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Gram Staining: A Brief Review

Ansar Ahmad Paray[1], Manju Singh[2], Mohsin Amin Mir[3], Amandeep Kaur[4]

[1]Ph.D Scholar, Centre for Interdisciplinary and Biomedical Research, Adesh University, Bathinda
[2]Assistant Professor Centre for Interdisciplinary and Biomedical Research, Adesh University Bathinda
[3]Pharm. D student, Adesh Institute of Pharmacy and Biomedical Sciences, Adesh University, Bathinda
[4]Professor, Department of Microbiology, Adesh Institute of Medical Science and Research, Adesh University, Bathinda

Corresponding Author: Ansar Ahmad Paray

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ABSTRACT

The Gram staining is one of the oldest and most important staining techniques used in microbiology first introduced in 1882 by Hans Christian Gram. The staining is the most crucial staining in microbiology and is the first step in differentiating bacteria. The gram-positive bacteria have thick cell wall because of thick layers of peptidoglycan, thus stain purple whereas gram-negative bacteria have thin layers of peptidoglycan and stains red/pink. Decolonization is most important step in gram staining as using decolorizing agent for long time can wash out all the stains from both types of bacteria Gram staining has been there used for over a century, now there have been several advancements in recent years to improve efficiency as well as accuracy of gram staining. Overall, these advancements in Gram staining have improved its efficiency, accuracy, and speed, making it a valuable tool in clinical microbiology and research laboratories. These advancements have enhanced our ability to identify and characterize bacteria, leading to better diagnosis and treatment of bacterial infections.

Keywords: Gram staining, staining techniques.

INTRODUCTION

The Gram staining is one of the oldest and most important staining techniques used in microbiology first introduced in 1882 by Hans Christian Gram (a Danish bacteriologist) mainly to identify organisms causing pneumonia at that time [1]. The staining is used for direct microscopic examination of clinical specimens and subcultures. The staining is the most crucial staining in microbiology and is the first step in differentiating bacteria. The staining distinguishes between Gram-positive and Gram-negative bacteria by coloring these cells into either red (gram negative) or violet (gram positive). The staining technique also gives a picture about morphological forms and arrangements of bacteria [2]. Besides Bacteria, gram staining is also used to identify various fungi and parasite

This staining technique involves four steps: staining with water-soluble dye called crystal violet, Use of Grams Iodine a mordant to bind the dye, decolourisation with acetone or 95% ethyl alcohol, and counterstaining, mainly with Safranin or diluted (1:10) Carbol fuchsin [1]

Principle of gram staining based on cell wall theory:

The gram-positive bacteria have thick cell wall because of thick layers of peptidoglycan, thus stain purple whereas Gram-negative bacteria have thin layers of peptidoglycan in cell wall also gram-negative bacteria have high lipid content.

In aqueous solutions crystal violet dissociates into Crystal violet positive (CV+) and Chloride negative (Cl –) ions. These ions penetrate via cell wall and membrane of both gram-positive and gram-

negative bacterial cells. The CV+ then interacts with negatively charged components of bacterial cells, thus staining the cells purple. When iodine is added, it interacts with CV+ to form large complexes of dye within the cytoplasm as well as in the outer layers of the cell. In the next step the decolorizing agent interacts with the lipids present in cell membranes of both gram-positive and gram-negative bacteria. The outer membrane of the gram-negative bacteria is lost from the cell, making the peptidoglycan layer exposed. Now the gram-negative cell walls become leaky thus allow the large crystal violet and iodine complexes to be washed from the cell.

In case of gram-positive bacteria, the highly cross-linked and multi-layered peptidoglycan is dehydrated by the addition of decolorizer thus trapping the large crystal violet and iodine complexes within the cell.

