EXERCISE 10

Miscellaneous Staining

OBJECTIVES

At the conclusion of the exercise, you should...

1. know what causes some bacteria to be "acid fast."
2. know the purpose of doing the acid-fast stain.
3. know what bacteria need to be stained using the acid-fast technique.
4. perform the acid-fast stain.
5. observe *Mycobacterium* species that have been acid-fast stained.
6. understand what is an endospore.
7. know what and why bacteria form endospores.
8. be able to perform the endospore stain.
9. learn the characteristics of Gram-positive, endospore-forming rods.
10. observe slides of *Bacillus* species with endospores.
11. know what and why bacteria form capsules.
12. observe slides of bacteria that have been stained with a capsule stain.
13. understand what is a negative stain.

Exercises Required
Exercise 1: The Brightfield Microscope
Exercise 6: Aseptic Technique
Exercise 7: Smear Preparation

INTRODUCTION

Bacteria are stained in order to see them easily (e.g., the simple stain) and to aid in their identification (e.g., the Gram stain, a differential stain in which more than one reagent is used and different bacteria react differently). A third use for staining bacteria would be to observe specific structures for identification purposes. Structural stains are used to stain specific structures. The endospore and capsule stains are examples of structural stains. The acid-fast stain is another example of a differential stain (using more than one reagent and the bacteria reacting differently to the reagents). In this exercise, you will perform and observe the acid-fast stain, the endospore stain, and observe a demonstration slide of the capsule stain.

MATERIALS

Cultures:

Mycobacterium smegmatis (acid-fast stain)

Staphylococcus aureus (acid-fast stain)
Bacillus megaterium (endospore stain)
Bacillus subtilis (endospore stain)
Klebsiella pneumoniae (milk culture for capsule stain)

Supplies:

Prepared slides showing:

1. Positive AFB (Acid-Fast Bacilli) sputum smears.
2. Endospore stains of *Bacillus* species.
3. Capsule stains of *Klebsiella pneumoniae*. Maneval's capsule stain.

A. ACID FAST STAIN (INTRODUCING THE GENUS *MYCOBACTERIUM*)

PROCEDURES

Technical Background

The acid fast stain is used most commonly to aid in the diagnosis of tuberculosis (TB), an infection caused by *Mycobacterium tuberculosis.* Mycobacteria can infect almost any tissue or organ of the body. Mycobacteria have a waxy substance called mycolic acid as part of the cell wall. This waxy substance makes the organism very slow growing and thus difficult to isolate and identify. Mycobacteria can be recovered optimally from clinical specimens when methods are used both to release them from body fluids and cells (called digestion) and to remove or sufficiently reduce competing organisms (called decontamination).

Specimens that are submitted for mycobacterial culture are of two categories: specimens of pulmonary origin (primarily to isolate M. tuberculosis) and specimens of extrapulmonary origin. The specimens of pulmonary origin (usually sputum) are almost always contaminated with normal flora. Because they can contain large numbers of normal contaminating flora and the normal consistency can trap the mycobacteria, they need to be decontaminated and digested, a process which also concentrates the bacilli.

After the digestion and decontamination procedures, the specimen is cultured and smears are prepared for staining. An acid-fast stain is performed, which uses acid-alcohol as the decolorizer. Because these organisms contain mycolic acid in their cell wall, they are resistant to decolorization and retain the original stain. The visualization of acid-fast bacilli in sputum or other clinical material should be considered only presumptive evidence of tuberculosis, since the stain does not specifically identify Mycobacterium tuberculosis. Culturing these bacteria allows for their identification.

Performing the Steps of the Acid-fast Stain

1. Using aseptic technique, prepare a slide of *Mycobacterium smegmatis* mixed with a loopful of *S. aureus.*
2. Air dry and then heat fix the smear.
3. Flood the slide with Kinyoun's TB Carbolfuchsin (KF) for 4 minutes.
4. Wash the slide gently with water.
5. Drip acid-alcohol on the slide until no more color drains from the smear.
6. Wash gently with water.
7. Counterstain with methylene blue for 30 seconds.

