Review Article

Diagnostic Utility of Gram Stain for Oral Smears – A Review

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Abstract

For rapid and successful treatment of infectious diseases, detection of the presence of microorganisms is essential. Traditional culture-based approaches are limiting and time consuming for microbial identification. The most popular staining technique for identifying Gram-positive and Gram-negative microorganisms in various tissues is called Gram staining. This method is utilized in both clinical practice and research. Gram staining of the oral smears is the preliminary step in the identification of any pathological shift in normal oral microbiota. This review discusses the principle of gram stain emphasizing its significance in diagnostic utility for oral smears.

Keywords: Bacteria, Gram-negative, Gram-positive, microorganisms, oral smear, staining

Introduction

Bacteria are prokaryotic organisms having characteristic shapes, often occurring in characteristic aggregates. Bacteria have a nuclear body rather than an enveloped nucleus. The functions of membranous organelles are performed by the plasma membrane since they lack membrane-bound organelles. Flagella, pili, and capsule constitute the surface structures. Flagella consists of a long filament present external to the cell surface and a basal body anchored in the plasma membrane which is responsible for locomotion. Pili are the proteinaceous appendages that help in adhesion to the host. Many bacteria express an outermost coat comprising S-layers and capsules composed of polysaccharides.[1]

The cell envelope is a complex multilayered structure that protects the bacteria and allows selective transport of nutrients and other products. Bacteria are classified into two groups based on their cell envelopes: those with a thin peptidoglycan cell wall surrounded by a lipopolysaccharide-containing outer membrane or those with a thick peptidoglycan layer but no outer membrane. Peptidoglycans consist of muramic acid, glucosamine, and peptide chains which may be highly cross-linked or partially cross-linked. Teichoic acids are anionic polymers threading through the layers of peptidoglycan which has a strong negative charge.[2]

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In 1884 Christian Gram accidentally found that alcohol decolorized gentian violet stain taken up by certain bacteria in lung sections after treatment with iodine-potassium iodide and also some bacteria exhibited resistance to decolorization. This is attributed to the difference in the cell envelope. Based on Christian Gram's observation, the cells which retained stain were referred to as Gram-positive organisms and others were Gram-negative organisms.[3] This review discusses the principle of gram stain emphasizing its significance in diagnostic utility for oral smears.

Staining of Bacteria

Bacteria appear opaque when observed unstained under a microscope. Staining is required to make the cells and their internal structures more visible under the light microscope by increasing the contrast between the organism and the background. Staining techniques are of two types [Figure 1]. In a simple staining procedure, only a single stain is used and the entire cell is stained with the same color. Bacterial cells have a slight negative charge. When basic stains having a positive charge, such as methylene blue or crystal violet are used, the

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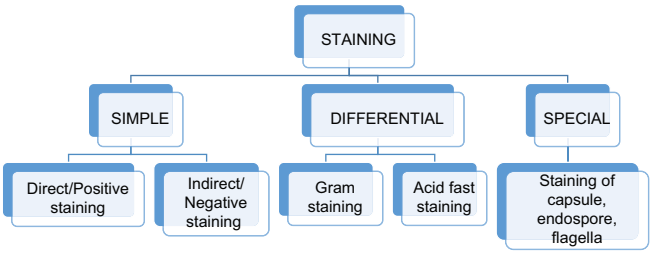

Figure 1: Classification of staining

bacterial cells are directly stained and this technique is called direct or positive staining. In indirect or negative staining, acidic dyes such as nigrosin or Indian ink are used, only the background is stained, and the organisms appear transparent. Differential staining uses contrast stains to differentiate two different types of bacteria. Gram staining and acid-fast staining are examples of differential staining techniques. Special stains are employed to stain-specific components of bacterial cells such as capsules, spores, or flagella.

