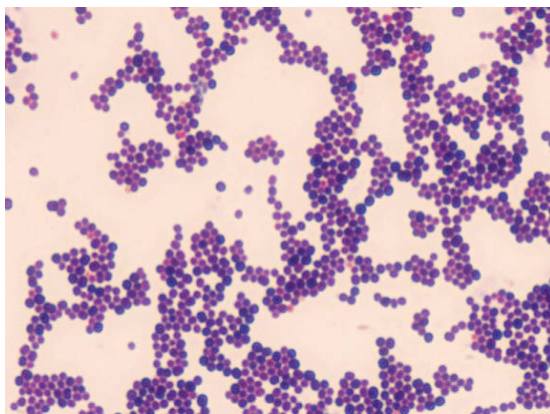


DIFFERENTIAL STAINING: GRAM'S STAINING

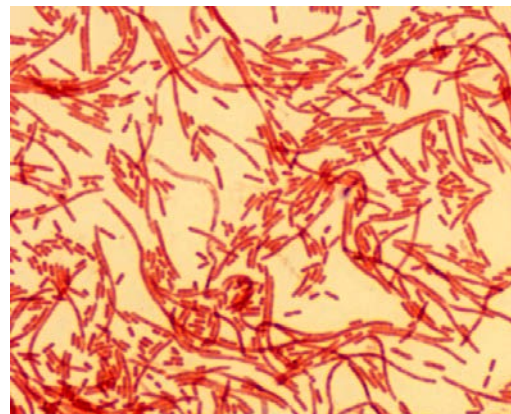
The Gram stain was first developed and used in 1884 by Hans Christian Gram. Gram was in search of a method to visualise cocci in tissue sections of lungs who had died of pneumonia.

In the original method of Christian Gram (1884), the smear was stained with aniline gentian violet treated with Lugol's iodine (Iodine 1g, KI 2g, water 300 ml), decolorized with absolute alcohol, and counterstained with Bismarck brown.

Certain bacteria when treated with basic para-rosaniline dyes such as methyl violet, Crystal violet (Hexamethyl-para-rosaniline 3 chloride) or gentian violet (Mixture of two preceding dyes), and then with iodine, 'fix' the stain so that subsequent treatment with a decolourizing agent - e.g. alcohol, acetone, does not remove the colour. Other organisms, however, are decolourized by this process. If a mixture of various organisms are thus stained and subjected to decolourization, it is found that some species retain the dye i.e., resist decolourization and these bacteria are termed as Gram Positive, whereas others are completely decolourized and takes the counter stain and are termed as Gram Negative bacteria.



Gram Positive Cocci



Gram Negative Rods

Grams staining was later on modified by Kupeloff and Beerman (1922- Decolourization with acetone), Burkes (1922- Decolourization with acetone), Jensen (Decolourization with alcohol), Weigert (Decolourization with aniline-xylol), Preston & Morrell (1962- Decolourization with iodine-acetone), (Gephardt et al., 1981- Use of Saffranin as counterstain) for better results.

Chemical Mechanism of Gram Reaction:

Many theories were put forth to explain why some bacteria resist decolourization and retain the primary dye and some bacteria get easily decolourized and take counter stain *i.e.*, Safranin. Theories include differences in cytoplasmic pH (Gram positive bacteria-2pH and in case of Gram negative-3 pH), and presence of Magnesium ribonucleoprotein in Gram positive bacteria and its absence in Gram negative bacteria have been proposed. But the thickness of Gram positive cell wall due to thick peptidoglycan layer and presence of more lipids in Gram negative cell walls have been accounted for the Gram reaction.

The theory stands as positively charged crystal violet passes through the cell wall and cell membrane and binds to negatively charged components inside the cell. Addition of negatively charged iodine (in the mordant) binds to the positively charged dye and forms a large crystal violet-iodine complex within the cell. Crystal violet (Hexamethyl-para-rosaniline 3 chloride) interacts with aqueous Potassium iodide-Iodine via a simple anion exchange to produce a chemical precipitate. The small chloride anion is replaced by the bulkier iodide, and the complex thus formed becomes insoluble in water. During decolorization, alcohol dissolves the lipid present in the outer membrane of Gram negative bacteria and it leaches the dye-iodine complex out of the cell. A thin layer of peptidoglycan does not offer much resistance either. The dye-iodine complexes are washed from the Gram negative cell along with the outer membrane. Hence Gram negative cells readily get decolorized. On the other hand Gram positive cells become dehydrated from the ethanol treatment, closing the pores as the cell wall shrinks during dehydration. The dye-iodine complex gets trapped inside the thick peptidoglycan layer and does not get decolorized.

