Exercise VI

Differential Staining: The Gram Stain

The Gram stain, discovered by Dr. Hans Christian Gram in 1884, is the most useful differential stain used to aid in identifying bacteria. It divides bacterial cells into two major groups, Gram positive and Gram negative, which makes it an essential tool for classification and differentiation of bacteria. The Gram stain is a differential stain that requires two stains in the procedure: a primary stain and a counterstain. The primary stain is crystal violet, which is followed by an iodine solution. The iodine is called a mordant, which is a substance (often a metallic component) that combines with a dye to form an insoluble colored compound. The insoluble precipitate is called the crystal violet-iodine complex. The mordant iodine is used to intensify the primary stain. Gram positive bacteria retain the primary stain in their cell walls. A decolorizing step occurs between the application of the primary stain and the counterstain. It removes the primary stain and decolorizes Gram negative bacteria. After decolorizing with 95% ethanol, a counterstain of safranin is applied to the smear. Depending on the components of the cell wall, bacteria will retain the primary stain during decolorizing or lose the primary stain and take up the counterstain. Organisms that resist decolorizing and retain the crystal violet-iodine complex appear purple or dark blue under the microscope and are called Gram positive (G+). Conversely, bacteria that decolorize, or give up the crystal violet-iodine complex will accept the safranin counterstain and appear red. They are Gram negative (G-) bacteria.

The basis for the differential Gram stain reaction is due to a difference in the permeability and structure and chemical components of the bacterial cell wall. Gram positive bacteria have a thicker cross-linked wall of peptidoglycan and are not lipid-rich. Gram negative bacteria have very little peptidoglycan, but do have an outer layer of lipopolysaccharide and they have a greater lipid content in their cell walls than Gram positive cells. Lipids are soluble in alcohol, which is used to decolorize Gram negative bacteria during the Gram stain procedure. Removal of the lipids by decolorizing is thought to increase the pore size of the cell wall, which accounts for the more rapid decolorizing of Gram negative bacteria. In Gram positive bacteria, the crystal violet penetrates the cell wall and the iodine then forms a large complex with the crystal violet inside the cell wall. This complex is too large to pass through the peptidoglycan of Gram positive bacteria. Gram positive cell walls also become dehydrated during the treatment with alcohol, decreasing the permeability so that the crystal violet-iodine complex is retained. Since the Gram negative bacteria lack thick walls of peptidoglycan and the alcohol disrupts the lipopolysaccharide layer, the complex is washed out of the cell wall with alcohol.
The most critical phase of the Gram stain procedure is the decolorization step, which is based on the ease with which the crystal violet-iodine complex is released from the cell. Over-decolorizing will result in the loss of the primary stain, causing Gram positive bacteria to appear Gram negative. Under-decolorizing, however, will not completely remove the crystal violet-iodine complex, causing Gram negative bacteria to appear Gram positive.

It is also important to note that Gram positive bacteria are not always constant in their reactions. Older cultures of some Gram positive organisms are subject to autolysis, or breakdown of the cell wall by enzymes produced by the bacteria as it ages. This causes older Gram positive bacteria to give a false Gram negative reaction. Gram negative-variable bacteria can appear both Gram positive and Gram negative on the same slide.

The Gram stain also allows for the recognition of the shape and pattern or arrangement of bacteria. The shapes are cocci (round), bacilli (rod), or spirilla (spiral). Based on how the bacteria divides during replication, different patterns or arrangements may be produced. The cellular morphology, or shapes, of the individual cells and their arrangement in pairs, chains, or clusters are useful in the identification of the bacteria.

