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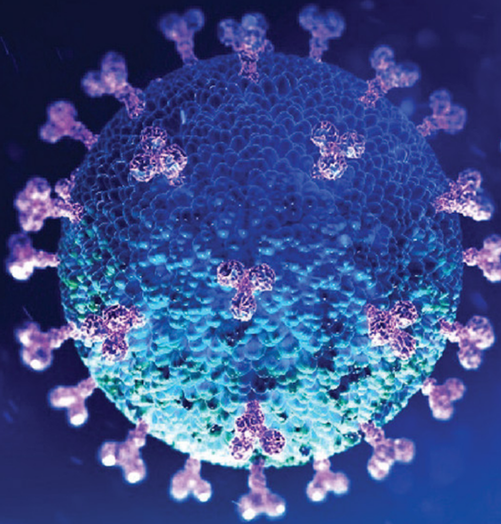
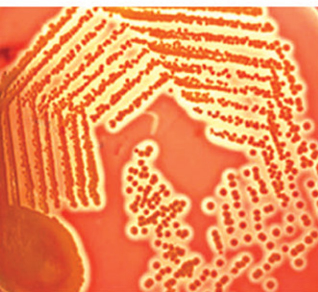
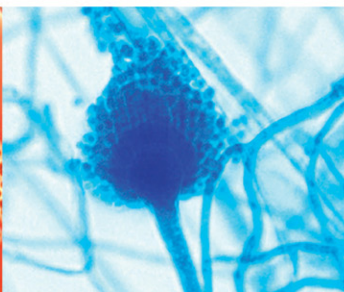
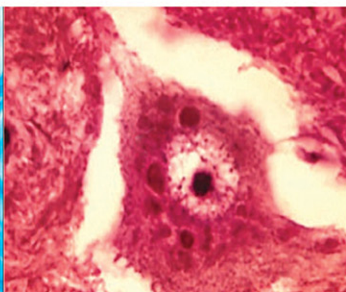


Essentials of PRACTICAL MICROBIOLOGY

As per the Competency Based Medical Education Curriculum (MCI)

Apurba S Sastry
Sandhya Bhat

2nd Edition

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CHAPTER 3.4

General Bacteriology: Direct Detection 2: Gram Staining

Problem Solving Exercise

Gram Staining

Case Scenario 1: A smear is provided, made from a sputum specimen of a 5-year-old child with acute onset of fever, productive cough and dyspnea for the past two days. Physical examination revealed dull note on percussion.

Case Scenario 2: A smear is provided, made from a CSF specimen of a two year old baby presented with high grade fever, vomiting and excessive crying for past three days.

Case Scenario 3: A smear is provided, made from a pus discharge of a postoperative wound infection of a 65-year-old man who has undergone an abdominal surgery.

Case Scenario 4: A smear is provided, made from a wound swab specimen of a foot ulcer of a diabetic patient.

1. Perform Gram stain of the smear provided.
2. Draw your observations with the help of a neat labeled diagram and give your interpretation.
3. Suggest which antibiotic can be started empirically for the treatment of this case?
4. What are various theories of Gram staining?
5. What are the various uses of Gram staining?

Explanation

The above clinical presentations are suggestive of a case of:

- Lobar pneumonia (case scenario 1)
- Pyogenic meningitis (case scenario 2)
- Surgical site infection (case scenario 3)
- Diabetic foot ulcer (case scenario 4)

The answers to the above questions are explained in this chapter.

Gram Staining technique was originally developed by Hans Christian Gram (1884) and it is the most widely used stain in diagnostic bacteriology.

PROCEDURE

Fixation

The smear is made on a clean glass slide from bacterial culture or clinical specimen. It is then air dried and heat fixed.

Step 1 (Primary Stain)

The smear is stained with pararosaniline dyes, such as crystal violet (or gentian violet or methyl violet) for one minute. Then the slide is rinsed with water. Crystal violet stains all the bacteria violet in color (irrespective of whether they are gram-positive or gram-negative).

