

Exercise 5

(Ed. Fall 2010)

THE PREPARATION OF A BACTERIAL SMEAR FOR STAINING:

The Simple stain, Gram stain, Acid Fast stain, Capsule stain and Endospore stain

INTRODUCTION:

Student Learning Objectives: At the end of this exercise, student will be able to:

1. Describe the methods of preparation of a bacterial smear from broth and solid media.
2. Describe the attributes of an ideal smear.
3. Identify the factors which can result in a less-than-ideal smear.
4. Define the purpose of the specific staining methods used to stain bacteria.
5. Prepare the specimen for staining (heat fixing).
6. Successfully perform the Simple stain, Gram stain, Acid Fast stain, Capsule stain, and Endospore stain on known cultures.
7. Define Quality Control and perform a QC test with the Gram stain
8. Gram stain an unknown culture.

Materials

- Broth cultures of *Streptococcus pyogenes*, *Micrococcus luteus*, and *Bacillus subtilis* (18 – 24 hours)
- Broth culture of *S. aureus* and *B. subtilis* over 48 hours old.
- Slant cultures of *E. coli*, *Bacillus subtilis*, *Neisseria sicca*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa*.
- Slant culture of *Staphylococcus aureus*.
- Slant culture of *Mycobacterium smegmatis* and *Corynebacterium sp.*
- Inoculating loop
- Bunsen burner
- Distilled water and medicine dropper.
- Microscope and slides
- Gram staining kit, Methylene Blue stain, India ink, Malachite Green, and Safranin (other than Gram's Safranin).
- Bibulous paper
- Wax pencils

Activities for PART 1:

1. Prepare bacterial smears on slides and heat fix where applicable.
2. Using methylene blue, prepare a simple stain of bacteria.
3. Using the Gram stain method, prepare a differential stain.

Activities for PART 2:

1. Using the India ink and Crystal Violet stains, prepare a differentail capsule stain.
2. Using Malachite Green and Safranin, prepare an endospore stain.

Introduction

To visualize bacteria under a light microscope, several staining methods are employed. However, the stain is as good as the starting material on the microscope slide, which is the bacterial smear. If the smear is less than ideal (Bad smear), then staining the specimen would not be productive and can yield inconclusive results at best. Since bacteria are grown in both liquid and solid media (broth and agar), specimens must be obtained using the appropriate tools and collecting the ideal amount for the smear. Too much can result in a very thick smear which can stain irregularly, and too little can result in a smear where bacteria will be too difficult to find. Another factor that can affect staining is the age of the culture; in the case of Gram positive bacteria, older cultures (>24 hours) tend to yield a non-uniform Gram stain, where although the culture is a pure culture, it will appear as if it were a mixed culture with both Gram positive (bleu) and Gram negative (reddish) organisms in the same smear. To avoid this, only “young” cultures are used for Gram-staining bacteria.

As you well know, bacteria can come in different shapes, sizes, and arrangements. Some may have extra cellular structures such as a capsule, while others may form a dormant structure such as an endospore when conditions are less than favorable for survival. In this exercise, you will use specific stains that will enable you to see these structures. Also, bacteria are known to grow in specific arrangements such as chains, bunches, diplococci, and tetrads. To be able to see the normal arrangement of these bacteria, it is best to obtain the smear from a broth culture where the bacteria are growing in a suspended medium. Obtaining a smear from a solid agar will usually result in rearrangement of their normal growth patterns.

Remember your aseptic technique for this exercise, and well as the fact that you will be handling potential pathogens. Staining is a messy procedure, so make sure to avoid spilling the stains on expensive clothing, shoes, and of course your eyes and skin. When you open the stain containers, or replace them back on the desk, please make sure that the spout is closed and is pointing away from you and others to avoid the stains from splashing into eyes. Wear eye protection if deemed necessary. Wear gloves if they do not get in the way of your procedures. Discard all waste stain material in the appropriate containers. Waste water goes in the designated buckets. Stained slides go in the beaker at the front of the room. Do not dump anything in the sink. If you spill it, clean it up before the stain sets. Check with your instructor if you are not sure.

