

# LAB 16: ISOLATION AND IDENTIFICATION OF NEISSERIAE, MYCOBACTERIA, AND OBLIGATE ANAEROBES

## A. THE NEISSERIAE

### DISCUSSION

The neisseriae are a group of **Gram-negative diplococci** 0.6-1.5  $\mu\text{m}$  in diameter (see Fig. 1A). Two species of *Neisseria*, *N. gonorrhoeae* and *N. meningitidis*, are considered as true human pathogens. Both of these organisms possess pili and adhesins for adherence to host cells, produce endotoxins, and resist destruction within phagocytes. *N. meningitidis* also produces a capsule to resist phagocytic engulfment.

- Scanning electron micrograph [Neisseria gonorrhoeae](#); courtesy of [Dennis Kunkel's Microscopy](#).
- [Scanning electron micrograph of Neisseria meningitidis](#). © Margaret Ketterer, author. Licensed for use, [ASM MicrobeLibrary](#).
- Transmission electron micrograph [Neisseria gonorrhoeae](#); courtesy of CDC.

For further information on virulence factors associated with *N. gonorrhoeae* and *N. meningitidis*, see the following Learning Objects in your Lecture Guide:

- [Endotoxin; Unit 3, Section C1b](#)
- [The Ability to Adhere to Host Cells: Pili and Adhesins; Unit 3, Section B2](#)
- [The Ability to Invade Host Cells; Unit 3, Section B3](#)
- [The Ability to Resist Phagocytic Engulfment; Unit 3, Section B5b](#)
- [The Ability to Resist Phagocytic Destruction and Serum Lysis; Unit 3, Section B5c](#)
- [The Ability to Evade Adaptive Immune Defenses; Unit 3, Section B6](#)
- [The Ability to Compete for Iron; Unit 3, Section B4](#)

***Neisseria gonorrhoeae* (the gonococcus)**, causes **gonorrhea**. It is estimated that there are approximately 2,000,000 cases of gonorrhea per year in the U.S. Infection usually occurs following sexual contact, with the incubation period averaging 2-7 days. In **males**, the gonococcus typically invades the anterior **urethra**, usually producing a **purulent discharge, pain upon urination, and a frequency of urination**. Approximately **5-10% of infected males are asymptomatic** but will still be infectious. The infection may spread up the reproductive tract, infecting the **prostate, vas deferens, epididymis, and testes**, causing painful inflammation and scar tissue formation that can result in **sterility**.

In **females, 30-50 percent** of those initially infected are **asymptomatic** or show mild symptoms. They are, however, still infectious. Initially, the organism invades the **cervix**, the **urethra**, and frequently the **rectum**. In about 15 percent of the cases, the organism spreads up the reproductive tract and infects the **fallopian tubes** causing **pelvic inflammatory disease (PID)**. The resulting inflammation and scar tissue formation may result in **sterility or abnormal (ectopic or tubal) pregnancies**.

The gonococcus may also cause **extragenital infections** such as **pharyngitis** (from oral-genital sex), **ophthalmia** (from inoculation of the eyes with contaminated fingers), and **proctitis** (from anal sex). In 1% - 3% of infected women and a lower percentage of infected males, the organism invades the blood and disseminates, causing a **rash, septic arthritis, endocarditis, and/or meningitis**. Dissemination occurs more frequently in females. **Congenital gonorrhea** is known as **ophthalmia neonatorum** and occurs as a result of the eyes of newborns becoming infected as the baby passes through the birth canal.

***Neisseria meningitidis* (the meningococcus) is the causative organism of meningococcal (epidemic) meningitis**. There are between 2000 and 3000 cases of meningococcal meningitis per year in the U.S. Approximately 50% of the cases occur in children between 1 and 4 years old. *N. meningitidis* infects the **nasopharynx** of humans causing a usually mild or subclinical upper respiratory infection. Colonization of the nasopharynx may persist for months. However, in about 15% of these individuals, the organism invades the **blood** and disseminates, leading **septicemia** and from there may cross the blood-brain barrier causing **meningitis**. A **petechial skin rash**, caused by endotoxin in the blood, appears in about 75 percent of the septic cases and fatality rates for meningococcal septicemia are as high as 30 percent as a result of the shock cascade. A fulminating form of the disease, called Waterhouse-Frederichsen syndrome, can be fatal within several hours due to massive intravascular coagulation and resulting shock, probably a result of massive endotoxin release. *N. meningitidis* is especially dangerous in young children.