Step 2 (Mordant)

Gram's iodine (dilute solution of iodine) is poured over the slide for one minute. Then the slide is rinsed with water. Gram's iodine acts as a mordant, binds to the dye to form bigger dye-iodine complexes in the cytoplasm.

Step 3 (Decolorization)

Next step is pouring of few drops of decolorizer to the smear, such as acetone (for 2–3 sec) or ethyl alcohol (20–30 sec) or acetone alcohol (for 10 sec). Slide is immediately rinsed with water. Decolorizer removes the primary stain from gram-negative bacteria while the gram-positive bacteria retain the primary stain.

Note: Decolorization is the most crucial step of Gram staining. If the decolorizer is poured for more time,

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even gram-positive bacteria lose color *(over decolorization)* and if poured for less time, the gram-negative bacteria do not lose the color of primary stain properly *(under decolorization).*

Step 4 (Counterstain)

Secondary stains, such as dilute carbol fuchsin or safranin are added for 30 seconds. They impart pink to red color to the gram-negative bacteria. Alternatively, neutral red may also be used as counterstain especially for gonococci. The slide is rinsed in tap water, dried, and then examined under oil immersion objective.

The steps of Gram staining and the color of gram-positive and gram-negative bacteria after each step are depicted in Figure 3.4.1.

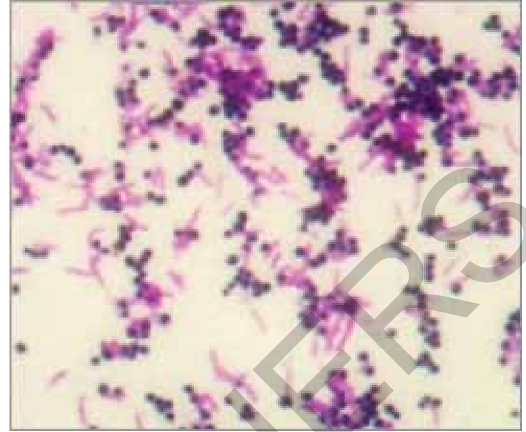
INTERPRETATION

Smear is examined under oil immersion objective (Fig. 3.4.2).

- Gram-positive bacteria resist decolorization and retain the color of primary stain, i.e., violet
- Gram-negative bacteria are decolorized and, therefore, take counterstain and appear pink.

MECHANISM OF GRAM STAINING

Though the exact mechanism is not understood, the following theories have been put forward.



Figs 3.4.2: Gram staining demonstrating violet-colored gram-positive cocci in clusters and pink-colored gram-negative bacilli in scattered arrangement.

Source: Department of Microbiology, Pondicherry Institute of Medical Sciences, Puducherry (*with permission*).

pH Theory

Cytoplasm of gram-positive bacteria is more acidic, hence can retain the basic dye (e.g., crystal violet) for longer time. *Iodine* serves as mordant, i.e., iodine combines with the primary stain to form a dye-iodine complex which gets retained inside the cell.

Cell Wall Theory

This is believed to be the most important postulate to describe the mechanism of Gram staining.