The mechanism of Gram reaction was determined in 1983 (Davies et al., 1983 and Beveridge and Davies, 1983). In aqueous solutions crystal violet dissociates into CV^+ and Cl^- ions which penetrates the cell wall and membrane of both Gram Positive and Gram Negative bacterial cells. The CV^+ interacts with negatively charged components of bacterial cells and stains bacterial cells purple. When Iodine is added Iodine (I^- or I_3^-) interacts with CV^+ to form large CVI complex within the cytoplasm and outer layers of the bacterial cell. The small Cl^- anion is replaced by the bulkier iodide, and the CVI complex thus formed becomes insoluble in water. On decolourization, decolourizer interacts with the lipids of the membranes of both Gram Positive and Gram Negative bacteria. The outer membrane of the Gram Negative bacterial cell is lost, leaving the peptidoglycan layer exposed. Gram Negative bacteria do have very thin layer of peptidoglycan, as compared to one to three layered thick peptidoglycan layer of Gram Positive bacteria (Dmitriev, 2004).

Due to action of decolourizer (ethanol) Gram Negative bacterial cell wall becomes leaky (Lipids get dissolved) and allow the large CVI complexes to be washed away from the bacterial cell. Due to thick multilayered nature of peptidoglycan layer in Gram positive bacteria along with the dehydration effect (due to ethanol), the large CVI complex is trapped in the cell wall of Gram positive bacteria. Hence, Gram positive bacteria resists decolourization and retains crystal violet-purple colour, whereas, Gram negative bacteria cell loses the purple colour and is only revealed when the counterstain, positively charged saffranin dye is added.

The length of the decolorization is critical in differentiating the gram-positive bacteria from the gram-negative bacteria. A prolonged exposure to the decolorizing agent will remove all the stain from both types of bacteria. Some Gram-positive bacteria may lose the stain easily and therefore appear as a mixture of Gram-positive and Gram-negative bacteria (Gram-variable).

The reagents for Gram's staining can be made or purchased commercially .

1. Primary Stain: Crystal Violet Staining Reagent.

Solution A

Crystal violet	2g
Ethanol, 95% (vol/vol)	20 ml

Solution B

Ammonium oxalate	0.8 g
Distilled water	80 ml

Mix A and B to prepare 100ml **crystal violet staining reagent**. Store for 24 h, filter through paper & use.

2. Mordant: Gram's Iodine

Iodine	1.0 g
Potassium iodide	2.0 g
Distilled water	300 ml

Grind the iodine & potassium iodide in a mortar and add water slowly with continuous grinding to dissolve the iodine completely. Store in amber coloured bottles.

3. Decolorizing Agent

Acetone	50 ml
Ethanol (95%)	50 ml

4. Counterstain: Safranin

Stock solution:

Safranin O	2.5g
Ethanol (95%)	100 ml

Working Solution:

10 ml Stock Solution
90 ml Distilled water

*Freshly made staining reagents are recommended.

Procedure: (Gram staining modified by Gephardt et al, 1981)

1. Fix the air dried smear by passing over the flame 2-3 times.
2. Flood the fixed bacterial smear with crystal violet staining reagent for 1 minute.
3. Wash the slide gently with distilled water for 2 seconds.
4. Flood the slide with Gram's Iodine(mordant) and wait for 1 minute
5. Wash the slide gently with distilled water for 2 seconds.
6. Flood the smear with decolourizing agent - by adding drop by drop - decolourizer, untill the smear becomes clear (15 seconds).
7. Wash the slide gently with distilled water for 2 seconds.
8. Flood the smear with counterstain, safranin. Wait for 30 seconds to 1 minute.
9. Pour off the stain from smear and wash the smear with tap water.
10. Air dry the stained smear and observe under oil immersion using a Brightfield microscope.

Interpretation: Gram Positive bacteria will stain purple , whereas , Gram Negative bacteria stains pink red colour.

KOH string test :

KOH string test may be used as a confirmatory test for the Gram Stain (Arthi et al., 2003). The formation of a string (DNA) in 3% KOH indicates that the isolate is a Gram-negative organism.

Procedure:

1. Place a drop of 3% KOH onto a glass slide.
2. Emulsify in KOH a loopful of the culture from a Blood agar incubated for 18-24 hours.
3. Continue to mix the suspension for 60 sec and by slowly lifting the loop, observe for the formation of a string.

Interpretation:

Gram-negative cells form a string within 60 seconds.

Gram-positive cells are not affected.

Exercise:

1. Draw well labelled diagrams of Gram negative and Gram positive bacterial cell wall.
2. Prepare the smear, fix and stain with Grams staining. Write the results.

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