14

STAINING TECHNIQUES FOR DEMONSTRATION AND IDENTIFICATION OF MICROORGANISMS

14.1 INTRODUCTION

Traditional methods for the demonstration of microorganisms in tissue sections can only be based upon staining characteristics and simple morphology because the organisms are fixed and dead. When it is suspected that a disease process may be caused by, or complicated by an infective agent, a sample of fresh tissue is normally provided for microbiological evaluation. The most effective means of isolating and identifying individual species of most organisms is to study their growth patterns and morphology in vitro. The study of these criteria has formed the basis for the identification and classification of microorganisms

OBJECTIVES

After reading this lesson, you will be able to:

- describe Ziehl-Neelsen stain
- demonstrate Fite acid fast stain
- explain periodic acid Schiff, Mucicarmine stain.

IDENTIFICATION OF BACTERIA

Most of the bacteria are demostrated by

Notes

14.2 GRAM STAIN

Some of the bacteria like mycobacterium do not get stained with Gram stain due to their lipid capsule.Ziehl-Neelsen stain is applied to identify mycobacteria which are stained pink to red in colour.

14.2.1 Gram Staining

This reaction may depend on the difference in permeability of cytoplasmic membrane.During staining a dye iodine complex is formed whithin the cell,which is insoluble in water.The complex diffuses freely from gram negative organisms but diffuses less in Gram positive bacteria due to poor membrane permeability.

14.2.2 Preparation of reagents

Crystal violet solution

Crystal violet	1.0gm
Absolute alcohol	20ml
Ammonium oxalate(1%)	80ml

Basic fuchsin

Basic fuchsin	1.0gm
Distilled water	100ml

Gram's iodine

Iodine crystal	1.0gm
Potassium iodide	2.0gm
Distilled water	300ml

14.2.3 Procedure

- Deparaffinize the section and bring it to water
- Put crystal violet for one minute
- Add Gram's iodine for 30 seconds
- Differentiate by dipping the section once or twice in alcohol
- Wash with water and counterstain with safranin for 45 seconds
- Wash with water

- Air dry and mount in DPX.RESULT _Gram positive bacteria stain blue black.
- Gram negative bacteria stain red or pink.
- Some of the bacteria like mycobacterium do not get stained with Gram stain due to their lipid capsule.Ziehl-Neelsen stain is applied to identify mycobacteria which are stained pink to red in colour .

14.3 ZIEHL-NEELSEN STAIN (ACID FAST STAIN)

Aim: To demonstrate Mycobacterium Tuberculosis in paraffin sections.

Principle: When Mycobacterium Tuberculosis are stained with a strong reagent like basic fuchsin in aqueous 5% phenol at high temperature the bacilii resist decolourization by strong acids (20% sulphuric acid). Any decolourized non AFB is counterstained with methylene blue or malachite green.

Control: Mycobacterium tuberculosis positive section

Reagents

1. Ziehl-Neelsen's carbol fuchsin

Basic fuchsin	1gm
Absolute alcohol	10ml
5% phenol (Aqueous)	100ml

Dissolve basic fuchsin in alcohol, and then add 5% phenol.

2. Methylene blue solution

Methylene blue	1gm
Distilled water	100ml

Procedure

- De-wax the sections in xylene and bring to water.
- Flood sections with carbol fuchsin and heat to steaming by intermittent flaming for 10 to 15 minutes or stain in coplin jar at 56°-60°C (oven or water bath) for 30 minutes.
- Wash in water to remove excess of stain.
- Differentiate with 20% sulphuric acid or in 3% hydrochloric acid in 70% alcohol until the tissue is very pale pink colour. Then washed in water (for 5 to 10 minutes).

Notes

- Wash in water.
- Counterstain in 1% methylene blue for 10 to 15 seconds.
- Wash in water.
- Dehydrate, clear in xylene and mount in DPX.

Result

Acid-Fast Bacilii	–	red
Nuclei	–	blue
Back ground	–	pale blue

14.4 FITE ACID FAST STAIN - LEPROSY

Aim: To demonstrate mycobacterium leprae (leprosy), in formaline fixed sections.

Principle: This technique combines peanut oil with xylene, minimizing the exposure of the bacteria's cell wall to organic solvent. Thus acid fastness of bacteria is retained.

Control: Leprosy positive tissue.

