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Expt → Identification of Bacteria Using Gram Staining.

Aim

To become familiar with:

- The chemical and theoritical basis of cell wall characteristics of bacteria.
- The differential staining to differentiate the bacterial morphology.
- The performance of the procedure for differentiating between the 2 principal groups of bacteria: Gram positive and Gram negative organisms.

Principle

Gram staining was introduced by Danish Bacteriologist Christian Gram in 1880. It is the most widely used differential staining in bacteriology which helps to classify bacteria as either Gram +ve or gram -ve.

Gram positive bacterial have cell walls composed of 60-90% peptidoglycan layer with peptide interbridges. Teichoic acid are covalently linked with peptidoglycan layer or the plasma lipids (lipo-teichoic acids).

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Teichoic acids appear to extend to the surface of peptido glycan layer & structurally it is integrated with PO_4 ions. Thus it is contributing - the negative charge on the gram positive bacterial cell surface and helps to bind with basic dye which is used to stain the cells. Gram positive bacterial Gram negative bacteria have a thin peptidoglycan layer of only 10-20% and an outer membrane composed of lipopolysachrids. The outer membrane consists of 3 components parts : Lipid A, core polysaccharides, ~~The outer~~ and O side chains. The core polysaccharide is integrated with PO_4 ions and thus contributing the surface negative charge to Gram-negative bacteria cell surface. This property is used for staining of gram -ve bacterial cell surface with basic dyes.

Differential staining requires the use of at least four chemical reagents that are applied sequentially to a heat-fixed smear - the ~~pt~~ primary stain, the mordant, the decolorizing agent and the counter-stain.

The procedure involves four steps :-

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- Bacterial Heat-fixed smear on the slide is first treated with a basic dye, crystal violet (CV). At this stage all the bacteria take up the primary stain and appear purple.

- In the second step, after washing, the smear is treated with iodine solution which acts as a mordant. Iodine conjures with crystal violet to form a CV-I complex inside the cell which intensified the color of the stain and all the cells will appear purple-black at this point.

- In the third step, smear is treated with alcohol to decolorize the stained cells. During this process, some bacteria will lose the CV-I complex whereas others will retain it.

- In the fourth step, after washing, smear is treated with a counter-stain, safranin. At this stage, all the bacteria which had lost the primary stain will take up the counter-stain and appear red. The rest, which retained the CV-I caplex will remain violet.

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Thus, after the completion of the above four steps, bacteria may appear either violet or red. The violet-colored ones are called Gram +ve and red-colored ones are called gram-negative.

Alcohol is a lipid solvent and it solubilizes some of the lipid content of the outer lipopolysaccharide layer of gram-ve bacteria leaving minute pores through which the CV-I complex oozes out. In gram-positive bacteria, this CV-I complex binds to cell wall more firmly as magnesium-ribonucleic acid-crystal violet iodine (Mg-RNA-CV-I) complex, which is difficult to remove.

Factors such as age of the culture, density of the smear, the length of the time involved in different steps etc. will affect the staining result.

Materials Required

- Cultures: 18-24 hours old nutrient agar slant cultures solutions
- Reagents: Crystal violet, Gram's Iodine, 95% ethyl alcohol, safranin
- Equipment: Inoculating loop, staining tray, glass

slides, blotting paper, microscope.

Preparation of reagents

1. Gram's Crystal violet (Hucker's modification)

Solution A

Crystal violet - 2.0 g

Ethyl alcohol - 120 ml

Dissolve the stain completely in solution A.

Solution B

Ammonium Oxalate - 0.8 g

Distilled water - 80 ml

Solutions A and B are mixed, filtered and stores in bottles.

2. Gram's Iodine

Potassium Iodine - 2.0 g

Distilled water - 10 ml

Dissolve the potassium Iodide in the distilled water. Add 1.0 g of Gram's iodine in 10 ml of distilled water and dissolve completely and make up to 300 ml.

→

Safranin solution :

Safranin

Distilled water - 100 ml

Dissolve the safranin in distilled water and filter.