[Concept map for Lab 16 - Organisms and Infections](#)

**A presumptive diagnosis of gonorrhea is made on the basis of two of the following three criteria:**

1. Typical Gram-negative diplococci inside of polymorphonuclear leukocytes seen in a Gram stain of urethral exudates in men and endocervical secretions in women (a positive GC smear; see below);
2. Growth of *N. gonorrhoeae* from the urethra of men or the endocervix of women on a culture medium with typical colony morphology, positive oxidase reaction, and Gram-negative diplococcal morphology in a Gram stain; and/or
3. Detection of *N. gonorrhoeae* by nonculture tests such as:
  - antigen tests, e.g., Gonozyne [Abbott]; an enzyme immunoassay (EIA) test for *N. gonorrhoeae* antigens detected from endocervical and male urethral swab specimens.
  - direct specimen nucleic acid probes, e.g., Pace 2C Assay [GenProbe]; a single-tube assay to screen for the presence of both *Chlamydia trachomatis* and *Neisseria gonorrhoeae* from endocervical and male urethral swab specimens.
  - nucleic acid amplification tests, e.g., LCR (ligase chain reaction) [Abbott]; DNA probes based on *N. gonorrhoeae* pilin gene sequences.

Antibiotic therapy may be started with a presumptive diagnosis.

**A definitive diagnosis of gonorrhea is made on the basis of:**

1. Isolation of *N. gonorrhoeae* from the site of exposure by culture, usually on a selective medium, demonstration of typical colony morphology, positive oxidase reaction, and Gram-negative diplococcal morphology in a Gram stain and
2. Confirmation of isolates by biochemical, enzymatic, serologic, or nucleic acid testing, e.g., carbohydrate utilization, rapid enzyme substrate tests, serologic methods such as fluorescent antibody tests, or a DNA probe culture confirmation technique.

**Isolation and Identification of *Neisseria gonorrhoeae***

**a. The GC smear**

A **GC smear (gonococcus smear)** is a Gram stain of urethral exudates in men and endocervical secretions in women and can be part of a presumptive diagnosis of gonorrhea. One looks for **Gram-negative diplococci with flattened adjacent walls that are seen both inside and outside of polymorphonuclear leukocytes** (see Fig. 1). This test is quite sensitive in symptomatic males but only 40-60% sensitive in symptomatic females. In asymptomatic males and females the Gram stain has a lower predictive value.

[Instructions for the Gram Stain from Lab 6.](#)

**b. Isolation of *Neisseria gonorrhoeae***

To diagnose genital gonorrhea in males, the sample to be cultured is taken from the **urethra**. In females, cultures are taken from the **cervix and the rectum**. In non-genital gonorrhea, the infected site is cultured. The gonococcus requires an enriched medium with increased carbon dioxide tension for growth. They are usually cultured on selective media such as **modified Thayer-Martin (MTM) Chocolate agar** or Martin-Lewis agar. Plates are then incubated at 35-37°C under increased carbon dioxide tension (5-10% CO<sub>2</sub>) such as that provided by a candle jar or a carbon dioxide incubator.

**MTM Chocolate agar is selective for *Neisseria*.**

The medium contains **enrichment factors** to promote the growth of *Neisseria*. In addition, it contains **antibiotics to inhibit normal body flora**: vancomycin to inhibit Gram-positive bacteria; colistin to inhibit Gram-negative bacteria; trimethoprim to suppress *Proteus*; and nystatin to inhibit yeast. The "chocolate" color is due to the hemoglobin enrichment added to the medium. Plates are then incubated under increased carbon dioxide tension as mentioned above. (Transgrow Medium is a convenient flask containing MTM Chocolate agar and CO<sub>2</sub>.) *N. gonorrhoeae* forms small, convex, grayish-white to colorless, mucoid colonies in 48 hours at 35-37°C (see Fig. 4).

**c. Identification of *Neisseria gonorrhoeae***

Once isolated, *N. gonorrhoeae* can be identified by the oxidase test, Gram-staining, carbohydrate utilization reactions, rapid enzyme substrate tests, serologic methods such as fluorescent antibody tests, or a DNA probe culture confirmation technique.

## 1. Oxidase test

All *Neisseria* are **oxidase positive**. The oxidase test can be performed as in Lab 13 using a **Taxo N® disc**. A moistened Taxo N® disc can be placed on a growing culture and a **blackening of the colonies surrounding the disc indicates a positive oxidase test** (see Fig. 2). All oxidase-positive cultures would be Gram stained to confirm Gram-negative diplococci.