After decolonization, the gram-positive cell remains purple in color, whereas the gram-negative cell loses the purple color (of primary stain) and takes the color of counter stain (Safranin, a positively charged dye). At the end gram-positive cell appears purple and the gram-negative cell pink/red. [3,4]

Preparation of smear; [5]
Requirements:

- Personnel Protective Equipment (PPE) such as Gloves, masks.
- Specimen (Clinical specimen such as body fluids etc/ Bacterial culture)
- Neat and Clean Microscopic glass slide
- Marker
- Sterile Bacterial loop
- Bunsen's Burner/Sprit lamp

Procedure;

1. Mark and area (oval shaped) on a clean glass slide using marker
2. Now the specimen is transferred using bacterial loop to the marked area on the slide, in case if specimen is bacterial colony from culture media add a normal saline or distilled water to the marked are and then pick up a colony and emulsify in the Normal saline or distilled water.
3. Now allow the specimen to air dry
4. Now the slide is heat fixed by passing over the flame 3-4 times facing smear upwards. Avoid overheating

Staining the smear:
Requirements:

- Prepared smear slide
- Crystal violet
- Grams Iodine
- Acetone or ethyl alcohol 70%
- 0.25% w/v Safranin or Diluted (1:10) Carbonyl Fuchsin
- Tissue paper
- Microscope
- Cedar wood Oil

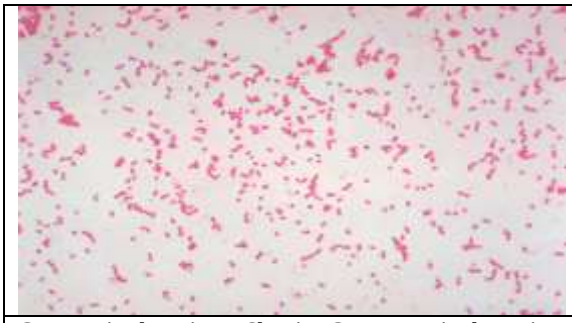
Staining Procedure:

1. Put the prepared smear on staining rack over the sink and add Primary stain i.e. Crystal violet on the smear for 1 minute
2. After 1 minute wash the smear under gentle tap water and add gram's Iodine for 1 minute.
3. After 1 minute again wash the smear under gentle tap water
4. Add decolorizer I.e., Acetone or ethyl alcohol 95% just for fewer than 10 seconds.
5. Again, wash the slide smear under gentle tap water and add Counter stain I.e., Safranin or Diluted (1:10) Carbonyl Fuchsin for <1 minute.
6. After 1 minute wash the smear under gentle tap water and allow to air dry
7. Clear the back side of smear with tissue paper, put a drop Cedar wood oil and focus on microscope using observe under Oil Immersion lens (100 X)

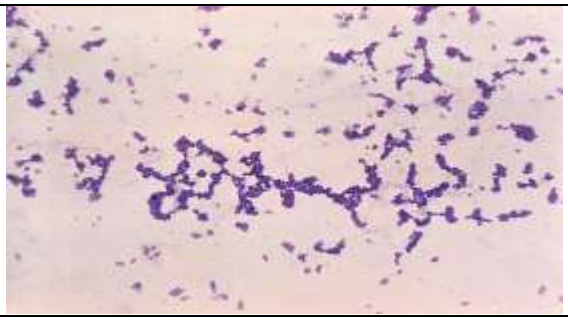
Note: Decolonization is most important step in gram staining as using decolorizing agent for long time can wash out all the stains from both types of bacteria [6].

Result interpretations: [5]

Gram positive bacteria	Dark Purple
Gram negative bacteria	Pink or red
Yeast cells	Dark Purple
Pus Cells/ White Blood cells	Pink
Erythrocytes and Tissue cells	Pink
Epithelial cells	Pale red

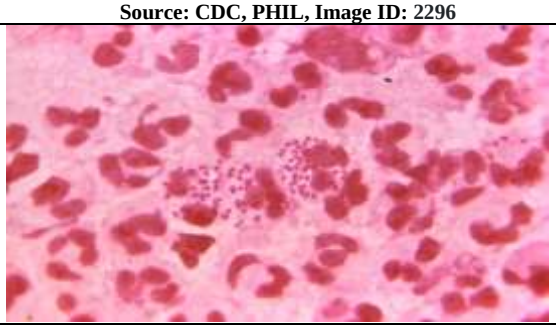


Gram-stained specimen, Showing Gram-negative bacteria (They retaining the counter staining dye called Safranin) Source: CDC, PHIL, Image ID: 1260



Gram-stained specimen, Showing Gram-Positive bacteria (retaining the primary dye called CV in the cell wall). Source: CDC, PHIL, Image ID: 2296