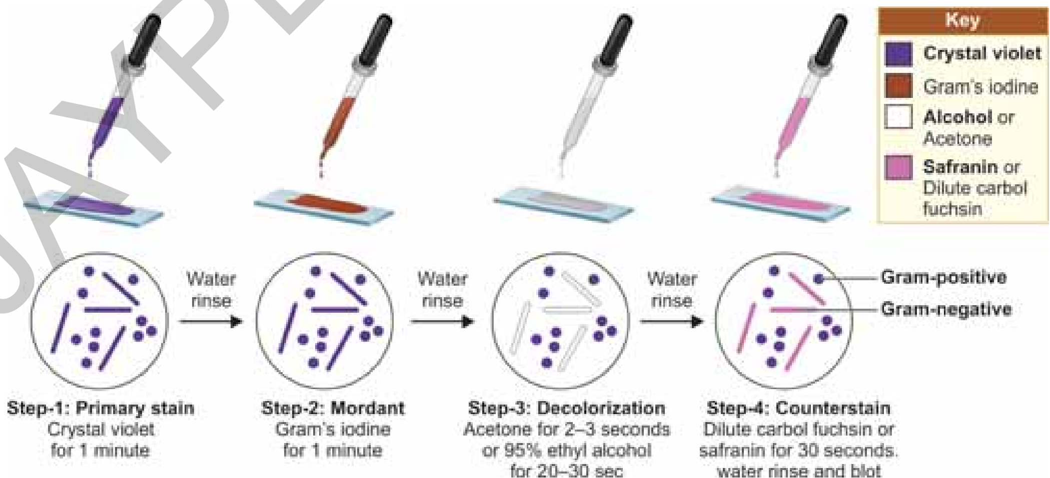


Fig. 3.4.1: Principle and procedure of Gram staining.

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Table 3.4.1: Differences between gram-positive and gram-negative cell wall.

Characters	Gram-positive cell wall	Gram-negative cell wall
Peptidoglycan layer	Thicker (15–80 nm), 50–100 layers, cross linked by pentaglycine bridge	Thinner (2 nm), 1–2 layers, directly linked, no pentaglycine bridge
Lipid content	Nil or scanty (2–5%)	Present (15–20%)
Lipopolysaccharide	Absent	Present (endotoxin)
Teichoic acid	Present	Absent
Variety of amino acids	Few	Several
Aromatic amino acids	Absent	Present

- Gram-positive cell wall has a thick peptidoglycan layer (50–100 layers thick), which are tightly cross linked to each other
- The peptidoglycan itself is not stained; instead it seems to act as a permeability barrier preventing loss of crystal violet (Table 3.4.1)
- Gram-negative cell wall is more permeable, thus allowing the outflow of crystal violet easily (Table 3.4.1). This is attributed to:
  - The thin peptidoglycan layer in gram-negative cell wall which is not tightly cross linked
  - Presence of lipopolysaccharide layer in the cell wall of gram-negative bacteria gets disrupted easily by the action of acetone or alcohol, thus allowing the primary stain to come out of the cytoplasm.
- After mordanting with Gram's iodine, bigger dye-iodine complexes are formed in the cytoplasm. Following decolorization, gram-negative bacterial cell wall (as more lipid content) gets dissolved leading to formation of larger pores through which the dye-iodine complexes escape. Due to less lipid in gram-positive bacterial cell wall, smaller pores are formed and dye-iodine complexes are retained.

MODIFICATIONS OF GRAM STAINING

There are few minor modifications of Gram staining which vary slightly from the method described earlier:

- **Kopeloff and Beerman's modification:** Primary stain and counterstain used are methyl violet and basic fuchsin, respectively
- **Jensen's modification:** This method involves use of absolute alcohol as decolorizer and neutral red as counterstain. It is useful for detection of meningococci and gonococci
- **Weigert's modification:** This modification is useful for staining tissue sections. Here, aniline-xylol is used as a decolorizer
- **Preston and Morrell's modification:** Here, iodine-acetone is used as decolorizer
- **Brown and Brenn modification:** This is used for Actinomycetes.