Activities for Part 1

1. Preparing a Smear from a Broth Culture

- Label two clean slides with your initials and the number 1 and 2.
- Using your wax pencil, make a circle about the size of a nickel on the top surface of these slides. Sometimes it is easier to make the circles if you warm the slide by putting it on the staining hot plate. The object of the circle on the slide is twofold. First, the circle serves to contain the specimen, so it doesn't fall off the slide if it is a liquid specimen. Second, the circle will help you focus your specimen area.
- Aseptically transfer one loopful of the *B. subtilis* broth culture and place it inside the circle of slide 1, and another loopful inside the circle of slide 2. Allow them to air-dry.
- Aseptically transfer a second loopful to slide 2 and place it on top of the first dried loopful. Allow it to air-dry.
- Turn on your Bunsen burner and quickly pass the slides through the flame 2 or three times. Heat fix the specimen kills the bacteria and causes them to stick to the glass surface so they don't wash off during the staining procedure.
- The slides should be very warm but not too hot to touch with the back of your hand.
- Allow the slides to cool off before staining.

By using at least two different amount of the broth, you will be able to tell which is the ideal amount for the smear after staining. Although you cannot count the bacteria in the broth culture, the higher turbidity usually indicates a higher number of bacteria suspended in the medium, compared to a medium with little turbidity.

2. Preparing a Smear from a Solid Medium (agar slants)

- Label two clean slides with your initials and the numbers 3 and 4.
- Draw a circle with your wax pencil as indicated above.
- Place a small drop of water (2 – 3 loopfuls) in the circle of each slide.
- Aseptically transfer a small amount of *E. coli* from the slant culture using the needle and mix it with the water on slide 3. Allow it to air dry.
- Aseptically transfer an even smaller amount by barely touching the surface of the slant and mix it with the water on slide 4. Allow it to air dry.
- Heat fix both slides and allow to cool off before staining.

By using at least two different amounts of cells from the slant culture, you will be able to tell which amount is ideal for making a good smear after staining. You can thin out the smear by adding more water and spreading the bacteria over a larger area, or by using less bacteria in the first place.

Now you are ready to stain the slides. Start your procedure by clearing off any unwanted items from your desk area. Place all the needed stains within reach, so you don't have to run off to find the stains. Make sure your stainless steel waste water receptacle and the staining rack are in place. Have enough paper towels at hand in case of spills. Read the staining procedures and understand the steps BEFORE you start staining your slides.

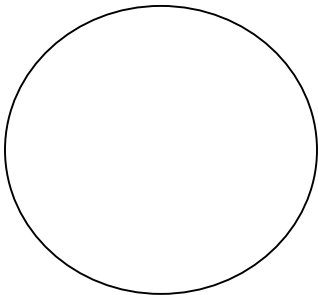
3. The Simple Stain

- Place slide 1 from above exercise on the staining rack and flood with Methylene Blue.
- Allow to stain for 1 minute.
- Using the staining tongs/forceps, tilt at an angle to allow stain to drain off slide.
- Wash/rinse off excess stain from slide and blot with bibulous paper.

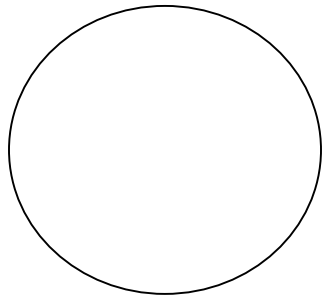
To blot, you may place the slide inside the booklet and gently press on it. Allow slide to dry out completely before viewing. Repeat the same procedure with the other three slides.

After viewing the slides under high power and oil immersion, determine which of these had the ideal amount of specimen to make a good smear. This will help you determine the amount of specimen (concentration of cells) you need to make good smears for the rest of the course. Now proceed to the next stain, and perhaps the most important stain in your Microbiology course, the Gram Stain.

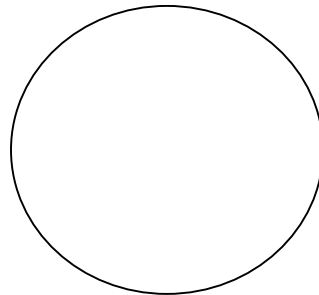
Draw your findings in the circles below and complete the information in the table.



