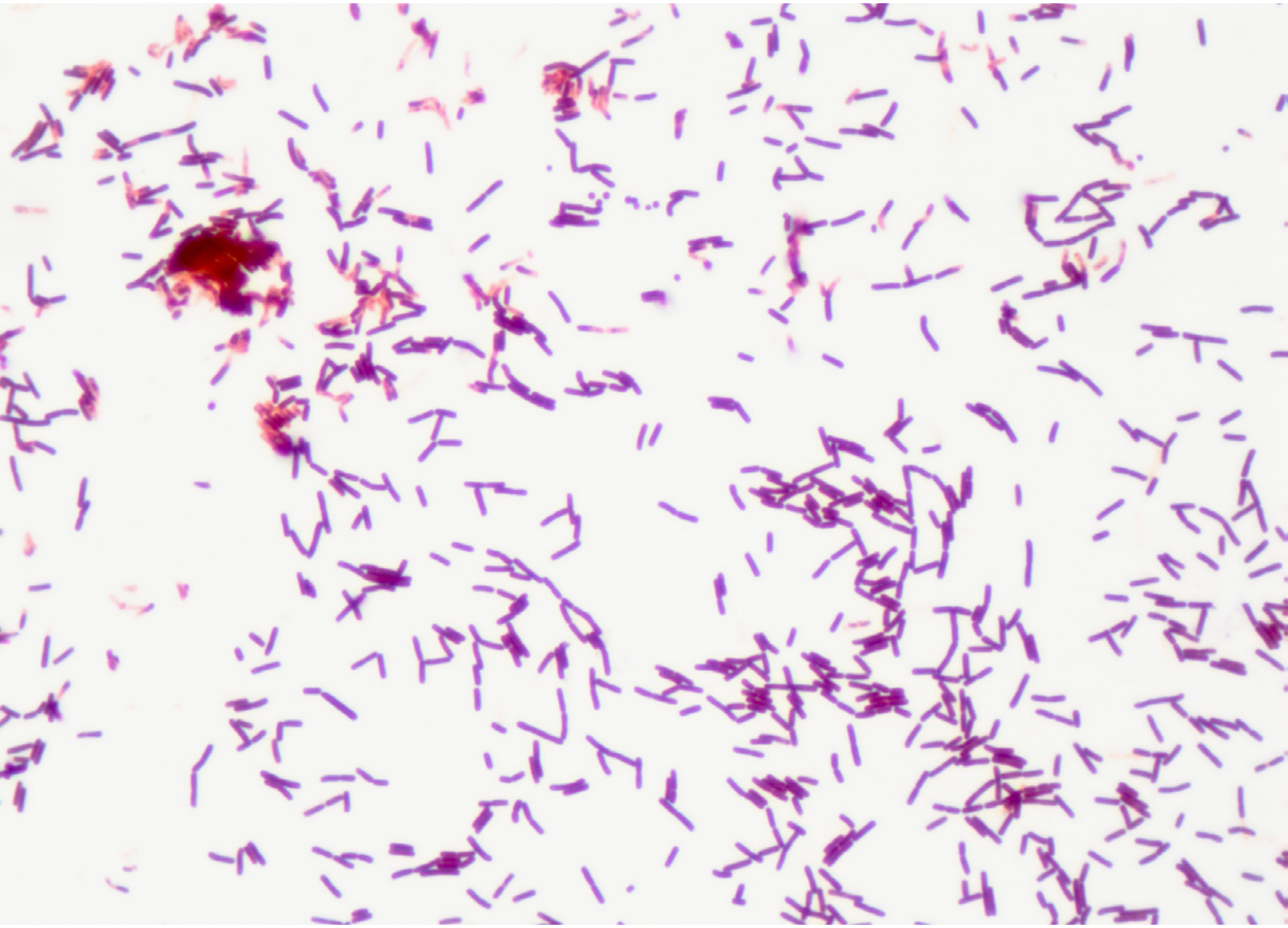
Application Note



Gram Staining to Differentiate between Bacteria in Medical Microbiology

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Gram Staining to Differentiate between Bacteria in Medical Microbiology

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Gram staining is a method of staining bacteria that was developed in 1884 by the Danish bacteriologist Hans Christian Gram. The distinctive feature of this technique is that the staining makes it possible to differentiate between gram-positive and gram-negative bacteria. This process makes it possible to identify the different types of bacteria and visualize their morphology with an optical microscope. Gram staining is an important part of the diagnosis of infectious diseases, since gram-positive and gram-negative bacteria are often treated using different antibiotics.