Properties of Gram-Positive and Gram-Negative Bacteria

The difference in physical and chemical properties of bacteria is responsible for differential staining. Gram-positive bacteria have a cell envelope composed of an outer thick peptidoglycan layer along with cell wall-associated polymers such as teichoic acids, teichuronic acids, and other neutral or acidic polysaccharides and the inner cytoplasmic membrane. Membrane-associated and cell wall-associated proteins are displayed on the cell surface which serves as anchoring mechanisms for other macromolecules such as S-layer, pilus, and flagella.[3] Gram-negative cell envelope consists of three layers – the outer membrane, the peptidoglycan cell wall, and the cytoplasmic or inner membrane. The outer membrane is a lipid bilayer with an outer leaflet composed of glycolipids, principally lipopolysaccharide, and an inner leaflet containing phospholipids. The outer membrane contains proteins such as lipoproteins, porins, and β-barrel proteins. Gram-positive bacteria lack this lipopolysaccharide-containing membrane but are surrounded by layers of peptidoglycan many times thicker than is found in Gram-negative bacteria. The peptidoglycan cell wall of Gram-negative bacteria is made up of repeating units of the disaccharide N-acetyl glucosamine-N-acetyl muramic acid, which are cross-linked by pentapeptide side chains. Without peptidoglycan, cells lose their characteristic shape and are called spheroplasts. The inner membrane is a phospholipid bilayer containing membrane proteins. The outer membrane and inner membrane delimit an aqueous cellular compartment called the periplasm. The periplasm is densely packed with proteins and it is more viscous than the cytoplasm [Table 1].

Table 1: Difference between gram positive and gram-negative bacteria

Properties	Gram-Positive Bacteria	Gram -negative Bacteria
Cell membrane	Thick	Thin
Layers	2 layer: Inner-Cytoplasmic membrane Outer-Thick peptidoglycan	3 layer: Inner –cytoplasmic membrane Thin peptidoglycan Outer-lipopolysaccharide
Lipid content	Low	High
Periplasmic space	Absent	Present
Teichoic acid	Present	Absent
Porin channel	Absent	Present
Mesosomes	Well developed	Less prominent
Endotoxin	Absent	Present

Gram Stain Components

Dr. Christian Gram's original formulation included aniline gentian violet, Lugol's Iodine (potassium tri-iodide in water), and ethanol to remove the dye. Gram-negative bacteria, which did not retain the stain, appeared colorless. Later, Carl Weigert included safranin staining as the last step. The Gram-negative bacteria were contrasted using the safranin solution as a counterstain. The components of Gram stain are as follows:[4]

- Primary stain: these are positively charged basic dyes containing chromatophores. Basic dyes stain all bacterial cells as a result of attraction between chromatophores and bacteria. Some of the basic dyes that can be used as primary stains are crystal violet, methyl violet, and gentian violet
- Mordant: iodine acts as a mordant and it is added to increase the affinity between bacteria and the dye. It forms a complex with crystal violet an insoluble substance in the cytoplasm and thereby fixes the dye onto the slide
- Decolorizer: it removes the primary stain from stained cells. Acetone is the most rapid decolorizer used followed by ethanol
- Counterstain: it is a basic dye of contrast color to the primary stain. Safranin, dilute carbol fuchsin, and neutral red can be used as counterstain.

Principle of Gram Staining

The exact mechanism is not very well understood and various theories that have been suggested:[5]

- Acid protoplasmic theory: According to this theory, the protoplasm of Gram-positive bacteria is more acidic than Gram-negative bacteria, resulting in resistance to decolorizer
- Lipid theory: This theory is based on the fact that Gram-negative bacteria contain more lipids which get subsequently dissolved and the dye-iodine complex leak from the cell during the decolorizing step. The Gram-positive bacteria contain more protein which gets dehydrated during decolorization and shrinking the pore size
- Magnesium ribonucleate theory: magnesium ribonucleate present in Gram-positive bacteria forms a complex with dye-iodine which is insoluble in decolorizer
- PH theory: the pH of Gram-positive bacteria protoplasm is low and hence it retains the basic primary stain

- Cell wall permeability theory: This theory is the most accurate among others and widely accepted. It states that the primary stain is embedded in the thick peptidoglycan layer of Gram-positive bacteria and is retained even after the application of decolorizer. Whereas in Gram-negative bacteria, the primary stain gets fixed to the outer lipopolysaccharide layer, which is soluble in organic solvents and gets dissolved in decolorizer. As a result, the primary stain is removed from the bacteria and they subsequently take up the counterstain.