## 2. Carbohydrate utilization

The various species of *Neisseria* can be differentiated according to carbohydrate utilization patterns. *Neisseria* species do not produce acid from carbohydrate fermentation but rather by an oxidative pathway. These tests are done using a media with single carbohydrates ( **glucose, maltose, sucrose, or lactose**) and a pH indicator to detect acid. The medium seen today uses phenol red as a pH indicator. If the sugar is utilized, acid end products cause the **phenol red pH indicator to turn yellow**. *N. gonorrhoeae* produces acid from **only glucose** (see Fig. 5) whereas *N. meningitidis* produces acid from **glucose and maltose** (see Fig. 6).

There are a variety of *Neisseria* species and other genera with similar morphology that often colonize the body. By testing for lactose and sucrose utilization as well as performing other tests such as rapid enzyme substrate tests, serologic methods, or DNA probes, *N. gonorrhoeae* and *N. meningitidis* can be definitively identified. Typical sugar utilization reactions can be seen in [Table 1](#).

### d. Serologic and Nucleic Acid Tests to Identify *N. gonorrhoeae*

**Serologic tests are also available for rapidly identifying *N. gonorrhoeae***. These include an ELISA test to detect gonococci in urethral pus or on a cervical swab, as well as a direct serologic test using [fluorescent monoclonal antibodies](#) to detect *N. gonorrhoeae*. Serologic testing will be covered in Labs 17 and 18.

**Nucleic acid probes and nucleic acid amplification tests** are also available for identifying *N. gonorrhoeae*.

[Concept map for Lab 16 - Identification](#)

**Highlighted Bacterium: *Neisseria gonorrhoeae***

Click on this link, read the description of *Neisseria gonorrhoeae*.

## 2. Isolation and Identification of *Neisseria meningitidis*

### a. Gram stain

A presumptive diagnosis of meningococcal meningitis often made by doing a **Gram stain of cerebrospinal fluid or petichial skin lesions and looking for Gram-negative diplococci seen both inside and outside of polymorphonuclear leukocytes**. This can be followed by serologic tests, nucleic acid probes, or culturing.

### b. Serologic identification

There are at least 12 different serological groups of *N. meningitidis* based on their capsular polysaccharides, but over 90 percent of meningococcal meningitis cases are caused by five serologic groups: A, B, C, Y, and W135. Serogroups A and C usually causes the epidemic form of meningitis. Serogroup C is the most common serogroup in North America whereas B is the most common in Europe and Latin America. Serogroups A and C are common in China, the Middle East, and parts of Africa. Serogroup Y has been increasing in the U.S., Israel, and Sweden. Direct serologic testing to detect meningococcal capsular polysaccharides can be performed on cerebrospinal fluid or on organisms from skin lesions for rapid identification. Direct serologic testing will be discussed in more detail in Lab 17.

### c. Nucleic acid identification

A polymerase chain reaction test to amplify meningococcal DNA can also be used to detect *N.*

*meningitidis* in cerebrospinal fluid or blood.

#### d. Isolation of *Neisseria meningitidis*

To isolate *N. meningitidis*, cultures are taken from the **nasopharynx, blood, cerebrospinal fluid, and skin lesions**. Typically cultures are done on an enriched, non-selective medium such as **blood agar or chocolate agar** grown in 3-7% carbon dioxide. MTM Chocolate agar is also sometimes used for isolation. Medium to large, blue-gray, mucoid, convex, colonies form in 48 hours at 35-37°C.

#### e. Identification of *Neisseria meningitidis*

Once isolated, *N. meningitidis*, like *N. gonorrhoeae* discussed above, is identified by the oxidase test, Gram staining, and carbohydrate utilization reactions. *N. meningitidis*, like all neisseriae, is **oxidase-positive** and appears in a Gram stain as **Gram-negative diplococci**. In carbohydrate utilization tubes, ***N. meningitidis* produces acid from both glucose and maltose** (see Fig. 6) but not from lactose and sucrose (see Table-1). The acid end products turn the phenol red pH indicator from red to yellow. *N. meningitidis* also produces gamma-glutamylaminopeptidase, an enzyme that can be detected by biochemical testing.

[Concept map for Lab 16 - Identification](#)

**[Highlighted Bacterium: \*Neisseria meningitidis\*](#)**

Click on this link, read the description of *Neisseria meningitidis*.