Bacteria

Microbiological analysis with an optical microscope is an integral part of the work in a medical laboratory. The goal of such an investigation is to search for and detect the presence of infectious agents in patient samples. Infectious agents include fungi, viruses, parasites, and bacteria. Certain types of bacteria can cause serious diseases such as plague and cholera. As a result, detecting them is crucial to determining which therapy to recommend to the infected patient.

Bacteria are small, single-cell microorganisms with an average size of between 0.5 and 10 micrometers. Thanks to their morphology, they can be easily detected with a microscope. Bacteria exist in a number of shapes, including rods, spheres (cocci), threads, and spirals (spirochetes) (Fig. 1).

Although bacteria have a nucleus-like region that contains the genome (called a nucleoid), they do not have a true nucleus (prokaryotes) and usually no organelles. Bacterial chromosomes consist of the bacteria's largest circular DNA molecules and float freely in the cytoplasm of the cell. The cytoplasm also contains further small DNA molecules, called plasmids, which are usually ring-shaped and double-stranded. Plasmids are separate from the chromosomes, however, and can be transmitted from one bacterium to another. Ribosomes are required for the translation process within the scope of biological protein synthesis. The cytoplasm is surrounded by the cell membrane (also known as plasma

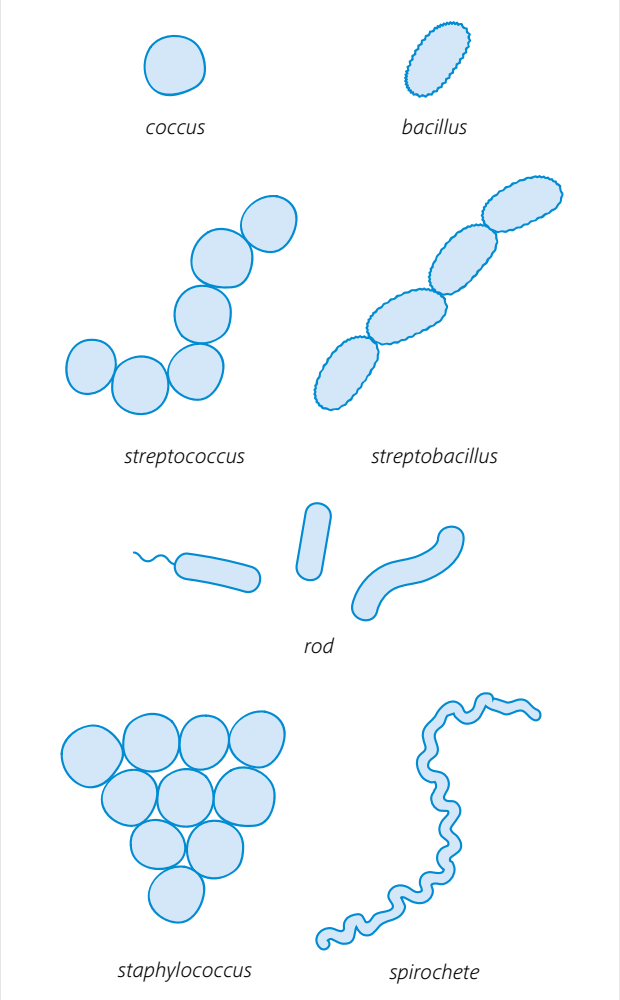

Figure 1 *Morphology of bacteria*

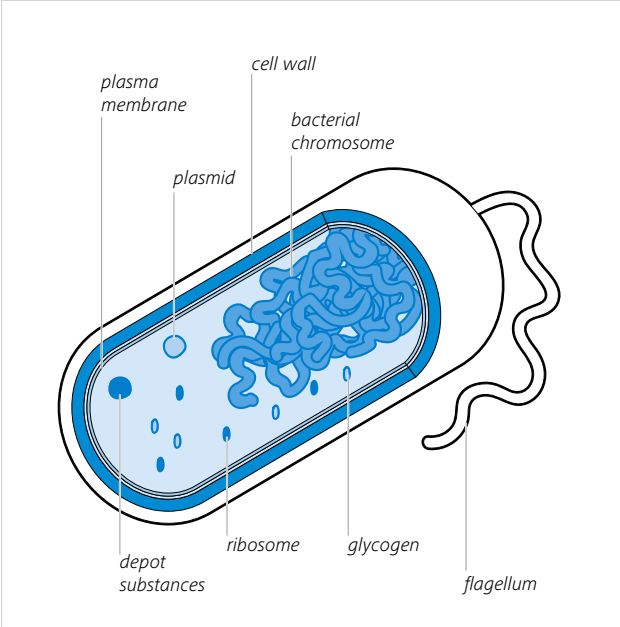

Figure 2 *Structure of a bacteria cell*

membrane), which is responsible for controlling the movement of substances between the inside and the outside of the cell. Outside the plasma membrane lies the cell wall, which is responsible for ensuring that the cell retains its shape. It also protects the cell from the turgor pressure resulting from the differing concentrations inside and outside the cell, which would cause the cell to burst. The cell wall is surrounded by a layer of mucus that protects the bacteria from drying out. Numerous pili (cell appendages) are found on the outside of the cell. They can attach themselves to solid substances, nutrients from the environment, or other bacteria, for example. A number of strains of bacteria also contain a flagellum. The primary role of this thread-shaped appendage made of proteins is locomotion (Fig. 2).