USES OF GRAM STAIN

Gram staining has the following uses:

- **Differentiation of bacteria into gram-positive and gram-negative:** It is the first step towards identification of bacteria
- **To start empirical treatment:** Gram staining of clinical specimen gives preliminary clue about the bacteria present (based on the shape and Gram staining property of the bacteria) so that the empirical treatment with broad spectrum antibiotics can be started early, before the culture and susceptibility report is available
- **For identification:** Gram staining from bacterial culture gives an idea to put the required biochemical tests for further identification of bacteria
- **For fastidious organisms,** such as *Haemophilus* which takes time to grow in culture, Gram staining helps in early presumptive identification based on their morphology (pleomorphic gram-negative bacilli)
- **Anaerobic organisms**, such as *Clostridium* do not grow in routine culture. Therefore, organisms detected in Gram stain, but aerobic culture-negative gives a preliminary clue to perform an anaerobic culture of the specimen
- **Yeasts:** In addition to stain the bacteria, Gram staining is useful for staining certain fungi, such as *Candida* and *Cryptococcus* (appear gram-positive budding yeast cells)
- **Quality of specimen:** Gram staining helps in screening the quality of the **sputum specimen** before processing it for culture. Presence of more pus cells and less epithelial cells indicates good quality specimen.

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General Bacteriology: Direct Detection 3: Special Staining (Acid-Fast Stain, Albert Stain) and Other Methods

CHAPTER 3.5

ACID-FAST STAIN

Problem Solving Exercise 1

Acid-fast Staining

A smear is provided, made from a sputum specimen of a 15-year-old boy presented with fever, productive cough and hemoptysis for past two weeks.

1. Perform acid-fast staining of the smear provided.
2. Draw your observations with the help of a neat labeled diagram and give your interpretation.
3. Suggest the treatment regimen given in this case.
4. What is RNTCP grading and its implications?
5. Which is the conventional culture medium used and describe the colonies grown?
6. What is the recommended molecular method available for rapid and accurate identification? Mention its advantages?

Explanation

The above clinical presentation is suggestive of a case of pulmonary tuberculosis. The answers to the above questions have been explained in this chapter and also in Chapter 35.

The acid-fast staining was discovered by Paul Ehrlich and subsequently modified by Ziehl and Neelsen. This staining is done to identify acid-fast organisms, such as *Mycobacterium tuberculosis* and others (Table 3.5.1). Acid-fastness is due to presence of mycolic acid in the cell wall.

Table 3.5.1: Acid-fast organisms or structures and percentage of sulfuric acid suitable for staining (for decolorization).

Acid-fast organisms/structures	Sulfuric acid (%) needed for decolorization
Mycobacterium tuberculosis	25
Mycobacterium leprae	5
Nocardia	1
Acid-fast parasites, such as *Cryptosporidium, Cyclospora, Cystoisospora*, Microsporidia*, *Taenia saginata* segments, hooklets of hydatid cyst	0.5–1
Bacterial spore	0.25–0.5

*Microsporidia are now considered to be evolved from fungi.

Ziehl-Neelsen Technique (Hot Method)

Smear Preparation

Smear measuring 2 × 3 cm in size is prepared in a new clean grease free, scratch free slide from the yellow, purulent portion of the sputum.

Heat Fixation

The smear is air dried and then heat fixed by passing over the flame. Coagulation of the proteinaceous material in the sputum will facilitate fixing of the smear.

Step 1 (Primary Stain)

Smear is poured with carbol fuchsin (1%) for 5 minutes. Intermittent heating is done by flaming the underneath of the slide until the fumes appear. Heating helps in better penetration of the stain.

- Care must be taken to ensure that the smear does not dry out. To prevent drying, more carbol fuchsin stain is added to the slide and then the slide is reheated
- Rinse the slide with tap water, until all free carbol fuchsin stain is washed away. At this point, the smear on the slide looks red in color (Fig. 3.5.1).