8. Wash gently with water.
9. Blot dry very gently.
10. Air dry.
11. Observe the slide under oil and compare to the figure in the Atlas manual.
12. Interpretation: The acid-fast *Mycobacterium smegmatis* should be rod shaped and appear dark pink to bright red. The *S. aureus* should be cocci and appear blue.
13. Observe the positive AFB sputum smear. Typical acid-fast bacilli (AFB) appear as purple to red, slightly curved, short or long rods (2-8 µm) against the tissue which stains with the blue counterstain. Note the different appearances between your slide and the stained sputum slide.

B. ENDOSPORE STAINS (INTRODUCING THE GENUS BACILLUS)

PROCEDURES

Technical Background

During extreme environmental conditions, such as heat or drought, certain bacteria are capable of forming a specialized cell structure called an **endospore**. This **spore** is unique to certain genera of bacteria. Two of these genera are ***Clostridium*** and ***Bacillus***. Endospores are highly durable, dehydrated cells with thick cell walls. They can survive extreme heat, lack of water, and many toxic chemicals and radiation. Because of their nearly impenetrable cell walls, the Gram-stain method will not stain endospores; therefore, specialized staining methods are necessary. Most of the methods utilize heat to drive the stain into the endospore cell wall. One method, called the Schaeffer-Fulton Method, uses malachite green stain with heat and safranin for a counterstain. The endospore will stain green and the surrounding vegetative cell will stain pink.

There is a cold endospore staining method that utilizes malachite green by flooding the slide for at least 10-15 minutes and then counterstaining with safranin. The endospores will appear a lighter green than when heat is used. This method is safer and cleaner to use.

Performing the Steps of a Cold Endospore Stain

1. Prepare a slide of *Bacillus* megaterium and a slide of *Bacillus subtilis*.
2. Air dry and heat fix the slides.
3. Flood the slide with malachite green for 10-15 minutes.
4. Rinse the slide with water.
5. Counterstain with safranin for 30 seconds.
6. Rinse with water and blot dry.
7. Air dry the slide.
8. Examine the slides with oil immersion.
9. Also, observe the prepared slides of *Bacillus megaterium* endospores.
10. Draw and label your observations in the evaluation portion of the Data section.

C. CAPSULE STAINS

PROCEDURES

Technical Background

Many bacteria produce a slimy layer that surrounds and adheres to the cell. When the slime is loosely bound to the bacterium, it is called a **slime layer**. When highly symmetrical and organized, this layer is called a **capsule**. Capsules play a role in the **virulence (disease-causing ability)** of some bacteria. Capsules are composed of polysaccharides and are water-soluble. Simple stains will not adhere to the capsule; to visualize them, the background and bacteria must be stained while the capsule remains unstained. This process is called **negative staining**. One method of negative staining uses nigrosine (or India ink), which stains the background black. Crystal violet is used as a counterstain to stain the bacterial cell, thus making the capsule visible as a **clear halo** around the cell. Another method (Maneval's) uses Congo red for the background, where the bacterial cells are stained reddish-brown and the capsules are unstained.

Performing the Steps of a Capsule Stain

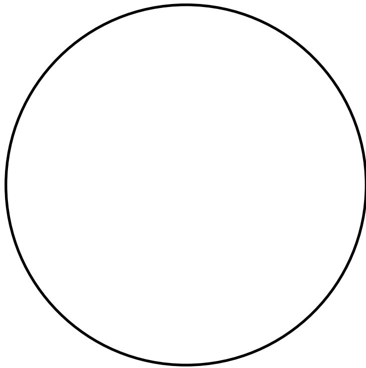
1. Place a drip of Congo red on a slide.
2. Transfer some of the bacteria to be stained from a milk culture to the drop of Congo red on the slide and mix.
3. Spread the mixture of Congo red and bacteria over the slide to about the size of a quarter.
4. Allow the slide to air dry. DO NOT HEAT FIX THE SMEAR.
5. Flood the slide with the Maneval's stain (second solution) for 1 minute.
6. Rinse the slide with water.
7. Air dry.
8. Examine under oil immersion. The capsules appear unstained, the background is purple-red, and the bacterial cells are stained red to reddish-brown, depending on the thickness of the smear.
9. Observe the prepared slides of Klebsiella pneumoniae capsules.
10. Draw your and label your observations in the evaluation portion of the Data section.