Gram-stained specimen, Showing Candida (Gram-Positive) Source: CDC, PHIL, Image ID: 21793



Gram-stained specimen, Showing Gram-Negative bacteria (Intracellular) with numerous white blood cells (WBCs), also known as polymorphonuclear leukocytes/Cells, or PMNs. Source: CDC, PHIL, Image ID: 15018

Importance and uses of gram staining: [1, 5]

- Divides bacteria into two groups, Gram-positive and Gram-negative, on the basis of their cell wall and cell membrane permeability.
- The staining also reveals the morphology (shape, size and arrangement) of bacteria.
- The staining is used to direct microbiologic examination of specimens such as body fluid or biopsy and yields results more quickly than culture. Direct Gram stain smears thus guides the clinicians on the initial choice of antibiotics, pending the results of culture and sensitivity, Judges Specimen quality.
- Direct Gram stain smears also Contributes to the selection of culture media, especially with mixed flora is there in specimen and provides internal quality control when direct smear results are compared to culture results
- The staining also provides preliminary information to the clinician regarding presumptive bacterial pathogens
- The staining also characterizes the type of bacteria growing in culture media.
- Besides bacteria yeasts and fungi, can be also seen on a Gram-stained smear. Yeast takes gram positive color.

Common mistakes while Gram Staining:

Mistake	Results	Corrective Action
Using excessive heat during fixation of smear	The morphology of cell gets altered making the cells to get decolorized more easily	Avoid excessive heating of smear
Using Inappropriate or low concentration of	The cells to get decolorized more easily	Use proper concentration of Crystal Violet (2%)

Crystal Violet		
Excessive decolonization	Even the gram-positive cell will lose the primary stain	Proper decolonization
Prolonged or excessive washing between steps	Crystal violet (CV) stain is susceptible to wash-out with water (but not the CV-iodine complex).	Do not use more than a 5 s water rinse at any stage of the procedure
Improper exposure with Mordant I.e., Grams Iodine	This will lead improper formation of CV-Iodine complex hence cell gram positive will lose primary stain while washing and decolorizing. A closed bottle (0.33% starting concentration) at room temperature loses >50% of available iodine in 30 days, an open bottle >90%. Loss of 60% iodine results in erratic results	Use of proper concentration of iodine
Prolonged counterstaining (use of Safranin)	Counter stain is also a basic dye, so it is possible to replace the CV-iodine complex in Gram-positive cells with an over-exposure to the counter stain.	The counter stain should not be left on the slide for more than 30 s

Various causes of errors in gram staining.
Inappropriate specimen sampling, specimen processing, preparation of smear, as well as prior antibiotic therapy are the various having adverse impact on Gram stain result. The inherent nature few organisms can also produce misleading results, such as *Acinetobacter* species may stain Gram positive, whereas *Bacillus* spp. species may appear Gram negative. False-negative gram stains can also occur due to inadequate specimen or smear preparation or because of failure to examine an adequate number of fields. Moreover, training and maintenance of proficiency for Gram staining remains challenging.[7]

- **Advancements in gram Staining:**
  Gram staining has been there used for over a century, now there have been several advancements in recent years to improve efficiency as well as accuracy of gram staining.
  Modified Gram Staining Protocols: Modified gram staining protocols have been developed to enhance the detection and differentiation of certain bacteria. For example, the Hucker's modification of the Gram stain uses a different decolonization step, allowing for the differentiation of Gram-negative bacteria with a higher degree of accuracy [8,9]
- **Fluorescence Light-Up Gram-Staining Technique for Living Bacterial Differentiation**
  This newer technique had been developed based on surface components of pathogenic bacteria. Here in this technique of fluorescence light-up Gram-staining using two bio-orthogonal fluorescence turn-on probes TPPB and BPTZ are used. This Gram-staining technique selectively label gram negative bacteria by TPPB, while BPTZ can label both Gram positive and Gram-negative bacteria, thus offer a clear differentiation of the living gram positive and gram-negative bacterial populations with high selectivity and sensitivity. [10]

Automation in Gram staining [11, 12, 13]

- **Automated Gram Stainers:** Traditionally Gram staining involves a series of manual steps, which can be time-consuming and prone to human error. Automated Gram staining systems have been developed to streamline the process and provide consistent results. These systems automate the staining steps, reducing variability and increasing the throughput of samples
- **Digital Imaging and Analysis:** Advances in imaging technology and digital analysis have made it possible to automate the interpretation and quantification of Gram-stained slides. High-resolution digital imaging systems capture images of stained slides, which can be analyzed using image processing algorithms. These algorithms can detect and classify bacterial cells, providing quantitative data on cell morphology, distribution, and other parameters.