Bacteria reproduce through cell division. A cell first doubles its organelles, and then divides in half. This results in two identical cells with the same genome.

Gram Staining

Gram staining [1] is a method developed by the Danish bacteriologist Hans Christian Gram (1853 – 1938) to distinguish between bacteria for the purpose of a microscopic analysis. This method of staining is based on the difference in the composition of bacterial cell walls, which was not known at the time, since the bacteria's resulting coloration is based on its chemical and physical properties. This method classifies bacteria into two groups: gram-positive and gram-negative. Not all strains of bacteria can be classified using this method, however. As such, gram-variable and gram-indeterminate strains also exist.

Most strains of bacteria use a macromolecule (peptidoglycan) in their cell wall structure to enclose the cell and give it structural strength. This molecule is known as murein. Murein is extremely stable and determines the morphology of most bacteria as a result of its different levels of occurrence [2].

The two principle types of bacteria can be differentiated from one another based on the thickness of the murein layer. Gram-positive bacteria have a thick cell wall that consists primarily of a 15 to 80 nm thick layer of up to 40 individual sheets of murein. In contrast, gram-negative bacteria have only a 10 to 20 nm thick layer of murein that consists of only one or a few sheets. Furthermore, they have a second outer membrane composed of lipids that is similar to the cell membrane on the outside of the cell wall (Fig. 3).

Gram Staining Process (Fig. 4), [2]

The bacteria to be stained are affixed to a slide and dried, and crystal violet dye is subsequently applied for a period of two to three minutes. Crystal violet is a positively charged aniline dye. The specimen is then briefly rinsed with distilled water and subsequently treated with Lugol's iodine (potassium iodide-iodine complex in water). This results in a crystal violet-iodine complex forming in the murein sheets. The specimen is then once again rinsed with distilled water. The dye complex is water-insoluble. The actual differentiation takes place in the next step. For this purpose, the specimen is washed with a differentiation solution (96% alcohol) until no more dye is released. This differentiation does not cause gram-positive bacteria to decolorize as strongly, since the crystal violet-iodine complex forms an extremely stable bond