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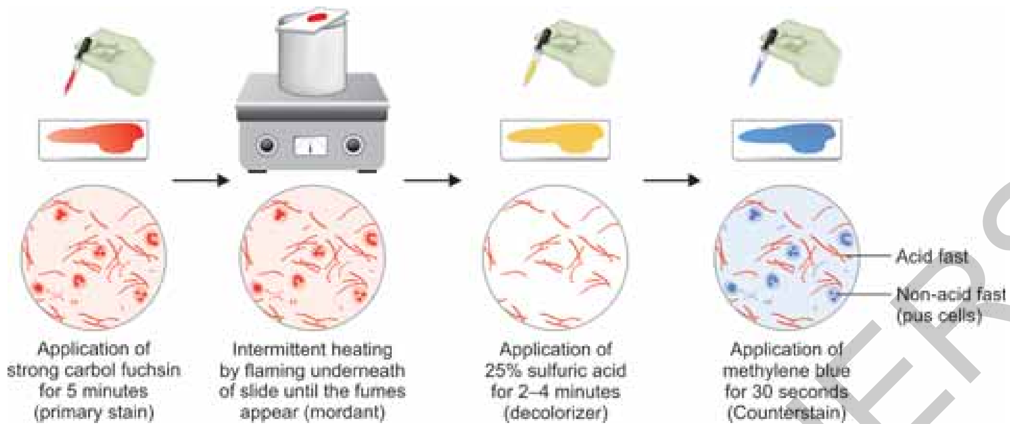

Fig. 3.5.1: Ziehl-Neelsen technique.

Step 2 (Decolorization)

It is done with 25% sulfuric acid for 2–4 minutes. Repeat decolorization for 1–3 minutes if the slide is still red. Then the slide is gently rinsed with tap water and tilted to drain off the water. The back of the slide is wiped clean with a swab dipped in sulfuric acid.

Step 3 (Counterstaining)

It is done with methylene blue (0.1%) for 30 seconds. Slide is rinsed in tap water, dried, and then examined under the binocular microscope using low power objective (10×) to select a suitable area and then screened under oil immersion field (100×). Contaminated materials/slide should be discarded in jar containing 5% phenol.

Interpretation

Mycobaterium tuberculosis appears as long slender, straight or slightly curved, beaded, less uniformly stained, red-colored acid-fast bacillus. Other non-acid fast organisms present in the smear, pus cells and the background take up the counterstain and appear blue (Fig. 3.5.2).

Fig. 3.5.2: Ziehl-Neelsen staining of sputum smear showing long slender, straight or slightly curved, beaded red colored acid–fast bacilli.

Source: Department of Microbiology, JIPMER, Puducherry (*with permission*).

Modifications of Acid-fast Staining

Hot method (Ziehl-Neelsen technique) is the most commonly done acid-fast staining technique. Other modifications include:

- Cold method *(Kinyoun's method)*: It differs from Ziehl-Neelsen stain in that—
  - Heating is not required
  - Phenol concentration in carbol fuchsin is increased
  - Duration of carbol fuchsin staining is more.
- Decolorization can be done with acid-alcohol (3 mL of HCl and 97 mL of ethanol)
- Malachite green can be used as counterstain
- Concentration of sulfuric acid may vary depending on the acid-fastness of the structure to be demonstrated. More the content of mycolic acid in the cell wall, more is the acid-fastness, hence more is the percentage of sulfuric acid needed (Table 3.5.1).

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ALBERT STAIN

Problem Solving Exercise 2

Albert Staining

A smear is provided, made from a throat swab specimen of a 15-year-old boy presented to an ENT OPD with fever, sore throat and difficulty in swallowing for past two days. On examination, a dirty grey membrane was observed over the tonsils.

1. Perform a suitable staining of the smear provided.
2. Draw your observations with the help of a neat labeled diagram and give your interpretation.
3. Suggest which antibiotic can be started empirically for treatment in this case
4. How can this clinical condition be prevented?

Explanation

The above clinical presentation is suggestive of a case of faucial diphtheria. The answers to the above questions have been explained in this chapter and also in Chapter 33.

Albert stain is used to demonstrate the metachromatic granules of *Corynebacterium diphtheriae*.

Composition of Albert Stain

Includes:
- Albert I: Comprises of toluidine blue, malachite green, glacial acetic acid, alcohol (95% ethanol), and distilled water
- Albert II: Contains iodine in potassium iodide.

