EDUCATIONAL COMMENTARY – GRAM STAIN UPDATE

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Learning Outcomes
Upon completion of this exercise, participants will be able to:

• list four uses of the Gram stain.
• explain the theory behind the Gram stain procedure.
• discuss sources of error in performing the Gram stain.
• delineate ways to prevent error in performing the Gram stain.

Developed in the 1880s by Danish pathologist Christian Gram, the Gram stain divides bacteria into two broad groups: gram-positive organisms, which retain the primary dye and stain blue or purple; and gram-negative organisms, which lose the primary dye and stain red or pink. In the laboratory, the Gram stain is used to:

1. Directly examine clinical specimens.
2. Obtain preliminary information about the identity of pathogenic bacteria.
3. Characterize bacteria growing in culture media.
4. Assess the quality of sputum specimens submitted for culture.

Principle and Procedure
The differential staining seen with the Gram stain occurs because gram-positive and gram-negative bacteria differ in cell wall composition. The cell walls of gram-positive bacteria contain thick peptidoglycan, high numbers of cross-linked teichoic acids, and little lipid. This makes them less permeable to organic solvents. As a result, the primary dye does not wash out during the decolorization step, and the bacteria appear blue.

The cell walls of gram-negative bacteria, on the other hand, have a thinner peptidoglycan layer and higher lipid content which increase permeability to organic solvents. This increased permeability allows the primary stain to wash out during the decolorization step. The cell walls then take up the counterstain and the bacteria appear red.
The basic Gram stain procedure consists of five steps:

1. The material to be stained is fixed to a glass slide, either by heating or by using 70% to 95% methanol. Of the two methods, many microbiologists prefer methanol fixation because it better preserves the morphology of host cells, red blood cells, and bacteria. Methanol fixation also leaves a clearer background and prevents liquid specimens from washing off the slide. Finally, methanol fixation makes bacteria more resistant to decolorization, thus providing greater control over this step.

2. The slide is flooded with the primary crystal violet stain, which attaches to the cell walls.

3. The slide is gently rinsed with water and then covered with iodine. Iodine is a mordant, which means it helps attach the crystal violet to the cell wall. Instead of the classic Gram iodine, many laboratories today use iodine that has been stabilized with polyvinylpyrrolidone (“stabilized iodine”). The advantage of stabilized iodine is that the available iodine in the solution remains constant, whereas the available iodine in Gram iodine solution deteriorates rapidly. However, under-decolorization of gram-negative enteric bacteria has been reported with stabilized iodine.

4. The crystal violet–iodine complex is rinsed with water and then decolorized. Microbiologists can choose among three decolorizers: acetone, alcohol, or an acetone-alcohol mixture. Pure acetone decolorizes most quickly, whereas pure ethanol decolorizes most slowly. The rate at which an acetone-alcohol mixture decolorizes depends on the relative concentrations of each component.

5. Last, the slide is rinsed with water and flooded with the counterstain. The traditional Gram stain method uses safranin as a counterstain, but carbolfuchs in or 0.8% basic fuchsin are sometimes substituted because they better stain anaerobes and other weakly-staining gram-negative bacteria. Another modification to the counterstaining step adds a tartrazine-fast green stain immediately before counterstaining with safranin to provide better contrast between gram-negative bacteria and background material.

Sources of Error

Sources of error in performing the Gram stain fall into three categories:

1. Due to inherent limitations, the Gram stain will not detect all bacteria; and some bacteria may not stain true to their cell wall. For example, the Gram stain will not detect organisms that exist within host cells (such as Chlamydia), organisms with no cell wall (such as Mycoplasma, Ureaplasma, and Rickettsia), and bacteria that are too small to be seen with light microscopy (such as Treponema). Also, Neisseria, Moraxella, and Acinetobacter are prone to under-decolorization and can appear gram-positive despite the Gram stain reagents used, the culture medium, or the clinical material from which they are cultured.
2. Conditions that damage the bacterial cell wall can cause gram-positive bacteria to appear gram-negative or gram-variable. For example, bacteria taken from cultures older than 48 hours or from patients on antibiotic therapy are prone to cell wall damage. An inflammatory response at the infected site of a patient's body and ingestion of bacteria by phagocytes can also injure the cell wall. Finally, some bacteria (such as, *Streptococcus pneumoniae*) have autolytic enzymes that can damage the cell wall.

3. Technical errors that occur during preparation or staining are a source of unreliable Gram stain results:
   - **Smears that are too thick or unevenly thick** are impossible to read accurately because the bacteria are hard to see and because uneven staining can make organisms appear gram-variable.
   - **Fixing the smear with excessive heat** makes bacteria more susceptible to over-decolorization. Excessive heat also alters the morphology of background cells, making it more difficult to determine whether bacteria have been phagocytized by neutrophils. Altered cell morphology can also make it harder to assess the adequacy of sputum specimens.
   - **Low concentrations of crystal violet** can lead to over-decolorization. A 0.3% concentration of crystal violet requires only 5 to 10 seconds of decolorization, whereas higher concentrations of crystal violet (up to 2%) can accommodate longer decolorization times.
   - **Prolonged decolorization**, especially with acetone, can cause over-decolorization.
   - **Insufficient decolorization** can make gram-negative bacteria appear gram-positive.
   - **Insufficient exposure to iodine** and **lack of available iodine** can cause gram-positive bacteria to appear gram-negative.
   - **Insufficient counterstaining** can fail to stain gram-negative bacteria and background material.
   - **Excessive counterstaining** can cause gram-positive bacteria to appear gram-negative.
   - **Excessive rinsing** between any of the steps can cause over-decolorization.

**Reporting Results**

A Gram stain of clinical material (that is, a “direct smear”) should be evaluated both for information about bacterial cells present and for the presence of inflammatory cells, other cells, and debris. Inflammatory cells indicate an infection, and squamous epithelial cells in sputum specimens can indicate contamination from the mouth. The presence of tissue debris can confirm that the smear was properly fixed and stained.
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The Gram stain report should note the Gram reactions, morphologies, and arrangements of bacteria seen on the slide. The presence of host cells and debris should be noted as well. Finally, if bacteria or host cells are not present, this should be reported.

Conclusion
The Gram stain can quickly provide useful information about likely pathogens and specimen quality. However, it also has inherent limitations, and it is prone to technical errors if performed by inexperienced personnel. To maximize the quality of Gram stains, laboratories should evaluate several adaptations of the method and then choose the procedure that works best for them.

Suggested Reading


Strand CL. Positive blood cultures: can we always trust the Gram stain? Am J Clin Pathol. 2006;126:671-672.

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