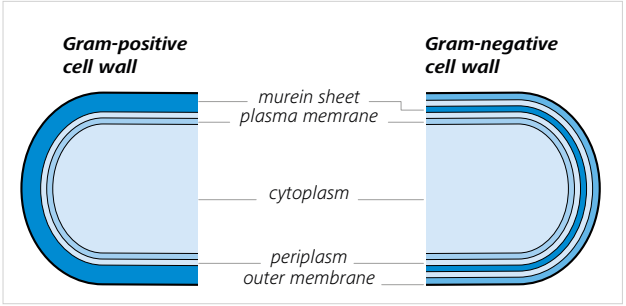

Figure 3 *Gram-positive vs. gram-negative bacteria*

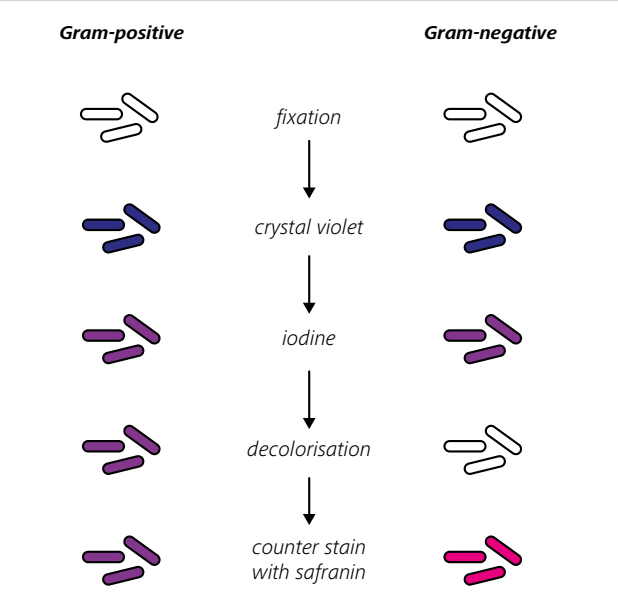

Figure 4 *Gram stain of gram-positive and gram-negative bacteria*

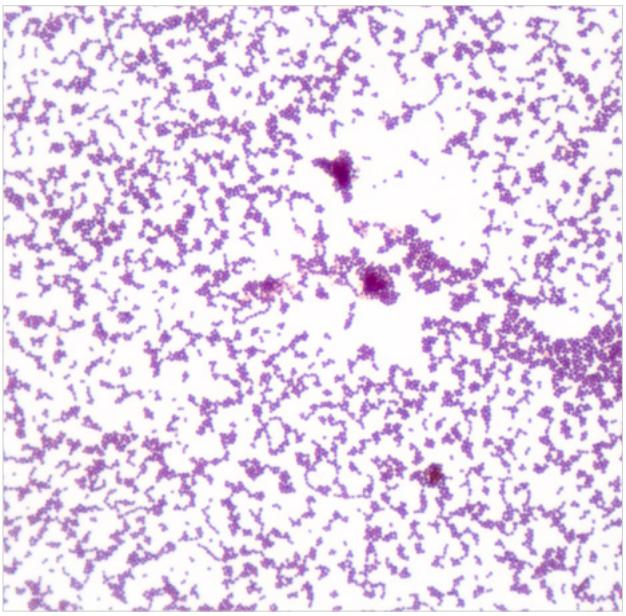

Figure 5 *Staphylococcus, gram-positive, 100× oil immersion*

in the murein sheets and the alcohol cannot penetrate through the gram-positive bacteria's thick cell wall. In contrast, gram-negative bacteria can be easily decolorized with alcohol. The gram-negative bacteria is then marked though a subsequent one-minute counterstaining with safranin, which turns it red or red-orange (Fig. 5 and Fig. 6).

When carried out correctly, gram staining is a very informative and fast method of differentiating between different types of bacteria. Correctly carrying out the method includes, among other things, only completely decolorizing the gram-negative bacteria during differentiation. This is be achieved by carrying out the differentiation step for the optimum amount of time. If the differentiation step is not carried out long enough, the gram-negative bacteria also remain dyed with crystal-violet. If this step is carried out for too long, it will end up decolorizing both the gram-negative as well as the gram-positive bacteria. The use of a test specimen to determine how to correctly carry out this staining method is recommended [2].