Procedure for Staining the Smear

- Specimen collection: Gram staining is commonly performed on sputum, blood, cerebrospinal fluid, ascetic fluid, synovial fluid, pleural fluid, as well as urine. A wooden spatula could be used to obtain a smear of exfoliated cells in the oral cavity
- Smear preparation: Patients should be asked to rinse their mouths before getting a smear to remove any food debris or necrotic slough, and the most representative site must be chosen for the smear. Before using, the wooden spatula should be lightly moistened. Then, gently scrape the mucosa with the spatula. It is important to ensure that the smear is neither too thick nor too thin
- Fixation: The smear should be fixed before staining to kill the microorganisms by coagulating the protoplasm of the cell and for the adherence of the smear to the slide, thereby the sample takes up stain readily. Fixation can be accomplished by holding the slide three to five times for 3–4 s over a Bunsen flame, or by allowing the slide to dry in the air for 15–30 min
- Gram staining procedure: The slide should be submerged in 1% crystal violet for 15 s, after which any remaining dye is removed with tap water. Lugol's iodine was added to the slide. After 30 s, the excess is poured off. The slide is placed at an angle and acetone is added so that acetone drains readily. The slide is washed with tap water after 2–5 s until no more crystal violet comes out. Finally, the smear is counterstained with dilute carbol fuchsin for 20 s and washed with water. The smear is viewed under an oil immersion microscope after it is dry [Figure 2].[6]

Interpretation of Gram Staining Results

Gram-negative bacteria will stain red while Gram-positive bacteria stain purple. After staining, some Gram-positive bacteria might appear to be Gram-negative. Such bacteria are called Gram-variable bacteria. There are two groups of Gram-variable bacteria. The cell walls of the *Actinomyces-Arthrobacter-Corynebacterium-Mycobacterium-Propionibacterium* group of bacteria become fragile during cell division, releasing their cytoplasmic components and crystal violet-iodine complex, resulting in gram variability. In the *Bacillus-Butyrivibrio-Clostridium* group of bacteria, the cell wall underlying the S-layer becomes thinner and diffuses as the culture ages and lyses during gram staining.[7]

Modification for Paraffin Sections: (Modified Brown-Brenn Method)

This staining method is a modification of Taylor's modification of Brown-Brenn stain for bacteria. In this method, ammonium oxalate is added to the primary stain to prevent precipitation of the crystal violet and to reduce stain deposit on the slide, Gram's iodine is used which is a combination of 2 g iodine and 4 g potassium iodide, wherein potassium iodide is used to attain high concentrations of iodine in aqueous solution [Figure 3]. Ethanol is used as a decolorizer for tissue sections to prevent over-decolorization. Since bacteria are small when compared to the thickness of the section, by the time the differentiating fluid has fully penetrated the whole section, some bacteria may be over-differentiated. The basic fuchsin is used as a counterstain to stain Gram-negative organisms and nuclei of other cells as red. Picric acid-acetone is used to stain other tissue components yellow and Acetone-xylene solution is used for the differentiation step.[8] The tissue section should not be allowed to dry out at any point during the staining procedure. If the section is allowed to dry out after treatment with the modified Grams of iodine, decolorization with alcohol-acetone will be difficult and inadequate. If the section is allowed to dry out after staining with basic fuchsin, differentiation with picric acid acetone will be inadequate.

