effects of treatment on these patients. Collect stool specimens in a sterile wax-free container.

### Urine

Urine is the next most commonly encountered specimen that requires processing before culture. The genitourinary tract, one of the most common sites of extrapulmonary human tuberculosis, accounts for nearly 20% of the extrapulmonary tuberculosis cases in the United States, and the incidence shows little evidence of decreasing. About 35% of patients with genitourinary tuberculosis also have tuberculosis elsewhere. Other mycobacteria act as noninvasive contaminants in the genitourinary tract and rarely as pathogens. To minimize excessive contamination of urine specimens, the external genitalia is washed before the specimens are collected, and the urine is processed immediately or refrigerated. A first morning midstream or catheterized specimen is collected for three consecutive days. At least 40 ml are required for testing. The total amount of urine collected should be centrifuged at  $3600 \times g$  for 30 minutes. If the total amount is more than 40 ml, aliquot into equal amounts then centrifuge. Plate the sediment and make a smear. From 50 to 70% of the cases are positive by smear examination, with 25 to 95% positive by culture. Pooled urine samples, catheter bag specimens, and specimens less than 40 ml are not acceptable.

### Plating and incubation requirements

To date, the ideal media for culturing mycobacteria has yet to be formulated. Such an ideal media would possess the following characteristics: (1) support rapid growth from a small amount of inoculum, (2) inhibit the growth of contaminants, (3) permit preliminary separation of mycobacteria based upon pigment production and colonial morphology, (4) be easy to prepare, and (5) be economical. Because mycobacteria require a long incubation period for growth, it is important that the medium inhibit bacteria and fungi. These contaminants will overgrow the mycobacteria. Consequently, the plate or tube is of no use and must be thrown away if this occurs. There are many different types of media available for recovery of mycobacteria. They include nonselective and selective (containing antimicrobial agents) media. All mediums comprise malachite green to suppress the growth of bacteria. The two basic media are egg-based and agar-based. Egg-based media consist of whole eggs or egg yolks, potato flour, salt, and glycerol. The most popular egg-based media is the Löwenstein-Jensen (L-J) and the agar-base formulas are Middlebrook 7H10 and 7H11. Agar-based media are transparent and allow for early detection of microscopic colonies.

| Solid Media                       | Liquid Medium         |
|-----------------------------------|-----------------------|
| <b>Agar-based</b>                 | Middlebrook 7H9 Broth |
| —Middlebrook 7H10                 | Dubos Tween Albumin   |
| —Middlebrook 7H11                 | BACTEC 12B medium     |
| —Mitchison's selective 7H11       | Septi-Check AFB       |
| <b>Egg-based</b>                  |                       |
| —Löwenstein-Jensen (L-J) with RNA |                       |
| —L-J with pyruvic acid            |                       |
| —Wallenstein                      |                       |

### Processing samples

If the total volume of fluid specimens is 10 ml or more, the specimen should be centrifuged at  $3600 \times g$  for 30 minutes. The sediment is plated on one or more of the mediums shown in the above table. A smear is also made. Incubate cultures in the dark at 35 to 37°C in 5 to 10% CO<sub>2</sub> with high humidity. Candle jars are unacceptable for recovery of mycobacteria. Liquid broth mediums are examined weekly and a slide is prepared to check for the presence of AFB. Once the smear is made, invert the liquid medium (this encourages small numbers of mycobacteria to multiply) and re-incubate.

**CAUTION:** Make sure the cap is tightly closed before inverting! When weekly smear examinations of the inoculated broth reveal the presence of AFB, subculture the liquid medium to solid agar and/or egg based media for growth and identification of the organism. If the inoculated broth is still smear-negative after four to six weeks, centrifuge the entire volume (in aliquots, if necessary) at  $3600 \times g$  for 30 minutes and streak all the sediment to both egg- and agar-based media. Even with these

enriched media, the cultures should be incubated for at least eight weeks and eight to 12 weeks if *M. ulcerans* is suspected. Plates are incubated inverted in CO<sub>2</sub>-permeable polyethylene bags or sealed with CO<sub>2</sub>-permeable shrink-seal or cellulose bands. Remember to examine the plates or tubes every three to four days. Caps on the tube media are loosened and then tightened to allow gas exchange. Media for the mycobacteria are usually purchased, because of the labor and expense of preparation. These commercially prepared media work well, but each lot should be checked with a strain of *M. tuberculosis* having known growth characteristics. Even under the optimum conditions, the cultivation of mycobacteria may present some difficulties.

### **Decontamination and digestion**

Sterile fluids or tissues normally do not require decontamination. However, most specimens received in the laboratory contain an abundance of microorganisms other than mycobacteria. These microbes generally divide much faster than mycobacteria and will overgrow them on culture media. It is necessary to treat specimens with chemical agents that not only kill contaminating microorganisms, but also in the case of sputum, liquefies the specimen for easier handling. The majority of specimens received in the laboratory fall into this group; for example, sputum, bronchial washings, and gastric lavage. Among extrapulmonary specimens expected to be contaminated are those *not* handled aseptically during or after collection and clean-catch urine specimens. Process these specimens as you would sputum samples.

The mucin in certain specimens trap mycobacterial cells and protect contaminating bacteria from the decontamination. The mucin must be broken down or reduced to eliminate the contaminating bacteria while releasing the mycobacteria trapped in the mucin and cells. We are using sputum as a typical specimen in this discussion; however, the digestion and concentration techniques are equally applicable to bronchial and gastric washings. Conventional chemical methods of decontamination and digestion result in the destruction of a large percentage of tubercle bacilli. Thus, there is a need for a milder decontamination and digestion procedure using a good mucolytic agent. The best yield of tubercle bacilli is expected from the use of the mildest digestion that gives sufficient control of contaminants. The two most popular techniques are the N-acetyl-L-cysteine-sodium hydroxide (NALC-NaOH) and the Benzalkonium chloride (Zephiran)-trisodium phosphate.

### ***NALC-NaOH method***

The mucolytic agent, N-acetyl-L-cysteine, provides a mild decontamination and liquefaction process for viscous sputums. The NALC procedure, considered by many authorities as the method of choice or “gold standard”, was advocated by the National Communicable Disease Center. Mucoproteins in sputum and mucus specimens are readily dissolved by NALC in a weak NaOH solution. The main advantage of the NALC technique is greater survival of the tubercle bacillus during the digestion process because of the lower concentration of alkali present. Add an equal volume of the digestant solution to the sputum in a tube and wait until the specimen clears (five to 30 seconds).

**CAUTION:** Digestant should be dispensed with a fresh, sterile pipette for each specimen. Take every precaution to avoid contaminating the digestant. After digestion is complete, neutralize the NaOH with 5 mol/liter of sterile phosphate buffer and centrifuge at  $3600 \times g$  for 15 minutes. Pour off the supernatant fluid into a disinfectant solution such as 5% phenol, being careful not to disturb the sediment. Then add 1.0 to 2.0 ml of sterile water or bovine serum albumin. This serves as a buffer against pH changes during growth of the culture. Mix well, inoculate culture media, and prepare a slide for staining.

### ***Zephiran-trisodium phosphate method***

Zephiran-trisodium phosphate method requires digestion with trisodium phosphate for one hour. (The older method required a 12 to 24 hour exposure. This is no longer an acceptable procedure, due to the low survival rate of mycobacteria after the lengthy exposure to the digestant.) Begin by mixing the specimen with an equal amount of benzalkonium chloride-trisodium phosphate in a 50 ml disposable leak-proof centrifuge tube. Vortex for 30 seconds. Allow to stand at room temperature for 15 minutes;

then centrifuge for 30 minutes at  $3600 \times g$ . Decant the supernatant fluid into a disinfectant, observing necessary precautions. Neutralize the sediment with 20 ml of neutralizing buffer (obtained by Difco Laboratories) and centrifuge for 20 minutes at  $3600 \times g$ . Decant the supernatant and inoculate the sediment onto egg-based media and prepare a slide.

#### **409. Staining and examining smears for acid-fast bacilli**

The physician depends on the laboratory for information that will help him or her to make a preliminary diagnosis of tuberculosis to effectively treat the patient. The staining and examination of smears is an important step for early intervention.

##### **Principle**

Members of the genus *Mycobacteria* and certain species of *Nocardia* and *Rhodococcus*, cyst of *Cryptosporidium* and *Isospora* species, and *Legionella micdadei* possess relatively large amounts of lipids, fatty acids, and waxes within their cell wall. Such substances withstand penetration and staining of the cytoplasm by ordinary methods; for example, Gram-stain. When these resistant forms are heated during exposure to a high concentration of a basic dye in phenol, staining is more readily accomplished. Phenol serves as a carrier for the dye in penetrating the lipid layer. Once stained, the organisms resist decolorization with acid-alcohol; even with prolonged exposure. They are thus said to be “acid-fast”. Although all bacteria are stained in this manner, only the aforementioned organisms (with rare exceptions) resist decolorization with the acid-alcohol solution. The tubercle bacillus in a stained smear will appear pink to red because of the absorption of carbol fuchsin. The nonacid-fast bacteria, having been decolorized by acid-alcohol, show up as blue due to the methylene blue counterstain.

##### **Staining smears**

Recent reports indicate that increased recovery rates of acid-fast bacteria can be made by using a cytocentrifuge, increasing the relative centrifugal force during the centrifugation steps to  $3800 \times g$ , or by using polycarbonate membrane filters to concentrate the specimens. Smears for examination have to cover about a 1 by 2 cm area of the microscope slide. Carefully heat-fix the smears at  $80^{\circ}\text{C}$  for 15 minutes or at  $65$  to  $70^{\circ}\text{C}$  for two hours on an electric hot plate. In labs performing smears only, one can minimize the infectious hazard from aerosol production by using an equal volume of household bleach with the sputum. Mix the sample and let it stand for 15 minutes and then prepare a smear.

**NOTE:** Bleach can cause disintegration of the AFB if it is allowed to stand longer than 15 minutes. *Mycobacteria* stain poorly or with difficulty when the conventional Gram-staining procedure is used. Two common methods for selective staining of mycobacteria discussed are: (1) the carbol fuchsin stains, of which Ziehl-Neelsen and modified Kinyoun methods are examples, and (2) the fluorochrome staining method.

##### ***Ziehl-Neelsen and modified Kinyoun***

The Ziehl-Neelsen or modified Kinyoun staining techniques are used routinely for examining specimens or cultures for the presence of AFB. Both use basic dyes in conjunction with other constituents that increase the penetrability of the dyes. Carbol fuchsin is the primary stain, steam is the mordant, 3%  $\text{H}_2\text{SO}_4$  in 95% ethanol is the decolorizer, and methylene blue is the counter stain in the Ziehl-Neelsen method. The only difference in the modified Kinyoun procedure is that phenol replaces the steam. Once the cell is stained, acid-alcohol decolorizer does not remove the light pink (acid-fast) color from the stained cell. The background is stained with methylene blue, which serves as a color contrast for the pink tubercle bacilli. In the Ziehl-Neelsen procedure, time and temperature of heating the smear are somewhat critical. Overheating diminishes the color of the organisms from a pink to brown and can distort the shape of the cell. The Kinyoun “cold” method is more advantageous in that it is less cumbersome and requires less time. Using oil immersion magnification, examine at least 300 fields of the slide before reporting the smear as negative.

### **Fluorochrome stains**

Since the early 1960s, acid-fast fluorochrome stains for mycobacteria have been gaining greater popularity. Auramine O or auramine O with rhodamine are the most commonly used fluorochrome stains. Most laboratories employing auramine O-rhodamine for identification of mycobacteria on smears or in tissue, find it to be more sensitive than the carbol fuchsin stains. Although auramine O-rhodamine is no more specific for mycobacteria than carbol fuchsin, the organisms are more easily seen. They stain bright yellow to gold against a contrasting dark background. Because of this contrast it is possible to screen larger areas of the slide using a lower magnification objective and only a minimum of 30 fields are examined. Because of the low magnification used, it is possible for you to scan the entire slide. In this technique, you are looking for “stars” on a dark background by using the lower power objective and then switching to the high dry objective to confirm that the “stars” seen have bacillary morphology. The use of fluorochrome stains usually requires special high-intensity light sources with strong monochromatic lighting derived from mercury-vapor or halogen light. Light of the proper wavelength excites the fluorochrome stain to emit the yellow color characteristic of auramine O-rhodamine. Fluorescent antibody stains for different species of mycobacteria have been developed but have had little practical use. Fluorochrome staining is used, if available, to provide the advantages of ease, speed, and thoroughness of observation. As previously indicated, low-power objectives are used to permit inspection of a large area in a short period of time. Other advantages are better contrast, minimal eyestrain, and relative unimportance of the color acuity of the technicians. However, all negative fluorescent smears should be confirmed with the Ziehl-Neelsen or modified Kinyoun stain. The procedure chosen for use in your laboratory may be made on the basis of personal preference, or the equipment available for use. We must emphasize that the fluorescent method is not a fluorescent antigen-antibody technique. The stain, a combination of auramine O plus rhodamine or auramine O alone, is a lipophilic dye. The failure to detect certain strains of mycobacteria other than *M. tuberculosis*, especially rapid growing organisms, is one disadvantage of the fluorochrome procedure. The fluorochrome procedure also stains organisms that have been rendered nonviable by chemotherapy; therefore, laboratories employing this technique should expect to occasionally see positive smears and negative cultures on patients who have been receiving adequate chemotherapy for a few weeks. Fluorochrome-stained smears are observed within 24 hours of staining because of the possibility of fading fluorescence. Stained smears are stored at 5°C overnight to minimize the loss of fluorescence, but if at all possible, do not stain smears unless they can be observed within 24 hours. We reiterate that the Ziehl-Neelsen and Kinyoun methods are still perfectly acceptable procedures for smear examination, but a little slower than the fluorochrome technique.

### **Examination**

The examination of stained smears is the most rapid procedure available for the detection of AFB. You can examine air-dried, heat-fixed direct smears made from the specimen or concentrated sediment. This technique provides “same day” information to clinicians concerning the status of their patients. The smear examination has been neglected somewhat in recent years, perhaps because the procedure is thought to be too time-consuming or because a more sensitive culture technique is in use. The value of microscopy should not be underestimated as a means for a rapid, presumptive diagnosis of mycobacterial infection and for observing a patient’s response to chemotherapy. Patients with positive sputum smears are the greatest threat to the community since they are the infectious reservoirs for the spread of tubercle bacilli. On the other hand, smear negative patients, especially those receiving adequate chemotherapy, are less likely to spread disease. For these reasons, bacteriology laboratories should develop a high degree of skill in the preparation, staining and examination of clinical specimens by microscopy for the presence of mycobacteria. The observation of acid-fast organisms in a stained smear does not provide identification of the mycobacterial species. To do this, you must obtain the organisms by culture. Also, failure to observe AFB in a smear does not rule out tuberculosis or other mycobacterial infections. It has been estimated that from 10,000 to 100,000 AFB per ml of sputum must be present to detect them in stained preparations.

### Self-Test Questions

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

#### 408. Processing specimens for mycobacteria isolation

1. What one piece of safety equipment is essential if a laboratory is attempting to isolate mycobacteria?
2. Why is a sputum specimen most often submitted to the laboratory for the detection of mycobacteria?
3. In general, what is the preferred type of sputum specimen for the detection of mycobacteria?
4. When and where should gastric washings be collected?
5. What is used to neutralize gastric fluid in order to prevent the death of mycobacteria?
6. How are the chances of isolating mycobacteria from body fluids enhanced?
7. How is aseptically collected fluid for the isolation of mycobacteria prevented from clotting?
8. What blood culture techniques can be used to successfully recover *M. chelonae*, *M. avium* complex, and *M. tuberculosis* from AIDS patients?
9. Next to sputum, what is the next *most commonly* processed specimen for the recovery of mycobacteria?
10. What is the preferred specimen for isolation of mycobacteria from a suspected case of genitourinary tuberculosis?
11. What are the two basic media used for isolation of mycobacteria?
12. How are fluid specimens processed if their volume is 10 ml or more?
13. What are the incubation requirements and time of incubation required for mycobacteria?



14. Why is it necessary to treat most specimens with chemical agents?
15. What are the two most popular techniques used for decontamination and digestion of mycobacterium specimens?
16. Which procedure is considered by many authorities as the method of choice or “gold standard” that was advocated by the National Communicable Disease Center?

**409. Staining and examining smears for acid-fast bacilli**

1. What substances are present in the cell wall of the genus *Mycobacterium* and certain *Nocardia spp.* that enable them to withstand penetration and staining?
2. What is the action of phenol in an acid-fast stain?
3. What causes the bacillus in a stained smear to appear pink to red?
4. Recent reports indicate that increased recovery rates of acid-fast bacteria can be made by using what procedure?
5. How is the infectious hazard from aerosol production during the preparation of AFB smears minimized?
6. What are two common methods for selective staining of mycobacteria?
7. What are the reagents and their purpose in the Ziehl-Neelsen method?
8. What is the major difference between the Ziehl-Neelsen and the modified Kinyoun acid-fast staining techniques?
9. What two fluorochrome stains are used in the fluorochrome staining procedure?

