

**Lab Exercise #3 - INSTRUCTIONS****Identification of Unknown Bacteria****Differential Staining and Specialized Bacterial Growth Media****I. OBJECTIVES:**

- ✓ Provide the student with an opportunity to perform Gram stain
- ✓ Evaluate and interpret the results of the Acid fast and Endospore stains.
- ✓ Compare and contrast these three procedures and differentiate between the clinical information obtained from performing these stains.
- ✓ Proper microscope focusing techniques and microscope care.
- ✓ Use terminology correctly.

**II. TERMINOLOGY:** Students should define and use the following terms:

Acid Fast	Endospore stain	not acid fast
Acid Fast Stain	Enterobacteraceae	positive control
bacilli	<i>Escherichia coli (E. coli)</i>	primary stain
<i>Bacillus subtilis (B. sub)</i>	Gram negative	spirillia
cocci	Gram positive	sporulation
counterstain	Gram stain	<i>Staphylococcus epidermidis (S. epi)</i>
Dichotomous key	mordant	vegetative cell
differential stain	<i>Mycobacterium smegmatis (M. smeg)</i>	
endospore	negative control	

**III. INTRODUCTION:**

In this lab your job will be to perform Gram, Acid fast and Endospore stains on the slides that you created last week. You will examine these stains through the microscope and use the results of your stains and streak plates to identify your bacterium using a dichotomous key.

**Gram Stain**

Refer to lab #2a for information regarding the Gram stain.

**Acid Fast Stain**

The **Acid-fast stain** is used in the identification of bacteria of the genera *Mycobacterium*, which are bacilli. *Mycobacterium tuberculosis* is the causative agent of tuberculosis and *Mycobacterium leprae* causes leprosy. The acid-fast stain is most commonly used on the **clinical sample** sputum when tuberculosis is suspected. In lab we will use *Mycobacterium smegmatis (M. smeg)*, which is a non-pathogenic bacterium in this genus. *Mycobacteria* have a cell wall that is different from both Gram-negative and Gram-positive bacteria. *Mycobacteria* can stain either Gram-positive or Gram-negative depending on the age of the culture. They are said to be "**Gram variable.**" The cell wall of *Mycobacteria* contains a waxy substance called **mycolic acid**. The presence of this waxy material means that it is difficult to get stains into and out of *Mycobacteria*. In an acid-fast stain, the primary stain carbolfuchsin is forced into *Mycobacteria* with heat. *Mycobacteria* are not easily decolorized with acid-alcohol after staining with hot carbolfuchsin; they are said to be **acid fast**. (An old term for "won't fade in the wash" was color-fast.) **Non-acid-fast**

**bacteria** will decolorize under this rigorous regime. After the decolorization process, the cells are counterstained with crystal violet. All cells that were decolorized will now stain blue/purple. Cells that decolorize with the acid alcohol are known as 'non acid fast' bacilli/cocci. The most important clinical use of acid-fast staining is in the diagnosis of tuberculosis.

### Endospore Stain

*Clostridium* and *Bacillus* are two genera of bacteria that produce endospores. Endospores are resistant structures made through a process known as sporulation by members of these genera in response to extreme environmental conditions. Extreme environments include high temperatures and lack of food. The endospores are inert structures meaning they do not metabolize and do not reproduce. They simply exist, much like plant seeds, until placed in appropriate environmental conditions. When environmental conditions are favorable, the endospore germinates. Upon germination, the cell returns to its normal metabolic state, capable of reproduction.

Normal staining techniques will not stain the resistant endospores. The stain, malachite green, is forced into the spore with heat much like the carbol fuchsin was forced through the waxy mycolic acid layer of *Mycobacterium*. Once the endospore is stained, the counter stain, Safranin, provides color for the vegetative (i.e. metabolically active) cells. At the end of this differential staining process the vegetative cells are pink and the endospores, if present, are green.