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## B. THE MYCOBACTERIA

### DISCUSSION

The mycobacteria are **rod-shaped** bacteria generally 0.4 by 3.0 µm in size that are said to be **acid-fast**. This means that because of their unique cell wall (see Fig. 7), when they are stained by the acid-fast procedure (Appendix C), they will resist decolorization with acid-alcohol and stain red (see Fig. 8, Fig. 12A, and Fig. 12B), the color of the initial stain, carbol fuchsin. With the exception of a very few other acid-fast bacteria such as *Nocardia*, all other bacteria will be decolorized and stain blue (the color of the counterstain, methylene blue). The acid-fast stain is an important test for the genus *Mycobacterium*. Fluorescent microscopy staining may also be used to identify *Mycobacterium*.

The most common species of *Mycobacterium* to cause human infections are *M. tuberculosis*, *M. leprae*, *M. avium complex*, *M. kansasii*, *M. fortuitum*, *M. chelonae*, and *M. abscessus*. ***M. tuberculosis*** is the causative organism of **tuberculosis**, ***M. leprae*** causes **leprosy**, and ***M. avium complex* (MAC)** frequently causes systemic infections in people with HIV/AIDS. The other species of *Mycobacterium* occasionally cause tuberculosis-like infections, especially in the debilitated or immunosuppressed host.

- [Scanning electron micrograph of \*Mycobacterium tuberculosis\*](#); courtesy of CDC

It is estimated by the World Health Organization that worldwide, there are over 8,800,000 million new cases of tuberculosis each year and nearly 3,000,000 deaths. One-third of the world's population - approximately 1.9 billion people - are thought to be infected with *M. tuberculosis*. In the U.S. there are approximately 20,000 new cases of tuberculosis a year. *M. tuberculosis* is typically acquired by inhalation of aerosolized infectious particles. In the vast majority of people, primary infection is asymptomatic or minimally symptomatic. Approximately 5% of people exposed to *M. tuberculosis* develop active disease within 2 years; another 5% - 10% develop disease sometime later in life. Typical symptoms are malaise, cough, weight loss, and night sweats.

**Primary infection** typically occurs after inhalation of the organism and subsequent generation of a peripheral lung lesion. The body responds with what is termed delayed hypersensitivity to form characteristic lesions called **granuloma** or tubercles (see Fig. 3). The formation of granuloma is actually the result of cell-mediated immune responses attempting to "wall-off" and localize infections that the body cannot effectively remove with macrophages. Although primary infection may be self-limiting, progression of the localized lesion may lead to pneumonia. The organisms may eventually die within the granuloma, or the tissue may undergo caseation, liquification, and cavitation. This can result in bronchogenic spread of the *M. tuberculosis*. In rare instances, the

organism may enter the blood causing disseminated miliary tuberculosis.

**Secondary infection** is usually due to a relapse of either self-resolved lesions or a previously treated disease. Approximately 90% of tuberculosis in immunocompetent patients is a result of secondary infection. *M. tuberculosis* is relatively resistant to many disinfectants and most antibiotics and is also able to resist destruction within phagocytic macrophages.

### Concept map for Lab 16 - Organisms and Infections

For further information on virulence factors associated with *M. tuberculosis* and *M. leprae*, see the following Learning Objects in your Lecture Guide:

- [Acid-Fast Cell Wall; Unit 1, Section B2c](#)
- [Acid-Fast Cell Wall Components; Unit 3, Section C1d](#)
- [The Ability to Resist Phagocytic Destruction and Serum Lysis; Unit 3, Section B5c](#)
- Ways in Which Cell-Mediated Immunity Protects the Body
  - [Section III B1: Cytotoxic T- Lymphocytes \(CTLs\)](#)
  - [Section III B2: Activating Macrophages and NK Cells](#)
  - [Section III B3: Stimulating Cells to Secrete Cytokines](#)

### Diagnosis of tuberculosis

The diagnosis of tuberculosis is traditionally based on clinical findings, chest radiographs, a sputum or tissue smear showing acid-fast bacilli or fluorescing mycobacteria after using fluorochrome staining. Confirmation can be made using cultures, DNA probes, and polymerase chain reactions to amplify mycobacterial DNA.

