

Pink Bugs/Purple Bugs

A Study of Gram Positive and Negative Bacteria



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Gram stains are used every day in Histology to distinguish bacterial classifications, such as gram-positive bacteria from gram-negative bacteria. But, why? Is there a difference between these bacterium? If so, what does that mean to those who are helping patients fight infections? How are these bacteria stained, and who came up with the staining procedures? How do we, as Histotechs, troubleshoot these stains? And why are controls important? My goal is to successfully answer these questions for you.

History

In 1884, Hans Christian Gram, a Danish bacteriologist, developed a new staining technique (by critiquing procedures that already existed), which enabled him to distinguish two groups of bacteria. While examining lung tissue from patients who had died of pneumonia, he discovered that certain stains were preferentially taken up and retained by bacterial cells.⁹ In his technique, Gram first treated bacteria with an aqueous violet solution, then with a solution of iodine in potassium iodide. When the bacteria were next washed with alcohol, some bacteria retained the original violet stain, but others lost their color.² Therefore, the gram-positive bacteria are purple in color because they retain the violet color after alcohol decolorization, and the gram-negative bacteria are pink in color because they retain the color of the counterstain, either saffranin (used for microbiological cultures) or basic fuchsin (used on histology sections).

Gram-positive versus Gram-negative

In order to understand how these bacteria are stained, we need to first discuss their anatomy. The difference between gram-positive and gram-negative bacteria is their cell wall structure.

The gram-positive bacteria cell wall is made up of a thick sheath of peptidoglycan. It also contains tightly bound acidic polysaccharides, including teichoic acid and lipoteichoic acid. Teichoic acid is a polymer of ribitol or glycerol and phosphate embedded in the peptidoglycan sheath. Lipoteichoic acid is similar in structure but is attached to the lipids in the plasma

membrane. These molecules function in cell wall maintenance for the gram-positive bacteria.⁴ In contrast to the gram-positive bacterial cell wall, the gram-negative bacterial cell wall is much more complex. It consists of a rigid peptidoglycan layer, which is much thinner than that found in the gram-positive cells, overlaid by an outer membrane containing a diversity of structures.⁶ Between the cytoplasmic membrane and the outer membrane is the periplasmic space containing a gel-like periplasm in which resides the cell wall peptidoglycan as well as various enzymes. In addition to phospholipids, the outer membrane contains unique gram-negative lipopolysaccharides and various proteins and lipoproteins.⁶

The peptidoglycan of the gram-negative cell is chemically similar to, but not identical with, the peptidoglycan of the gram-positive cell. The major difference between the two cell types is in the thickness of the peptidoglycan layer rather than the chemical makeup. When the bacterial cell wall is placed in the environment of the human body as part of a viable microorganism, there is a diversity of functions/effects that can be noted. Some of these are specific for gram-negative organisms (due to the relative complexity of their cell walls) and some are general.⁶

Staining Procedures

Some of the staining techniques we use in histology are the Brown and Brenn, the Brown and Hopps, and the Gram-Twort procedures. The Brown and Hopps and the Brown and Brenn staining procedures are very similar, the difference being in the differentiation and counterstaining steps. The end results are always the same: the gram-positive bacteria are blue to purple to black, and the gram-negative bacteria are pink-red to violet.

When doing these stains, it is important to focus on what you are doing. The steps are time sensitive so if you are doing more than one stain, it is important to prioritize and coordinate procedures. If you have more than a couple of slides to stain with the gram procedure, its best to only do a couple at a time, so splitting them into groups might be worth while.

The first step in the procedure is to cover the slide with the Crystal Violet stain for one minute. All tissue components and microorganisms will be stained a blue/violet color. Rinse for about ten seconds with running water. The next step is to add the mordant, the Iodine, for one minute. This

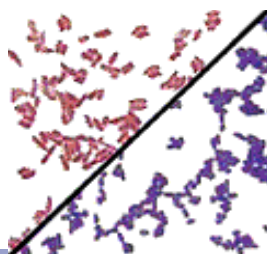


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step creates a dye-lake effect in the bacteria. The Crystal Violet and the Iodine combine to create a large molecule (CV-I₃) that is insoluble in water. This molecule is too big to be easily washed out of the gram-positive bacteria because of the bacteria's thick outer wall. Thus the gram-positive bacteria retain the crystal violet. Once the distilled water rinse is complete, the decolorization step is next. To decolorize, an organic solvent such as acetone or alcohol is used. The decolorizer is a fast, one to two second long step. The purpose of the decolorization step is to separate the gram-positive bacteria from the gram-negative bacteria. The decolorizing mixture causes dehydration of the multilayered peptidoglycan in the gram-positive cell wall, thus decreasing the space between the molecules and causing the cell wall to trap the crystal violet-iodine complex within the cell. In the gram-negative bacteria, the decolorizing mixture acts as a lipid solvent and dissolves the outer membrane of the gram-negative cell wall. The thin layer of peptidoglycan is unable to retain the crystal violet-iodine complex and the gram-negative cell is decolorized.¹

At this point of the procedure, each staining method finishes slightly differently from each other.

