Lab Exercise #3b
Identification of Unknown Bacteria:

- Differential Staining (Gram, Acid-fast, Endospore)
- Interpret Unknown on MacConkey's & Mannitol Salt Media
- Identification of Unknown

I. OBJECTIVES:

- ✓ Provide the student with an opportunity to perform Gram, Acid fast and Endospore stains.
- ✓ Compare and contrast these three procedures and differentiate between the clinical information obtained from performing the two stains.
- ✓ Provide practice using a dichotomous flow chart for identification of an unknown bacterium
- ✓ Proper microscope focusing techniques and microscope care.
- ✓ Read and interpret growth on selective and differential media.
- ✓ View and understand demonstration of API 20 E bacterial test.
- ✓ Use terminology correctly.

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

API20E	*Escherichia coli (E. coli)*	*Mycobacterium smegmatis (M. smeg)*
Bacillus subtilis (B. sub	mordant	spirillia
Staphylococcus epidermidis (S. epi)	Enterobacteraceae	sporulation
Dichotomous key	Endospore stain	vegetative cell
Acid Fast	counterstain	differential stain
vegetative cell	endospore	negative control
Acid Fast Stain	Gram negative	not acid fast
bacilli	Gram positive	positive control
cocci	Gram stain	primary stain

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

In the late 1800's, Danish bacteriologist Christian Gram developed a method for staining bacterial cells that seemed to separate the cells into two groups. These groups, known as Gram negative and Gram positive, were separated on the basis of the color of the bacteria after a series of stains were applied to them. The Gram negative cells stain red and the Gram positive cells stain purple, thus the Gram stain is a **differential stain**, (i.e. it illustrates differences between bacterial cells). Today, the identification of an unknown organism begins with a Gram stain. Gram stains quickly tell not only if a bacterium is Gram-positive or Gram-negative, but will also tell you the shape of the bacterium (its cell morphology). Clinically, Gram stain results allow for rapid intervention with appropriate antibiotics.

The Gram stain procedure involves first staining all cells purple with the **primary stain**, crystal violet. In the next step, the cells are treated with Gram's iodine, which is a mordant. A **mordant** is a chemical that

fixes in place a dye already present. In this case, the Gram's iodine causes the crystal violet to clump together, or to precipitate. It is thought that this occurs in a layer of the cell wall called the peptidoglycan. Next, the cells are washed with acetone alcohol, which is able to wash the precipitated crystal violet out of Gram-negative cells but not Gram-positive cells, perhaps because the thick peptidoglycan of Gram-positive cells traps the precipitates efficiently. Last, the cells are counterstained with safranin. The safranin enters all cells, and stains the Gram-negative cells pink. Since the Gram-positive cells are already dark purple, the safranin is not visible in them. A **counterstain** is a stain used to visualize cells that would otherwise have no stain at the end of a procedure. Without a counterstain, they would not be visible.

Acid Fast Stain

The second differential stain you will perform is an **Acid-fast stain**. This 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 fuschsin 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.

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.

API-20E Diagnostic Test

If the microbe is a Gram negative bacillus, the diagnostic test most frequently employed is the API20E. This particular API product is a system of 20 individual, miniaturized tests used to determine the metabolic capabilities and, thus, the genus and species of organisms in the family Enterobacteraceae. Your instructor will demonstrate this test. You will NOT be using this test in lab today.

Cupule

Tube

Figure 1: API Cups

Used with permission from Dennis Tauch - Biomerieux

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

Bacterial smears from previous lab
Streak plates from previous lab
Microscope
API 20 E test strip, incubation chamber, results form, and ID book
Gram stains: Crystal violet, Acetone alcohol, Gram's Iodine, Safranin
Acid Fast stains: Carbol Fuchsin, Acid Alcohol, Crystal Violet
Endospore stains: Malachite Green, Safranin
Hot plates & water baths
Slide holders, Slide-shaped blotting paper

V. PROCEDURES:

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

Gram Stain

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

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.
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.

Endospore Stain

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 remian.
5. Counterstain with safranin for about 20 seconds. Rinse and blot dry.
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.

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 #3b.

B. Examine the media plates that you inoculated last week for differential and selective information that will help you identify your unknowns.

C. Identify your unknown bacteria using the dichotomous key.
   1. Observe the growth of your unknown on the MacConkey's and Mannitol salt plates.
   2. Compare the results of the Gram, acid fast and endospore stains.
   3. Use this information as needed to follow the dichotomous key.
   4. Write the identification of your unknown (number and scientific name) on your Lab Report 3b. Question #1.

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.