

Practical No.6

A- Special Staining Techniques:

This procedure is called so, when certain bacteria, or some of their structures could not be stained or seen or being differentiated with other bacterial cells when using the ordinary staining techniques. Such procedures are; staining of: spores, DNA, flagella, capsule,....etc., or staining of *Mycobacterium tuberculosis*, or *M.leprae*, Nocardia, or Actinomyces.

1- Ziehl Neelsen stain (acid fast stain):

This technique is used for staining of *Mycobacterium tuberculosis* (the causative agent of tuberculosis), *Mycobacterium leprae* (the causative agent of leprosy), Nocardia and Actinomyces (the causative agents of nocardiosis and actinomycosis respectively). These bacteria when stained with strong basic dyes, such as, strong carbol fuchsin (with steaming) they retain the stain even when strong decolourizing agents are used, such as; 20% H₂SO₄ (in case of *M. tuberculosis*) hence are called acid fast bacteria, all other bacteria will loose the stain, and then could be stained with the counter stain, therefore this stain could also be called differential stain.

The above mentioned bacteria possess a complex lipids in their cell wall which is called wax D, composed of two molecules of mycolic acid and one molecule of the sugar trehalose, which mainly gives the resistance to staining by ordinary stains, and also lead to the formation of what is called serpentine cords when a smear is taken from a colony on Lownstein Jensen medium.

Procedure for Acid Fast Stain;

- 1) the specimen is taken from either a suspected patient with tuberculosis, or from formalin killed culture, or from a non pathogenic species of Mycobacterium, such as *M. phlei*. Make a heat fixed smear from the specimen or the bacteria you obtain (as shown earlier).
- 2) Flood with strong carbol fuchsin stain, and steam for 5 minutes
- 3) Leave to cool down, hold the slide with a forceps in an angle position and add 20% H₂SO₄ or acid alcohol until no more color runs off.
- 4) Wash with water and add the counter stain which is methylene blue.
- 5) Wash with water, leave to dry in an angle position, examine by oil immersion lens for red bacilli.

Exercise; examine and draw what you see.

2- Spore stain;

Bacterial spore is not a reproductive stage as that occurs in case of fungi, but rather it is some sort of protective stage that some bacteria is passing through when facing certain abnormal or hard conditions. Bacterial sporulation goes in one direction, in other words, as soon as the bacteria start its sporulation process it will not go back to the vegetative form even when the good conditions for growth is resumed and there is no any factor affecting growth. Each cell forms one spore, and each spore will germinate to one cell.

During sporulation process, the cell forms several layers; **1) the core**; which contains its DNA and some ribosomes and dipicolinic acid, and some other most important factors that keep the cell resistant to most adverse conditions **2) the cortex** (consist of double layer of peptidoglycan , differs from that of the cell wall), **3) the spore coat**, consist of solid resistant protein, **4) the exosporium**, consist of keratinized protein.

The above layers give the spore protection against several hard environmental conditions, at the same time, become difficult to stain the spore with the ordinary staining techniques; therefore its staining requires a special procedure such as steaming in order to allow the stain to penetrate into the spore.

Procedure for spore staining;

- 1) Prepare a heat fixed smear of a spore forming bacteria such as, *Bacillus subtilis* or any species of Clostridium.
- 2) Flood with malachite green, and steam for 5 minutes
- 3) Leave to cool down and wash with water
- 4) Add safranin , as a counter stain, for 1 minutes
- 5) Wash with water, leave to dry in upright position, examine under the oil immersion lens.

Exercise; Examine and draw what you see under the microscope

3- Capsule stain;

Capsule is a gelatinous spherical or oval shape surrounding certain species of bacteria as a protective extracellular structure, protect the cell from phagocytosis by phagocytic cells, therefore this structure is considered, in several species of bacteria, as a virulence factor, and its loss leads to the loss of the virulence in that bacteria.

The capsule is consisting of 97-98% water, and only 2-3% of dissolved solids. These solids differ with the different species, may be consisting of polysaccharides, glycoproteins, polysaccharides with certain lipids....etc.

Since the capsule consisting of large amount of water, it requires a special procedure for staining.

One of the procedures is called Hiss method;

- 1) Prepare a smear for bacteria that possess a capsule, such as; Klebsiella or Pneumococci, and heat fix very gently on the flame to avoid damaging of the capsule by excess heat.
- 2) Flood the smear with 1% aqueous solution of crystal violet.
- 3) Steam very gently for 2-3 minutes
- 4) Leave to cool and rinse with 20% copper sulfate.
- 5) Leave to dry, and examine under the oil immersion lens.

Another procedure: staining with India ink:

- 1) Place a loop-full of capsulated bacteria on one end of the slide.
- 2) Add a drop of India ink on the bacteria.
- 3) With another slide withdraw the mixture to make a thin film.
- 4) Allow to dry, and examine under the oil immersion.