10. What are the advantages of the fluorochrome stains over the carbol fuchsin stains for identifying mycobacteria?
11. What kind of light source is usually required to examine fluorochrome stains?
12. What is one disadvantage of the fluorochrome procedure?
13. Of what value is microscopic examination of stained smears for the detection of AFB?

## 2-2. The *Mycobacteriaceae* Family

The family *Mycobacteriaceae* contains only one genus—*Mycobacterium*. DNA studies show that the mycobacteria are similar to the other mycolic acid-producing bacteria: *Nocardia*, *Rhodococcus*, and *Corynebacterium* species. In the future, this similarity may support the consolidation of these genera into a single family.

### 410. *Mycobacterium* species

There are 54 recognized species of mycobacteria with more than 27 species isolated in human clinical specimens. Some species cause only human infections, others have been isolated only from animals, yet, most species are saprophytes which are found in soil and water. *Mycobacterium* species can be tentatively classified into the following four groups: (1) obligate pathogens, (2) facultative pathogens, (3) opportunistic pathogens, and (4) saprophytic species.

#### General characteristics

The most striking characteristic of the mycobacteria is the enormous amount of lipid present in their cell walls. This comprises 40% of the total dry weight; thus, these cells grow as extremely rough colonies that do not readily absorb water. The mycobacterial cell wall consists of a polypeptide layer with free lipids (waxes, mycosides, and cord factor), arabinogalactan mycolate layer, peptidoglycan layer with mycolic acids, and then the cytoplasmic membrane. The structural foundation is the peptidoglycan skeleton with the mycolic acids as the major lipids in the cell wall. They are aerobic, nonspore-forming, nonmotile, slightly curved or straight bacilli, and are 0.2 to 0.6 by 1.0 to 10  $\mu\text{m}$  in size. The bacilli sometime form branched, filamentous, or mycelium-like rods, but they can be easily disrupted into shorter rods or coccoid elements. Microscopically, individual cells of *M. tuberculosis* are rod shaped, 2 to 5  $\mu\text{m}$  or more in length and 0.4 to 0.6  $\mu\text{m}$  wide. After dividing, the multiplying cells tend to form tightly packed cords that appear to pile up at the center, giving the colonies a characteristic rough, corded appearance.

#### Clinical significance

Out of the 27 species isolated from human clinical specimens, 95% of all human infections are caused by eight species or groups: *M. tuberculosis*, *M. leprae*, MAC, *M. intracellulare*, *M. kansasii*, *M. fortuitum* complex, *M. scrofulaceum*, and *M. xenopi*.

#### *Mycobacterium tuberculosis*

*M. tuberculosis* (TB) is the species most commonly isolated from suspected pulmonary tuberculosis. *M. tuberculosis* ranks as one of the top 10 pathogenic organisms of humans. According to the World

Health Organization (WHO), tuberculosis remains a major global public health problem. WHO estimates that 8 million new cases and 3 million deaths are directly attributable to the disease each year. This makes tuberculosis the leading cause of death due to a single infectious agent. In the United States, tuberculosis is unevenly distributed among the population.

There are certain subgroups that are at a higher risk for *exposure* and *latent* infections of tuberculosis than those in the general population. These groups are the medically under-served ethnic minorities, homeless persons, prison inmates, alcoholics, IV drug users, the elderly, foreign-born persons from areas where tuberculosis infections are high, and those who come in contact with persons with active tuberculosis. Tuberculosis infections are now migrating from these lower socioeconomic groups to those health care team members who treat these patients, and sometimes subsequently members of their families.

Groups with a higher likelihood of developing *active* tuberculosis are those with chronic renal failure, compromised immune system, body weight greater than 10% below ideal weight, and diabetes mellitus. Others include children less than four years old, persons who have been infected with tuberculosis within the past two years, persons with fibrotic lesions on chest X-ray, and those with HIV infection—the greatest known risk factor.

Tuberculosis is spread from person-to-person by the inhalation of the infective airborne particle known as “droplet nuclei”. These particles are from 1 to 5  $\mu\text{m}$  in diameter and travel through the airway to the lungs. Once attached to the alveolar walls, alveolar macrophages engulf the organisms and transport them to other parts of the body. In the healthy host, a cell-mediated immune response limits further multiplication and spread of *M. tuberculosis*. However, some bacilli may remain viable, but dormant, for many years after initial exposure. Patients with latent infections have a positive purified protein derivative (PPD) skin test but are otherwise asymptomatic and are usually not infectious. Of the persons who have latent infections, 15 to 20% will develop the active disease. Pulmonary tuberculosis is a slowly progressive inflammatory process. Symptoms are coughing, weight loss, low-grade fever, dyspnea, and chest pain. Other clinical manifestations include cervical adenitis, skin infections, pericarditis, synovitis, and meningitis.

Tuberculosis in AIDS patients quickly disseminates. One of the most distressing problems to arise from the rebirth of tuberculosis among AIDS patients and the homeless population is the increasing frequency of multiple drug resistance isolates of *M. tuberculosis*. These isolates are also known as MDR-TB. Another contributor to MDR-TB is patient non-compliance with the treatment regimen. Often this is due to the fact that the treatment involves a long-term commitment for cure, and these patients are at risk of not completing the course of therapy.

### ***Mycobacterium leprae***

*M. leprae* is the infectious agent of leprosy (a.k.a. Hansen’s disease), which is a chronic granulomatous disease. Leprosy is relatively rare in the United States, but cases have been reported in California, Hawaii, Texas, and Louisiana. However, more than 12 million cases are reported worldwide with 90% of these infections occurring in Asia and Africa. Most of the infections now seen in Europe and North America are acquired abroad. The reservoir for *M. leprae* is not confirmed, although naturally occurring infections of the nine-banded armadillo have been documented in Texas and Louisiana. Clinical manifestations are due to the patient’s immune reaction and ranges from the tuberculoid (low infectivity) to the lepromatous (high infectivity) forms. Tuberculoid leprosy produces lesions characterized by anesthetic macules with hypopigmentation and peripheral nerve damage resulting in complete sensory loss. Lepromatous leprosy produces many erythematous macules, papules, or nodules. These nodules cause extensive tissue destruction to nasal cartilage, bone, and ears with diffuse nerve involvement and patchy sensory loss. *M. leprae* differs from the other mycobacteria because it cannot be grown in cell-free cultures. Therefore, laboratory confirmation of leprosy is usually made through histological examination of material taken from a skin lesion or earlobe that reveal AFB, and the skin test reactivity to lepromin. Leprosy is spread from

person-to-person through respiratory secretions that are exposed to the skin, the inhalation of infectious aerosols, contact with wound exudates, and possibly through an arthropod vector. Large numbers of AFB can be observed in the nasal secretions of patients with lepromatous leprosy.

### ***Mycobacterium avium complex and Mycobacterium intracellulare***

*M. avium* and *M. intracellulare* are usually considered together as *M. avium* complex or MAC, because differentiation by physiological parameters is difficult and their diseases in humans are identical. This group of organisms was historically an uncommon human pathogen—until recently. In many clinical laboratories they are the most common mycobacteria isolated, since they are the major mycobacterial pathogen in AIDS patients treated in the United States. These organisms are isolated from soil, water, infected poultry, swine, and other animals. AIDS patients seem to acquire the infection primarily by ingestion of contaminated food or water. The mode of transmission by the inhalation of the organisms is probably less common and person-to-person spread does not occur. *M. avium* complex isolated from asymptomatic patients frequently represents transient colonization; still, it can cause a pulmonary disease indistinguishable from tuberculosis. When observed in the immunocompromised patient (AIDS patients, patients with underlying malignancy, or inherited immunodeficiency) the disease is typically disseminated. The magnitude of these infections is striking, with virtually no organ spared. The tissues of some patients are literally filled with hundreds to thousands of mycobacteria bacilli. Disseminated MAC infections may be the cause of the wasting syndrome experienced by many AIDS patients. MAC is also the leading cause of mycobacterial lymphadenitis in children less than 12 years of age.

### ***Mycobacterium kansasii***

*M. kansasii* has occasionally been isolated from water samples from various parts of the world; however, its natural reservoir is still unknown. It causes chronic pulmonary infections resembling classic tuberculosis. In the United States, nearly all cases are reported from California, Texas, Louisiana, Florida, Illinois, and Missouri.

### ***Mycobacterium scrofulaceum and Mycobacterium xenopi***

*M. scrofulaceum* is mostly associated with cervical lymphadenitis in children, and rarely associated with pulmonary disease, disseminated disease, conjunctivitis, osteomyelitis, meningitis, and granulomatous hepatitis. It accounts for approximately 2% of the mycobacterial infection in AIDS treated in the United States. *M. xenopi* was not recognized as a potential human pathogen until 1965. It has been isolated from potable water, including water storage tanks in hospitals. Human infections with *M. xenopi* have been primarily reported from Great Britain, France, Denmark, Australia, and the United States. In Great Britain, birds are considered a possible natural reservoir. Clinical manifestations include pulmonary (most common), extrapulmonary, and disseminated disease.

### **Antimicrobial susceptibility and treatment**

Antimicrobial susceptibility testing for mycobacteria, if done properly, provides important information to the physician for determining antimicrobial therapy. It is not routinely done in most military microbiology laboratories because it is a time-consuming procedure that requires meticulous care in preparation of the medium, colony selection, and inoculum standardization. It also involves labor-intensive quality control and thorough knowledge of result interpretation. The Center for Disease Control and Prevention recommends that initial isolates from all patients be tested for drug susceptibility to confirm the anticipated effectiveness of antimicrobial therapy. Therefore, isolates should be sent to a reference laboratory for susceptibility testing if not performed in your laboratory. The first-line drugs in the treatment of tuberculosis are isoniazid (INH), rifampin, pyrazinamide, ethambutol, and streptomycin. The second-line drugs are *para*-aminosalicylic acid, ethionamide, cycloserine, capreomycin, kanamycin, amikacin, ciprofloxacin, ofloxacin, and rifabutin. They should be used if resistance or toxicity occurs to first line drugs. The current regimen for therapy includes INH plus rifampin for nine months for drug-susceptible tuberculosis. Drug-resistant tuberculosis has become a serious concern and is on the rise in the United States. MDR-TB are resistant to INH or

most effective antituberculosis drugs) or both and some strains are reported to be resistant to seven different drugs. The mortality for MDR-TB is 72 to 89% and death occurs between four to 16 weeks after diagnosis.

#### **411. Laboratory identification**

The question that naturally arises is, “What services should a clinical laboratory offer to assist the health care provider in the diagnosis and management of tubercular infections?”

##### **Services**

Four services are needed to fulfill this requirement: (1) the detection and isolation of mycobacteria in clinical samples, (2) identification of the organisms isolated, (3) susceptibility testing of the isolates, and (4) monitoring of the patient’s response to treatment. Not all laboratories should attempt to provide all of these services in-house and may require the assistance of a reference laboratory to meet some or all of them. In response to concerns over the adequacy of laboratory diagnostic services, the American Thoracic Society recommends three levels of laboratory service or types of laboratories be designated. It is impractical for all laboratories to provide all four services. It is suggested that the majority of clinical laboratories concentrate their efforts on isolation and identification of the primary pathogen of the family *Mycobacteriaceae*—*M. tuberculosis*. The Center for Disease Control and Prevention suggests, as a rule of thumb, that proficiency may be maintained by microscopic examination of 10 to 15 smears per week, or by routine digestion and culture of 20 clinical specimens per week. This volume of work exceeds that experienced by many clinical laboratories. Another facet of the problem of maintaining proficiency largely is the fact that test procedures used to identify mycobacterium have not been simplified. These tests are labor intensive and their correct performance and interpretation require a high degree of proficiency on the part of the laboratory technician. Therefore, procedures requested infrequently should be referred to a laboratory offering a higher level of service.

##### **Isolation**

*M. tuberculosis* is the most common species of *Mycobacterium* found in clinical specimens from patients suspected of having tuberculosis. “Mycobacteria other than tuberculosis” (“MOTT”) are also found in these specimens, and some may even be associated with disease. The majority of Air Force laboratories are able to identify *M. tuberculosis*, and all are capable of shipping AFB to a reference laboratory for precise characterization. Most mycobacterial pathogens may be identified by rate of growth, pigmentation, colony morphology, and one or two other properties. Nevertheless, the results of a single test must never be depended upon for identification of the organism. The mycobacteria are aerobic and will not grow under strict anaerobic conditions. An incubation temperature of 35 to 37°C is the optimum. Growth is slow, usually requiring two weeks or longer. The tendency of human, avian, and bovine species to form colonies only at elevated temperatures (no growth at 25°C) is significant in distinguishing them from the atypical groups.

##### **Growth rate**

Growth rate is defined as the number of days of incubation required for colonies to become visible without the aid of magnification. Rapid growers usually mature within seven days. Slow growers require more than seven days. On primary isolation, even rapidly growing mycobacteria may take more than seven days to appear on the culture medium; consequently, the rate of growth should always be determined by using as inoculum a fresh, *liquid* subculture diluted sufficiently to yield isolated colonies on whatever solid medium (usually egg-based) is inoculated. The liquid medium most commonly used is enriched Middlebrook 7H9 broth. Once the purity of the unknown culture growing on primary isolation medium has been determined (by smear examination), several colonies are aseptically picked and inoculated into 5 ml of 7H9 broth. Broth cultures are incubated at 35 to 37°C for five to seven days. Shaking the broth cultures daily (by hand) will increase the amount of growth achieved. A word of caution—make sure aerosols are not created when shaking the culture.

At the end of the incubation period, the bacterial suspension is diluted on the basis of the extent of turbidity in the broth, and 0.1 ml of several 10-fold dilutions are inoculated onto each of several tubes of egg-based medium. The inoculated media is then incubated at 35 to 37°C and observed for the growth of *M. tuberculosis*. If other species of mycobacteria are suspected, incubation can be carried out at higher or lower temperatures. Examine the tubes after five to seven days of incubation and weekly thereafter. *M. tuberculosis* is grouped with the slow-growing mycobacteria because it takes more than seven days of incubation for mature colonies to be visible to the unaided eye. Tubercle bacilli also exhibit a restricted growth temperature range, growing best between 35 and 37°C. Growth of diluted inocula will not be seen in tubes incubated at 22 to 25°C, or at 42°C or above.

### ***Pigment production***

The mycobacteria may be divided into three groups on the basis of pigment production. Pigment formation is best observed in cultures that have isolated colonies. Yellow, orange, or red colonies are considered pigmented or chromogenic (CH). Pale yellow, pink, or tan colonies are recorded by color. White, cream, or buff colonies are considered nonpigmented or nonchromogenic (NC). To demonstrate pigment production, cultures should be grown initially in the dark, and later the young, actively growing colonies may be exposed to light. The following is a description of the three groups:

1. Photochromogens.
2. Scotochromogens.
3. Nonphotochromogenic.

#### *Photochromogens*

Cultures whose colonies are nonpigmented when grown in the dark, but turn yellow-orange after they are exposed to light for one or more hours and reincubated overnight.

#### *Scotochromogens*

Cultures whose colonies produce yellow-orange pigment when grown either in the dark or the light. Cultures grown in continuous or prolonged exposure to light may reveal more intense pigment than companion cultures protected from the light.

#### *Nonphotochromogenic*

Cultures whose colonies are nonpigmented or possess only pale shades of pigment (buff or light tan), and whose color is unaffected by exposure to light.