### MacConkey's Agar

MacConkey's is a **selective** media because it inhibits the growth of Gram positive bacteria due to the presence of crystal violet and bile salts. It is also **differential** meaning that it differentiates or distinguishes groups of bacteria on the basis of a color reaction based on whether or not the microbes growing on MacConkey's ferment lactose. MacConkey's contains two additives for this purpose: neutral red (a pH indicator) and lactose (a sugar). Bacteria, known as "lactose fermenters", metabolize the lactose to an acidic end product. This acidic metabolite causes the pH indicator, neutral red, to turn red. Thus the colonies of lactose fermenters will grow in pink/red colonies while non-lactose fermenters will be colorless (or more the color of the media prior to inoculation).

Members of the family Enterobacteriaceae (Gram negative bacilli typically found in the gastrointestinal tract of mammals) are the most frequently encountered bacteria isolated from many types of clinical specimens (e.g. urine). They are most commonly lactose fermenters.

### Mannitol Salt Agar

Mannitol Salt is a **selective** medium because it has a high concentration of NaCl (7.5%). Most bacteria cannot survive in this environment. The genus *Staphylococcus*, however, grows well in this media. The medium is also **differential**: It contains a dye that identifies pathogenic *Staphylococcus* (*S. aureus*) as it produces an organic acid from mannitol fermentation and changes the pH indicator from red to yellow.

### Using a Dichotomous Key to ID an Unknown

A **dichotomous key** is a way of dividing groups of bacteria based on their physical or metabolic attributes. "Dichotomous" means 'dividing into two parts'. A group of bacteria are divided into two groups on the basis of a certain characteristic (i.e. cellular morphology). This group is then subdivided into two groups on the basis of a second feature, and so on. Such a key, based on the stain results and media, is provided at the end of this exercise. You are directed to use the results of the differential stains (Gram stain, acid fast stain and endospores stain) and differential and selective media (MacConkeys and Mannitol Salt) to determine the identity of your unknown.

The number of unknowns in this case is limited to the seven types of bacterial cultures in stock in the lab. In the clinical setting the potential number of unknowns is many times this. Thus, much more than the few

characteristics of these cells is needed to identify the genus and species of the bacterium; the metabolic capabilities of the organism are studied in more detail.

Identifying an unknown is a fascinating process. In clinical medicine often times a pathogen is isolated from among a multitude of normal flora. Once that is accomplished, the bacterial staining techniques (Gram stains or acid-fast stains) are often used as a preliminary step in the identification of an unknown. After this preliminary data is collected the medical microbiologist will examine the physiological characteristics of the unknown, such as carbohydrate and nitrogen metabolism. An individual species is capable of certain metabolic pathways because of its genetic makeup. We use the biochemical capabilities of a microbe a phenotypic marker. We compare the phenotype (genetically expressed traits) of our unknown to the phenotype of known bacterial species. *Bergey's Manual of Systematic Bacteriology* contains the names and descriptions of most recognized species of bacteria.

Differential staining, as noted, provides preliminary information to the medical technologist charged with identifying the genus and species of a bacterium causing an infection disease in a patient. The med tech uses this information to decide which diagnostic test will be employed to determine the genus and species of the bacterium in question.

#### IV. MATERIALS (In addition to supplies found in your supply drawer):

- Bacterial smears from previous lab
- Streak plates from previous lab
- Microscope
- Gram stains: Crystal violet, Acetone alcohol, Gram's Iodine, Safranin
- Acid Fast stain slides for your unknown
- Endospore stain slides for your unknown
- Slide holders, Slide-shaped blotting paper
- Mannitol Salt media
- MacConkey's media

#### V. PROCEDURES:

##### A. Staining and Examining the Gram stain slid, Acid fast slide & Endospore slide

Today you will perform a Gram stain, if needed, and use the prepared slides of the Acid fast and Endospore stains to determine a preliminary identification of your unknown.