Reagents

1. **Xylene/Peanut Oil Solution:**

Xylene	50.0 ml
Peanut Oil	50.0 ml

   Mix well. Label with date, solution is stable for 1 year

   Caution: The solution is inflammable, irritant

2. **Ziehl-Neelsen Carbol-Fuchsin:**

   As described before

3. **1% Acid Alcohol**

4. **Methylene Blue:**

Methylene blue	1.0 gm
Distilled water	100 ml

Procedure

- De-paraffinize the section for two minutes in xylene and peanut oil mixture

- Drain, wipe excess of oil
- Place the slide in water until ready to stain
- Put Carbol - fuchsin, for 30 minutes, at room temperature
- Wash in tap water
- Dip acid alcohol until pale pink, dip until stain stops running
- Wash in tap water
- Counter-stain in methylene blue solution for 30 seconds
- Wash in tap water
- Blot and air dry
- Dip in xylene and mount with DPX and put coverslip

Results

Acid-fast bacilli – red

Background – blue

Note: Mineral oil may be substituted for peanut oil.

14.5 PERIODIC ACID – SCHIFF (PAS) STAIN

Periodic acid causes oxidation of 1:2 glycol groups in the tissues to di-aldehydes. The di-aldehyde reacts with fuchsin – sulfurous acid solution(Schiff's) to form a magenta colored compound.

Aim: To demonstrate glycogen, epithelial mucin, fungi, ameba and basement membrane.

Control: Liver and intestine

Reagents

Periodic acid 1%

Distilled water 100 m

Schiff's reagent

Basic fuchsin 1 gm

Distilled water 200 ml

1N hydrochloric acid 20 ml

Sodium or Potassium metabitesulfite1gm

Activated charcoal 2gm

Notes

- Dissolve basic fuchsin in boiling distilled water
- Shake for 5 minutes and cool to 50°C
- Filter and add 1N solution.
- Cool further and add sodium or potassium metabisulfite. Keep for 18 hours in dark.
- Add activated charcoal, shake well, filter and store the solution at 40°C.

Procedure

- Bring the sections to water.
- Dip the slide in Periodic acid solution for 5-10 minutes.
- Wash in tap water and rinse in distilled water.
- Put Schiff's reagent on the section for 20 minutes.
- Wash thoroughly in running water.
- Counerstain with Hematoxylin, dehydrate, clear and mount in DPX.

Result

- Glycogen (except non-sulfated acid mucopolysaccharide), basement membrane, fungi, parasites and other positive substances – magenta
- Nucleus – blue or violet

Giemsa stain

Aim: It is used to demonstrate

- Bacteria, Hematologic element, Bone marrow elements, Blood parasites

Reagents

Giemsa stain

Stock solution:

Giemsa powder	1.0 gm
Methyl alcohol	66 ml
Glycerin	66 ml

Add glycerin to Giemsa powder and put in oven at 60°C for 30 minutes to 2 hours (until the stain dissolves). Then add methyl alcohol.

Working solution:

Stock solution	1.25 ml
Methyl alcohol	1.50 ml
Distilled water	50 ml

Procedure

- Deparaffinize and take the section to water.
- Place the slide in working Giemsa solution overnight.
- Wash with tap water.
- Differentiate with 0.5% aquous acitic acid.
- Dehydrate and mount.

Result

Nuclei	–	blue
Malarial parasite	–	purple
Collagen and other elements	–	pink

Note:

1. Sections stained at room temperature for longer period show better results than sections stained at higher temperature for shorter period.
2. Differentiation with acetic acid removes only blue dye hence gives better red intensity.
3. Giemsa reagent improves with age.

14.6 MUCICARMINE STAIN

Aim: To demontrate mucin glycogen in tissues.

Principal: Aluminum is believed to form a chelation complex with carmine changing the molecule to a positive charge allowing it to bind with the acid substrates of low density such as mucus.

Reagents

1. Mucicarmine solution

Carmine alum lake	1.0 gm
Aluminum hydroxide	1.0 gm
50% alcohol	100 ml

- Mix well and add anhydrous Aluminum chloride 0.5 gm
- Boil gently for 2 to 3minutes, cool, filter and refrigerate (may be stored for 6 months)

Notes

2. Metanil yellow solution

Metanil yellow	0.25 gm
Distilled water	100 ml
Glacial acetic acid	0.25 ml

Mix well (may be stored for one year)

3. Harris hematoxylin

Procedure

- Deparaffize and bring the section to water.
- Mayer's hematoxylin for 10 minutes.
- Wash in running tap water for 5 minutes.
- Mucicarmine solution for one hour at room temperature.
- Rinse quickly in distilled water.
- Metanil yellow for 30 seconds to 1minute.(optional)
- Three changes of absolute alcohol.
- Dehydrate, clear and mount in DPX.