Application

- Differentiating Gram-positive and Gram-negative is the first step in the identification of bacteria with the help of which the corresponding biochemical tests can be selected to be used further for confirmation. In a study by Doel *et al*., Gram stain was used to compare the morphological features of bacteria obtained from dental plaque and tongue as a preliminary step[9]
- Gram staining can be used to establish the presence of an infection and to identify a microbiological etiology. Mahlen and Clarridge *et al*. reported a case of palatal abscess in a 66-year-old male patient. Gram staining of the purulent fluid from the abscess revealed pleomorphic Gram-negative rods and *coccobacilli* arranged in

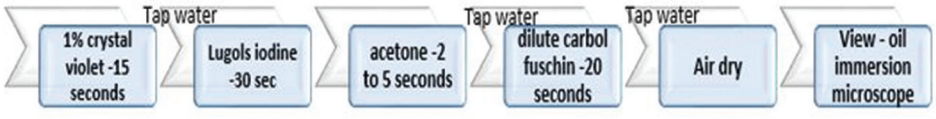

Figure 2: Procedure for staining

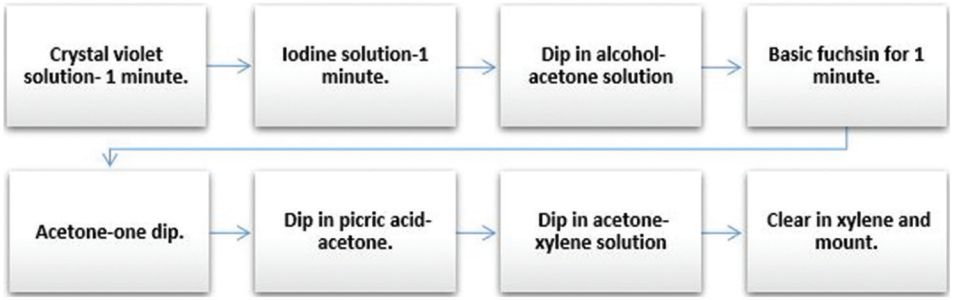

Figure 3: Procedure for modified Brown-Brenn method

packets of organisms which were then identified as *Campylobacter rectus*[10]

- Gram stain helps in the early presumptive identification of fastidious organisms like *Haemophilus* which takes time to grow in culture. Gram stain is more effective at a way to diagnose meningitis caused by *Streptococcus pneumoniae*, *Neisseria meningitidis*, and *Haemophilus influenzae*, according to Wu *et al*.[11]
- Gram stain gives a preliminary clue about the microorganism present so that the empirical treatment with broad-spectrum antibiotics can be started even before the culture report is available. According to Thass *et al*., tracheal aspirate samples can also be used to predict the provoking microorganisms by gram staining and initiate suitable therapeutic antimicrobial therapy[12,13]
- Gram stain can be used to screen the quality of clinical specimens such as sputum that should contain many pus cells and few epithelial cells
- Gram staining is also helpful in the identification of certain fungi such as *Candida* and *Cryptococcus* which appear as Gram-positive. In a study by Mohiddin *et al*., *Candida* and *Streptococci* in oral saliva and swab were identified based on gram stains.[14]

Role of Gram Stain for Oral Smears

The oral cavity is a unique ecosystem with different anatomic microniches comprising a diverse group of microorganisms including bacteria, archaea, fungi, protozoa, and viruses. The oral cavity is germ-free at birth and by the first year of life, the oral flora consists of streptococci, staphylococci, and Neisseria, together with some Gram-negative anaerobes such as *Veillonella* spp. After tooth eruption, the enamel surfaces are colonized by Gram-positive bacteria such as *Streptococcus mutans, Streptococcus sanguinis*, *Actinomyces* spp., *Lactobacillus*, and *Rothia* selectively and the crevicular tissue is colonized by Gram-negative anaerobic organisms such as *Prevotella* spp., *Porphyromonas* spp., *Neisseria*, and *Capnocytophaga*. As age advances, exogenous microbes colonize creating an imbalance in resident oral microflora.[15]

