EXPERIMENT 2

SIMPLE STAIN and the GRAM STAIN

In most microbiological staining procedures, the bacteria are first fixed to the slide by the **heat fixed smear** (Figure 1). In this procedure living, potentially pathogenic bacteria are smeared on the glass slide and allowed to air dry. Then the slide is heated so that the bacteria are killed and stuck (coagulation of surface proteins) to the slide. This renders the slide safe to use and keeps the bacteria stuck to the slide so that simple or complex staining procedures can be carried out on these bacteria.

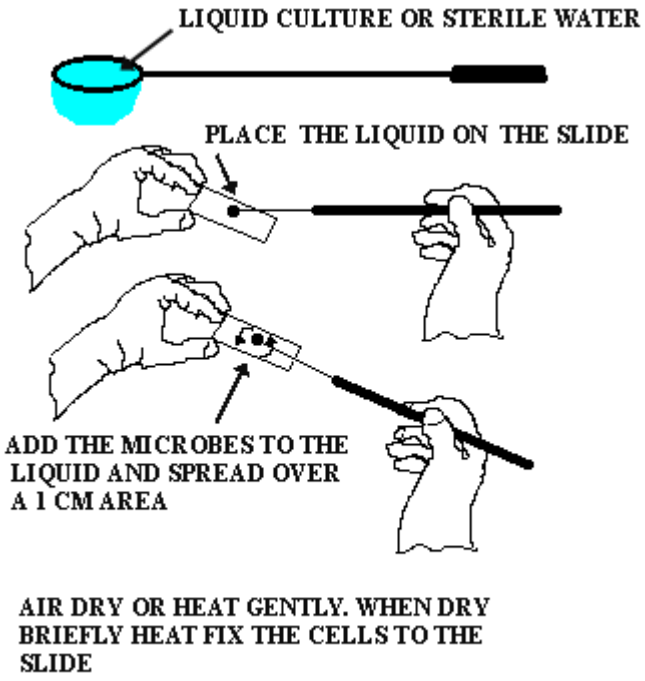


Figure 1. Preparation of the heat fixed smear

After preparation of the heat fixed smear, the cells may then be stained. The stains are dyes which stick to cells or cell parts by virtue of their charge or solubility properties. Most simple stains are dyes that have a strong positive charge; this is they are cations. Most proteins, carbohydrates, nucleic acids have a net negative charge. By adding the stain the cells, ionic attraction keeps the stain attached to the cells so that rinsing with water does not rinse off the stain attached to the cells, but does rinse off the stain covering the glass.

In 1883 Hans Christian Gram discovered an important differential staining method that is used extensively today. The stain is called the **Gram Stain** (Figure 2.). This staining procedure differentiates microbes into two basic groups: Gram positive microbes and Gram negative microbes.

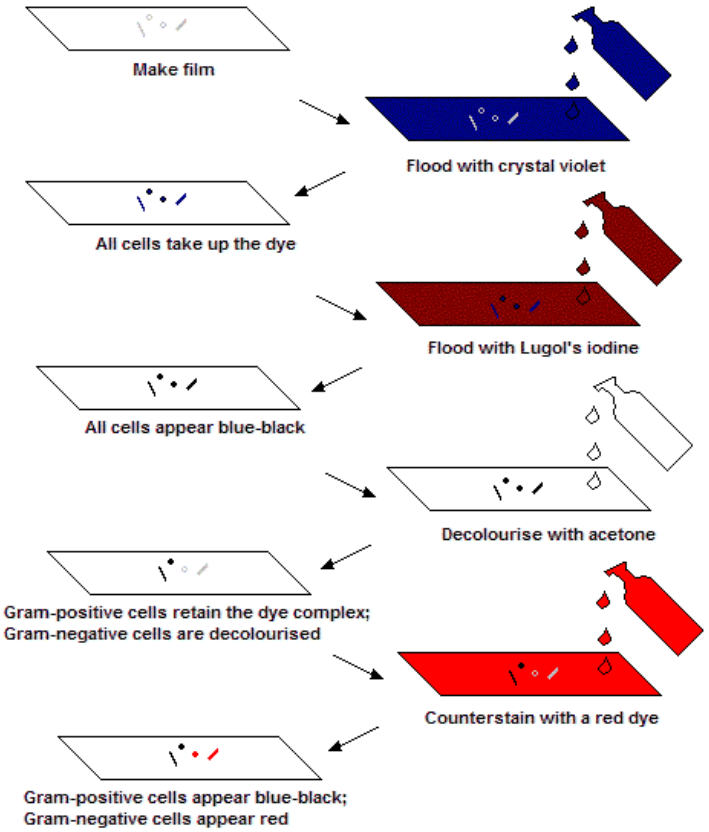


Figure 2. Gram Stain procedure

Differential stains render one type of microbe one color and other types of microbes another color. In the Gram stain, Gram positive organisms (Figure 3.) retain the primary dye complex (crystal violet-iodine) whereas Gram negative cells (Figure 4.) loose the primary dye complex during the challenge rinse.