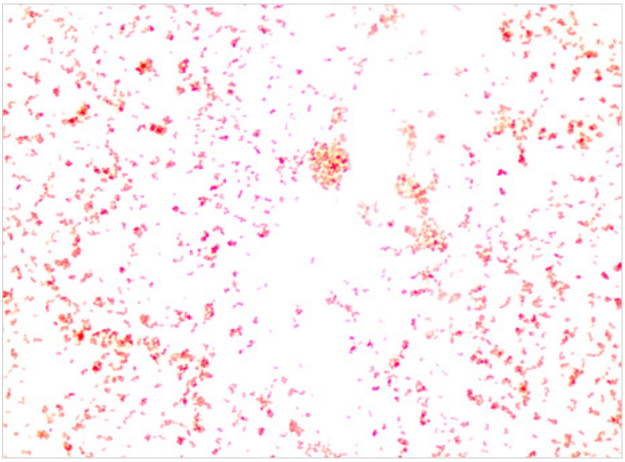

Figure 6 *Enterobacteriaceae, gram-negative, 100× oil immersion*

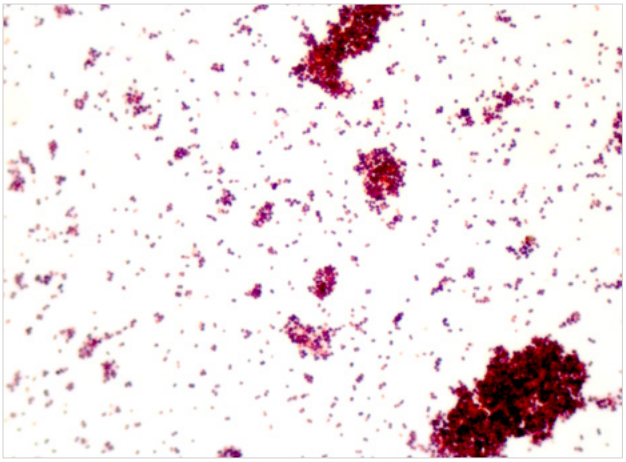

Figure 7 *Polychromasia in a gram stain: gram-positive bacteria (violet) are also stained negative (reddish-pink), 100× oil immersion*

Classification of Bacteria by Their Behavior during Gram Staining

Pathogens with Typical Gram-Staining Behavior	Gram-Positive Bacteria	Gram-Negative Bacteria
Cocci	■ *Staphylococcus* ■ *Streptococcus*	■ *Neisseria meningitidis*
Bacilli	■ Clostridium ■ Listeria ■ Actinomyces	■ Haemophilus influenzae ■ Brucella ■ Legionella pneumophila ■ Bartonella ■ *Enterobacter* ■ Yersinia ■ Shigella ■ Salmonella
Pathogens with Atypical Gram-Staining Behavior		
Mycobacteria Mycoplasma Spirochaetes ■ Borrelia ■ Leptospira ■ Treponema Chlamydia		

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Figure 8 *ZEISS Axio Lab.A1*

Recommended Microscopy Configuration

Gram-stained bacteria are viewed in brightfield using an optical microscope (Fig. 8). A high-aperture and high-magnification lens is required to view the small bacteria in a microscope; this can be achieved with a 100× oil immersion objective like ZEISS N-Achroplan, for example. In order to correctly identify the bacteria, being able to reliably differentiate between blue/violet and red shades is important, which means the objectives used need to offer excellent color correction. The abbreviation “Achro” in the name of the objective refers to this correction. It is only possible to truly tap an objectives full potential when the condenser can also perform this color correction – like the ZEISS #424225-9070-000 achromatic-aplanatic condenser. A microscope camera with a wide dynamic range is recommended for documenting the images.

Summary

An optical microscope can be used to identify and differentiate between different types of bacteria in a medical laboratory through the use of suitable, differentiating stains. Gram staining is one of the most common staining methods, which can be used to easily distinguish between different types of bacteria based on the structure of their cell wall. A carefully prepared specimen and a high-aperture, high-magnification lens should be used to reliably interpret the visual data.

References

[1] https://en.wikipedia.org/wiki/Gram_staining

[2] Maria Mulisch, Ulrich Welsch (ed.). Romeis Mikroskopische Technik, 18. 2010 Edition, pp. 243.

[3] Dörries, Hof. Medical Microbiology, Thieme Publishing Company, 6th Edition (2014), pp. 284.

Footnote 1:

N-acetylmuramic acid and N-acetylglucosamine are what is known as “C2 amino sugars” and form an alternating copolymer (β-1,4-glycosidic bond) of this macromolecule. This chain is then cross-linked by peptide via the N-acetylmuramic acid’s hydroxycarbonyl function. Several antibiotics work by destroying the murein layer of the many types of bacteria. For example, penicillin blocks murein biosynthesis [Lit. 3, p. 301].

Footnote 2:

Bacteria also exist without a pepdidoglycan layer such as chlamydia. Legionella are also gram-negative and must be identified through differential diagnosis with the antibody immunofluorescence.

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