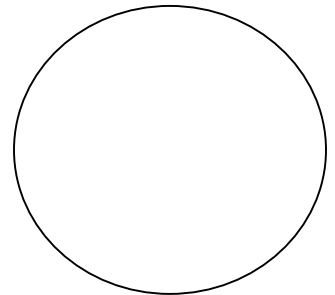
Slide 1



Slide 2



Slide 3



Slide 4

Smear/ Slide #	Smear (Describe)	Evaluation (Too few, Good, Too many)

4. The Gram stain

This activity to be done in pairs. Everyone should become proficient at this staining method. Prepare the following bacterial smears as you have done in the previous exercises from the following sources:

- Broth cultures of *Streptococcus pyogenes* and *Micrococcus luteus* (18 – 24 hours)
- Broth culture of *S. aureus* over 48 hours old.
- Slant cultures of *E. coli*, *Bacillus subtilis*, *Neisseria sicca*, *Staphylococcus aureus*, and *Streptococcus pyogenes*.

Procedure:

Heat fix your slides. What does heating the slide do?

Staining the Slides.

- Place the slide on the staining rack.
 - Flood the slide with Crystal Violet for 1 minute.
 - Tilt the slide to drain excess stain into receptacle and rinse gently with water while holding at an angle.
 - Replace the slide on the staining rack.
 - Flood the slide with Gram's Iodine for at least 1 minute.
 - Drain and rinse gently with water while holding at an angle.
 - Holding the slide at an angle, decolorize with alcohol/acetone-alcohol quickly by dripping the decolorizer for about 3 – 5 seconds, and rinse IMMEDIATELY for several seconds. This is the critical step in the Gram stain. Do not over decolorize.
 - Replace the slide on the staining rack
 - Flood the slide with Gram's Safranin for 30 – 60 seconds.
 - Holding the slide at an angle, drain excess Safranin and rinse with water.
 - Blot with bibulous paper and allow to completely dry before viewing.
- Repeat the procedure for all slides. You may stain more than one at a time.

Remember that de-colorization is the critical step! Do not over-decolorize!

Look up this information in your text and lab atlas. In your own words, describe what is happening at each step:

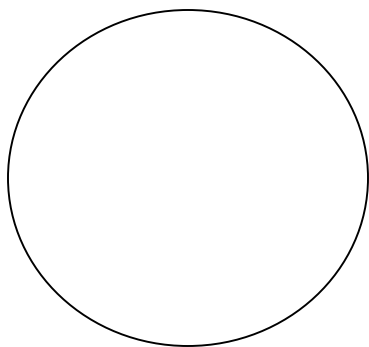
Crystal violet _____

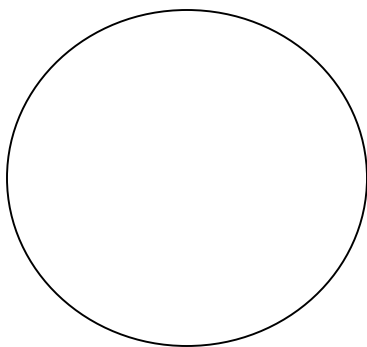
Gram's iodine _____

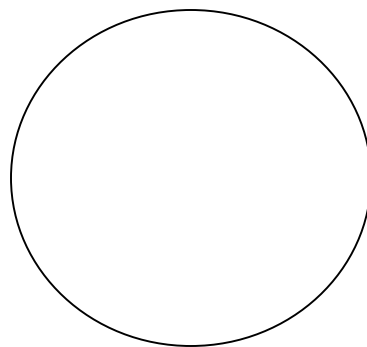
Alcohol
Decolorizer _____

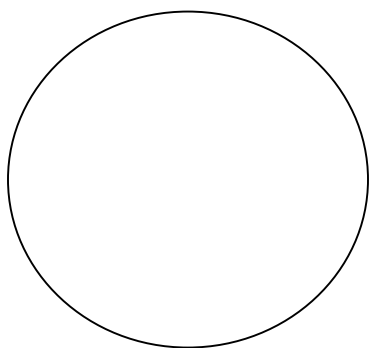
Safranin _____

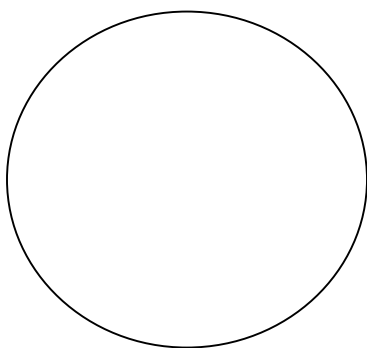
5. Draw your findings below. Make detailed drawings of your stains at 1000X (Oil).