Procedure :

- Microscopic slides were cleaned thoroughly with detergent and water, followed by wiping with alcohol.
- Using sterile inoculation loop, a smear of the test bacteria was prepared the cells were spread by means of a circular motion by the inoculation loop.
- The smear of allowed air to dry & then the heat fixing of smear was done.
- Crystal violet stain was applied to the smear and left undisturbed for one minutes
- Gram's iodine was decolorized with 95% ethyl alcohol, the alcohol was added drop wise until the crystal violet fails to get washed away from the smear.
- After washing, the smear was stained with the counter stain safranin and left undisturbed for one minute.

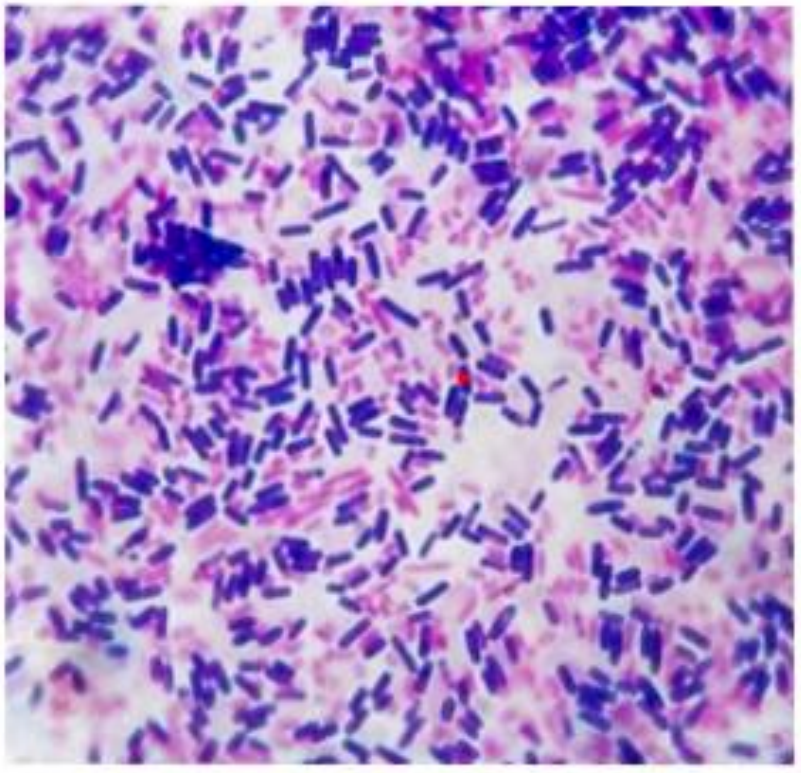
. The slide was air-dried after washing with tap water and observe under the microscope.