The arrangements or patterns found in cocci are:

- a. diplococci – pairs
- b. streptococci – chains
- c. staphylococci – grape-like clusters
- d. tetrads – four in a square
- e. sarcina – eight in a cube

Bacilli vary in size from very long oblong to short rods. They may even have squared off ends or one end larger than the other, forming a club. The patterns or arrangement they form are:

- a. diplobacilli – pairs
- b. streptobacilli - chains

Spiral bacteria usually occur singly. Some are very tightly coiled, whereas others are long and slightly curved or only curved at one end.
Materials Needed

prepared slides of Gram positive and Gram negative cocci, bacilli, and spiral bacteria
culture of *Staphylococcus epidermidis*
culture of *Escherichia coli*
2 clean microscope slides
crystal violet
safranin
Lugol’s iodine
ethanol
Bunsen burner
striker
inoculating loop
wax pencil
microscope
immersion oil
staining tray
bibulous paper
dropper bottle of distilled water
stop watch
clothespin
goggles
gloves
spray bottle of disinfectant
paper towels

Procedure 1: Observation of prepared slides

1. Observe slides of Gram positive and Gram negative cocci, bacilli, and spiral bacteria.
2. Be able to identify the different shapes and patterns or arrangements.

Procedure 2: Gram Stain

1. Obtain a clean microscope slide.
2. Place a drop of distilled water in the center of the slide.
3. Using aseptic technique, transfer a sample of *Staphylococcus epidermidis* to the drop of water using an inoculating loop.
4. Prepare a heat-fixed smear.
5. Using a clothespin, hold the slide over the staining tray.
6. Flood the slide with the primary stain crystal violet and allow it to stand for 1 minute.
7. Tilt the slide at a 45 degree angle and drain the excess crystal violet into the tray.
8. Continue to hold the slide at a 45 degree angle and gently rinse with a dropper bottle of distilled water.
9. Hold the slide level and flood with Lugol’s iodine and allow to stand for 1 minute.
10. Tilt the slide at a 45 degree angle and allow the iodine to drain into the tray.
11. Immediately rinse the slide with a dropper bottle of distilled water.
12. With the slide at a 45 degree angle, decolorize quickly by allowing the ethanol to run over and off the slide into the tray. Decolorizing should take no more than 15 seconds, but the drippings should be clear. Do not decolorize too much. This is the most critical step.
13. Rinse the slide immediately with the dropper bottle of distilled water to halt the decolorizing process.
14. Hold the slide level and flood with the counterstain safranin. Allow it to stand for 1 minute.
15. Tilt the slide at a 45 degree angle and drain the excess safranin into the tray.
16. Rinse the slide with the dropper bottle of distilled water.
17. Blot the slide with a piece of bibulous paper. Do not rub the slide.
18. Observe the slide under oil immersion.
19. Dispose of the slide in a beaker of bleach.
20. Dispose of the stains in the tray by placing them in the container under the hood.
21. Repeat the procedure with *Escherichia coli*.

A number of factors can result in variable Gram stain reactions:

1. Improper heat-fixing of the smear. If a smear is heated too much, the cell wall can rupture causing Gram positive cells to release the primary stain and accept the counterstain.
2. Cell density of the smear. An extremely thick smear may not decolorize as rapidly as one of ordinary density.
3. Concentration and freshness of the Gram stain reagents.
4. Length and thoroughness of washing after crystal violet, and the amount of water remaining on the slide when iodine is applied.
5. Concentration and amount of decolorizing alcohol applied.
6. Age of bacterial cultures. Gram stain reactions are reliable only for cultures up to 24 hours old. Variability of Gram stain reactions in old cultures is often related to cell wall integrity and permeability.

**Clean-up Procedure**

1. Clean the microscope objectives with lens paper and ethyl alcohol.
2. Clean oil off of the microscope with Kim wipes.
3. Return the microscope, Bunsen burner, inoculating loop and other materials to the appropriate place.
4. Spray and wipe the table top with disinfectant.
Cocci (round shape)

Singles (no arrangement)  Diplococcus (pairs)

Streptococcus (chains)  Staphylococcus (grape-like clusters)

Tetrad (4)  Sarcina (cube of 8)

Bacterial Shapes and Arrangements (patterns)
Bacilli (cylindrical or rod shape)

Singles (no arrangement)          Diplobacillus (pairs)

Streptobacillus (chains)          Coccobacillus

Spirilla (spiral shape)

Spirillum or Spirochete (loosely and tightly coiled)          Vibrio (curved end)

Bacterial Shapes and Arrangements (patterns)