#### 1. Presumptive tests for tuberculosis

a. The **PPD skin test** (or other presumptive skin tests) detects **delayed hypersensitivity** to purified protein from the cell wall of *M. tuberculosis*. A positive skin test, assessed at 48-72 hours, indicates that the person has developed a cellular immunity to the organism as a result of either a **previous or a current infection**. Recommendations for interpretation of positive skin tests are based on the size of induration in relationship to other risk factors the patient may have:

- **Induration of 5 mm or more**

- Contacts of persons known to be infected
- Patients with abnormal chest film
- Organ-transplant recipients
- HIV-positive patients
- Other immunosuppressed patients

- **Induration of 10 mm or more**

- Residents of prisons, nursing homes, institutions
- Recent immigrants from countries with a high incidence of tuberculosis
- Healthcare workers
- Children aged <4 years or infants, children, and adolescents exposed to high-risk adults
- Patients with other high-risk medical factors (eg, diabetes, renal failure, cancer, silicosis)

- **Induration of 15 mm or more**

- No risk factors.

b. **Chest X-rays** are used to detect confluent granuloma formation in the lungs and possible cavitation, which could be a result of past or present infection with tuberculosis or with some other pulmonary infection that may be mistaken for tuberculosis.

c. An **Acid-fast stain of the sputum** may indicate **acid-fast bacilli**, which is a presumptive test for active tuberculosis. In reporting acid-fast slide results, the slide should be observed for 10-15 minutes before considered negative. Results are reported as positive or negative for acid-fast bacilli. Sometimes the amount of acid-fast bacilli are indicated, with 3-9 per slide reported as rare, 10 or more per slide reported as few, and more than one per oil immersion field reported as numerous.

- Photomicrograph showing a positive [acid-fast stain of \*Mycobacterium tuberculosis\* in sputum](#).
- Photomicrograph showing a positive [acid-fast stain of \*Mycobacterium tuberculosis\* in sputum](#).

#### 2. Confirmation of Active Tuberculosis

##### a. Cultures

**Active tuberculosis** is confirmed by **culturing the organism**. Sputum is usually treated with sodium hydroxide, which is cidal for contaminants but not for *M. tuberculosis*. The liquified sputum is then neutralized, centrifuged, and the sediment is inoculated onto special enrichment media such as Lowenstein-Jensen agar slants, Middlebrook agar, or 7H 10 Oleic acid agar plates. Felsen Quadrant plates with agar containing different antimicrobial agents are also inoculated to determine drug sensitivity.

Plates are incubated in a carbon dioxide atmosphere. It takes from **3-8 weeks** for colonies to form. In recent years, **more rapid culture methods** for detection of *M. tuberculosis* have been developed. One involves adding radioisotopic substrates to a broth growth medium in order to detect the release of carbon dioxide during mycobacterial growth. Another uses fluorometric technology to detect oxygen consumption during growth. These techniques usually detect mycobacterial growth in **less than two weeks**.

#### b. Nucleic acid tests

**Polymerase chain reactions** to amplify mycobacterial DNA, **DNA probes**, and **probes to detect ribosomal RNA** from *M. tuberculosis* are also being used with increased frequency to confirm tuberculosis.

The above procedures represents a very simplified outline for the diagnosis of tuberculosis. Culturing of *M. tuberculosis*, atypical mycobacteria, and other clinically significant mycobacteria involves a complicated series of complex procedures carried out only in large, well-equipped labs by experienced personnel.

[Concept map for Lab 16 - Identification](#)

**Highlighted Bacterium: *Mycobacterium tuberculosis***

Click on this link, read the description of *Mycobacterium tuberculosis*.

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## C. THE OBLIGATE ANAEROBES

### DISCUSSION

**Obligate anaerobes** are organisms that **grow only without oxygen** and, in fact, oxygen inhibits or kills them. They obtain energy from anaerobic respiration or fermentation. A variety of obligate anaerobic bacteria, which are usually **normal flora** of the body, may cause human infections. Obligate anaerobes primarily cause **infections of anaerobic wounds**, although they may participate in all varieties of infections and involve any tissue or organ. Five organisms or groups of organisms account for about two-thirds of all clinically significant anaerobic infections. These are *Bacteroides fragilis*, *Bacteroides melaninogenicus*, *Fusobacterium nucleatum*, *Clostridium perfringens*, and the anaerobic cocci.

- ***Bacteroides fragilis*** is the **most common cause of anaerobic infections** in humans. It is also a **predominant organism of the normal human intestinal tract**. It mainly causes **wound infections**. *B. fragilis* is a Gram-negative bacillus.
- *Bacteroides melaninogenicus* is normal flora of the upper respiratory, gastrointestinal, and genitourinary tracts.
- *Fusobacterium* species are normal flora of the upper respiratory, gastrointestinal, and genitourinary tracts.
- ***Clostridium perfringens***, as well as other clostridial species, are normal flora of the intestinal tract of various animals and may cause **gas gangrene**. *C. perfringens* is a Gram-positive bacillus. *C. tetani* causes tetanus and *C. botulinum* causes botulism.
- Anaerobic cocci such as *Peptostreptococcus*, *Peptococcus*, and *Veillonella* are also normal flora of the body.