The following table divides the *Mycobacterium* species into groups based on pigment production. It also provides an overview of pathogenicity and growth characteristics at 25 and 37°C.

| Organisms and Groups                             | Pathogenicity        | Colony Formation at 25°C     | Colony Formation at 37°C    |
|--|----------------------|------------------------------|-----------------------------|
| <b><i>Mycobacterium Tuberculosis Complex</i></b> |                      |                              |                             |
| — <i>M. tuberculosis</i>                         | Strictly pathogenic  | No growth                    | 14 to 29 days               |
| — <i>M. bovis</i>                                | Strictly pathogenic  | No growth                    | 21 to 56 days               |
| — <i>M. africanum</i>                            | Strictly pathogenic  | No growth                    | 21 to 35 days               |
| — <i>M. ulcerans</i>                             | Strictly pathogenic  | 21 to 35 at 32°C             |                             |
| — <i>M. leprae</i>                               | Strictly pathogenic  | Only grows in cell cultures. |                             |
| <b>Runyon Group I Photochromogens</b>            |                      | As a Group<br>14 to 28 days  | As a Group<br>10 to 21 days |
| — <i>M. kansasii</i>                             | Usually pathogenic   |                              |                             |
| — <i>M. marinum</i>                              | Usually pathogenic   |                              |                             |
| — <i>M. simiae</i>                               | Usually pathogenic   |                              |                             |
| — <i>M. asiaticum</i>                            | Sometimes pathogenic |                              |                             |

| Organisms and Groups                           | Pathogenicity        | Colony Formation at 25°C    | Colony Formation at 37°C    |
|--|----------------------|-----------------------------|-----------------------------|
| <b>Runyon Group II<br/>Scotochromogens</b>     |                      | As a Group<br>14 to 28 days | As a Group<br>10 to 21 days |
| — <i>M. szulgai</i>                            | Usually pathogenic   |                             |                             |
| — <i>M. scrofulaceum</i>                       | Sometimes pathogenic |                             |                             |
| — <i>M. xenopi</i>                             | Sometimes pathogenic |                             |                             |
| — <i>M. goodii</i>                             | Nonpathogenic        |                             |                             |
| — <i>M. flavescens</i>                         | Nonpathogenic        |                             |                             |
| <b>Runyon Group III<br/>Nonphotochromogens</b> |                      | As a Group<br>14 to 28 days | As a Group<br>10 to 21 days |
| — <i>M. haemophilum</i>                        | Strictly pathogenic  |                             |                             |
| — <i>M. malmoense</i>                          | Strictly pathogenic  |                             |                             |
| — <i>M. shimoidei</i>                          | Strictly pathogenic  |                             |                             |
| — <i>M. avium</i>                              | Usually pathogenic   | No growth                   |                             |
| — <i>M. intracellulare</i>                     | Usually pathogenic   | No growth                   |                             |
| — <i>M. gastri</i>                             | Nonpathogenic        | No growth                   |                             |
| — <i>M. nonchromogen</i>                       | Nonpathogenic        |                             |                             |
| — <i>M. terrae</i> complex                     | Nonpathogenic        |                             |                             |
| <b>Runyon Group IV Rapid Growers</b>           |                      | As a Group<br>7 days        | As a Group<br>3 to 5 days   |
| — <i>M. abscessus</i>                          | Sometimes pathogenic |                             |                             |
| — <i>M. fortuitum</i>                          | Sometimes pathogenic |                             |                             |
| — <i>M. chelonae</i>                           | Sometimes pathogenic |                             |                             |
| — <i>M. phlei</i>                              | Nonpathogenic        |                             |                             |
| — <i>M. smegmatis</i>                          | Nonpathogenic        |                             |                             |
| — <i>M. vaccae</i>                             | Nonpathogenic        |                             |                             |

### Colony morphology

On culture media inoculated with bacterial suspensions, diluted to yield isolated colonies, individual colony types may be observed and described. They prove to be helpful in identifying the organism. The same descriptive terms used to describe bacteria are also used for mycobacterial colonies (see volume 1 unit 3). Young colonies can be observed under the 10 X objective of a stereoscope and morphology described. Middlebrook 7H10 or 7H11 is best because they are transparent. Colonies are smooth, rough, or intermediate in roughness, they may be thin or transparent, and have filamentous extensions. Colony morphology can tentatively identify species of mycobacteria. Colonies of *M. tuberculosis* are nonpigmented, grow best between 35 and 37°C, and usually require two weeks or more to form macroscopic colonies. On most egg-based media the colonies are rough, dry, and easily crumble. They are usually heaped up in the center, and have a thin, almost transparent, erose edge. Occasionally, the colonies are flat, rough, and spreading, with only a slight central elevation. Combinations of colony forms between these extremes may be seen. On 7H10 or 7H11 agar-based media, *M. tuberculosis* grows as flat, rough, spreading, nonpigmented colonies, with only slight elevations in the center. When viewed through a dissecting microscope (stereoscope), by transmitted light, the colonies appear dark, rough, and roped or corded. This is due to the multiplication of cells that are closely aligned in a side-by-side or end-to-end arrangement. You should know that *M. tuberculosis* colonies *do not look alike* on egg-based agar and Middlebrook agar. Although colonies of tubercle bacilli may, on rare occasions, absorb the dye from an agar on which they are growing and appear green (some cultures absorb the malachite green dye from L-J slants during incubation, or when they are stored in the refrigerator), they do not appear bright yellow and orange as do some other mycobacteria. Colonies of *M. tuberculosis* are always rough, never smooth and hemispherical.

If a tube of medium has an excessive amount of water in it, this may flow over the colonies and make them appear smooth, but if the caps are loosened and the moisture dries up, the colonies again look rough.

### **Biochemical tests**

Although the features are characteristic and suggestive of *M. tuberculosis*, they are not unique to that species. By adding to these features the niacin, nitrate reduction, and 68°C catalase tests, the laboratory should be able to identify *M. tuberculosis* with a high degree of reliability. The appearance of slow-growing, rough colonies on culture media, and the detection of niacin in culture extracts lends to a presumptive identification of this species. However, you must still perform additional tests to arrive at a definitive identification. For definitive identification, each culture isolate should be transferred to a liquid medium and then subcultured to a solid medium not only to determine rate of growth, pigment production, and colony morphology, but also to perform the niacin, nitrate reduction, and 68°C catalase tests.

#### ***Niacin test***

Niacin in the culture is detected by a color reaction with cyanogen bromide and aniline. To test for niacin production, pipette 1.0 ml of sterile saline into a three-week old (or older) culture slant. Be sure there is no niacin-containing additive, such as penicillin, in the medium. In order to extract the niacin, puncture the slant around the growth with the dropper pipette tip. The colonies should be covered with the saline and allowed to stand for about 30 minutes. Transfer a portion of the saline to a small screw-capped test tube. Add an equal portion of aniline and cyanogen bromide solution to the saline extract. If niacin is present, a yellow color will appear almost instantly. **CAUTION:** Cyanogen bromide is a tear gas, so perform the test in a well-ventilated area or a fume hood.

#### ***Nitrate reduction test***

The ability of mycobacteria to reduce nitrate ( $\text{NO}_3$ ) to nitrite ( $\text{NO}_2$ ) provides a differential tool that aids in species identification. The test can be performed by using chemical reagents or reagents-impregnated paper strips, or it can be combined with a test for niacin detection. *M. tuberculosis* is one of the strongest reducers of nitrate among the mycobacteria, a trait that makes the paper strip test especially valuable for this species. Other mycobacteria may give results in the paper strip test which conflict with those obtained by the classical procedure with chemical reagents. If reliable results are to be expected, a heavy suspension of the organism must be used. A light suspension may yield a questionable reaction.

#### ***Catalase test***

Except for some isoniazid-resistant mutants of *M. tuberculosis*, most mycobacteria possess catalase enzymes that break down hydrogen peroxide to water and oxygen. When suspended in pH 7 buffer and heated to 68°C for 20 minutes, several species (most notably *M. tuberculosis*) lose this enzymatic activity. The hot catalase test is a valuable adjunct to the other in vitro tests described for specifically identifying *M. tuberculosis*. It may be especially helpful with strains of tubercle bacilli that give negative or only weakly positive results in the niacin test. To check the catalase activity at room temperature, prepare a 1:1 mixture of 10% Tween 80, and 30% hydrogen peroxide. Add 0.05 ml of the mixture to a Löwenstein-Jensen slant. Bubbling indicates a positive-catalase test. To test catalase activity at 68°C, add several loopfuls of mycobacteria from a slant to 0.5 ml of a phosphate buffer solution (pH 7.0) in a test tube. Incubate at 68°C in a water bath for 30 minutes. Add 0.5 ml of the Tween-hydrogen peroxide mixture to the buffer-growth solution and observe for the bubbling indicative of a positive reaction.



Key biochemical test for identification of the suggested groupings of mycobacteria.

| Species Group                  | Key Tests  |
|--------------------------------|--|
| <i>M. tuberculosis</i> complex | Niacin, nitrate reduction, 68°C catalase   |
| Nonphotochromogens             | Nitrate and tellurite reduction, semiquantitative and 68°C catalase, Tween 80 hydrolysis |
| Photochromogens                | Nitrate reduction, Tween 80 hydrolysis, semiquantitative catalase, urease                |
| Rapid Growers                  | Arylsulfatase, nitrate reduction, iron uptake, growth on MacConkey agar                  |
| Scotochromogens                | Nitrate reduction, Tween 80 hydrolysis, urease, 5% NaCl tolerance                        |

### Self-Test Questions

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

#### 410. *Mycobacterium* species

1. What are the general characteristics of the *Mycobacterium* species?
2. What is the most common species of mycobacterium found in clinical specimens from patients suspected of having pulmonary tuberculosis?
3. What are the subgroups of the population that are at a higher risk for exposure or latent infections of tuberculosis?
4. Who are those who have a higher likelihood of developing active tuberculosis?
5. How is tuberculosis spread?
6. What is one of the most distressing problems to arise from the rebirth of tuberculosis among AIDS patients and the homeless population?
7. What are the two clinical manifestations caused by the two forms of *M. leprae*?
8. How is the laboratory confirmation of leprosy usually made?
9. In many clinical laboratories, what organisms are now the most common mycobacterial isolates related to AIDS patients treated in the United States?

**411. Laboratory identification**

1. What are the four services needed to fulfill the requirement of diagnosing and managing tubercular infections?
2. Most mycobacterial pathogens may be identified by what characteristics?
3. What tendency of human species of mycobacteria is significant in distinguishing them from atypical groups?
4. How is growth rate defined?
5. Into what three groups can mycobacteria be divided on the basis of pigment production?
6. What is the usual amount of time required for *M. tuberculosis* to form macroscopic colonies at 37°C?
7. What are the characteristic colony morphology of *M. tuberculosis*?
8. What tests are required for the definitive identification of *M. tuberculosis*?
9. What color does a positive test for niacin produce?
10. What is the basis of the nitrate reduction test?
11. What type of organisms are the exception to the rule that most *M. tuberculosis* are catalase positive?
12. What hot catalase (68°C) reaction would you expect from an isolate suspected of being *M. tuberculosis*?

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## Answers to Self-Test Questions

### 408

1. Any laboratory that attempts to isolate mycobacteria must be equipped with a properly operating class I or II BSC.
2. Eighty-five percent of mycobacterioses in the United States involve the lungs.
3. 5 to 10 ml of sputum expectorated immediately on arising in the morning for three to five successive days.
4. Gastric washings are collected in a hospital immediately after the patient awakens.
5. 10% sodium carbonate.
6. By immediate inoculation into a liquid medium such as Middlebrook 7H9 broth.
7. By adding sterile heparin or EDTA.
8. Lysis-centrifugation and the radiometric BACTEC 13A blood culture bottle.
9. Urine.
10. A first morning midstream or catheterized specimen is collected for three consecutive days. At least 40 ml are required for testing.
11. The two basic media are egg-based and agar-based.
12. If the total volume of fluid specimens is 10 ml or more, the specimen should be centrifuged at  $3600 \times g$  for 30 minutes and the sediment is plated on one or more appropriate mediums.
13. Incubate cultures in the dark at 35 to 37°C in 5 to 10% CO<sub>2</sub> with high humidity for at least eight weeks and eight to 12 weeks if *M. ulcerans* is suspected.
14. It is necessary to treat specimens with chemical agents that not only kill contaminating microorganisms, but in the case of sputum, liquefies the specimen for easier handling. The mucin must be broken down or reduced to eliminate the contaminating bacteria while releasing the mycobacteria trapped in the mucin and cells.
15. The two most popular techniques are the NALC-NaOH and the Zephiran-trisodium phosphate.
16. The NALC procedure.

### 409

1. They possess relatively large amounts of lipids, fatty acids, and waxes within their cell wall.
2. Phenol serves as a carrier for the dye in penetrating the lipid layer.
3. The tubercle bacillus in a stained smear will appear pink to red because of the absorption of carbol fuchsin.
4. Recent reports indicate that increased recovery rates of acid-fast bacteria can be made by using a cytocentrifuge, increasing the relative centrifugal force during the centrifugation steps to  $3800 \times g$ , or by using polycarbonate membrane filters to concentrate the sputum specimen.
5. In labs performing smears only, one can minimize the infectious hazard from aerosol production by using an equal volume of household bleach with the sputum. Mix the sample and let it stand for 15 minutes and then prepare a smear.
6. Two common methods for selective staining of mycobacteria discussed are: (1) the carbol fuchsin stains, of which Ziehl-Neelsen and modified Kinyoun methods are examples, and (2) the fluorochrome staining method.
7. Carbol fuchsin is the primary stain, steam is the mordant, 3% H<sub>2</sub>SO<sub>4</sub> in 95% ethanol is the decolorizer, and methylene blue is the counter stain.
8. The only difference in the modified Kinyoun procedure is that phenol replaces the steam.
9. Auramine O and auramine O with rhodamine.
10. Although auramine-rhodamine is no more specific for mycobacteria than carbol fuchsin, the organisms are more easily seen when auramine-rhodamine is used, staining bright yellow to gold against a contrasting dark background. Because of this contrast, it is possible to screen larger areas of the slide using a lower magnification objective than the oil immersion objective required for examination of carbol fuchsin acid-fast stains and only a minimum of 30 fields are examined.
11. Examination of fluorochrome stains usually requires special high-intensity light sources with strong monochromatic lighting derived from mercury-vapor or halogen light.

12. The failure to detect certain strains of mycobacteria other than *M. tuberculosis*, especially rapid growing organisms, is one disadvantage of the fluorochrome procedure. The fluorochrome procedure also stains organisms that have been rendered nonviable by chemotherapy.
13. This technique provides “same day” information to clinicians concerning the status of their patients.

**410**

1. They are aerobic, nonspore-forming, nonmotile, slightly curved or straight bacilli, and are 0.2 to 0.6 by 1.0 to 10  $\mu\text{m}$  in size. The bacilli sometime form branched, filamentous or mycelium-like rods, but they can be easily disrupted into shorter rods or coccoid elements.
2. *M. tuberculosis*.
3. These groups are the medically under-served ethnic minorities, homeless persons, prison inmates, alcoholics, IV drug users, the elderly, foreign-born persons from areas where tuberculosis infections are high, and those who come in contact with persons with active tuberculosis. Also, healthcare industry workers are at increasing risk.
4. Groups with a higher likelihood of developing active tuberculosis are those with chronic renal failure, compromised immune system, body weight greater than 10% below ideal weight and diabetes mellitus. Others include children less than four years old, persons who have been infected with tuberculosis within the past two years, persons with fibrotic lesions on chest X-ray, and those with HIV infection—the greatest known risk factor.
5. From person-to-person by the inhalation of the infective airborne particle that is known as droplet nuclei.
6. The increasing frequency of multiple drug resistance to *M. tuberculosis* (MDR-TB) isolates.
7. Infections range from the tuberculoid (low infectivity) to the lepromatous (high infectivity) forms.
8. Histological examination of material taken from a skin lesion or ear lobe that shows reactivity to lepromin.
9. *M. avium-intracellulare* complex or MAC.

**411**

1. Four services are needed to fulfill this requirement: (1) the detection and isolation of mycobacteria in clinical samples, (2) identification of the organisms isolated, (3) susceptibility testing of the isolates, and (4) monitoring of the patient’s response to treatment.
2. Rate of growth, pigmentation, and colony morphology and other properties.
3. Tendency to grow only at temperatures above 25°C.
4. The number of days of incubation required for colonies to become visible without the aid of magnification.
5. Photochromogens, scotochromogens, and nonphotochromogens.
6. Two to four weeks or 14 to 29 days.
7. Colonies of *M. tuberculosis* are nonpigmented, grow best between 35 and 37°C, and usually require two weeks or more to form macroscopic colonies. On most egg-based media the colonies are rough, dry, and easily crumble. They are usually heaped up in the center, and have a thin, almost transparent, erose edge. Occasionally, the colonies are flat, rough, and spreading, with only a slight central elevation. Colonies of *M. tuberculosis* are always rough, never smooth and hemispherical. Combinations of colony forms between these extremes may be seen. Slow-growing, rough colonies that are niacin positive. Moisture in the tube can give the false appearance of smooth colonies, but once removed the colonies will return to their rough state.
8. Determination of growth rate, pigment production, niacin, nitrate, and 68°C catalase tests.
9. Yellow.
10. The ability of mycobacteria to reduce nitrates to nitrite.
11. Isoniazid-resistant mutants of *M. tuberculosis*.
12. Negative reaction.