In the *Brown and Hopps* procedure, once the water rinse is complete (after the decolorization step), the counterstain is applied. In this procedure the counterstain is a percentage (such as 1%) Basic Fuchsin, and is left on the tissue for about five minutes. A running water rinse afterwards is needed to remove the excess stain from the slide. In the next step, a Gallego solution is used to further differentiate the stains. This solution is made up of formaldehyde, acetic acid, and distilled water, and acts in removing (decolorizing) the red basic fuchsin counterstain from the background, and helps sets the basic fuchsin into the gram negative bacteria. After another running water rinse, the slides are dehydrated and cleared through the next few steps. Acetone is used as the dehydrant in this procedure, followed by a few minutes in a picric acid-acetone mixture. This mixture removes more of the basic fuchsin from the background, and also acts as a pale yellow counterstain to the background. An acetone-xylene mixture is used next to aid in the ease of the slides into the clearing step of xylene. The prospective results: gram-positive bacteria: blue/purple/black; gram-negative bacteria: pinkish-red to violet; and the background: yellow/orange.⁷

In the *Brown and Brenn* gram staining procedure, the CV-I₃ acetone decolorization step is followed by a water rinse, which is followed by the counterstain application. In this procedure, the counterstain is a working solution made up from a 1% Basic Fuchsin solution and distilled water (20mL each). A picric acid-acetone mixture is used once again and acts as to remove excess red basic fuchsin from the background as well as being a yellow counterstain to the background. The dehydration step follows with an application of acetone, and the slides are then cleared in xylene. The results are similar to that of the Brown and Hopps procedure, with the gram negative bacteria being slightly more red than violet.⁷

The end results are always the same: the gram-positive bacteria are blue to purple to black, and the gram-negative bacteria are a pink-red to violet.

Often, pathologists will ask for both the Brown and Hopps as well as the Brown and Brenn on the same patient's tissue. In general, gram negative bacteria are slightly easier to find on the Brown and Hopps procedure, due to their violet color against the yellow/orange/red background. Conversely, gram-positive bacteria are slightly easier to find on the Brown and Brenn procedure, due to its paler less red background.

Last, but not least, is the *Gram-Twort* staining method. Once the initial CV-I₃ staining and organic solvent decolorization steps are completed, the slide is counterstained with a combination of Neutral red and Light green. The results from this staining method are as follows: gram-positive bacteria: blue/black; gram-negative bacteria: red; background, which includes connective tissue and muscle: green.⁷

Control Blocks

It is important to run a control with each stain you perform. When it comes to doing the gram stain, it is essential to have a control block that contains both gram-positive and gram-negative bacteria. The two bacteria can be in the same piece of tissue, or can be in two separate tissues from two different patients, depending upon pathologist's preference. Both types of bacteria are needed to determine that the results are accurate with both gram-positive staining along with gram-negative staining. It will also play a part in the troubleshooting process if a stain goes wrong.

Trouble-shooting

With this stain being time sensitive, it is easy to go too far or not far enough with decolorization, and you could end up with inaccurate results. Here are some helpful hints.

- 1) Over-decolorization: What if, while looking under the microscope at your control tissue, all you see is pink-red bacteria? You have most likely left the slide too long in the acetone (decolorizing step after the CV-I₃). If the acetone is left on too long, it can actually dissolve the thick peptidoglycan layer in the gram-positive cell wall. As a result, the Crystal Violet-Iodine complex gets washed out of not only the gram-negative bacteria but also the gram-positive bacteria. This allows the red basic fuchsin to be taken up by the gram-positive bacteria, instead of it remaining blue/purple/black. When this happens a misdiagnosis of a gram-negative infection (instead of a gram-positive infection) could be made by the pathologist.⁸
- 2) Under-decolorization: If all you see in your control are bacteria that are blue/purple/black, and you know there should also be red gram-negative bacteria present, you most likely did not leave the slide in acetone long enough after the CV-I₃ step. The gram-negative bacteria have retained the crystal violet-iodine complex in their cell walls, as the acetone has not had enough time to successfully decolorize the gram-negative bacteria in order for the basic fuchsin to be retained later on in the procedure. When this happens, the gram-negative bacteria will stain blue/purple/black instead of red, and a misdiagnosis of a gram-positive infection could be made.⁸
- 3) Increased Background: If the tissue is not counterstained in the picric acid-acetone long enough, then not enough basic fuchsin is being removed from the background, and not enough yellow of the picric acid is staining the background. In cases where the bacteria are very small, or very few in number, it is very important that the background be pale, and have more yellow in it than red. If there is too much red in the background tissue, this could obscure the red gram-negative bacteria from being observed with the microscope, and possibly also hinder the visualization of the blue/purple/black gram-positive. It is therefore possible that the bacteria are overlooked, and a misdiagnosis of no bacteria infection is made.