***Clostridium difficile*** causes severe **antibiotic-associated colitis** and is an opportunistic Gram-positive, endospore-producing bacillus transmitted by the fecal-oral route. ***C. difficile* is a common healthcare-associated infection (HAIs) and is the most frequent cause of health-care-associated diarrhea.** *C. difficile* infection often recurs and can progress to sepsis and death. CDC has estimated that there are about 500,000 *C. difficile* infections (CDI) in health-care associated patients each year and is linked to 15,000 American deaths each year.

Antibiotic-associated colitis is especially common in older adults. It is thought that *C. difficile* survives the exposure to the antibiotic by sporulation. After the antibiotic is no longer in the body, the endospores germinate and *C. difficile* overgrows the intestinal tract and secretes toxin A and toxin B that have a cytotoxic effect on the epithelial cells of the colon. *C. difficile* has become increasingly resistant to antibiotics in recent years making treatment often difficult. There has been a great deal of success in treating the infection with fecal transplants, still primarily an experimental procedure. Polymerase chain reaction (PCRs) assays, which test for the bacterial gene encoding toxin B, are highly sensitive and specific for the presence of a toxin-producing *Clostridium difficile* organism. The most successful technique in restricting *C. difficile* infections has been the restriction of the use of antimicrobial agents.

Although anaerobic procedures are no more difficult than those used in aerobic bacteriology, strict adherence to proper technique is necessary to ensure recovery of the organism. There must be proper selection of the specimen, proper specimen collection, proper specimen transport to the lab, and provision of a proper anaerobic environment. Common ways of **culturing obligate anaerobes in the lab** include:

### 1. Brewer anaerobic jar with GasPak®

A GasPak® is a commercially-produced disposable hydrogen and carbon dioxide generator envelope. When water and catalyst are added, hydrogen and carbon dioxide are produced. The hydrogen then combines with oxygen to form water, thus creating an anaerobic atmosphere. The cultures are placed in a Brewer jar, water is added to the GasPak®, the lid of the jar is sealed, and the jar is placed in an incubator.

2. **Media containing reducing agents** Media such as **Thioglycolate medium** (see Fig. 9) and Anaerobic agar contain chemicals which function as reducing agents. The reducing agents absorb oxygen and create a reduced environment required by anaerobes.

3. **Carbon dioxide incubators** Carbon dioxide incubators are frequently used to culture anaerobes. After the cultures are placed in the incubator, the air is evacuated and replaced by carbon dioxide gas.

## Isolation and Identification of *Clostridium perfringens*

### 1. Direct microscopic examination of exudates

Gram stains of purulent exudates from gas gangrene show **stout Gram-positive bacilli** frequently surrounded by a capsule. Endospores are usually not produced on ordinary culture media or in tissues.

### 2. Isolation on Blood agar

When inoculated onto Blood agar plates and grown anaerobically, *C. perfringens* produces smooth, glossy colonies which are usually surrounded by a **double-zone hemolysis** (see Fig. 10). The double-zone hemolysis appears as a narrow zone of beta-type hemolysis (due to theta toxin) near the colony surrounded by a wider zone of incomplete hemolysis (due to alpha toxin).

### 3. Identification in Litmus Milk medium

In anaerobically-grown Litmus Milk cultures, enzymes of *C. perfringens* will attack the proteins and carbohydrates of the milk producing a **"stormy fermentation"** with **acid production** (litmus turns pink), **clotting of milk proteins, and gas formation** (see Fig. 11). For further information on virulence factors associated with *C. perfringens*, see the following Learning Objects in your Lecture Guide:

- [Endospores; Unit 1, Section B3e](#)
- [Toxins that Damage Cell Membranes; Unit 3, Section C2c](#)

[Medscape](#) article on infections associated with organisms mentioned in this Learning Object. Registration to access this website is free.