## Unit Review Exercises

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

29. (408) Safety in the mycobacteriology laboratory is important because many of the techniques employed have the potential for creating hazardous aerosols of infectious
  - a. spores.
  - b. viral particles.
  - c. droplet nuclei.
  - d. elementary bodies.
30. (408) As a safety measure, what solution is the disinfectant for cleaning the laboratory following mycobacteria processing?
  - a. 0.5 percent sodium hypochlorite.
  - b. 1.0 percent sodium hypochlorite.
  - c. 0.5 percent sodium hydroxide.
  - d. 1.0 percent sodium hydroxide.
31. (408) What biosafety level practices are required for labs performing identification and susceptibility testing of mycobacterial isolates?
  - a. Biosafety level 1.
  - b. Biosafety level 2.
  - c. Biosafety level 3.
  - d. Biosafety level 4.
32. (408) Direct smears of feces for AFB is especially useful for evaluating what mycobacteria AIDS patients?
  - a. *M. chelonae*.
  - b. *M. tuberculosis*.
  - c. *M. intracellulare*.
  - d. MAC.
33. (408) What is the second most common specimen encountered in the laboratory for diagnosis of mycobacterioses?
  - a. Blood.
  - b. Urine.
  - c. Feces.
  - d. Sputum.
34. (408) Which of the following are the most popular egg-based media and agar-based media for recovery of mycobacteria?
  - a. Dubos Tween albumin and Wallenstein.
  - b. L-J and Wallenstein.
  - c. Dubos Tween albumin and Middlebrook 7H10 and 7H11.
  - d. L-J and Middlebrook 7H10 and 7H11.
35. (409) The only difference in the modified Kinyoun procedure from the Ziehl-Neelsen is the
  - a. mordant.
  - b. decolorizer.
  - c. counter stain.
  - d. primary stain.

36. (409) Which is the most commonly used fluorochrome stain for identification of mycobacteria on a smear?
- a. Acridine orange.
  - b. Calcofluor white.
  - c. Auramine O-rodamine.
  - d. FITC.
37. (410) The Mycobacterium are
- a. aerobic, spore-forming, motile bacilli.
  - b. anaerobic spore-forming, motile bacilli.
  - c. aerobic, nonspore-forming, nonmotile bacilli.
  - d. anaerobic nonspore-forming, nonmotile bacilli.
38. (410) What Mycobacterium is associated with a chronic granulomatous (Hansen's) disease?
- a. *M. avium*.
  - b. *M. leprae*.
  - c. *M. kansasii*.
  - d. *M. intracellulare*.
39. (411) Which one of the following is not a method of identifying most mycobacterial pathogens?
- a. Growth rate.
  - b. Pigmentation.
  - c. Growth at 25°C.
  - d. Colony morphology.
40. (411) Rapidly growing Mycobacterium usually mature within
- a. 2 days.
  - b. 7 days.
  - c. 14 days.
  - d. 21 days.

**Please read the unit menu for unit 3 and continue ➔**

## Unit 3. Introduction to Medical Mycology

|  |             |
|--|-------------|
| <b>3–1. General Characteristics of Fungi and Specimen Collection .....</b> | <b>3–1</b>  |
| 412. Morphology characteristics and classification of fungi .....          | 3–1         |
| 413. Collecting, processing, and shipping mycology specimens.....          | 3–8         |
| <b>3–2. Laboratory Identification .....</b>                                | <b>3–17</b> |
| 414. Macroscopic and microscopic examination.....                          | 3–17        |

**M**EDICAL mycology is the study of fungi that can invade the human body and produce disease. In medical mycology, clinical laboratory procedures are directed toward the demonstration, isolation, and identification of pathogenic fungi found in body tissue and fluids. Fungi are microorganisms without chlorophyll, roots, stems, or leaves. Some fungi can be seen without a microscope; for example, mushrooms. Historically, the fungi were regarded as relatively insignificant causes of infection. However, with the expansion of the immunocompromised patient population the list of opportunistic fungal pathogens is increasing. It is becoming apparent that the clinical laboratory must be able to recognize and identify potential fungal pathogens. This unit discusses the general characteristics, structures, and basis for classifying fungi, collecting and processing specimens, safety precautions, and laboratory identification of fungi.

### 3–1. General Characteristics of Fungi and Specimen Collection

The term *fungi* is a general term used for a group of eukaryotic protists which includes mushrooms, yeasts, rusts, molds, smuts, etc. Fungi lack chlorophyll and are probably degenerate offsprings of chlorophyll-bearing ancestors, most likely the algae. Fungi are important components in the energy cycle where they function as decomposers. Thus, in nature they are valuable saprophytes. Of the estimated 250,000 species, less than 150 are known to be primary pathogens in humans.

#### 412. Morphology characteristics and classification of fungi

Due to the importance placed upon macroscopic and microscopic morphology, identification of fungi can be frustrating. Learning basic structures and principles of classification can result in the ability to recognize and properly identify most medically important fungi. The International Code of Botanical Nomenclature adopted by the 15th International Botanical Congress, Tokyo, 1993, governs the nomenclature of fungi.

##### General characteristics

The medically important fungi are grouped simply based on macroscopic morphology as either a yeast or mold. These are descriptive, not formal taxonomic terms. Macroscopically, yeasts produce moist, creamy, opaque, or pasty colonies, and molds produce fluffy, cottony, woolly, or powdery colonies. Under certain circumstances, some slow-growing pathogenic fungi are dimorphic and produce a mold form at 25 to 30°C and a yeast form at 35 to 37°C. Microscopically, yeasts are characterized by simple budding, and molds are recognized by production of innumerable filamentous hyphae. Their rigid cell wall contains chitin (glucose and mannose polymers), mannans, and/or cellulose. They may be unicellular (yeast) or multicellular (molds). They differ significantly from bacteria because they have a definite nucleus or nuclei with a nuclear membrane. From the standpoint of size, the fungi are relatively large in comparison to the bacteria. The yeasts (nonfilamentous fungi) generally reach a diameter of about 5 to 6 µm, almost as large as an average red blood cell. Filamentous mold hyphae normally range in width from 5 to 50 µm and may attain a length of several millimeters. The microscopic detail of fungal structures and the fungi life cycle will be discussed in the next area.

***Monomorphic (one-form) fungi***

Certain fungus species of medical importance are known to multiply only by budding, regardless of whether they are incubated at 25 or 35°C. Fungi that propagate only by budding are designated monomorphic (one-form) yeasts. Fungi that are in mold form whether cultured at 25 or 35°C (if growth appears at 35°C) are defined as the monomorphic (one-form) molds.

***Dimorphic (two-form) fungi***

Another group of fungi, including those that cause some of the most serious mycotic infections in man, possess the unique capability of multiplying at 35°C in the form of the monomorphic yeast, and at 25°C in the form of the monomorphic molds. By exhibiting this temperature-dependent diphasic phenomenon, a given species of the group can exist either as a saprophytic mold in nature, or as a parasitic yeast form in human tissues. These morphological variations are reversible when incubation temperatures are adjusted, and many times this reversibility assists in accurate diagnosis. Those fungi capable of existing in two forms are known as dimorphic (two-form) fungi.

**Structures and life cycle**

The fungi have extremely simple fundamental structures: thallus, hyphae, mycelium, and spores or conidia. With these basic structures they live, multiply, and carry out the other functions characteristic of microorganisms in general. The life cycle of the fungi can be separated into two phases: (1) vegetative, and (2) reproductive.

***Vegetative phase***

The vegetative phase is characterized by elongated, branching filaments. These tubular filaments are known individually as hypha, or hyphae, when more than one is being considered. Hyphae are threadlike structures of the fungal thallus. The thallus is the actively growing vegetative portion or body of the fungus. The hyphae have cell walls of varying thickness lined with layers of cytoplasm. The thickness of the walls may increase greatly as the fungus matures. Chitin is the primary constituent of the cell walls of most of the higher fungi, while in some forms cellulose is probably the chief ingredient. The hyphae may be nonseptate (without partitions), septate (with transverse walls or septa), or pectinate (with comb-like lateral projections). Terminal coils or spirals on hyphae occur in some species. If septate are formed, the hypha is divided into cells, and each cell may contain one, two, or several nuclei. When septate walls are not formed (nonseptate), the entire hyphal filament is made up of a single cell with many nuclei embedded in the peripheral cytoplasm and scattered uniformly throughout its mass. A mass of hyphae is referred to as a mycelium. The mycelium is a mass composed of countless intertwined hyphae with two forms and functions—vegetative, and aerial.

***Vegetative mycelium***

The vegetative mycelium penetrates into the substrate and exchanges water and absorbs food for growth. It also anchors the fungus to the host either by penetration of simple hyphal filaments or, as in the case of the genus *Rhizopus*, by means of root-like rhizoids. The rhizoids permit better adherence to the host and provides a greater surface area for direct diffusion of enzymes from the fungus into the host. The enzymes convert nutrient material into simpler compounds that can be absorbed by the fungus for growth and reproduction.

***Aerial mycelium***

The aerial mycelium projects above the surface of the substrate and produces the fruiting bodies from which the spores are borne. The aerial mycelium may also be called sporangiophore or conidiophore, which is stem-like. Air currents to propagate the species then disseminate the spores.

***Reproductive phase***

The reproductive phase usually commences when the fungus reaches maturity or when it faces unfavorable environmental conditions, such as temperature variations or lack of nourishment. The

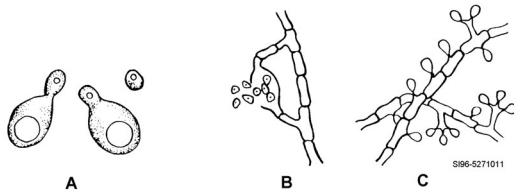


reproductive organ is called a spore. Spores may be asexual or sexual. Asexual sporulation is through mitosis (a process of cell reproduction from a single nucleus that forms two daughter cells with exactly the same chromosome and DNA content as that of the original cell). Sexual sporulation is through meiosis (a process of cell reproduction by the joining of two different or separate nuclei followed by rapid nuclear division and the formation of new cells with half of the chromosomes and DNA from each of the two different nuclei). Some fungi are capable of only asexual sporulation, whereas others have the capacity for both asexual and sexual spore formation. The term *spore* is reserved only for those reproductive structures that arise from meiosis or mitosis within a sporangium. All other asexual “spores” are called *conidia*. The spore may be either unicellular or multicellular; and when released from the mother plant, it is capable of duplicating the species. Although primarily reproductive in function, spores additionally serve to disperse the fungus and protect it from extinction during adverse environmental conditions. Along with characteristic morphology, the way in which they reproduce is an important means of classifying and identifying fungi.

#### *Asexual spores or conidia*

Sporangiospores, as shown in figure 3-1, are asexual spores produced or borne within a sac-like membrane (a sporangium) and are often motile. Asexual conidia, as shown in figure 3-2, include arthroconidia, blastoconidia, and chlamydoconidia.

Arthroconidia develop within a septate hyphal filament and they are disseminated by a breaking up of the spore chain thus formed.



**3-2. Examples of (A) Arthroconidia, (B) Blastoconidia, and (C) Chlamydoconidia.**

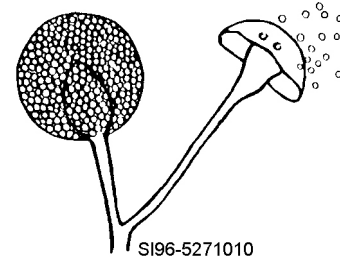
Blastoconidia arise from simple

budding of the yeast or yeast-like fungi. They may also develop directly from true or pseudomycelium.

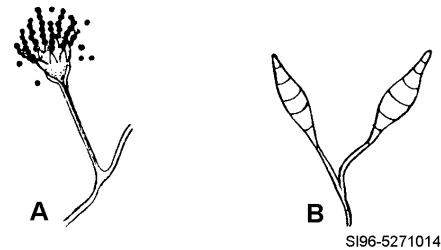
The chlamydoconidia are rounded, thick-walled bodies formed by a vegetative hyphal segment. It is a

highly resistant, resting spore capable of survival long after the vegetative mycelium has lost its viability.

Another variety of asexual conidia are formed either directly on the mycelium or on a modified supportive structure (for example, conidiophore, sporangiophore). Conidia are always borne free (no sac), vary greatly in size and shape, and are nonmotile. The small, unicellular type, as shown in figure 3-3, is called microconidium, while the larger, multicellular type is termed macroconidium.



**Figure 3-1. Asexual sporangiospores.**



**Figure 3-3. Examples of (A) Microconidia and (B) Macroconidia.**

#### *Sexual spores*

Sexual spores involve meiosis only. Sexual spores are divided into oospores, as shown in figure 3-4; zygospores, as shown in figure 3-5; basidiospores, as shown in figure 3-6; and ascospores, as shown in figure 3-7.

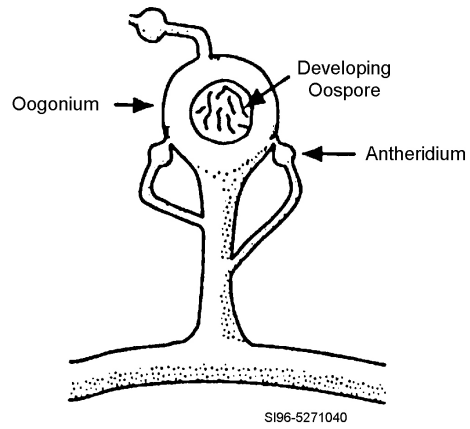


Figure 3-4. Example of oospores.

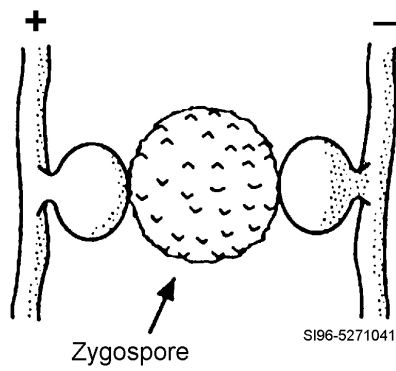


Figure 3-5. Example of zygospores.

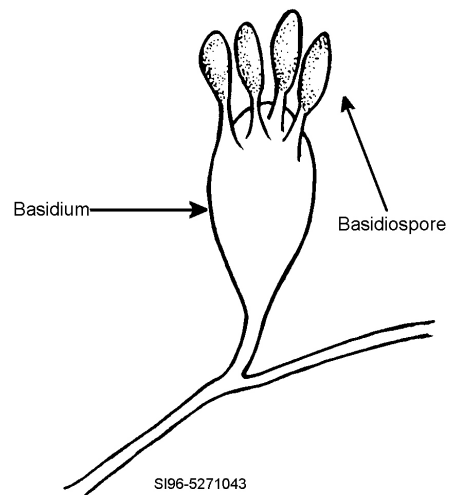


Figure 3-6. Example of basidiospores.

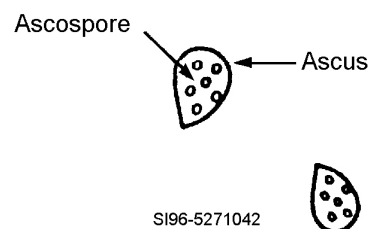


Figure 3-7. Example of ascospores.

The table below reviews the different types of spores or conidia and their characteristic.

| Asexual Spores                            | Characteristics                                      | Sexual Spores | Characteristics   |
|---|--|---------------|---|
| Sporangiospores                           | Produced in a large sac-like structure               | Oospores      | Formed by the union of 2 sexually undifferentiated cells                    |
| Arthroconidia                             | Hyphae segment into rectangular, thick-walled cells  | Zygospores    | Formed by two independent or different strains of sexually compatible types |
| Blastoconidia                             | Developed by budding                                 | Basidiospores | Produced in a fixed number within a club-shaped structure (basidium)        |
| Chlamydoconidia                           | Resistant spores, surrounded by a thick wall         | Ascospores    | Produced in a fixed number (usually 4 to 8) within a sac                    |
| Conidia<br>—Microconidia<br>—Macroconidia | Small, single-celled Large, multi-celled (segmented) |               |   |

The microscopic morphology of the fungi shown in the preceding figures is an important aid to identification. The type of hyphae, spores or conidia, and spore-bearing structure, and the manner in which these organs relate to each other in many instances will give a direct, accurate diagnosis of the mycotic agent under investigation.

### Classification

Many classification systems are available for the fungal groups, but most of them are complicated and not very practical. Fungi were classified with plants in the Plant Kingdom, but the organisms have been transferred to a separate fifth kingdom on the bases of cell structure.

#### Class Zygomycetes

This class contains the lower fungi. Most members produce broad, sparingly septate hyphae. They reproduce asexually by forming sporangia that contain sporangiospores that are large, ornamented cells, as shown back in figure 3-1. Sexual reproduction, when present, is by means of zygospores, as shown back in figure 3-5. However, zygospores are rarely seen in the clinical laboratory, because two different independent strains of compatible mating types are required for the production of sexual spores. This group contains only a few human pathogens, for example *Rhizopus* and *Mucor* species.

#### Class Ascomycetes

This class, along with the classes *Basidiomycetes* and *Deuteromycetes*, represents the higher fungi. The sexual spore for this class is the ascospore, identified by its production within an ascus sac, and shown back in figure 3-7. Under favorable conditions, sexual ascospores are formed. Four to eight ascospores develop within each ascus. The asci break open to release ascospores. This is illustrated in view B of figure 3-8. The asexual conidia in this class are morphologically different. The first type has unicellular, round, or oval forms reproducing asexually by the simple budding, as shown in view A of figure 3-8. The perfect yeast, genus *Saccharomyces*, represents this type. Note in view C of figure 3-8 that another type of *Ascomycetes* has separate hyphae producing solitary lateral conidia. Others produce asexual macroconidia as seen in view B of figure 3-3.