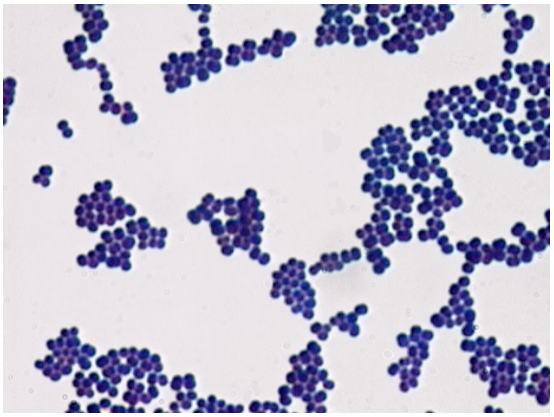


Figure 3. Gram positive cocci

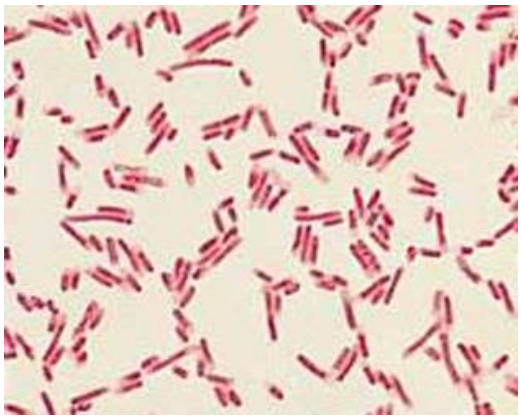


Figure 4. Gram negative rods

Most differential stains have a challenge step that follows staining with a primary dye. In the Gram stain the challenge step is a rinse with either ethanol or acetone (either may be used). This step dehydrates and tightens the cell wall of Gram positives (mainly peptidoglycan) such

that the rinse does not enter the cell. Gram negatives have mainly a lipid cell wall (even though they do contain peptidoglycan) that allows the challenge rinse to penetrate the cell and rinse out the crystal violet-iodine complex rendering the Gram negative cell colorless. Thus, the Gram negative cells must be stained to be seen, and this is done with the counter stain.

The counter stain used in the Gram stain is Safranin (pink). This is applied and stains the Gram negatives pink. To a Gram positive cell the counter stain stains the cell, but the cells are already so heavily stained by crystal violet that the addition of pink does not change the dark purple color of the cells. Thus Gram positives appear deep purple and Gram negatives appear pink.

Note that the success of the Gram stain relies upon the integrity of the cell wall. Gram positive bacteria that have been overly heat fixed resulting in destruction of all or parts of their cell wall can appear to be pink (Gram negative) or have pink areas. This is an artifact! Further, old moribund cultures of Gram positive cells can appear pink. This is because the cell wall has allowed the challenge rinse to enter the cell. Successful Gram stains should be done on young, growing cells.

Many experiments in this course will utilize the Gram stain. You should KNOW THE GRAM STAIN.

MATERIALS

1. Slant cultures of *Escherichia coli, Micrococcus roseus,* or other bacteria.
2. Inoculating loops, Slides.
3. Gram staining reagents in dropping bottles.
4. Toothpicks.
5. BHI plate from Experiment I.

PROCEDURE

Heat Fixed Smear

1. Obtain six **dry clean glass slides**. If the slides are not clean (hold it up to the light and look) clean them with detergent and water, then dry them. In this experiment you will need to prepare six heat fixed slides. Two will be for simple stain, and the rest will be for Gram stain. The following directions are for one slide.

2. Place on slide one drop of water from one loopful of water. The instructor will demonstrate this.

3. Flame sterilize a loop.

4. With your other hand pick up the slant, open the top holding the cap with the little finger of the hand holding the loop. Flame off the top of the tube.

5. With the sterile loop, touch the bacterial growth on the slant. Flame off the tube and replace the cap.

6. Smear the bacteria on the end of the loop into one of the drops of water on the slide.

7. Repeat steps 4-7 for each slant culture.

8. For broth cultures (yeast suspension), after flame sterilizing your loop, simply place a loopful of culture directly onto the slide (not into a drop of water).

9. Allow the smears to air dry. **CAUTION:** Do not use air jets on the Lab Console to dry your slides - This will make an aerosol of the bacteria from your slide! Furthermore, this air is dirty: it comes from an oil pump - compressed air source. We all have to breathe this air.