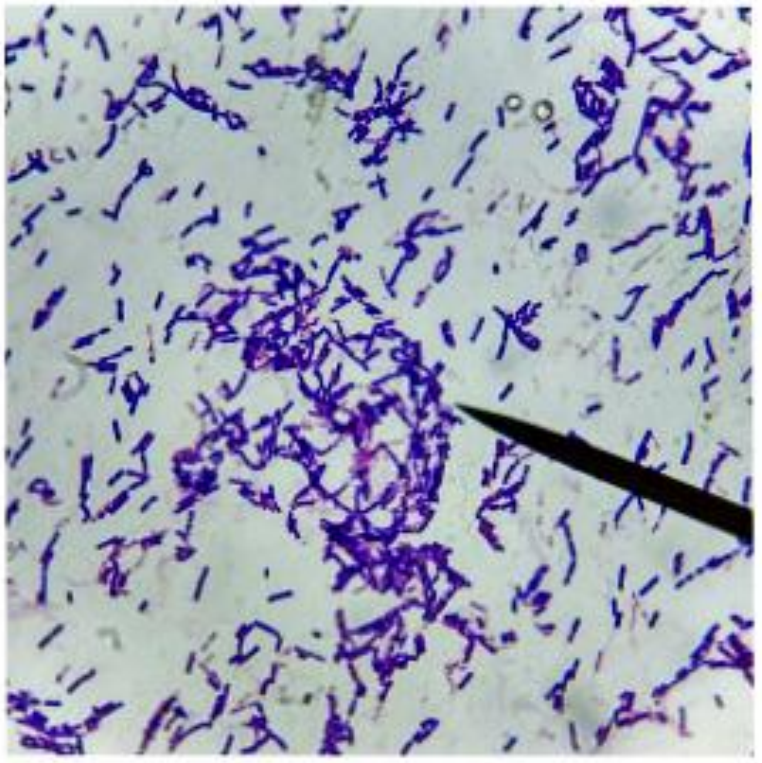
Precautions

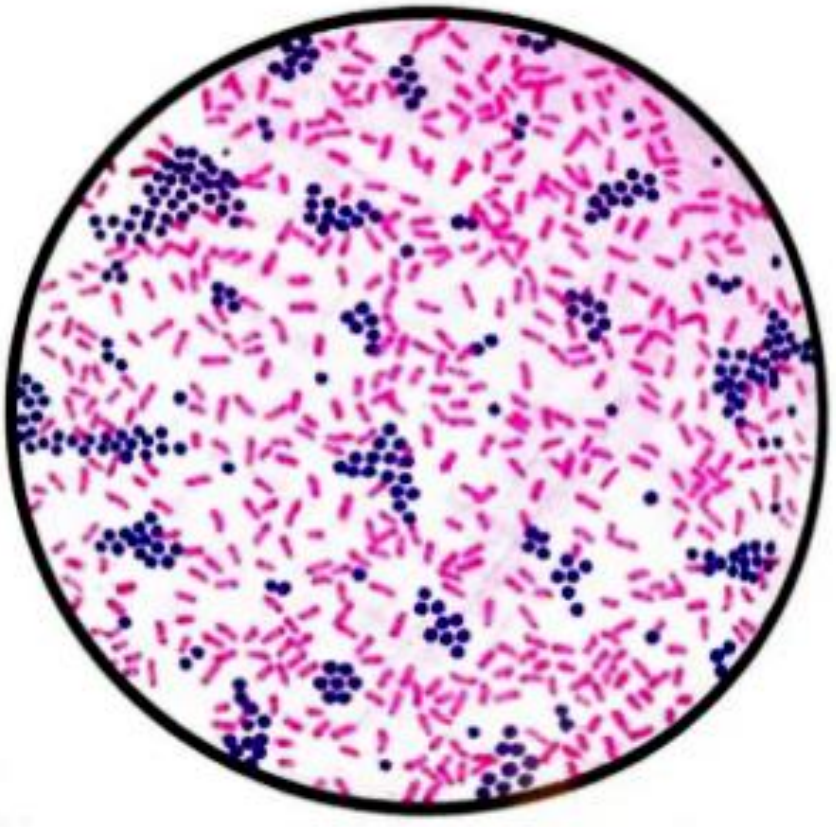
The most critical phase of the procedure is the decolorization step, which is based on the ease with which the CV-I complex is released also and caused them to appear Gram -ve under decolorization will not completely remove the CV-I complex and cause Gram -ve bacteria to appear Gram +ve strict adherence to all instructions will help remedy past of the difficulty but individual practice and experiment experience is the key to obtain proper decolorization.

It is imperative that the slide be throughey washed under running tap water between application ~~and prepare~~ of the reagents. This removes excess reagent and prepare the slide for application of the subsequent reagent.

The best gram stained prepared are made with fresh cultures i.e., those not older than 24 hours. As culture age, especially in the case of gram +ve cells, the bacteria lose their ability to retain the primary stain and may appear Gram-variable, i.e. ~~Gram~~ some cells will appear purple while others will appeared.







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Observations :

Violet colored, spherical shaped bacteria in singles %, pairs tetrades, short chains & irregular grape like cluster seen along with pink colored, rod shaped bacteria that are present haphazardly. If the bacteria stays purple, they are Gram +ve, & the gram -ve cell is pink to red.

Inference

The given smear contain both the gram +ve cocci and the gram -ve bacilli.

Result

The staining technique used gram +ve bacteria will appear purple under a microscope and gram -ve bacteria will also provide information about your infection and will appear pink, the shape, size and quantity of bacteria will provide information about your infection.

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