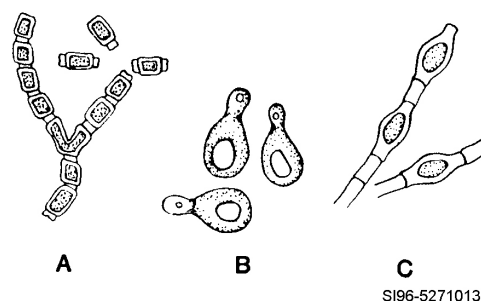


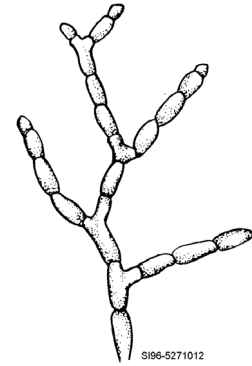
Figure 3-8. Reproductive forms of the Class *Ascomycetes* (A) asexual budding, (B) sexual ascospores, and (C) asexual solitary lateral conidia.

**Class Basidiomycetes**

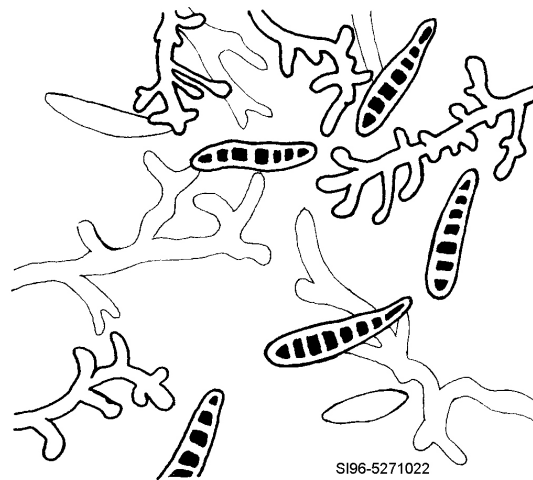
In this class, the hyphae are septate. Sexual reproduction is by means of basidiospores developed on club-shaped structures called basidia. Each basidium usually bears four exogenous basidiospores resembling toes on a foot. Figure 3-6 shows these characteristics. This group includes the rusts, smuts, and fleshy mushrooms. Except in mushroom poisoning, they play no role in disease production in humans.

**Class Deuteromycetes**

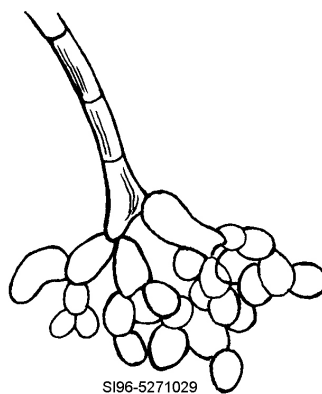
Class Deuteromycetes (“Fungi Imperfecti”) contains, with a few exceptions, most of the fungi pathogenic to man. These fungi lack demonstrable means of sexual reproduction and, therefore, are considered imperfect. Two morphologically distinct types represent them: septate; a filamentous, mold form; and imperfect yeast form resembling the perfect yeast form. The dimorphic fungi are in this class. This class of organisms reproduces by means of asexual conidia. Figures 3-9, -10, and -11 illustrate the various shapes of conidia in the “mold form” *Deuteromycetes*. The *Deuteromycetes* are important because they include many known disease-producing fungi.



**Figure 3-9.** Flask-shaped fruiting body containing conidia.



**Figure 3-10.** Broad-based, segmented conidia (macroconidia).



**Figure 3-11.** Budding or blastic type conidia.

### ***Class Oomycetes***

The *Oomycetes* undergo a special mating process characterized by the production of specialized structures, resulting in oospores contained in an oogonium. Refer back to figure 3-4. To date, there is only one medically important fungus in this group—*Pythium* species. The table below is a relatively simple scheme that can be used to classify the microorganisms within the Kingdom Fungi (Myceteae).

| <b>Taxonomic Designation</b>            | <b>Common Genera in the Class</b>   |
|---|---|
| Division: Amastigomycota                |   |
| —Subdivision: Zygomycotina              |   |
| ——Class: Zygomycetes                    |   |
| ————Order: Mucorales                    | <i>Rhizopus</i> , <i>Mucor</i> , <i>Rhizomucor</i> , <i>Absidia</i> ,<br><i>Cunninghamella</i> , <i>Saksenaea</i>   |
| ————Order: Entomophthorales             | <i>Basidiobolus</i> , <i>Conidiobolus</i>   |
| —Subdivision: Ascomycotina              |   |
| ——Class: Ascomycetes                    |   |
| ————Subclass: Hemiascomycetidae         |   |
| ————Order: Endomycetales                | <i>Saccharomyces</i> , <i>Pichia</i> , and some <i>Candida</i> species  |
| ————Subclass: Plectomycetidae           |   |
| ————Order: Onygenales                   | <i>Arthroderma</i> , some <i>Trichophyton</i> and <i>Microsporum</i><br>species, <i>Ajellomyces</i> , and some <i>Histoplasma</i> and<br><i>Blastomyces</i> species |
| ————Order: Eurotiales                   | Some <i>Aspergillus</i> and <i>Penicillium</i> species  |
| —Subdivision: Basidiomycotina           |   |
| ——Class: Basidiomycetes                 |   |
| ————Subclass: Holobasidiomycetidae      |   |
| ————Order: Agaricales                   | <i>Amanita</i> , <i>Agaricus</i>  |
| ————Subclass:<br>Heterobasidiomycetidae |   |
| ————Order: Filobasidiales               | <i>Filobasidiella</i> and some <i>Cryptococcus neoformans</i>   |
| —Subdivision: Deuteromycotina           |   |
| ——Class: Deuteromycetes                 |   |
| ————Subclass: Blastomycetidae           |   |
| ————Order: Cryptococcales               | Imperfect yeast: <i>Candida</i> , <i>Cryptococcus</i> , <i>Trichosporon</i> ,<br><i>Pityrosporum</i>  |
| ————Subclass: Hyphomycetidae            |   |
| ————Order: Moniliales                   |   |
| ————Family: Moniliaceae                 | <i>Epidermophyton</i> , <i>Coccidioides</i> , <i>Paracoccidioides</i> ,<br><i>Sporothrix</i> , <i>Aspergillus</i>   |
| ————Family: Dematiaceae                 | <i>Phialophora</i> , <i>Fonsecaea</i> , <i>Exophiala</i> , <i>Wangiella</i> ,<br><i>Xylohypha</i> , <i>Bipolaris</i> , <i>Exserohilum</i> , <i>Alternaria</i>       |
| ————Subclass: Coelomycetidae            |   |
| ————Order: Sphaeropsidales              | <i>Phoma</i>  |

| <b>Taxonomic Designation</b>       | <b>Common Genera in the Class</b> |
|------------------------------------|-----------------------------------|
| Division: Mastigomycota            |                                   |
| —Subdivision: Diplomastigomycotina |                                   |
| —Class: Oomycetes                  | <i>Pythium</i>                    |

### **413. Collecting, processing, and shipping mycology specimens**

An in-depth study of specimen collection, processing, and shipping was covered in volume 1 of this course. The techniques used during collection of specimens for bacterial cultures also apply for mycology specimens. In this lesson, we will briefly review only the differences between the specimens submitted for fungal cultures. A review of safety precautions is also important when processing mycology specimens. Therefore, safety rules are listed.

#### **General guidelines for specimen selection**

For the most part, there are four common factors to consider in selecting clinical specimens for isolation of fungi:

1. Site of the infection.
2. Age and immunocompetence of the patient.
3. Manifestation of the infection.
4. Stage of infection.

#### ***Site of the infection***

The site of infection will dictate the type of specimen that can be obtained. Common sense dictates that the specimen should be collected from the active sites of the infection. For example, in a situation where the skin is infected, samples in the form of skin scrapings from the outer active edge of the infection should be collected. These outer edge samples are preferred since they are most apt to contain identifiable fungal elements.

#### ***Age and immunocompetence of the patient***

The age of the patient oftentimes determines how the infection is manifested or even if an infection will occur. Adults and children vary in immunocompetence depending on their age and overall health. Recipients of organ transplants and prosthetic devices, patients with chronic renal failure, diabetes, leukemia, and other hematologic malignancies, and AIDS patients are considered immunocompromised. An immunocompromised adult or immunoincompetent child may succumb to an agent that for the average person would not pose a threat. The patient's immunologic status may also affect the choice of procedures you would use to confirm a diagnosis. It may be impossible to diagnose a fungal infection by serological means in a patient who is no longer able to produce antibodies to infectious agents.

#### ***Manifestation of the infection***

How has the mycosis manifested itself? Fungi can be categorized as to the type of tissue for which they have a natural affinity. The following four categories are fungal diseases:

1. Superficial mycoses— infections confined to the outermost layers of the skin and hair.
2. Cutaneous mycoses— infections that reach deeper into the epidermis, as well as invasive hair and nail diseases.
3. Subcutaneous mycoses— infections involving the dermis, subcutaneous tissues, muscle, and fascia.
4. Systemic mycoses— deep-seated infections that begin primarily in the lung but may spread to many organ systems.

### ***Stage of infection***

Lastly, the specimen to be collected may be determined by what stage of infection the patients are in when they first seek medical assistance. Mycotic infections, in their acute stage, are generally quite localized, and the specimen should be collected from the focal point of infection. If the disease is disseminated, the opportunity exists to collect specimens from multiple sites.

### **Collecting specimens**

As with all the other microorganisms, specimen collection and processing is the key for successfully recovering fungi. All specimens should be collected under aseptic conditions, transported in sterile leakproof containers, and processed as soon as possible. If swabs are used for collection, always submit two swabs. Use one swab for cultures and the other for preparing a slide for direct examination. It is extremely important for the physician to communicate with the laboratory about patient status and presumptive diagnosis for proper processing.

### ***Hair, skin, and nail scrapings***

Hair, skin, and nail scrapings are primarily used to diagnose superficial and cutaneous mycotic infections. Two superficial mycotic agents and certain cutaneous agents may be isolated from hair. It is best to examine the suspect area of the patient's scalp under a Wood's lamp (ultraviolet light) in a dark room to detect hairs that fluoresce with a bright, yellow-green color. Hairs infected by certain fungi may fluoresce when placed under a filtered ultraviolet light. Using forceps, pluck some of the fluorescing hairs and place them in a sterile Petri dish until time for examination. Remember, however, that tinea capitis (ringworm), caused by most species of *Trichophyton*, and many infections with *Microsporum gypseum*, do not show fluorescence. If no fluorescent hair is seen, re-examine the patient under ordinary light and pluck hair from the edge of the patches of infection. Skin or nail samples are obtained by scraping the skin around the lesion or the nail with a scalpel blade or a microscope slide after wiping affected area with an alcohol swab. These specimens are placed in a sterile Petri dish or tube for transport to the laboratory. **NOTE:** Do not refrigerate these specimens.

### ***Body fluids and other specimens***

The specimens that fall into this category are usually submitted for identification of subcutaneous mycoses. Body fluids, exudates, and tissues samples require special mention. Spinal fluid should be placed in sterile, screw-capped tubes. Blood cultures are used for identifying disseminated fungal infections. Use the same collection procedure for obtaining blood for bacterial cultures. Place 5 to 10 ml of blood in the appropriate automated culture bottle system used by your laboratory. Bone marrow specimens are useful for the isolation of certain fungal agents, such as *Histoplasma capsulatum*. These specimens should be placed in a sterile anticoagulant immediately upon collection, or placed in blood culture bottles. Urine samples should be processed as soon as possible after collection. Twenty-four hour urine and catheter bag samples are unacceptable for fungal cultures. Collect exudates and pus from the active margin of open abscesses or ulcers. Material from closed lesions aspirated with a syringe by a physician should be inoculated directly to appropriate media or placed in sterile screw-capped vials or tubes for later inoculation. Tissues for fungal examination should be collected aseptically. The tissue should be placed in sterile, wide-mouthed containers, and delivered to the laboratory for examination and culture as soon as possible. Care should be taken to prevent dehydration of the specimen. A small amount of sterile water or saline may be added to the sample for this purpose. Cornea scrapings from patients with suspected mycotic keratitis are usually collected in the operating room and directly inoculated to fungal media in Petri dishes or tubes.

### ***Sputum***

Sputum is the specimen most frequently submitted to the laboratory for the diagnosis of systemic mycosis. A 5 to 10 ml first-morning specimen is collected in a clean, sterile, wide-mouthed, screw-capped container. Two problems need to be overcome if a quality sputum sample is to be collected—normal flora contamination of the specimen and an improperly collected specimen. Many saprophytic fungi may be present in the mouth and their presence may make the recovery of clinically significant

fungi less likely. Although pathogenic fungi isolated from sputum are generally systemic, we should recall that repeated isolation of saprophytes, such as *Aspergillus* or *Mucor* species, may be of clinical significance. Instructing the patient to brush his or her teeth and use a mouthwash before collecting the first morning specimen will significantly decrease saprophytic fungi. The second problem encountered in collecting sputum samples—improper collection by the patient—can be corrected by giving the patient clear instructions, both verbal and written. These instructions inform the patient that sputum samples are lower respiratory tract secretions and not saliva from the oropharynx. In very young, incapacitated, or uncooperative patients who swallow these secretions, gastric lavage or bronchoscopy specimens may have to be collected. Upper respiratory swabs can also be collected.

### Cultivation and isolation requirements

Like bacteria, the fungi are lacking in chlorophyll and must rely on some source of available organic material for food. There are fungi that can produce enzymes capable of breaking down nearly all the organic substances known to man. In some cases, their food requirements include complex sugars, proteins, and fats. Other fungi have little need for preformed nutrients and can grow in pickle brine or organic acids when the necessary minerals are present. It is not atypical to find fungi growing on fingerprints left on camera lenses. Unlike the bacteria, which prefer a slightly alkaline pH, fungi grow best at an acid pH, normally around pH 5.6.

### Media

Since the fungi and many bacteria can use identical substances for growth, antibiotics are often incorporated into fungal mediums to inhibit contaminating bacteria and saprophytic fungi. There is one disadvantage, however. Numerous fungi of medical importance are also inhibited and may not be recovered from initial plating if antibiotics are present. Chloramphenicol or cycloheximide, or both of these inhibitory substances, may be added to the medium to retard growth of contaminants that include saprophytic fungi and contaminating bacteria. The table below examines the different mediums used for the recovery and identification of most medically important fungi.

| Media  | Purpose  |
|--|--|
| Primary Recovery Media:                        |  |
| Brain-heart infusion agar (BHI)                | Supports growth of fastidious thermally dimorphic fungi, recovery of saprophytic and pathogenic fungi                  |
| Buffered-charcoal yeast extract agar           | Primary recovery of <i>Nocardia</i> species  |
| Dermatophyte test medium (DTM)                 | Recovery of dermatophytes, use for screening only  |
| Inhibitory mold agar (IMA)                     | Enriched media containing chloramphenicol or gentamicin, supports growth of almost all fungi while inhibiting bacteria |
| Mycosel agar                                   | Recovery of dermatophytes, inhibits some saprophytes   |
| Potato Dextrose and Potato Flake agar          | Stimulates conidia production, used in preparation of microslide cultures  |
| Sabouraud dextrose agar (SDA) with antibiotics | Recovery of saprophytic and pathogenic fungi, inhibits bacteria  |
| SABHI agar                                     | Primary recovery of saprophytic and pathogenic fungi   |
| Yeast-extract phosphate with Ammonia           | For recovery of <i>Histoplasma capsulatum</i> and <i>Blastomyces dermatitidis</i> from contaminated specimens          |
| Differential Test Media:                       |  |
| Ascospore agar                                 | Detection of ascospores in ascosporogenous yeasts ( <i>Saccharomyces</i> species)                                      |
| Birdseed (Niger Seed) agar                     | Identification of <i>Cryptococcus neoformans</i>   |
| Casein agar                                    | Differentiation of aerobic actinomycetes and characterization of some dematiaceous fungi                               |
| Cornmeal agar with Tween 80                    | Differentiation of yeast and various species of <i>Candida</i> , identification of <i>C. albicans</i>                  |



| Media                                       | Purpose  |
|---|--|
| Czapek's agar                               | Recovery and differentiation of <i>Aspergillus</i> species                     |
| Rice medium                                 | Identification of <i>M. audouinii</i>  |
| Sabouraud dextrose agar (SDA) with 15% NaCl | Testing tolerance to 15% NaCl for identification of dematiaceous (black) fungi |
| <i>Trichophyton</i> agars 1 – 7             | Differentiation of <i>Trichophyton</i> species                                 |
| Urea agar                                   | Differentiation of yeast-like fungi  |

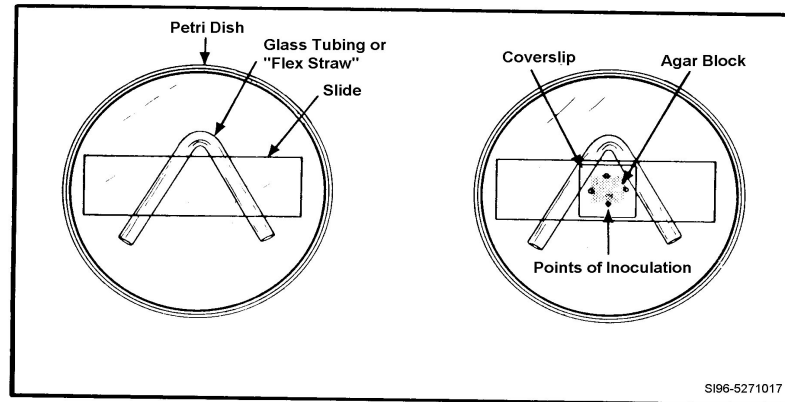
From a review of the table, it is readily seen that mycologists have a number of options in the selection of media for primary recovery and differential testing of fungi. These decisions must be based on individual needs. A possible battery of primary culture media may include Sabouraud dextrose agar (SDA), SDA with chloramphenicol and cycloheximide, inhibitory mold agar (IMA), and brain-heart infusion agar (BHI). SABHI agar is a combination of SDA and BHI with or without antibiotics and is one of the most useful media for the isolation of pathogens. The different mediums can be placed in Petri dishes, screw-capped tubes, or on slides.