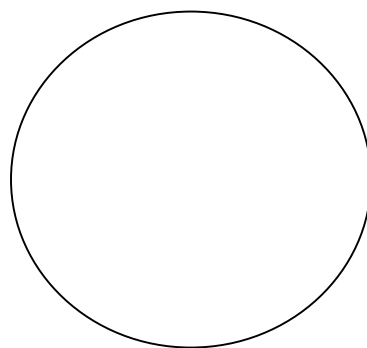


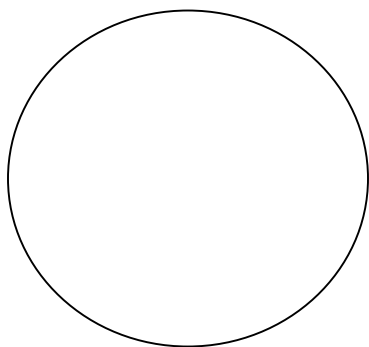


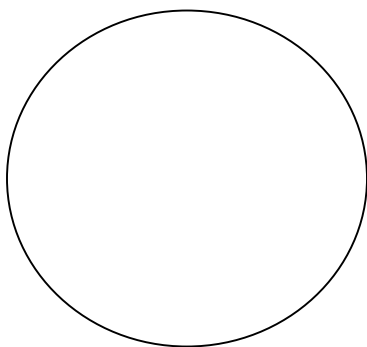


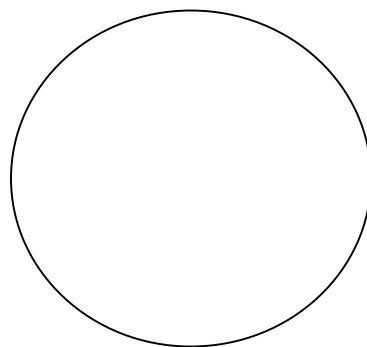












6. Based on your observations, lecture notes, and readings, answer the questions below:

1. Did you observe any differences between the older cultures of *S. aureus* and the younger cultures of *S. aureus*?

2. Which cultures would you use to get conclusive results? Why?

3. Which culture would you use to determine the normal arrangement/cultural characteristics of *Streptococcus pyogenes*? Why?

4. If you were staining a known Gram positive species, and the slide revealed reddish-pinkish organisms, what errors do you think may have occurred?

5. Other than staining bacteria from a culture medium, how can the Gram stain be used in a clinical setting?

6. Why is the Gram stain referred to as a "Differential Stain"?

7. List five pathogenic Gram Positive and five pathogenic Gram negative organisms and the diseases they cause. Does the Gram reaction affect treatment with antibiotics?

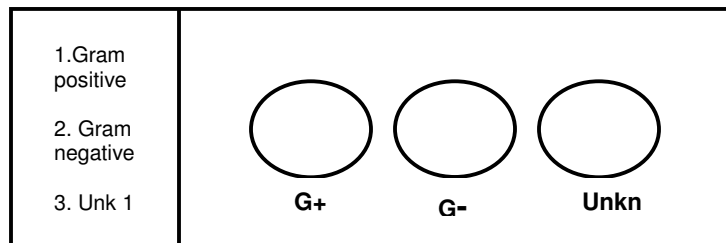
Gram (+) Organism	Disease	Gram (–) Organism	Disease
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<hr/>	<hr/>	<hr/>	<hr/>
<hr/>	<hr/>	<hr/>	<hr/>

7. Unknown slides and Quality Control

You will be given an unknown culture to Gram stain. You will also learn to perform a Quality Control Test to ensure the validity of the procedure and reagents. Follow the instructions below and document your findings.

Performing Quality Control tests.

Gram Stain- When we are gram staining an unknown, we place a known Gram-positive and Gram-negative organism on the same slide. That way, all three organisms are stained under the same conditions, to ensure the validity (accuracy) of the procedure.



Example: Quality Control Slide for Gram Stain (QC)

Stain your unknown and report your results to your instructor.