Overall, these advancements in Gram staining have improved its efficiency, accuracy, and speed, making it a valuable tool in clinical microbiology and research laboratories. These advancements have enhanced our ability to identify and characterize bacteria, leading to better

diagnosis and treatment of bacterial infections.

Some potential areas of research and innovation on Gram staining could include:

1. **Enhancement of staining techniques:** Develop improved staining protocols or modifications to existing methods to enhance the clarity and accuracy of Gram staining results. This could involve optimizing the concentration or composition of the staining reagents, exploring alternative staining dyes, or introducing innovative techniques such as fluorescent labeling.
2. **Automation and standardization:** Explore automation technologies and robotic systems to streamline the Gram staining process, reduce human error, and ensure consistent results. This could involve developing automated staining platforms or integrating image analysis algorithms to automate the interpretation of stained samples.
3. **Rapid and point-of-care testing:** Investigate the development of rapid and portable Gram staining methods that can be performed at the point of care, such as in resource-limited settings or during emergencies. This could involve simplifying the staining procedure, utilizing micro fluidic devices, or employing novel staining approaches that provide quick and reliable results.
4. **Antibiotic susceptibility testing:** Explore the integration of Gram staining with antibiotic susceptibility testing to enable simultaneous identification and determination of bacterial resistance patterns. This could involve developing innovative staining techniques that differentiate between susceptible and resistant bacteria based on staining characteristics or exploring the use of additional markers to assess antibiotic susceptibility.
5. **Image analysis and artificial intelligence:** Develop advanced image analysis algorithms and machine learning techniques to automate the interpretation of Gram-stained samples. This could involve training AI models to classify bacteria based on staining patterns or developing algorithms to analyze multiple staining parameters for more comprehensive bacterial characterization.
6. **Alternative staining methods:** Investigate alternative staining methods that can complement or improve upon the limitations of Gram staining. This could include exploring novel staining dyes, molecular-based staining approaches, or other imaging techniques that provide additional information about bacterial structure, metabolism, or virulence.
7. **Microbiome analysis:** Explore the application of Gram staining techniques in studying microbial communities and analyzing complex microbiomes. This could involve adapting staining protocols to handle diverse microbial populations or combining Gram staining with other molecular methods to gain insights into the composition and function of microbiota.
8. **Environmental and industrial applications:** Investigate the adaptation of Gram staining for environmental monitoring, industrial processes, or quality control. This could involve developing staining techniques to identify and characterize specific bacteria in various settings, such as wastewater treatment plants, food production facilities, or pharmaceutical manufacturing.

Overall, these research areas aim to improve the accuracy, speed, automation, and versatility of Gram staining, expanding its applications and enhancing its impact in various scientific, medical, and industrial fields.

CONCLUSION

Gram staining though old but is still widely used method in bacteriology for differentiation of bacteria into gram-positive

or the gram-negative group. This differentiation is usually achieved through the use of different staining solutions. Gram stain is a simple procedure. and can be performed quickly and easily but preparation and interpretation of the smear requires considerable experience and training and can therefore be prone to errors. Reporting Gram stain results of direct smears are highly reliable and useful when samples are from sterile Identification of some bacteria (*Acinetobacter spp*, Nocardia spp and Actinomycetes spp etc.) can be Gram variable which is a challenging problem. The quality of the results and confidence in the reports can be improve with the qualified and experienced laboratory professionals and with the clinical history of patient. As the health care system moves toward a greater transparency and focus on the reduction of the potential for harm due to medical errors, laboratories cannot begin to improve if we do not first know where we stand. Monitoring of Gram stain error rates is an essential first step in order to reduce the errors in diagnosis.

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