#### *Tube culture method*

Tubed culture media or “slants” are routinely used for isolating fungi from clinical specimens. Screw-capped, 18 × 150 mm test tubes afford a larger butt and are more resistant to drying. Hence, this culture method is preferred because of the longer period of incubation often necessary for adequate fungal growth. The slants should be thick, and after inoculation, the caps should be screwed on but left slightly loose. Be sure that all tubes are properly labeled before incubation. The clinical material is inoculated with the 22-gauge nichrome needle by making two or three deep cuts into the medium at about midpoint of the slant surface. Culture tubes are more easily stored, require less space, obviously safer for small laboratories, and have a lower dehydration rate than culture plates. Leave screw caps of the tubes slightly loose to permit oxygen to enter and allow the slant surface to remain dry through air exchange with the outside. A dry surface gives better sporulation and better pigment production. Laboratories staffed with personnel experienced in handling fungal cultures may use Petri dishes. Although somewhat more hazardous, the advantages of Petri dishes include better aeration of cultures, a larger surface area which provides better isolation of colonies, greater ease of examination, and subculture of fungal colonies.

#### *Slide culture method*

Slide cultures for monomorphic mold permit the microscopic observation of the undisturbed relationship of spores to hyphae—one of the main criteria for identification of pathogenic or saprophytic monomorphic molds. This is the best method for preserving and observing the actual structures of a fungus. A medium conducive to sporulation or mold should be used in this culture. Thus, if a teased or Scotch tape mount fails to yield sufficient information for identification, or in the event that permanent study slides are desired, the slide culture for monomorphic molds can be set up. This procedure, shown in figure 3-12, uses a Petri dish containing a bent glass rod or a “flex straw”, a coverslip, and an ordinary microscopic slide. All (except flex straw) have been wrapped in metal foil and sterilized. With a sterile scalpel blade, cut approximately 1 square centimeter of medium from a plate of potato dextrose agar or other appropriate agar. Place it aseptically on the center of the microscopic slide atop the bent glass rod within the Petri dish. Inoculate each of the four sides of the square of medium by making a cut of 1 millimeter into the medium. Cover the square of inoculated medium with the coverslip and add 8 to 10 drops of sterile water to the bottom of the Petri dish.



**Figure 3-12. Procedure for slide culture method.**

Replace the top of the Petri dish and incubate the culture at 25°C until growth appears. The slide culture may be examined microscopically without disturbing the coverslip. When the desired stage of growth is reached, a lactophenol cotton blue preparation may be made by gently lifting the coverslip (with its adhering fungus) and laying it on a microscopic slide holding 1 to 2 drops of dye. Make permanent mounts by blotting the excess dye around the edges of the coverslip and sealing the coverslip with fingernail polish or varnish. Highly virulent molds should not be grown on slide cultures because of the danger of infection to persons handling them.

### **Processing specimens**

Before we go over processing specimens, we must review safety precautions.

### ***Safety precautions***

Every laboratory performing diagnostic work in mycology should be equipped with a biological safety cabinet operating under negative air pressure to draw fungus spores away from the technician. Many fungi produce spores that are very light and easily become airborne. Thus, precautions are necessary to prevent contamination of the laboratory and infection of personnel. The principles of major precautions for the mycology laboratory are essentially the same as those for any other clinical laboratory. The following is a list of the safety precautions:

1. Do not eat, drink, or apply cosmetics in the laboratory.
2. Wear a laboratory coat, gloves, and/or face shield when working in the laboratory.
3. Work in a biologic safety cabinet class I or II when processing specimens other than hair, skin, or nail scrapings.
4. Do not mouth pipette.
5. Wipe up all spills and splatters with disinfectant (10% bleach or Amphyl).
6. Wipe bench tops with disinfectant when work is completed.
7. Wash hands with soap and water after handling mycology specimens and before leaving laboratory.

### ***Precautions that specifically apply to the mycology laboratory***

Probably a good rule of thumb is to handle each specimen as if it contained a highly virulent mycotic agent. In the event of an accident, such as splashing of infectious material into the eye or open skin, report this immediately to a physician. If you drop a culture, cover the debris with disinfectant. As with any other type of accident in microbiology, use good judgment and be prepared to institute decontamination measures immediately.

1. Do not set up a slide culture of a mold until it has been examined in a teased preparation.
2. Do not set up or use the slide culture method for identification purposes of *Histoplasma*, *Blastomyces*, *Coccidioides*, *Paracoccidioides* species or *Xylohypha bantiana*.
3. Do not make Scotch tape preparations of pathogenic mold (excluding dermatophytes). All such preparations may easily contaminate the work environment.
4. Discard supernatants and all other unneeded specimens into containers with disinfectant.
5. Do not work with *H. capsulatum*, *C. immitis*, and *B. dermatitidis* without a biological safety cabinet. These are considered biosafety level 3 organisms if you are propagating or manipulating cultures of these organisms.
6. Biosafety level 2 precautions are to be followed while processing clinical specimens.
7. All fungus cultures to be discarded must first be sterilized by autoclaving.
8. Tubed slants of media should be used for primary isolation. Petri dishes (plates) should *never* be used if (a) *Coccidioides immitis* is suspected or (b) if a culture is to be mailed.
9. Avoid splattering when you sterilize the wire needles or loops in the incinerator.
10. *Never* sniff or waft a fungal culture to determine whether it has an odor.

### ***Hair, skin, and nail scrapings***

Superficial dermatophyte infections can often be diagnosed by demonstrating the characteristic delicate hyphal forms in KOH mounts of infected hair, skin scales, or nail scrapings. Regardless of the presence or absence of fungal structures microscopically; hair, skin, and nail scrapings should be cultured on media for primary isolation of dermatophytes and pathogenic fungi. If infections with *Piedraia hortae* (black piedra) or *Trichosporon* species (white piedra) are suspected, SDA without chloramphenicol and cycloheximide should be inoculated, because both fungi are sensitive to these antibiotics.

### ***Body fluids and other specimens***

Body fluids and tissue, such as peripheral blood, urine, synovial fluid, bone marrow, or biopsy material collected under aseptic conditions, may be inoculated directly to culture media. Large volumes that require centrifugation for concentration or specimens that cannot be immediately processed can be temporarily stored in sterile, screw-capped tubes or bottles at 4°C. The culture techniques used for exudates, body fluids, and tissues are essentially the same as those for sputum.

### ***Sputum***

Centrifuge the sputum and examine a drop of the sediment directly on a clean glass slide under coverslip. First, scan under low power, then high power, using reduced illumination. Because more than one mycotic agent may give the same clinical signs, several routine identifying steps should be followed. After direct examination, use a Gram-stain to demonstrate hyphae or blastospores. Fungal structures stain gram-positive. An acid-fast stain will demonstrate the mycelium of *Nocardia* species. The Giemsa or Wright stain is useful for *Histoplasma capsulatum*. An India ink mount will reveal the encapsulated budding or non-budding blastospores of *Cryptococcus neoformans*. Yeast extract-phosphate agar is an excellent medium for contaminated respiratory specimens since bacteria and saprophytic yeasts are inhibited, whereas the systemic fungi are not.

### ***Incubation requirements***

The setting up of separate cultures at 25°C and 37°C has been an expensive and time-consuming practice as reflected by the relatively low rate of recovery of the dimorphic species in most laboratories. It is now recommended that the mold form of these fungi be recovered first at 25 or 30°C, and any isolates suspected of being dimorphic be secondarily cultured to the yeast form by incubating subcultures at 35 to 37°C on special enriched media. This technique differs from past practices of setting up fungal cultures at 37°C to recover the yeast form of the dimorphic fungi. The

cultures should be examined at least three times a week for visible evidence of fungal growth. All cultures should be incubated at 30°C (optimal) for a minimum of 30 days before being discarded. Six weeks of incubation is considered optimal, but space limitations can often preclude this.

### **Preparation of specimens for shipment**

For a number of reasons, sometimes it's necessary to send a clinical specimen or an unidentified fungus isolate to a reference laboratory for further study.

### ***Packaging***

Breakage and possible contamination of the container can be avoided by proper attention to packaging. Cultures should be submitted in screw-cap glass tubes that permit tight closure, and sealed with adhesive tape. Wrap specimen container in cotton or other absorbent material and insert into a metal container. Test tubes must always be wrapped individually in cotton or paper before placing them in a metal container. Any free space in the metal container is then stuffed with cotton or other packing material to prevent breakage. The specimen container is capped and placed in a cardboard, corrugated fiberboard, wood, or shipping container. Any free space in the shipping container should be stuffed with packing material. Fluid specimens obtained under aseptic conditions (for example, blood, spinal fluid, or aspirated pus) may be shipped if they are tightly sealed in sterile vials or tubes and carefully packed. Hair, skin, and nail scrapings or pure cultures of suspected pathogens may also be shipped; but urine, bronchial washings, or biopsied tissue usually become quickly overgrown with contaminants in transit, making the isolation of pathogenic fungi improbable. If sputum samples must be shipped by mail, overgrowth with contaminating microorganisms may be controlled by adding penicillin, streptomycin, or chloramphenicol. Cotton swabs should never be mailed because they will dry out. Of course, Petri dishes are subject to breakage and leakage and; thus, should not be used for mailing purposes.

### ***Labeling***

Labeling information needed by the reference laboratory includes the patient's name, age, and sex; identification number or registration number; name of the requesting physician and the submitting facility; specimen origin and date of collection; and preliminary diagnosis or suspected organism. When cultures are submitted, such information as type of medium, date of inoculation, and incubation temperature are extremely helpful. Additional data that can be of value, when relevant, are the patient's record of residency and travel in the United States and foreign countries, and the results of any skin or serological tests. Follow basic guidelines for shipment of bacteriological specimens in volume 1, unit 2.

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## **Self-Test Questions**

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

### **412. Morphology characteristics and classification of fungi**

1. What are the macroscopic morphology of yeasts and molds?
2. What does it mean when a fungi is "dimorphic"?
3. What are the microscopic morphology of yeasts and molds?

4. What are the fundamental structures of a fungus?
5. What are the two phases of the fungus life cycle?
6. What are the three forms of hyphae?
7. What is asexual sporulation?
8. What is sexual sporulation?
9. What are the two types of conidia?
10. Match each class of fungi 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, more than once, or not at all.

*Column A*

- \_\_\_\_ (1) They include many, if not most of the known disease producing fungi.
- \_\_\_\_ (2) The sexual spore is the ascospore.
- \_\_\_\_ (3) They develop club-shaped structures.
- \_\_\_\_ (4) This class contains the lower fungi.
- \_\_\_\_ (5) Contains *Rhizopus* and *Mucor* species.
- \_\_\_\_ (6) Contains the "Fungi Imperfecti".
- \_\_\_\_ (7) Contains the *Pythium* species.

*Column B*

- a. Class *Zygomycetes*.
- b. Class *Ascomycetes*.
- c. Class *Basidiomycetes*.
- d. Class *Deuteromycetes*.
- e. Class *Oomycetes*.

**413. Collecting, processing, and shipping mycology specimens**

1. What are the four common factors to consider in selecting clinical specimens for isolation of fungi?
2. What is superficial mycoses?
3. What is cutaneous mycoses?

4. What is subcutaneous mycoses?
5. What is systemic mycoses?
6. What specimens are used to diagnose superficial and cutaneous mycotic infections?
7. Bone marrow specimens are useful for isolation of what fungal agent?
8. What two problems must be overcome to collect quality sputum samples for fungal cultures?
9. What is the one disadvantage of using media with antibiotics?
10. What are the two most common antibiotics used in fungal mediums?
11. What is a possible battery of primary culture media for isolation of fungi?
12. Why are screw-capped, 18 × 150 mm test tubes preferred?
13. Why is the slide culture method the best method for identification of fungal structures?
14. Which organisms should *not* have slide cultures set up for identification purposes?
15. Which suspected organism should *never* be inoculated to Petri plates or mailed?
16. What two stains are useful for observing *Histoplasma capsulatum*?
17. An India ink mount will reveal the encapsulated budding or non-budding blastospores of what species?

18. What is the recent recommendation for incubating fungal cultures?

19. How long should cultures be kept before discarding?

20. What labeling information is needed by the reference laboratory?

## 3-2. Laboratory Identification

The techniques used in medical mycology are similar, in many respects, to those used in medical bacteriology. That is to say, we attempt to plant an organism on artificial culture media and grow it out in isolated colony form. The culture can then be studied from the standpoint of its morphology, physiological properties, and immunologic characteristics. With the mycotic agents, however, much greater emphasis is placed on differences in colony appearance (macroscopic) and details of cell structure and arrangement (microscopic). Less reliance is placed on fermentation reactions and serological tests.

### 414. Macroscopic and microscopic examination

In this section, we will mention some of the techniques designed to make morphological studies of the fungi easier. When direct morphological studies are not sufficient, special techniques must be used. These techniques will be discussed in detail in subsequent lessons of this text.

#### Macroscopic examination

Gross-examination of inoculated tubes should be carried out on a routine daily basis. If this is not possible, they should be examined at least three times a week for visible evidence of fungus growth. The following growth characteristics aid appreciably in the final identification of the fungus; rate of growth, colony size, elevation, shape and margin, texture, and surface pigmentation on both front and reverse sides of the slant. There is a considerable difference in the growth rate between the fungi and bacteria. When culturing bacteria, colony examination and biochemical testing can usually be accomplished 24 to 48 hours after the specimen is inoculated to media. The fungi, particularly the pathogenic forms, may require three to five weeks, or longer, to reach a growth state that permits definitive diagnosis. Rapid growers usually appear within five to 10 days and slow growers within three to four weeks. See volume 1 of this CDC for terms used to describe colony elevation, shape, and margin. Yeast-like, creamy, moist, smooth, wrinkled, glabrous, powdery, granular, velvety, fluffy, and cottony can be used to describe colony texture. Pigmentation can be white, cream, tan, beige, yellow, light gray, dark gray, orange, green, pink, reddish, deep red, purple, brown, black, and nonpigmented.

#### Microscopic examination

Microscopic examinations can be done directly on the patient specimens and fungal colony. Direct microscopic examination of the specimen is in addition to, not instead of, a culture. It provides the physician with early information regarding possible therapy needs. Trained personnel should perform interpretations on direct examinations because some fungal elements are part of our normal flora and our environment. A number of stains and procedures can be used to detect fungal elements in direct examination of patient samples. Each stain and procedure has its own advantages and disadvantages; therefore, each laboratory should survey its needs and capabilities and choose the best procedures for its laboratory. Following is a brief discussion of the different stains and procedures.

### *Wet mounts*

The technique of wet mounting is used for preparing specimens from either patients or cultures for microscopic viewing. Once a yeast or mold has been recovered on primary isolation medium, it is necessary to prepare mounts for microscopic study of the colonies.