Result

Mucin	–	deep rose
Nuclei	–	black
Other tissue elements	–	yellow (if metanil yellow is used)
	–	colorless (if metanil yellow is not used)
Capsule of cryptococci (fungus)	–	deep rose

Control small intestine

14.7 GROCOTT-GOMORI'S METHANAMINE SILVER STAIN

Aim: To demonstrate fungi and bacteria particularly to stain carbohydrate. Cell wall of fungi like Pnemocystis Jiroveci is outlined by black stain.

Reagents

- 0.5% aqueous periodic acid

- Methanamine silver stock solution

3% methanamine	100ml
5% silver nitrate	5ml

- Add the silver nitrate solution to methanamine solution and mix it properly. A white precipate will form and redissolve on shaking. Filter the stock solution in brown bottle (stable for 6 months at 4°C).

  Methanamine silver working solution-

Stock solution	50ml
5% sodium borate	5ml

1. Mix well and filter.
2. Preheat for 10 to 20 minutes at 60°C prior to actual use.
3. 0.2% gold chloride
4. 3% sodium thiosulfate
5. Light green

Procedure

- Deparaffinze slides to distilled water.
- Oxidize in 0.5% periodic acid for 15 minutes at room temperature.
- Rinse three time in distilled water.
- Incubate the slides in methanamine silver working solution for 30 minutes to one hour at 60°C.
- Rinse in hot distilled water and check microscopically.
- Rinse in distilled water at room temperature.
- Tone in gold chloride solution for one minute.
- Rinse in distilled water.
- Treat with sodium thiosulfate solution for 2 minutes.
- Wash in running tap water for 10 minutes.
- Counterstain in nuclear fast red or light green for 5 minutes.
- Dehydrate, clear in xyline and mount in DPX.

Result

Basement membrane	black
Fungi cell wall	black

Notes

Background	pink or green (depends on counterstain nuclear fast red or light green)
Control	kidney or skin

INTEXT QUESTIONS 14.1

1. Mycobacterium Tuberculosis in paraffin section is demonstrated by ....
2. .... or .... is used as counter stain
3. Periodic acid Schiff stain is used to demonstrate ...., ... & ....
4. In periodic acid Schiff stain the nucleus appears .... & glycogen, fungi, parasites appear ....
5. Mucin in tissue is demonstrated by .... stain
6. .... stain is used to demonstrate fungi and bacteria
7. In Grocott-Gromori's methanamine silver stain the fungal cell wall and basement membrane appears .... & background appears ....
8. The control used in Grocott-Gromori's Methanamine silver stain is .... or ....

WHAT HAVE YOU LEARNT

- Ziel-Neelson stain is used to demonstrate mycobacterium tuberculosis in paraffin sections
- Methylene blue or malachite green is used as counter stain
- Fite acid fast stain is used to demonstrate mycobacterium leprae in fromaline fixed sections and this technique combines peanut with xylene
- Periodic acid schiff stain is used to demonstrate glycogen, epithelial mucin, fungi and amaeba
- Liver and intestine is used as control in periodic acid Schiff stain
- Giema stain is used to demonstrate bacteria and blood parasites

- In Giema stain nuclei appears blue, malarial parasite appears purple and collagen and other elements appear pink
- Mucin in tissue is demonstrated by mucicarmine stain
- Grocott-Gomori's Methanamine silver stain is used to demonstrate fungi and bacteria particularly to stain carbohydrate

TERMINAL QUESTIONS

1. What are the stains which may be used to demonstrate fungi?
2. What is the principle of silver methanamine staining?
3. What is the difference in Acid Fast staining for Mycobacterium Tuberculosis and Leprae?
4. What are the uses of Giemsa stain?
5. What control is used in PAS stain?

ANSWERS TO INTEXT QUESTIONS

14.1

1. Ziel-Neelson stain
2. Methylene blue or Malachite green
3. Glucogen, fungi & ameba basement membrane
4. Blue or violet, Magenta
5. Mucicarmine
6. Grocott-Gomori's Methanamine Silver stain
7. Black, Pink or green
8. Kidney or skin