In the study by Derafshi *et al*., the oral bacterial flora of patients with removable dentures was evaluated and Gram stain was used for the identification of Gram-negative rod bacteria subsequent to bacterial culture in MacConkey agar. Non-oral pathogenic bacteria like *Enterobacter cloacae* and Gram-negative bacilli were more commonly detected from the saliva of the denture wearers suggesting the fact that oral health measures in patients with removable dentures should be adopted to decrease the risk of cross infection.[16]

Plaque biofilm is found on dental surfaces and appliances, especially in the absence of oral hygiene. In the early stages of biofilm formation, there is a shift of microorganisms from aerobic and *Facultatively anaerobic* species like *Streptococcus* to a situation with predominant facultative and obligate anaerobic, Gram-negative *cocci* and rods such as *Fusobacteria*, *Spirochetes*, and *Actinobacteria*.[15] Supragingival plaque is usually dominated by Gram-positive bacteria, including *Streptococcus mutans*, *Streptococcus salivarius, Streptococcus mitis*, and *Lactobacillus* in healthy individuals. In dental caries, cariogenic microorganisms such as *S. mutans*, *Lactobacillus,* and *Actinomycetes* are predominant in the supragingival plaque.[17] Subgingival microflora of chronic gingivitis and periodontitis consists predominantly of Gram-negative anaerobic bacteria such as *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, *Fusobacterium nucleatum*, and *Bacteroides* species.[18] Hence, gram staining of the oral smears plays an important role in the identification of any pathological shift in normal microbial flora.

Factors Affecting Staining

- The age of the bacterial culture: Cultures with older cells (those that have been in existence for longer than 24 h) immediately lose their Gram-positivity and appear Gram-negative
- Heat: Excessive heat during fixation can result in the loss of Gram-positive cells. Excessive heat degrades the cell wall. Gram-positive microorganisms begin to resemble Gram-negative microorganisms
- Cell overcrowding: Overcrowding of cells can lead to improper decolorization, which has an impact on the gram staining process. Thin smears discolor more quickly than thick smears when the cell is crowded, which provokes discoloration. Whenever the cell wall is destroyed, Gram-positive bacteria become Gram-negative bacteria.

Common Error

- Low crystal violet concentration: Crystal violet concentrations of up to 2% are effective, but at lower levels, stained cells appear to decolorize relatively quickly
- Excessive washing between steps: Water is prone to removing the crystal violet stain (but not the crystal violet-iodine complex). Use no more than a 5-s water rinse at any time while performing the procedure
- Insufficient iodine exposure: The amount of mordant necessary for the formation of the crystal violet-iodine complex must be present. Lower concentrations (0.33%–1% typically used) are easier to decolorize. In addition, air exposure and high temperatures hasten the loss of Gram's iodine from the solution, making reagent quality control crucial
- Longer decolorization: Experts with training can use the acetone-alcohol mixture, while inexperienced technicians should use 10% ethanol because it decolorizes more gradually. It takes skill to determine when decolorization is complete
- Excessive counterstaining: Upon overexposure to the counterstain which is a basic dye, it is possible that the crystal violet—iodine complex in gram- positive cells will be replaced by the counterstain. Hence, the counterstain should not be left on the slide for more than 30 seconds.[19]

Disadvantage of Gram Stain

- The interpretation of slides can be difficult if the microscopic smear is thick and clumped. Decolorization time should have very close monitoring to avoid under-decolorization or over-decolorization
- Thicker smears require longer decolorizing time. Similarly, cultures should undergo evaluation while they are still fresh
- Old cultures tend to lose the peptidoglycan cell walls, which predisposes Gram-positive cells to be Gram-negative or gram variable
- Gram stain is not useful for organisms without a cell wall like *Mycoplasma* species, and for smaller bacteria such as *Chlamydia* and *Rickettsia* species.[20]

Conclusion

For the majority of bacteria, the Gram stain is still a useful microbiological method. The staining outcome is still the most crucial taxonomic tool for dividing the two different types of bacterial cells, and it is a fundamental characteristic for describing a distinct species. Gram staining of the oral smears can serve as the preliminary step in the identification of pathogenic microorganisms.

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Conflicts of interest

There are no conflicts of interest.

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