#### *Potassium hydroxide (KOH) mount*

The potassium hydroxide wet mount is perhaps the most widely used of all wet mounts. Let's assume that a patient with a lesion on his arm has been sent to the mycology laboratory. Wash the lesion area thoroughly with 70% alcohol to remove any dirt particles, bacteria, and medication, which might interfere with microscopic examination. Then, scrape the outer, active margin of the lesion with a sterile scalpel blade. Deposit the scrapings directly into a sterile Petri dish. Transfer a small fragment of the material into a drop or two of 10% KOH on a clean glass slide and coverslip it. Let the preparation sit at room temperature from 15 to 30 minutes so that tissue and debris will dissolve in the KOH. Then, examine the slide under low, then high magnification. To save time, you may heat the slide gently for a few seconds over a flame. **NOTE:** Do not boil the KOH. The strong alkali serves as a clearing agent, making the fungal elements (spores, conidia, and/or mycelium) more easily discernible. The KOH mount can only provide information as to the presence or absence of fungal elements in the tissue scrapings, but does nothing to provide towards the identity if present. A positive identification requires culturing and additional observation of the ensuing growth. You can easily identify hyphal forms of fungi associated with infection in skin or nails after warming epithelial scraping or nail clippings gently in 10% potassium hydroxide, which acts as a keratolytic agent. The use of potassium hydroxide for the identification of fungi from nonkeratinized specimens (e.g., sputum) is inappropriate and has no advantage over saline. Phase microscopy is found to be most helpful in the direct examination of sputum or other fluid specimens, with fungal spores and hyphae appearing in sharp contrast to the background. Cotton swabs should not be used in preparing slides because cotton strands can be mistaken for hyphae. Also, note that the KOH preparation is not a permanent mount since the reagent will eventually destroy the fungal elements.

#### *Lactophenol cotton blue (LPCB) mount*

Lactophenol cotton blue is a mounting fluid and is the basic stain used in mycology. It serves a fourfold purpose—the lactic acid acts as a clearing agent; phenol serves as a fungicidal or killing agent; glycerol prevents drying; and cotton blue (Poirrier's or aniline blue) lends color to the structures and makes them more readily observable. Lactophenol cotton blue with polyvinyl alcohol (PVA) added is excellent for making permanent mounts of fungal wet preparations or slide cultures.

#### *Tease mount technique*

Using either the teased or cellophane tape wet mount, we can now examine the growing colony microscopically. Ordinarily, the specimen used for making teased, wet mount preparations is obtained from a fungus growing on the surface of a culture medium. The mycologist uses a stiff, nichrome wire (22 gauge) with the last 4 or 5 mm bent at right angles to the main shaft. The wire is fixed firmly in a metal handle for ease of transferring mycelial growth. A pair of short, stiff teasing needles are useful in pulling apart dense mycelium for microscopic examination. Remove a small quantity of the mycelium with a stiff needle and scrape it into one or two drops of lactophenol cotton blue mounting fluid placed on a clean glass slide. Next, gently pull the mycelium apart so that individual structures will be clearly visible. Rough handling will destroy the all-important spore-hyphal relationship, or natural arrangement, and make identification difficult. The slide may now be coverslipped and examined under the microscope using low and high dry power. The slide can be preserved for later use by sealing the edges with fingernail polish or varnish. The most significant drawback of the tease mount method is the difficulty in preserving continuity between the spore, fruiting structures, and hyphae after such rough handling. This fault can be critical when exact microscopic definition is required for differential identification. Transfer of cultures should be done over absorbent paper (paper toweling) moistened with a fungicidal agent. It is also wise to use a small vial of sand



moistened with a fungicidal agent to remove excessive fungal material from the wire before incinerating. This prevents dangerous spattering of infectious material as the wire is sterilized.

#### *Cellophane or scotch tape mount technique*

Some workers prefer the clear cellophane tape wet mount to the teased preparation because the cellophane tape technique is relatively simple. It subjects the fungus to considerably less trauma, and preserves the structural relationship essential for accurate identification. There are two disadvantages to this technique: (1) visual acuity is partially lost due to the tape itself, regardless of its clarity, and (2) tape does not lend itself to the preservation of slides for future study because there is a tendency for the tape to loosen. A strip of clear cellophane tape approximately 1 1/2 inch in size is used. Uncap the tube containing the fungal culture and touch the sticky surface of the tape to the mycelium with forceps. The tape is then placed (sticky surface down) on top of 1 to 2 drops of lactophenol cotton blue on a clean glass slide. Press the tape gently but firmly against the glass slide. The mount may then be examined microscopically.

#### *India ink preparation mount*

The India ink wet mount technique is used primarily to reveal the encapsulated budding or non-budding blastospores of *Cryptococcus neoformans* in spinal fluid. However, other body fluids may be similarly examined. After centrifuging the specimen, transfer a loopful of the sediment to a clean glass slide and mix the material with a small drop of undiluted India ink. After coverslipping, examine the slide microscopically. Since the India ink is unable to penetrate the large capsule surrounding *Cryptococcus neoformans*, the capsule appears as a clear disc against a black background. Also, a centrally located dense, single, or budding cell within the disk will be seen. Figure 3-13 provides an example. The sole purpose of the India ink is to reveal the capsule of *C. neoformans* as an aid to accurate diagnosis. If the wire loop is used for mixing spinal fluid and ink, allow the wire to cool sufficiently to prevent precipitation of the ink by heat. Sputum and pus samples should be cleared with KOH and heat, and then mixed with India ink.

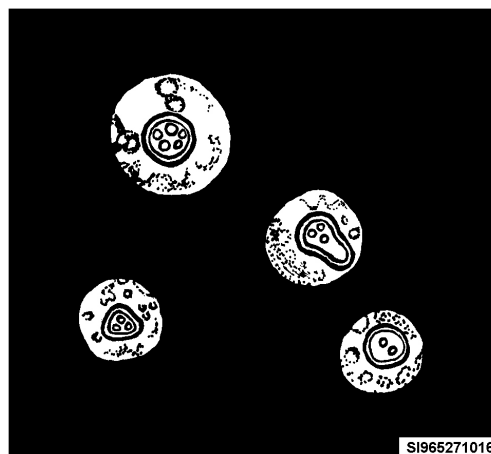


Figure 3-13. India ink preparation of *Cryptococcus neoformans*.

#### *Stained preparations*

Stained preparations are useful for demonstrating a range of microscopic features and may be used, when indicated, as an aid in identification of certain fungi. Thus, wet mounts, dried slides, and fixed films may be stained. Fungal elements and structures can be seen in fixed materials with the following different stains.

#### *Gram stain*

Gram stain is useful in demonstrating mycelial elements and yeast cells in a variety of specimens. The mycologist uses this method when examining granules or other specimens for presence of an

actinomycete. The stain is not primarily used to demonstrate the presence of fungi in specimens. All fungi are gram-positive, often staining so heavily that it is not possible to observe morphological features. Be aware that common Gram-stain artifacts can appear as yeast cells.

#### *Acid-fast stain*

An acid-fast stain is used to detect the partially acid-fast hyphal segments of *Nocardia* species, *Blastomyces dermatitidis*, and ascospores. Acid-fast organisms stain red.

#### *Wright stain or Giemsa stain*

Wright stain or Giemsa stain is used for detecting intracellular yeast forms of *Histoplasma capsulatum* in blood and bone marrow, since the organism is too small to notice readily without staining.

#### *The periodic acid-Schiff (PAS), Methenamine silver, and Papanicolaou stains*

The PAS and Methenamine silver stains are most often used in histopathology for demonstrating fungi in tissue sections. Yeast forms and hyphae of molds are readily distinguished by the use of either of these stains. The dyes included in these stains specifically stain the carbohydrate-rich constituents of the fungal cell wall. Papanicolaou stain allows the cytotechnologists to detect fungal elements in secretions and body fluids.

#### *Calcofluor white*

Calcofluor white is a relatively new stain that binds to beta 1–3 and 1–4 polysaccharides such as cellulose and chitin. As we have already discovered, cellulose and chitin are present in the fungal cell wall. This stain is extremely useful in the clinical mycology laboratory because it can rapidly (in one minute) detect fungal elements through bright fluorescence and it can be mixed with KOH if clearing is required. Of course, this stain requires a fluorescence microscope. This stain replaces the older traditional KOH preparation in laboratories that have a fluorescence microscope.

### **Non-culture-dependent methods**

Rapid, accurate, non-culture-dependent methods for diagnosis of life-threatening fungal infections need to be developed. Methodologies with potential for providing rapid identification of fungi include chemical detection of fungal macromolecules, detection of fungus-specific metabolites, and detection of fungus-specific nucleic acid sequences. Direct examination of the specimen and detection of circulating antigens are the only rapid diagnostic procedures currently available. Antigen detection assays are available for Cryptococcal and Histoplasma antigens and they are widely accepted. Tests for aspergillosis, candidiasis, and coccidioidomycosis are now available in Europe and, in the near future, should be available in the United States if in fact they aren't already at the time of publication of this volume. Antigen specific tests and assays will be covered in 4T051C, volume 2, *Immunology*.

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## **Self-Test Questions**

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

### **414. Macroscopic and microscopic examination**

1. What are the growth characteristics that aid appreciably in the final identification of the fungus?
2. When do rapid growers and slow growers usually appear on media?

3. Microscopic examinations can be performed on what type of materials?
4. Which wet mount is perhaps the most widely used of all wet mounts?
5. What is the wet mount specimen collection procedure?
6. Why do you allow the specimen and the 10% KOH sit at room temperature from 15 to 30 minutes?
7. What kind of information does the KOH wet mount provide?
8. What is the basic stain used in mycology and its fourfold purpose?
9. The teased or cellophane tape wet mount is used for what type of specimen?
10. What is the most significant drawback of the tease mount method?
11. What are the two disadvantages to the cellophane tape wet mount?
12. What is the sole purpose of the India ink when used to detect *Cryptococcus neoformans*?
13. What should be done to sputum and pus samples before mixing with India ink?
14. What are the stains that can be used for detecting fungal elements in specimens?
15. Why is the calcofluor white an extremely useful stain?

16. What are some of the methodologies with potential for providing non-growth, rapid identification of fungi?
17. What antigen detection assays are currently available and widely accepted?

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### Answers to Self-Test Questions

#### 412

1. Macroscopically, yeasts produce moist, creamy, opaque, or pasty colonies, and molds produce fluffy, cottony, woolly, or powdery colonies.
2. Under certain circumstances, some slow-growing pathogenic fungi are dimorphic and produce a mold form at 25 to 30°C and a yeast form at 35 to 37°C.
3. Microscopically, yeasts are characterized by simple budding and molds are recognized by production of innumerable filamentous hyphae.
4. Thallus, hyphae, mycelium, and spores or conidia.
5. Vegetative and reproductive.
6. Nonseptate (without partitions), septate (with transverse walls or septa), or pectinate (with comb-like lateral projections) and terminal coils or spirals in some species.
7. Asexual sporulation is through mitosis (a process of cell reproduction from a single nucleus, which forms two daughter cells with exactly the same chromosome and DNA content as that of the original cell).
8. Sexual sporulation is through meiosis (a process of cell reproduction by the joining of two different or separate nuclei followed by rapid nuclear division and the formation of new cells with half of the chromosomes and DNA from each of the two different nuclei).
9. The small, unicellular type is called microconidium, while the larger, multicellular type is termed macroconidium.
10. (1) d.  
(2) b.  
(3) c.  
(4) a.  
(5) a.  
(6) d.  
(7) e.

#### 413

1. Site of the infection, age and immunocompetence of the patient, manifestation of the infection, and stage of infection.
2. Superficial mycoses—infections confined to the outermost layers of the skin and hair.
3. Cutaneous mycoses—infections that reach deeper into the epidermis, as well as invasive hair and nail diseases.
4. Subcutaneous mycoses—infections involving the dermis, subcutaneous tissues, muscle, and fascia.
5. Systemic mycoses—deep-seated infections that begin primarily in the lung but may spread to many organ systems.
6. Hair, skin, and nail scrapings.
7. *Histoplasma capsulatum*.
8. Normal flora contamination of the specimen and an improperly collected specimen.

9. Numerous fungi of medical importance are also inhibited and may not be recovered from initial plating if antibiotics are present.
10. Chloramphenicol or cycloheximide.
11. SDA, SDA with chloramphenicol and cycloheximide, IMA, and BHI.
12. Screw-capped, 18 × 150 mm test tubes afford a larger butt and are more resistant to drying.
13. It permits the microscopic observation of the undisturbed relationship of spores to hyphae—one of the main criteria for identification of pathogenic or saprophytic monomorphic molds.
14. Do not set up slide cultures of *Histoplasma*, *Blastomyces*, *Coccidioides*, *Paracoccidioides* species or *Xylohypha bantiana* for identification purposes.
15. *Coccidioides immitis*.
16. Giemsa or Wright stain.
17. *Cryptococcus neoformans*.
18. It is now recommended that the mold form of these fungi be recovered first at 25 or 30°C, and any isolates suspected of being dimorphic be secondarily cultured to the yeast form by incubating subcultures at 35 to 37°C on special enriched media.
19. For a minimum of 30 days. Six weeks of incubation is considered to be optimal, but space limitations do not always make this possible.
20. (1) The patient's name, age, and sex; (2) identification number or registration number; (3) name of the requesting physician and the submitting facility; (4) specimen origin and date of collection; and (5) preliminary diagnosis or suspected organism.

#### 414

1. Rate of growth, colony size, elevation, shape and margin, texture, and surface pigmentation on both front and reverse sides of the slant.
2. Rapid growers—five to 10 days, and slow growers—three to four weeks.
3. Patient specimens and the fungal colony.
4. Potassium hydroxide.
5. Wash lesion with 70% alcohol, scrape the outer, active margin with sterile scalpel blade, and place scrapings in a sterile Petri dish.
6. To let the tissue and debris dissolve.
7. It informs us only of the presence or absence of fungal elements.
8. Lactophenol cotton blue; (1) the lactic acid acts as a clearing agent; (2) phenol serves as a fungicidal or killing agent; (3) glycerol prevents drying; and (4) cotton blue (Poirrier's or aniline blue) lends color to the structures and makes them more readily observable.
9. The microscopic examination of a growing colony.
10. The difficulty in preserving continuity between the spore, fruiting structures, and hyphae after such rough handling.
11. (1) Visual acuity is partially lost due to the tape itself, regardless of its clarity, and (2) tape does not lend itself to the preservation of slides for future study because there is a tendency for the tape to loosen.
12. To reveal the capsule of *C. neoformans* as an aid to accurate diagnosis.
13. Cleared with KOH and heat.
14. Gram-stain, acid-fast stains, Wright, Giemsa, PAS, methenamine silver, papanicolaou stains, and calcofluor white.
15. It is extremely useful in the clinical mycology laboratory because it can rapidly (in one minute) detect fungal elements through bright fluorescence and it can be mixed with KOH if clearing is required.
16. Chemical detection of fungal macromolecules, detection of fungus-specific metabolites, and detection of fungus-specific nucleic acid sequences.
17. Cryptococcal and *Histoplasma* antigens.

**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. 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.**

41. (412) Which one of the following is not a simple fundamental structure of the fungi?
  - a. Hyphae.
  - b. Thallus.
  - c. Conidia.
  - d. Rhizoids.
42. (412) The vegetative phase of the fungi is characterized by
  - a. round, segmented spores.
  - b. round, branching filaments.
  - c. elongated, segmented spores.
  - d. elongated, branching filaments.
43. (412) The two specified environmental conditions that could activate the reproductive phase of the fungi life cycle are
  - a. constant temperature and lack of nourishment.
  - b. temperature variations and lack of nourishment.
  - c. constant temperature and abundance of nourishment.
  - d. temperature variations and abundance of nourishment.
44. (412) Which of the following classes of true fungi include the rusts, smuts, and poisonous fleshy mushrooms?
  - a. Ascomycetes.
  - b. Zygomycetes.
  - c. Basidiomycetes.
  - d. Deuteromycetes.
45. (412) What class of fungi contains most of the fungi pathogenic to man?
  - a. Ascomycetes.
  - b. Zygomycetes.
  - c. Basidiomycetes.
  - d. Deuteromycetes.
46. (413) Normally, fungi grow best around what acid pH?
  - a. 3.5.
  - b. 4.6.
  - c. 6.5.
  - d. 6.8.
47. (413) Differentiation of yeast and various species of *Candida*, identification of *C. albicans* can be accomplished using
  - a. cornmeal agar with Tween 80.
  - b. SDA.
  - c. buffered-charcoal yeast extract agar.
  - d. yeast-extract phosphate with ammonia.

48. (413) Systemic mycoses are deep-seated infections that may spread throughout the body but primarily begin in the
- lung.
  - intestines.
  - cutaneous tissue.
  - reproductive organs.
49. (413) The following are advantages of culture tubes over Petri dishes for fungus cultures except
- easy storage.
  - safer for small labs.
  - lower dehydration rate.
  - better aeration of the cultures.
50. (414) Macroscopic examination of fungus growth characteristics includes but is not limited to
- rate of growth, texture, and pigmentation.
  - colony size, type of hyphae seen, and pigmentation.
  - rate of growth, type of spores produced, and pigmentation.
  - colony size, type of hyphae seen, and type of spores produced.
51. (414) Probably the most widely used of all wet mounts is
- hanging drop.
  - cellophane tape.
  - potassium hydroxide.
  - lactophenol cotton blue.
52. (414) Before collecting skin scrapings for a KOH mount, the lesion area is thoroughly washed with 70 percent alcohol to
- destroy fungus contaminants.
  - ensure a pure culture of pathogens.
  - destroy all saprophytic bacteria and fungi.
  - remove dirt particles, bacteria, and medications.
53. (414) The India ink wet mount reveals the encapsulated budding or non-budding blastospores of
- Nocardia* species.
  - Trichophyton* species.
  - Cryptococcus neoformans*.
  - Histoplasma capsulatum*.