##### **Gram Stain**

Complete the following only if your results from your Gram stain last week were inconclusive:

1. Take the heat fixed Gram slide to one of the staining racks located at the benches with sinks. Flood each slide with **crystal violet** and allow to stand for **one minute**. Rinse the slide with water.
2. Cover the smears with **Gram's iodine** solution and let stand for **one minute**. Rinse.
3. Flood the smear with **95 % acetone alcohol** for **15-20 seconds**; rinse. This is a critical step. Decolorization occurs when the alcohol flows colorless from the slide. Thick slide will require more time (closer to 20 seconds) than thin ones (10-15 seconds). Over decolorization may result and then all the bacteria will appear Gram negative.
4. Flood the slides with **safranin** for **one minute**. Rinse and blot dry.
5. Examine slide under oil immersion. See "Viewing Bacteria Under Oil Immersion" below, or the PPT slide of Instructions for Viewing Bacteria Under Oil Immersion.

### Acid-fast Stain

The following procedure for the acid fast stain **was already performed** on your unknown bacteria: First a bacterial smear slide was prepared with an acid-fast positive control (*Mycobacterium smegmatis*) in the circle on the left, and acid-fast negative control (*Staph epi*) in the circle on the right, and your unknown in the middle. The slide was heat fixed and then the acid fast staining procedure was performed.

1. Take your heat fixed Acid-fast slide to the boiling water bath in the hood marked "Acid-fast stain". Place the slide on the screen covering the boiling water and cover the smear with a strip of blotting paper; the paper should not extend beyond the edges of the slide.
2. Saturate the paper with Ziehl's carbolfuchsin. Allow the staining to continue for 3-5 minutes. Do not allow the slide to dry. If necessary, add more stain, but try not to add so much that the stain runs off the slide and into the boiling water.
3. At the end of the staining period remove the slide from the rack to a folded paper towel using a forceps. Use the forceps to remove the blotting paper and discard in biohazard. Carry the slide over the paper toweling to the staining sinks in the laboratory.
4. Rinse the cooled slide with tap water and then decolorize the smear for 10-30 seconds with acid alcohol. Exercise care that the smear is not over-decolorized. Rinse with tap water to stop the decolorization process.
5. Apply crystal violet for 30-45 seconds rinse and blot dry.

#### **This is where you come in:**

6. Examine slide under oil immersion. See "Viewing Bacteria Under Oil Immersion" below, or the PPT slide of Instructions for Viewing Bacteria Under Oil Immersion.
7. Record your results using the digital camera in the microscope. Make certain the capture a picture of the controls and your unknown.

### Endospore Stain

The following procedure for the Endospore stain **was already performed** on your unknown bacteria:

First a bacterial smear slide was prepared with an endospore positive control (*Bacillus subtilis*) in the circle on the left, and an endospore negative control (*E.coli*) in the circle on the right, and your unknown in the middle. The slide was heat fixed and then the endospore staining procedure was performed.

1. Take the heat fixed Endospore slide to the boiling water bath in the hood marked "Endospore stain."
3. Flood the smear with malachite green. Steam over boiling water for five minutes. Add additional stain if the stain evaporates, again, careful not to overrun the slide with stain.
4. Using the forceps, pull the stained slide off the screen and onto a folded paper towel. Allow the slide to cool and rinse with water until no dark green clumps remain.
5. Counterstain with safranin for about 20 seconds. Rinse and blot dry.

#### **This is where you come in:**

6. Examine slide under oil immersion. See "Viewing Bacteria Under Oil Immersion" below, or the PPT slide of Instructions for Viewing Bacteria Under Oil Immersion.
7. Record your results using the digital camera in the microscope. Make certain the capture a picture of the controls and your unknown.

### Viewing Bacteria Under Oil Immersion

You will be viewing your differential stain slides using the microscope. You will need to view each differential stain (Gram, Acid-fast and Endospore) using the oil immersion objective of the compound light microscope. When looking at your differential stain slides, always start by viewing the controls, since you know what these "knowns" should look like. Look at the

unknown after examining the controls. Below is a review of how to view objects under oil immersion.