10. Pass the dry slide slowly through the flame three times. The object is to heat the slide to about 70^{o}C. This can not be accurately measured, but can be tested. The slide should be very hot to the touch, but touchable.

11. For the tooth scrapping and BHI plate (bacteria from Experiment I), with a toothpick, scrape around and between your teeth and smear the material into one drop of water. Dispose of the toothpick in a Biohazard container. Flame sterilize a loop and transfer some bacteria from the BHI plate into the drop of water on the new slide. Allow the smears to air dry, then heat fix as above and save for Gram staining.

Simple Stain

1. Cover the *E. coli* and *S. aureus* smears with Crystal Violet for 1 minute.

2. Rinse with water.

3. Air dry. **CAUTION**: Remember, the slide must be completely dry before putting it on your microscope, but STILL DO NOT USE THE AIR JETS on your Lab Console.

4. Observe first with low power (10X) to locate a good field. Add a drop of oil and swing the oil immersion lens into the oil. Use only the fine focus to bring the image into clear focus.

Gram Stain

1. Stain the smears with Crystal Violet for 1 minute.

2. Rinse with water.

3. Stain the smears with Gram's Iodine for 1 minute.

4. Rinse carefully with acetone. Rinse only until blue color stops coming out of the smear.

5. Rinse with water.

6. Counter stain with Safranin for at least 1 minute.

7. Rinse with water, air dry. Remember, **the slide must be completely dry before putting it on your microscope**.

8. Use the low power (10X) lens for find a good field. Add a drop of oil and immerse the 100X oil immersion lens into the oil by rotating the nosepiece. Use only the fine adjustment to bring the image into clear focus.

Note:
1. Your Results pages should contain accurate drawings of each organism in the simple and Gram stain. Your drawings should be as exact as possible and the sizes of the cells and magnification should be noted.

2. You should be able to describe both the Gram reaction and cell morphology of the major types of bacteria in your teeth scrapping.

QUESTIONS

1. If your organisms were all blue, what is the Gram reaction?
   If your organisms were all pink, what is the Gram reaction?

2. If your organisms were a mixture of blue and pink, what is the Gram reaction? Why? How can that be proven?

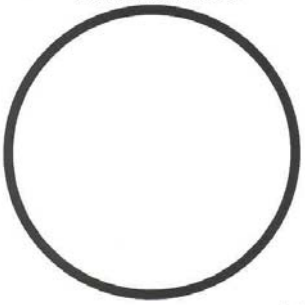
3. What would be the reason(s) for not finding any organisms on the slide?

HINTS

1. When heat fixing the smear, always make sure that the smear is not too thick and that it is on the top of the slide as you pass it through the flame.
2. Always wait until the slide is dry before heat fixing.
3. Make sure that loop is relatively cool before inserting it into any broth.
4. Always flame the inoculating loop after using it and before setting it down.

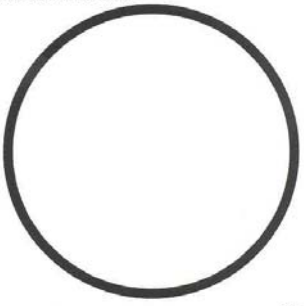
RESULTS

SIMPLE STAINS of
Escherichia coli



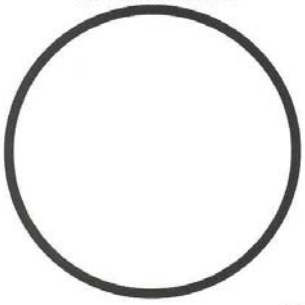
__X

Micrococcus roseus



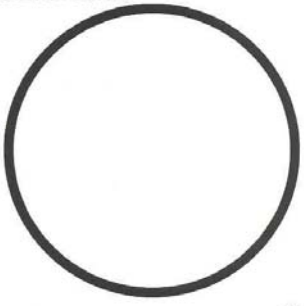
__X

GRAM STAINS of
Escherichia coli



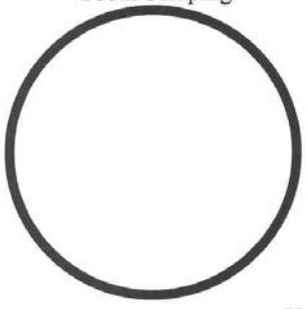
__X

Micrococcus roseus



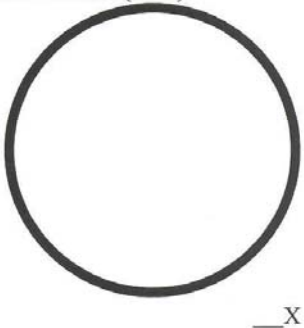
__X

Tooth Scraping



__X

BHI Plate Bacteria (Skin)



__X