## **Student Notes**



## Glossary

### Terms

|  |   |
|--|---|
| <b>abscess</b>                               | A localized collection of pus caused by infection buried in tissues, organs, or confined spaces.  |
| <b>antigenic</b>                             | Having the properties of an antigen, capable of eliciting an immune response.   |
| <b>antimicrobial</b>                         | Killing microorganisms, or suppressing their multiplication or growth.  |
| <b>antiserum</b>                             | Serum that contains antibodies; it may be obtained from an animal that has been immunized by injection of antigen into the body.  |
| <b>ascitic</b>                               | Effusion and accumulation of serous fluid in the abdominal cavity.  |
| <b>asymptomatic</b>                          | Showing or causing no symptoms.   |
| <b>cellulitis</b>                            | Inflammation of cellular tissue; especially purulent inflammation of the loose subcutaneous tissue.   |
| <b>cerebrospinal</b>                         | Pertaining to the brain and spinal cord.  |
| <b>chronic<br/>granulomatous<br/>disease</b> | An inherited disorder of WBC bactericidal or fungicidal function characterized by widespread granulomatous lesions of the skin, lungs, and lymph nodes.   |
| <b>colony</b>                                | A collection or group of bacteria or fungi in culture derived from the increase of an isolated single organism or group of organisms.   |
| <b>commensal</b>                             | Living on or within another organism, and deriving benefit without injuring or benefiting the other organism.   |
| <b>conjunctiva</b>                           | The delicate membrane that lines the eyelids and covers the exposed surface of the sclera.  |
| <b>counterstain</b>                          | A stain applied to render the effects of another stain more discernible.  |
| <b>cystitis</b>                              | 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. |
| <b>dyspnea</b>                               | Difficult or labored breathing.   |

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| <b>endocarditis</b>   | Inflammation of the endocardium; a disease generally associated with rheumatic fever, and sometimes with other acute febrile diseases.  |
| <b>endocervical</b>   | Pertaining to the interior of the cervix uteri.   |
| <b>endocervix</b>     | The mucous membrane lining the canal of the cervix uteri.   |
| <b>endogenous</b>     | Growing from within.  |
| <b>endospore</b>      | A spore produced in the hyphae or cell.   |
| <b>endotoxin</b>      | A heat-stable toxin present in the bacterial cell but not in cell-free filtrates of cultures of intact bacteria.  |
| <b>enterocolitis</b>  | Inflammation involving both the small intestine and the colon.  |
| <b>epididymitis</b>   | Inflammation of the epididymis.   |
| <b>epiglottitis</b>   | Inflammation of the epiglottis.   |
| <b>etiologic</b>      | Pertaining to the cause of a disease.   |
| <b>exogenous</b>      | Growing by additions to the outside; developed or originating outside the organism.   |
| <b>extracellular</b>  | Outside a cell or cells.  |
| <b>extrapulmonary</b> | Not connected with the lungs.   |
| <b>exudate</b>        | 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.                                |
| <b>filamentous</b>    | Composed of long threadlike structures; said of bacterial colonies.   |
| <b>fistula</b>        | An abnormal passage between two internal organs or leading from an internal organ to the surface of the body.   |
| <b>flagellum</b>      | 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. |
| <b>genitourinary</b>  | Pertaining to the genital and urinary organs; urogenital; urinosexual.  |
| <b>genome</b>         | The complete gene complement of an organism, contained in a set of chromosomes in eukaryotes, a single chromosome in bacteria, or a DNA or RNA molecule in viruses.   |
| <b>genotypic</b>      | Pertaining to or expressive of the genotype.  |

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| <b>gingivae</b>                 | 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.   |
| <b>glomerulonephritis</b>       | Inflammation of the capillary loops in the glomeruli of the kidney.   |
| <b>glomerulus</b>               | A tuft or cluster; used in anatomical nomenclature as a general term to designate such a structure.   |
| <b>immunodeficiency</b>         | A deficiency in immune response, either in that mediated by humoral antibody or in that mediated by immune lymphoid cells.  |
| <b>immunoglobulins</b>          | A protein of animal origin endowed with known antibody activity.  |
| <b>immunosuppression</b>        | The artificial prevention or diminution of the immune response, as by irradiation or by administration of antimetabolites, antilymphocyte serum, or specific antibody.  |
| <b>inclusion conjunctivitis</b> | Infection of the conjunctiva caused by an organism of the psittacosis-lymphogranuloma venereum- trachoma group.   |
| <b>intracellular</b>            | Situated or occurring within a cell or cells.   |
| <b>lake</b>                     | To undergo separation of hemoglobin from the erythrocytes, a phenomenon sometimes occurring in blood.   |
| <b>lavage</b>                   | The irrigation or washing out of an organ, such as the stomach or bowel.  |
| <b>lymphadenitis</b>            | Inflammation of one or more lymph nodes, usually caused by a primary focus of infection elsewhere in the body.  |
| <b>lymphogranuloma venereum</b> | A sexually transmitted infection due to specific strains of <i>Chlamydia trachomatis</i> , characterized by a primary cutaneous or mucosal lesion at the site of infection, which may be a papular, ulcerative, herpetiform, or erosive lesion. |
| <b>lyse</b>                     | To cause or produce disintegration of a compound, substance, or cell.   |
| <b>meatus</b>                   | A general term for an opening or passage; a general term for an opening in the body.  |
| <b>meningeal</b>                | Of or pertaining to the meninges.   |
| <b>meningitis</b>               | Inflammation of the meninges.   |
| <b>meningoencephalitis</b>      | Inflammation of the brain and meninges.   |
| <b>metachromatic</b>            | Staining differently with the same dye; said of tissues in which different elements take on different colors when a certain dye is applied.   |

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| <b>microbiota</b>    | The microscopic living organisms of a region; the combined microflora and microfauna of a region.   |
| <b>monoclonal</b>    | Derived from a single cell.   |
| <b>mucoid</b>        | Any one of a group of mucus-like conjugated proteins of animal origin.  |
| <b>mucosa</b>        | A mucus membrane.   |
| <b>mycetoma</b>      | A chronic, initially localized, slowly progressive, destructive infection of the cutaneous and subcutaneous tissues, fascia, and bone.  |
| <b>nasopharynx</b>   | The part of the pharynx which lies above the level of the soft palate.  |
| <b>necrosis</b>      | Death of tissue, usually as individual cells, groups of cells, or in small localized areas.   |
| <b>necrotizing</b>   | Causing necrosis.   |
| <b>nephropathy</b>   | Disease of the kidney.  |
| <b>nephrostomy</b>   | The creation of a permanent fistula leading directly into the pelvis of the kidney.   |
| <b>neutropenia</b>   | A decrease in the number of neutrophilic leukocytes in the blood.   |
| <b>neutrophil</b>    | 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. |
| <b>nosocomial</b>    | Pertaining to or originating in a hospital, as nosocomial disease.  |
| <b>nuchal</b>        | Pertaining to the nucha, or back of the neck.   |
| <b>ophthalmia</b>    | A severe inflammation of the eye or the conjunctiva, or deeper structures of the eye.   |
| <b>oropharynx</b>    | That division of the pharynx which lies between the soft palate and the upper edge of the epiglottis.   |
| <b>osteomyelitis</b> | Inflammation of bone caused by a pyogenic organism.   |
| <b>otitis media</b>  | Inflammation of middle ear.   |
| <b>papule</b>        | A small circumscribed, superficial, solid elevation of the skin.  |
| <b>pericardium</b>   | The fibroserous sac that surrounds the heart and the roots of the great vessels.  |
| <b>peritonitis</b>   | Inflammation of the pericardium.  |
| <b>pharyngitis</b>   | Inflammation of the pharynx.  |

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| <b>pleomorphic</b>                   | Occurring in various distinct forms.  |
| <b>pneumonia</b>                     | Inflammation of the lungs with consolidation.   |
| <b>polymicrobial</b>                 | Characterized by the presence of several species of microorganisms.   |
| <b>polymorphonuclear</b>             | Having cells of many forms.   |
| <b>prostate</b>                      | A gland in the male which surrounds the neck of the bladder and the urethra.  |
| <b>prosthesis</b>                    | An artificial substitute for a missing body part.   |
| <b>purulent</b>                      | Consisting of or containing pus; associated with the formation of or caused by pus.   |
| <b>pyogenous</b>                     | Caused by pus.  |
| <b>pyuria</b>                        | The presence of pus in the urine.   |
| <b>receptor-mediated endocytosis</b> | The uptake by a cell of material from the environment by invagination of its plasma membrane, it includes both phagocytosis and pinocytosis.  |
| <b>Reiter's syndrome</b>             | Arthritis associated with non-bacterial urethritis or cervicitis, conjunctivitis, and mucocutaneous lesions.  |
| <b>saprophytes</b>                   | Any organism, such as a bacterium, living upon dead or decaying matter.   |
| <b>septicemia</b>                    | Systemic disease associated with the presence and persistence of pathogenic microorganisms in the blood.  |
| <b>serotype</b>                      | The type of a microorganism as determined by the kinds and combinations of constituent antigens present in the cell.  |
| <b>serous</b>                        | Pertaining to or resembling serum.  |
| <b>subcutaneous</b>                  | Beneath the skin.   |
| <b>synovia</b>                       | 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. |
| <b>trachea</b>                       | The cartilaginous and membranous tube descending from the larynx and branching into the right and left main bronchi.  |
| <b>trachoma</b>                      | A chronic infectious disease of the conjunctiva and cornea, producing photophobia, pain, and lacrimation.   |
| <b>umbilicate</b>                    | Shaped like or resembling the umbilicus.  |
| <b>umbonate</b>                      | Knob-like; button-like; having a button-like raised center.   |
| <b>urethritis</b>                    | Inflammation of the urethra.  |

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| <b>viable</b>    | Capable of living.   |
| <b>virulence</b> | 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. |
| <b>zoonotic</b>  | Transmissible from animals to man under natural conditions.  |

## **Abbreviations and Acronyms**

|              |  |
|--------------|--|
| <b>AFB</b>   | acid-fast bacilli                              |
| <b>AIDS</b>  | acquired immunodeficiency syndrome             |
| <b>ATCC</b>  | American Type Culture Collection               |
| <b>ATP</b>   | adenosine triphosphate                         |
| <b>BHI</b>   | brain-heart infusion                           |
| <b>BKV</b>   | hemorrhagic cystitis-kidney                    |
| <b>BSC</b>   | biological safety cabinet                      |
| <b>CF</b>    | complement fixation                            |
| <b>CH</b>    | chromogenic                                    |
| <b>CMV</b>   | cytomegalovirus                                |
| <b>DNA</b>   | deoxyribonucleic acid                          |
| <b>DTM</b>   | dermatophyte test medium                       |
| <b>EB</b>    | elementary body                                |
| <b>EBV</b>   | Epstein-Barr virus                             |
| <b>ECM</b>   | erythema chronicum migrans                     |
| <b>ELISA</b> | enzyme-linked immunosorbent assay              |
| <b>G</b>     | gravity  |
| <b>HBV</b>   | hepatitis B virus                              |
| <b>HPV</b>   | human papilloma-virus                          |
| <b>HI</b>    | hemagglutination inhibition                    |
| <b>HIV</b>   | human immuno-deficiency virus                  |
| <b>HSV</b>   | herpes simplex virus                           |
| <b>ICTV</b>  | International Committee on Taxonomy of Viruses |
| <b>IFA</b>   | indirect immunofluorescence assay              |
| <b>IgM</b>   | immunoglobulin M                               |
| <b>IHA</b>   | indirect hemagglutination                      |
| <b>IMA</b>   | inhibitory mold agar                           |

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|-----------------------|--|
| <b>INH</b>            | isoniazid  |
| <b>JCV</b>            | leuko-encephalopathy                                 |
| <b>KOH</b>            | potassium hydroxide                                  |
| <b>LGV</b>            | lymphogranuloma venereum                             |
| <b>L-J</b>            | Löwenstein-Jensen                                    |
| <b>LPCB</b>           | Lactophenol cotton blue                              |
| <b>MAC</b>            | <i>M. avium</i> complex                              |
| <b>MAT</b>            | microscopic agglutination test                       |
| <b>MOTT</b>           | mycobacteria other than tuberculosis                 |
| <b>MPS</b>            | Mononuclear Phagocytic System                        |
| <b>NALC-NaOH</b>      | N-acetyl-L-cysteine-sodium hydroxide                 |
| <b>NC</b>             | nonchromogenic                                       |
| <b>NCCLS</b>          | National Committee for Clinical Laboratory Standards |
| <b>NGU</b>            | nongonococcal urethritis                             |
| <b>NO<sub>2</sub></b> | nitrite  |
| <b>NO<sub>3</sub></b> | nitrate  |
| <b>PAS</b>            | periodic acid-Schiff                                 |
| <b>PCR</b>            | polymerase chain reaction                            |
| <b>PID</b>            | pelvic inflammatory disease                          |
| <b>PPD</b>            | purified protein derivative                          |
| <b>PVA</b>            | polyvinyl alcohol                                    |
| <b>RIA</b>            | radioimmunoassay                                     |
| <b>RMSF</b>           | Rocky Mountain spotted fever                         |
| <b>RNA</b>            | ribonucleic acid                                     |
| <b>RSV</b>            | respiratory syncytial virus                          |
| <b>SDA</b>            | Sabouraud dextrose agar                              |
| <b>TB</b>             | tuberculosis   |
| <b>VSV</b>            | vesicular stomatitis virus                           |
| <b>VZV</b>            | varicella-zoster virus                               |
| <b>WHO</b>            | World Health Organization                            |
| <b>Zephiran</b>       | Benzalkonium chloride                                |

## **Student Notes**



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## **Student Notes**

**STUDENT FEEDBACK**  
**CAREER DEVELOPMENT COURSE (CDC): \_\_\_\_\_ VOL: \_\_\_\_\_**  
**ASSESSMENT SURVEY**

**STUDENT NAME (Optional): \_\_\_\_\_ DATE: \_\_\_\_\_**

**PURPOSE:** This survey is designed to obtain definitive and measurable feedback on the CDC volume you have just completed. It will provide us with your assessment of the quality of the training provided, identify areas where we may need to improve, and, with consolidation of the data, provide an overall assessment of how well we are doing in meeting your needs.

**INSTRUCTIONS:** Request you respond to the following statements. Please circle the appropriate response according to the following scale:

|   | 1                    | 2        | 3                    | 4                 | 5                 | 6     | 7                 |
|---|----------------------|----------|----------------------|-------------------|-------------------|-------|-------------------|
|   | Strongly<br>Disagree | Disagree | Somewhat<br>Disagree | Not<br>Applicable | Somewhat<br>Agree | Agree | Strongly<br>Agree |
| 1. The information provided in the CDC provided me with knowledge required to perform on the job.                           | 1                    | 2        | 3                    | 4                 | 5                 | 6     | 7                 |
| 2. The information was presented in a logical sequence and easy to read.  | 1                    | 2        | 3                    | 4                 | 5                 | 6     | 7                 |
| 3. The CDC volume was   |                      |          |                      |                   |                   |       |                   |
| a. well written,  | 1                    | 2        | 3                    | 4                 | 5                 | 6     | 7                 |
| b. provided sufficient details and examples,  | 1                    | 2        | 3                    | 4                 | 5                 | 6     | 7                 |
| c. emphasized key information, and,   | 1                    | 2        | 3                    | 4                 | 5                 | 6     | 7                 |
| d. the information was easily understood.   | 1                    | 2        | 3                    | 4                 | 5                 | 6     | 7                 |
| 4. Graphics provided consistently supported the text illustrating and/or clarifying principles, techniques, and procedures. | 1                    | 2        | 3                    | 4                 | 5                 | 6     | 7                 |
| 5. The format of the volume made information easy to read and reference or locate information.                              | 1                    | 2        | 3                    | 4                 | 5                 | 6     | 7                 |
| 6. Unit Review Exercises:   |                      |          |                      |                   |                   |       |                   |
| a. Questions were relevant to tasks performed in the workplace and knowledge required to perform on the job.                | 1                    | 2        | 3                    | 4                 | 5                 | 6     | 7                 |
| b. Questions and responses could be referenced to the text.   | 1                    | 2        | 3                    | 4                 | 5                 | 6     | 7                 |

Additional write-in comments/recommendations: \_\_\_\_\_

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**If you would like a response to this survey, be sure to include your name, address, and DSN.**

PLEASE RETURN THE SURVEY TO: **882 TRSS/TSOX  
939 MISSILE RD STE 2  
SHEPPARD AFB, TX 76311-2260**



## **Student Notes**

**AFSC 4T051**  
**4T051O 03 0506**  
**Edit Code 02**