- [Neisseria gonorrhoeae](#)
- [Neisseria meningitidis](#)
- [Mycobacterium tuberculosis](#)
- [Mycobacterium leprae](#)
- [Mycobacterium avium-intracellulare complex](#)
- [Bacteroides](#)
- [Clostridium tetani](#)
- [Clostridium perfringens](#)
- [Clostridium botulinum](#)
- [Peptostreptococcus](#)

## PROCEDURE

### A. *Neisseria gonorrhoeae* and *Neisseria meningitidis* Observe the following **demonstrations**:

1. **Positive GC smear** for *N. gonorrhoeae*. Note Gram-negative diplococci inside and outside of white blood cells.
2. *N. gonorrhoeae* growing on **MTM Chocolate agar**. Note small grayish-white convex, mucoid colonies. This medium is selective for pathogenic *Neisseria*.
3. Positive **oxidase test** for *N. gonorrhoeae* using a Taxo N® (oxidase) disc. Note oxidase-positive (black) colonies around the Taxo N® disc.
4. **Glucose, maltose, sucrose, and lactose sugar utilization tubes** inoculated with *N. gonorrhoeae* (note acid production is positive for glucose; negative for maltose, sucrose, and lactose) and *N. meningitidis* (note acid production is positive for glucose and maltose; negative for sucrose, and lactose).

### B. MYCOBACTERIA Observe the following **demonstrations**:

1. Positive acid-fast stain of the sputum from a person with active tuberculosis. Note acid-fast (red) rods. You must **look carefully** for the reddish acid-fast rods in the microscopic field. All other material in the sputum will pick up the blue counterstain.
2. 35mm projection slides showing normal guinea pig organs and organs with **granuloma**.
3. Positive **chest X-ray** for the presumptive diagnosis of tuberculosis.
4. Prepare an acid-fast stain of *Mycobacterium phlei*. (Ziehl-Neelsen Method)
  - a. Heat-fix a smear of *Mycobacterium phlei* as follows:
    1. Using the dropper bottle of distilled water found in your staining rack, place **1/2 of a normal sized drop of water** on a clean slide by touching the dropper to the slide.
    2. Aseptically remove a **small amount** of the *E. coli* from the agar surface and **gently touch it 2 - 3 times to the drop of water** until the water **just turns cloudy**. Flame the loop and let it cool.
    3. After the loop cools, **spread the bacteria/water mixture over the entire slide** to form a thin film.
    4. Allow this thin suspension to **completely air dry**.
    5. To heat-fix the bacteria to the slide, **pick up the air-dried slide with coverslip forceps and hold the bottom of the slide opposite the smear near the opening of the microincinerator for 10 seconds**. If the slide is not heated enough, all of the bacteria will wash off. If it is overheated, the bacteria structural integrity can be damaged.
  - b. Cover the smear with a piece of **blotting paper** and flood with **carbol fuchsin**.
  - c. **Steam for 5 minutes** by passing the slide through the flame of a gas burner.
  - d. **Allow the slide to cool and wash with water**.
  - e. **Add the acid-alcohol decolorizing slowly dropwise until the dye no longer runs off from the smear**.
  - f. **Rinse with water**.
  - g. Counterstain with **methylene blue for 1 minute**.
  - h. Wash with water, blot dry, and observe using oil immersion microscopy.
  - i. **Make sure you carefully pour the used dye in your staining tray into the waste dye collection container, not down the sink**.
  - j. Repeat using *Staphylococcus aureus*.

Acid-fast bacteria will appear red; non-acid-fast will appear blue.



### C. The Obligate Anaerobes

Observe the following **demonstrations** of *Clostridium perfringens*:

1. **Endospore stain** of *C. perfringens*.
2. **Blood agar** plate of *C. perfringens* grown anaerobically. Note double-zone hemolysis.
3. **Litmus milk culture** of *C. perfringens* grown anaerobically. Note stormy fermentation of milk.

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## RESULTS

### A. *Neisseria gonorrhoeae* and *Neisseria meningitidis*

#### 1. GC smear for *N. gonorrhoeae*

Make a drawing of the GC smear, noting the Gram reaction and arrangement of the bacteria and their association with the white blood cells.



[GC smear of \*Neisseria gonorrhoeae\*](#)

<b>Gram reaction</b> (purple = +; pink = -)	
<b>Shape and arrangement</b>	

#### 2. *N. gonorrhoeae* on MTM Chocolate agar with a Taxo N® disc.

<b>Growth on selective MTM chocolate agar</b> (positive or negative)	
<b>Colony description</b>	
<b><u>Oxidase reaction</u></b> (positive or negative)	

#### 3. Acid production from carbohydrate utilization

Organism	Glucose	Maltose	Sucrose	Lactose
<a href="#"><i>Neisseria gonorrhoeae</i></a> (positive or negative)				
<a href="#"><i>Neisseria meningitidis</i></a> (positive or negative)				

Positive = Acid (phenol red turns yellow)  
 Negative = No acid (phenol red remains red)

### B. *Mycobacterium tuberculosis*

#### 1. Acid-fast stain of sputum

Make a drawing of the acid-fast stain, noting the acid-fast reaction and the shape of the acid-fast bacteria.