Unknown # _____

Results: _____

PART 2

Activities for PART 2

8. Staining Acid-Fast Bacteria

While most bacteria can be stained using the Gram stain, others do not stain well due to the difference in the contents of the cell walls. A group of medically important organisms do not stain well with the Gram stain, so another stain is used to differentiate them from other bacteria especially if the smear is from material such as sputum or exudate that may contain other bacteria. Bacteria such as *Mycobacterium tuberculosis*, *M. leprae*, *M. avium-intracellulare*, and *Nocardia* sp. are all acid-fast organisms. The Acid Fast stain may also be used instaining some protozoa such as *Cryptosporidium*, *Isospora*, as well as *Cyclospora*, which you will observe in other exercises. In this exercise, you will be using a non-pathogenic species of *Mycobacterium*. However, use the aseptic technique and as with all bacteria, assume it is a pathogen.

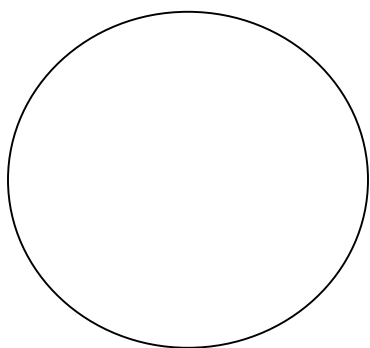
Mycobacteria are very slow growing organisms, and require special media, such as Lowenstein-Jensen medium, and are grown over several weeks in specialized incubators. The section of the lab or hospital in which *Mycobacterium tuberculosis* and other pathogenic *Mycobacteria* are grown and tested, are typically isolated by having their own air circulation systems (negative air systems), where no air from the lab is allowed to travel into the building, but always vented outside of the lab. Extra precautions are taken when manipulating these organisms due to their infectious and pathogenic nature.

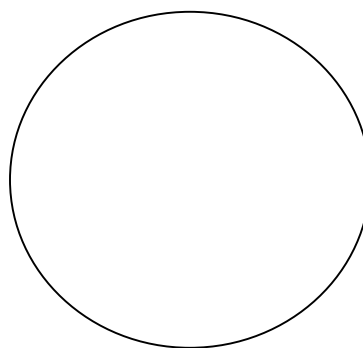
While there are several protocols for the Acid Fast stain, you will be using the Modified Kinyoun cold stain.

Procedure for the Cold Acid Fast Stain (Modified Kinyoun Stain)

- On a clean slide, draw two circles with your wax pencil and make a smear of *M. smegmatis* (Acid Fast) and *Corynebacterium* sp. or *S. epidermidis* (Non- Acid Fast Control) from the slant cultures.
- Prepare your slide exactly as you would for a Gram stain except heat fix your slide for 10 to 15 minutes using the hot plate at low heat (about 60C). Cool down before staining.
- Place the slide on a slide rack.
- Flood the slide with Carbofuchsin and allow to stain for 2 – 3 minutes.
- Drain and rinse with water for several seconds.
- Place the slide on the slide rack.
- Flood the slide with the Acid-Alcohol decolorizer for 15 – 30 seconds.
- Drain and add a few more drops of decolorizer until the red color stops coming off.
- Rinse with water for a few seconds.
- Place the slide on the slide rack.
- Flood the slide with Methylene Blue (or Brilliant Green) counter stain for 1 minute.
- Drain and rinse with water.
- Blot with bibulous paper and allow to dry.

Observe under oil, and draw your findings below.





Answer the following questions based on your readings and lecture notes.