Sometimes, to be certain that there are no bacteria present, a pathologist will also ask for a silver stain

such as Steiner and Steiner. This silver procedure will not differentiate gram-positive from gram-negative, but it will color the bacteria black and enlarge their size, thus making the bacteria easier to see against the background.

- 4) Dead/Treated Bacteria: If a biopsy is taken from a patient who is already being treated with antibiotics for a bacterial infection, the gram stain will be affected. The antibiotics damaged the cell walls of the bacteria, and the crystal violet-iodine complex will not be retained in the gram-positive cell walls. Therefore, these gram-positive bacteria may be misdiagnosed as a gram-negative bacteria infection.⁸

Medical Relevance

If the stains we do as histotechs come out correctly, our pathologists are better able to assist clinicians in fighting diseases. If it can be determined whether the bacterial infection that the patient has is gram-positive, or gram-negative, the better the chance the patient has being put on the correct treatment regimen. It is always interesting to see what gram-positive and gram-negative bacteria our medical facilities are fighting, and how those bacterium are treated.

Table 1 Gram Positive Bacteria

Gram-Positive	Typical Infection	Drug of Choice
Bacillus	Anthrax	Penicillin, doxycycline
Staphylococcus aureus	Abcess, skin, toxic shock	Penicillins, vancomycin
Streptococcus pyogenes	Strept throat, rheumatic fever	Penicillin, erythromycin
Clostridium	Gas gangrene	Penicillin, clindamycin
Corynebacterium	Diphtheria, rheumatic fever	Erythromycin, penicillin

Gram-negative bacteria are usually more dangerous as disease organisms, because their outer membrane is often hidden by a capsule which hides the antigens of the cell and so acts as “camouflage”. The human body recognizes a foreign body by its antigens. If the antigens are hidden, it becomes harder for the body to detect the invading microorganism.¹ Gram-negative bacteria have lipopolysaccharides in their outer

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membrane, an endotoxin which increases the severity of inflammation. This inflammation may be so severe that septic shock may occur. Gram-positive infections are generally less severe because the human body does not contain peptidoglycan. In fact, the human body produces an enzyme called lysozyme which attacks the open peptidoglycan layer of gram-positive bacteria. Gram-positive bacteria are also frequently more susceptible to antibiotics such as penicillin.

Table 2 Gram Negative Bacteria

Gram-Negative	Typical Infection	Drug of Choice
Neisseria gonorrhoeae	Gonorrhea	Ceftriaxone, quinolones
Neisseria meningitis	Meningitis	Penicillin G, ceftriaxone
Escherichia coli	Sepsis, diarrhea, uti	Cephalosporin, quinolones
Salmonella	Typhoid fever	Cefalosporin, quinolones
Pseudomonas	Lung/Burn infections	Ticarcillin, aminoglycoside
Legionella	Legionnaires disease	Erythromycin, rifampin
Influenzae	Meningitis	Cefotaxime, cepthriaxone

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MRSA

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MRSA stands for Methicillin-resistant Staphylococcus aureus, and is often pronounced as "mur'-sa", or spelled out as "M-R-S-A". *Staphylococcus aureus* is a gram positive cocci, often seen as grape-like clumps, and commonly referred to as just "staph". The name "aureus" means "golden", due to the golden-yellow colonies observed on blood agar plates in culture. *S. aureus* is found on the skin and nasal passages of healthy people. It usually doesn't cause any problems, but can enter the body and cause infections. Minor infections include pimples and boils, but the infections can become more serious, such as pneumonia, blood infections (sepsis), toxic shock syndrome (with rashes and fever), can effect major organs with necrotizing ("flesh eating") infections, and may even cause death.

Methicillin-resistant means that the bacterium is resistant to the most common antibiotics, including penicillin, methicillin and cephalosporins. Stronger antibiotics, such as vancomycin, must be given, often intravenously. These microorganisms are sometimes referred to as Multiply-resistant Staphylococcus aureus. The news media refer to them as the "superbug". MRSA bacteria were first discovered in the United Kingdom in 1961, and in the US in 1968. Since then, the number of *S. aurea* found to be resistant to methicillin in infected hospital patients has risen from 2% in 1974 to about 40% in 1997. A 2007 study by the CDC, and published in JAMA, implicated MRSA for 94,360 serious infections and associated with 18,650 hospital stay-related deaths in the US in 2005.

MRSA is spread by person-to-person contact via the hands, or from secretions from the infected skin lesions or nostrils. There appear to be two different strains, depending upon how acquired:

- **Hospital-acquired (HA-MRSA):** These are infections that occur nosocomially in patients with weakened immune systems, open sores, surgery, or invasive devices (such as catheters or dialysis). It is often caused by hospital staff who do not carefully wash their hands after changing dressings or are not wearing gloves when handling infectious body tissues.
- **Community-acquired (CA-MRSA):** These infections are found in healthy people who have not been in a hospital or in contact with any healthcare personnel. Outbreaks have been