EVALUATION OF RESULTS
EXERCISE 10: MISCELLANEOUS STAINS

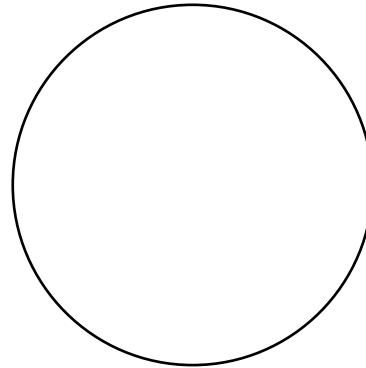
Purpose

Data

A. Acid-fast Stain **Prepared Slides**

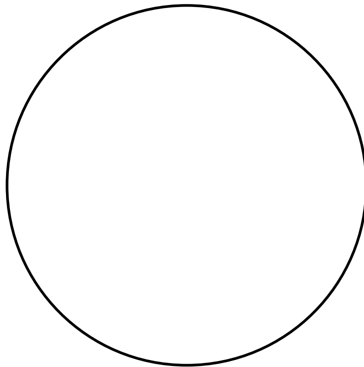


M. smegmatis + *S. aureus*

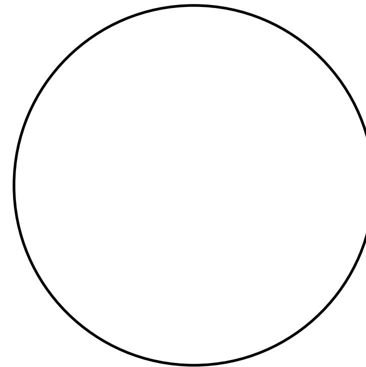


Sputum smear

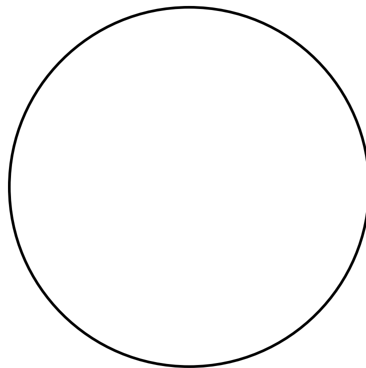
B. Endospore Stain



Bacillus megaterium

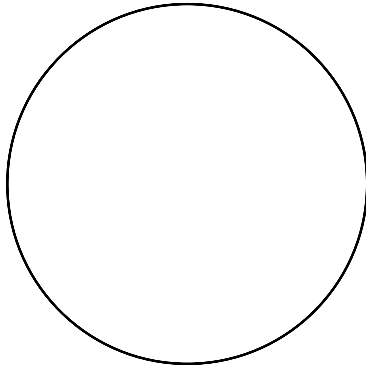


Bacillus megaterium (prepared slide)

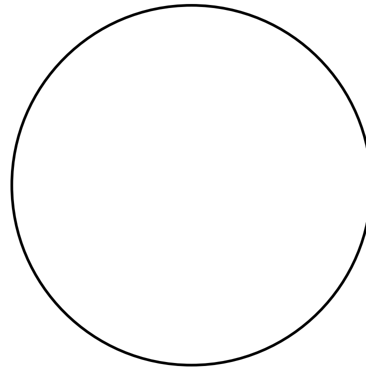


Bacillus subtilis

C. Capsule Stain



K. pneumoniae



K. pneumoniae– prepared slides

CONCLUSIONS, DISCUSSIONS, AND QUESTIONS

1. What is the decolorizing agent used in the acid-fast stain? How is this different from the decolorizing agent used in the Gram stain?

2. What diseases are diagnosed by using the acid-fast stain?

3. Why don't endospores stain with the Gram stain method?

4. Discuss some differences between the genus *Bacillus* and *Clostridium*, including their oxygen requirements, pathogenicity and endospore characteristics.

5. Explain how capsules contribute to the pathogenicity of some bacteria.

6. A postal worker shows his doctor a large, black, crusty sore on his hand. A Gram stain of the oozing liquid shows large Gram-positive rods in chains. Some of the rods are pink and have clear, oval shapes inside. What structural stain should be done immediately on a fresh smear of the oozing liquid? What is the reason for your answer?