*Acid-fast stain of Mycobacterium tuberculosis*

<b>Acid-fast reaction (red = +; blue = -)</b>	
<b>Shape</b>	

[2. Describe the granuloma seen in the guinea pig organs.](#)

### C. The Obligate Anaerobes

#### 1. *Clostridium perfringens*

a. **Blood agar** (anaerobically grown)

<b>Colony description</b>	
<b>Type of hemolysis</b>	

b. **Litmus Milk** (anaerobically grown)

<b>Color (pink or lavender)</b>	
<b>Stormy fermentation (positive or negative)</b>	

Pink = Acid (fermentation)

Blue = Alkaline (no fermentation)

Stormy fermentation = Clotting of protein and gas production

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## PERFORMANCE OBJECTIVES FOR LAB 16

After completing this lab, the student will be able to perform the following objectives: **A. THE NEISSERIAE**

### DISCUSSION

1. State the Gram reaction and the morphology of the neisseriae.
2. State the correct scientific names for the gonococcus and the meningococcus and indicate what disease each causes.
3. Describe how symptoms of gonorrhoea differ in the male and in the female.
4. State the possible urogenital complications of gonorrhoea in the male and in the female.
5. Name four possible extragenital *Neisseria gonorrhoeae* infections.
6. State how congenital gonorrhoea usually appears.
7. Give the normal habitat for *Neisseria meningitidis* and briefly describe how it reaches the meninges.

### ISOLATION AND IDENTIFICATION OF PATHOGENIC NEISSERIAE

1. Describe the appearance of a positive GC smear and indicate its significance in the diagnosis of gonorrhoea.

2. State where clinical specimens are obtained in the male and in the female for the isolation of *Neisseria gonorrhoeae*.
3. State where clinical specimens are obtained for the isolation of *Neisseria meningitidis* when it is causing meningitis.
4. Name a selective medium useful for the isolation of pathogenic *Neisseria* and describe how the resulting colonies will appear.
5. State the results of *N. gonorrhoeae* and *N. meningitidis* for the oxidase test and for acid production from oxidation of the sugars glucose, maltose, sucrose, and lactose.

## RESULTS

1. Identify a positive GC smear and state how you can tell it is positive.
2. Identify an organism as *N. gonorrhoeae* or *N. meningitidis* and state the reasons why when it is seen growing on MTM Chocolate agar with a Taxo N® (oxidase) disc and in carbohydrate utilization tubes containing the sugars glucose, maltose, sucrose, and lactose.

## B. THE MYCOBACTERIA

### DISCUSSION

1. Discuss one characteristic common to the genus *Mycobacterium* which allows us to distinguish this organism from most other genera of bacteria.
2. List two pathogenic species of *Mycobacterium* and name the infection that each causes.
3. State the significance of *Mycobacterium avium* complex (MAC).

### DIAGNOSIS OF TUBERCULOSIS

1. State three presumptive tests for the diagnosis of tuberculosis.
2. State two methods used for confirmation of active tuberculosis.
3. Describe the appearance of a positive acid-fast stain for tuberculosis.

## RESULTS

1. Identify a positive acid-fast stain and state how you can tell it is positive.
2. Recognize granuloma when shown a slide of a tuberculoid organ.

## C. THE OBLIGATE ANAEROBES

### DISCUSSION

1. Name the most common obligate anaerobe to cause wound infections in humans, state its normal habitat, and name the most common type of infections it causes.
2. Name the infection caused by *Clostridium difficile*, state its significance in health care-associated infections, and the significance of the overusage of antibiotics to its cause.
3. State three ways of culturing obligate anaerobes in the lab.
4. State the normal habitat of *Clostridium perfringens* and name an infection it may cause.

### ISOLATION AND IDENTIFICATION OF *CLOSTRIDIUM PERFRINGENS*

1. Describe the appearance of *C. perfringens* when it is anaerobically-grown on Blood agar and in Litmus Milk.

## RESULTS

1. Identify an organism as *C. perfringens* and state the reasons why when given anaerobically-

grown cultures of Blood agar and Litmus Milk.

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**SELF-QUIZ**

[Self-quiz](#)

[Answers](#)

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