1. How do acid fast organisms differ from others with regards to cell wall contents?

2. How does this affect their staining properties?

3. Name two Mycobacteria species and the human diseases they cause:

4. Which non-human animals can be infected with one of these?

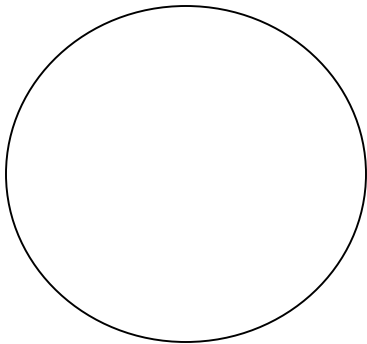
9. Capsule Stain

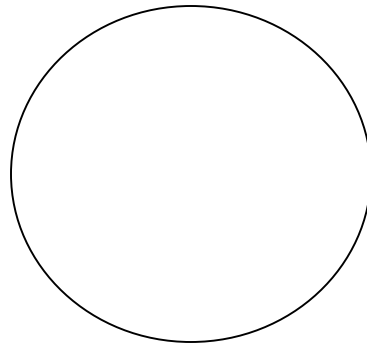
Some bacteria are able to secrete a polysaccharide slime layer or capsule which surrounds the organism and may provide protection from unfavorable environmental conditions, or from phagocytosis by cells of the immune system. This contributes to the cell's virulence factors and its ability to cause disease. Staining these organisms may prove difficult since the dyes may not bind to the polysaccharide capsule. In this case, negative staining methods are used. The capsule stain used in this lab is a modified Gin's method, whereby the capsule itself is not stained but the material around it and the cell itself are.

Procedure for the Gin's Capsule Stain

- On a clean slide, place a small drop of fresh India ink, and the same amount of water.
- Using a loop, transfer a small amount of *Klebsiella pneumoniae* from the slant to the slide and mix with the ink and water. Use a toothpick to mix well.
- Use another slide to spread/smear the mixture across the slide by placing the edge of the slide in the mixture and "pushing" the mixture across the slide toward the opposite end.
- Do not heat fix this slide. Allow to air dry.
- Flood the slide with Gram's Crystal Violet for 1 minute.
- Drain and gently rinse the slide.
- DO NOT BLOT. Place the slide at an angle to drain off and dry out on a paper towel. Since the specimen was not heat fixed, it will come off if you blot the slide.

Observe under oil immersion and draw your findings below.





Answer the following questions based on your readings and lecture notes.

1. Name two organisms that have capsules and the diseases they cause:

2. The _____ is the positive stain because _____.

3. The _____ is the negative stain because _____.

10. The Endospore Stain

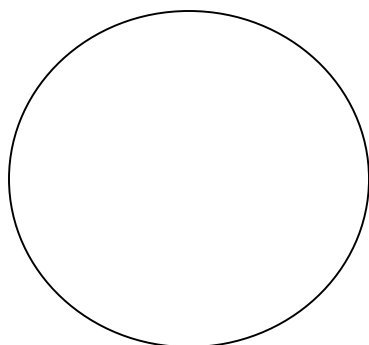
When some species of bacteria, mainly Gram positive rods, encounter harsh or unfavorable environmental conditions that can affect their survival, they form spores as a response to these conditions. Spores are able to survive for long periods of time, and able to withstand higher temperature and dessication, where vegetative cells would normally die. Spores are a smaller dormant form of the vegetative cells, but unlike fungi and plants, are not considered a form of reproduction. Spores allow the bacteria to survive these adverse conditions, until the favorable conditions return and the vegetative cells are able to live. Some spore forming organisms cause serious diseases in humans. Can you identify these organisms and the diseases they cause?

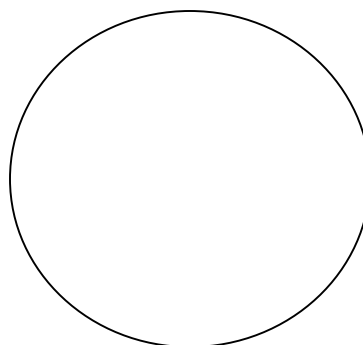
Organism	Disease
_____	_____
_____	_____
_____	_____

Procedure for the Schaeffer-Fulton Endospore Stain (can be very messy)

- Prepare a smear of *Bacillus subtilis*, and heat fix.
- Flood the slide with Malachite Green. Use the tongs or forceps to hold the slide.
- Heat the slide by passing the Bunsen burner flame over the slide. Remove as soon as steam starts to rise from the slide. Avoid breathing the steam.
- Periodically add more dye to the slide to prevent drying out. Stain for 5 minutes.
- Cool the slide on the staining rack. Add more dye as needed to prevent drying out.
- Drain the slide and rinse with water for 30 seconds.
- Flood the slide with Safranin for 1 minute.
- Drain and rinse for about 1 minute or until no more red color runs out.
- Blot with bibulous paper and allow to air dry before viewing under oil immersion.

Draw your findings in the circle below.





What color are the spores? _____