1. Place the specimen on the slide and secure the slide to the microscope using the stage clip. Check that the slide is held by the stage clip by moving it with the mechanical stage control.
2. Click the 10X objective lens (yellow band, total magnification 100XTM) in place.
3. Use the mechanical stage control to position the specimen over the light source.
4. Use the coarse adjustment to raise the stage to its highest position. Looking through the microscope ocular, turn the coarse adjustment slowly away from you, lowering the stage until your specimen comes into focus. Remember to adjust the iris diaphragm for the best image.
5. "Fine tune" your image with the fine adjustment knob.
6. Make sure the 40X objective (blue band, total magnification 400XTM) is covered with a finger cot to protect it from oil.
7. (**Note:** When focusing on a bacterial sample, always start at low power (100xTM) and work your way up. This not only helps find and focus on specimens quickly but also alleviates the potential of ramming a long, oil immersion objective through a slide when trying to focus with the course adjustment knob under high power. Pay attention to the **working distance** of the lens. This is the distance between the lens and the slide when the specimen is seen in sharp focus. The higher the magnification, the smaller the working distance. To avoid ramming a long objective into a slide, observe the **Working Distance Rule:** Use the coarse adjustment knob on low power only.)
8. You will use the oil immersion objective (100X) **after** you have focused your scope on your specimen using the 10X objective lens. If you have any difficulty finding a specimen, **always** go back to scanning power and begin the focusing process again.
  - a. Move the nosepiece so that you are in between the high power and oil-immersion objective.
  - b. Before moving the oil immersion objective into position, place a small drop of immersion oil on the slide over the area where the light source illuminates the specimen.
  - c. Click the oil immersion objective into place. When the objective is moved into position it should be in contact with the immersion oil. Check that this is so. Using **fine focus only**, sharpen the image. Other helpful hints:
    - do not use immersion oil that is cloudy
    - keep your eyepiece and objectives clean; use only lens paper when cleaning
    - do not tilt your scope

You should be very cautious when using oil immersion objectives and immersion oil. First, be sure you are using immersion oil, and not another kind of oil. Only the oil objective should come in contact with the oil. The other non-oil objectives on your microscope will not work properly if they get oil in them, which will happen if they are dragged through oil on a slide. Be sure you do not move from an oil immersion lens to a 40X objective, because the 40X objective will drag through the oil, and be ruined by the oil. The oil needs to be cleaned from the oil-immersion objective after use, and this is done using a special paper (lens paper) that will not scratch the lens.

9. Take micrographs of specimens viewed at 1000xTM where instructed to in your Lab Report #3.

### **Isolation Streak Plates of Unknown onto Specialized Media**

Retrieve the isolation streak plate of your unknown that you set up last week on TSY agar. We will use this

plate to perform an isolation streak plate on Mannitol salt and MacConkey's media for further identification of the unknown. Use the directions from lab #2 for performing the isolation streak.

1. Sterilize your inoculating loop.
2. Locate an isolated colony from the TSY streak plate.
3. Gently touch the center of the isolate colony with the sterile inoculating loop.
4. Transfer the bacteria on the loop to quadrant #1 on the Mannitol Salt plate.
5. Sterilize your inoculating loop and streak quadrant #2.
6. Sterilize your inoculating loop and streak quadrant #3.
7. Sterilize your inoculating loop and streak quadrant #4.
8. Repeat items 1-7 using the MacConkey's plate.
9. Write your initials, the unknown number and the date on the media side of the Petri plate.
10. Store the plates upside down in the green bin in the back of the lab. Again, they will be incubated at 37° C for 24 hours and then stored until you return next week.

**Always wash down your bench  
with disinfectant at the end of the lab.**

This material is adapted from the Applied Microbiology Laboratory Manual by Cynthia Schauer. For Power Point slides that correspond to this lab material, see the Virtual Microbiology Classroom of the [Science Prof Online](#) website.

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