Procedure

1. *Fixation*: The smear is heat fixed
2. Smear is covered with Albert I (Albert's stain) for 5 minutes, then the excess stain is drained out
3. Albert II is poured over the smear so as to cover it completely for 1 minute
4. Slide is washed in water, blotted dry and examined under oil immersion field.

Interpretation

Corynebacterium diphtheriae appears as green-colored bacilli arranged in Chinese letter or cuneiform pattern, with bluish-black metachromatic granules at polar ends (Fig. 3.5.3). These can be differentiated from diphtheroids which do not show granules and are arranged in palisade pattern. However, certain bacteria, such as *Corynebacterium xerosis* and *Gardnerella vaginalis* also possess metachromatic granules.

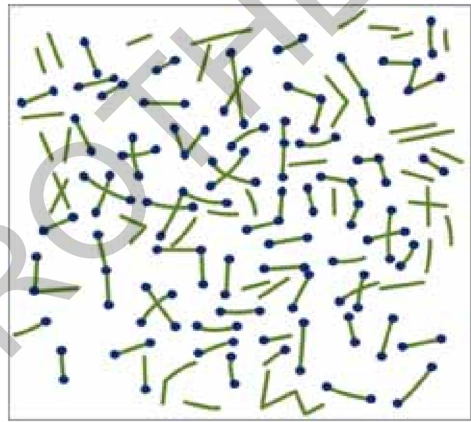

Fig. 3.5.3: Albert stained smear of *Corynebacterium diphtheriae* showing green bacilli with blue-black metachromatic granules (schematic).

NEGATIVE STAINING

A drop of bacterial suspension is mixed with dyes, such as India ink or nigrosin. The background gets stained black whereas unstained bacteria stand out in contrast. This is very useful in the demonstration of bacterial or yeast capsules which do not take up simple stains (Refer Fig. 41.3A, Chapter 41).

IMPREGNATION METHODS

Bacterial cells and structures that are too thin to be seen under the light microscope are thickened by impregnation of silver on their surface to make them visible, e.g.,

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Fig. 3.5.4: Silver impregnation staining demonstrating spirochetes.

Source: Public Health Image Library, ID# 836, Centers for Disease Control and Prevention (CDC), Atlanta (*with permission*).

demonstration of bacterial flagella and spirochetes (Fig. 3.5.4).

OTHER METHODS OF DIRECT DETECTION

Other Microscopic Techniques

Other microscopic techniques include:

- **Dark-ground and phase-contrast microscopy**—for demonstration of spirochetes in genital specimens (Refer Fig. 2.4, Chapter 2)
- **Hanging drop preparation for stool specimen**—for demonstration of darting motility; gives a clue about *V. cholerae* (discussed in Chapter 3.7).

Antigen Detection

Various immunological methods, such as latex agglutination test, immunochromatographic test are available which detect antigens directly from the clinical specimens.

- The classical example includes detection of capsular antigen of pneumococci, meningococci, *H. influenzae* in CSF specimen
- Urinary antigen detection for pneumococci and *Legionella*
- Direct fluorescent antibody test—for detection of *Treponema pallidum* from tissue sections or exudates.

Details about these antigen detection methods are discussed in Chapters 7 to 9.

Molecular Diagnosis

Bacterial DNA or RNA can be directly detected in the clinical specimens by various molecular methods, such as polymerase chain reaction (PCR). It is discussed in detail in Chapter 3.9.

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Essentials of
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  - Simple and lucid language—makes the understanding easy.

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They are also authors of other popular books which are extremely appreciated among faculty and students:

1. Essentials of Medical Microbiology, 3rd edition for MBBS
2. Essentials of Hospital infection control, 1st edition (Co-authored with Dr Deepashree R)
3. Essentials of Medical Parasitology, 2nd edition for MBBS
4. Review of Microbiology and Immunology, 9th edition for PG entrance examination
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