Staining of bacteria

- Microorganisms are transparent
- So, it is difficult to see them in an unstained state.
- When stained, they become opaque and the contrast between them & the surrounding medium is increased.
- they become clearly visible.

Importance of staining:

- To enable morphological features.
- To preserve them for future study.
- To differentiate bacteria.

- Preparation of smear:
- Smears should be spread evenly covering an area of about 15-20mm diameter on a slide.
- Fixation of smear: It is a process by which the internal and external structures of the cells and microorganisms are preserved & fixed in position. It also prevent smear being washed from slides during staining procedure.it also kills the microbe.

- There are two types (methods) of fixation:
- Physical Method: Heat fixation
- Allow the smear to air-dry completely.
- Rapidly pass the slide with smear upper most, three times through the blue flame of the spirit lamp or pilot flame of Bunsen burner. Allow the smear to cool before staining.
- Disadvantage: It can damage the organism & alter the staining reactions especially when excessive heat is used.
- It also damages leucocytes. So it is unsuitable for fixing smear which contains intracellular organisms such as N.gonorrhoeae & N.meningitidis.

- **Chemical fixation:**
- Chemicals – Alcohol , Acetic acid, Mercury chloride , Formaldehyde , Glutaraldehyde
- Method:

1. Allow the smear to air-dry completely.
2. Add one or two drops of chemicals.
3. Leave the alcohol on smear for a minimum 2 min, or until the chemical dries on the smear.
4. Advantage: Less damaging to microorganism than heat.
5. Pus cells are well preserved. So recommended for fixing the smear when looking for gram negative intracellular diplococci.

- It is more bactericidal than heat. So it is preferable to fix the smear prepared from sputum (M.tuberculosis)

Types of stain:

1) Simple stain:

- Single staining agent is used
- Basic dyes such as crystal violet, methylene blue, carbol fuchsin are used.

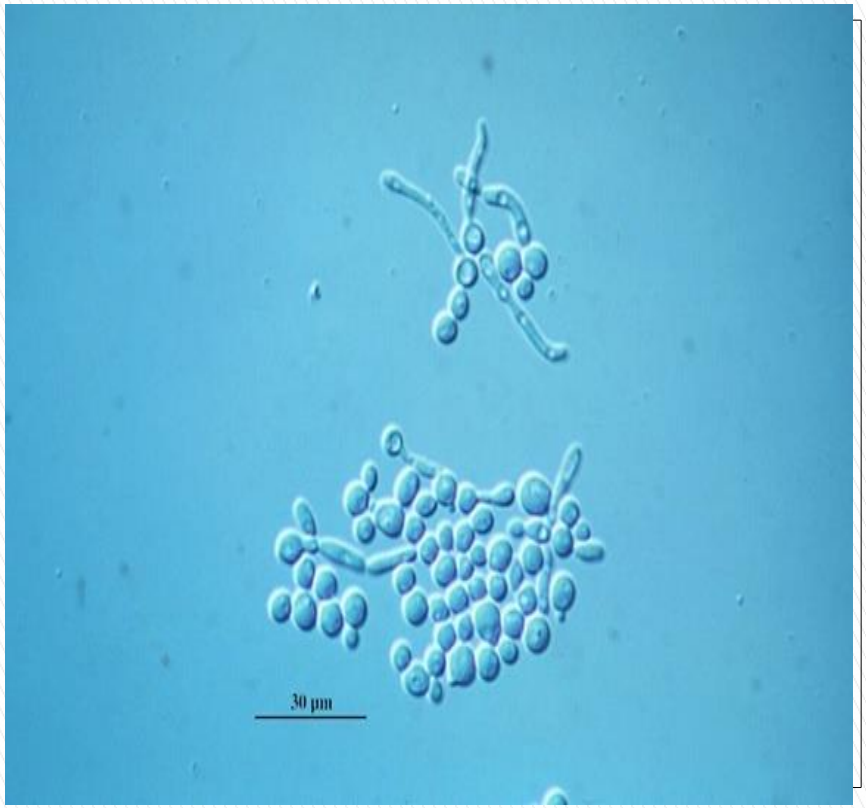
2) Differential stain:

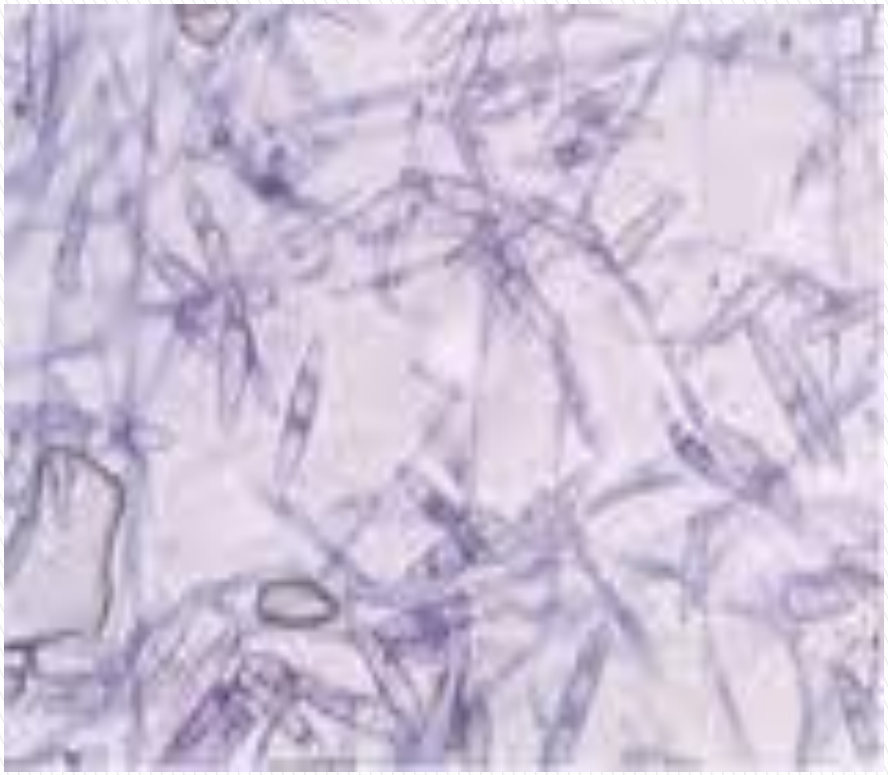
- Divide bacteria into separate groups based on staining properties. Gram stain, Acid fast stain

3) Stains used for specific structures

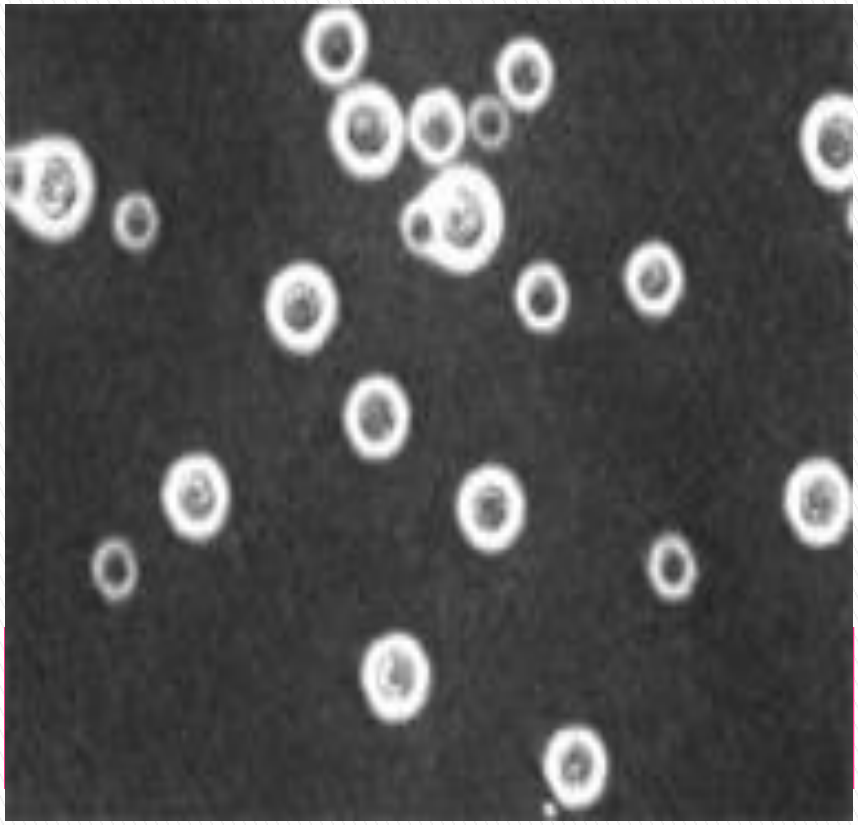
- Negative staining – for capsule demonstration dye such as India ink or nigrosine used
- Impregnation stain – Bacterial flagella are thin, thread like structures which cannot be seen under light microscope. They are thickened by coating them to see under light microscope.
- Modified acid fast stain — for spore demonstration
- Albert stain — for metachromatic granules

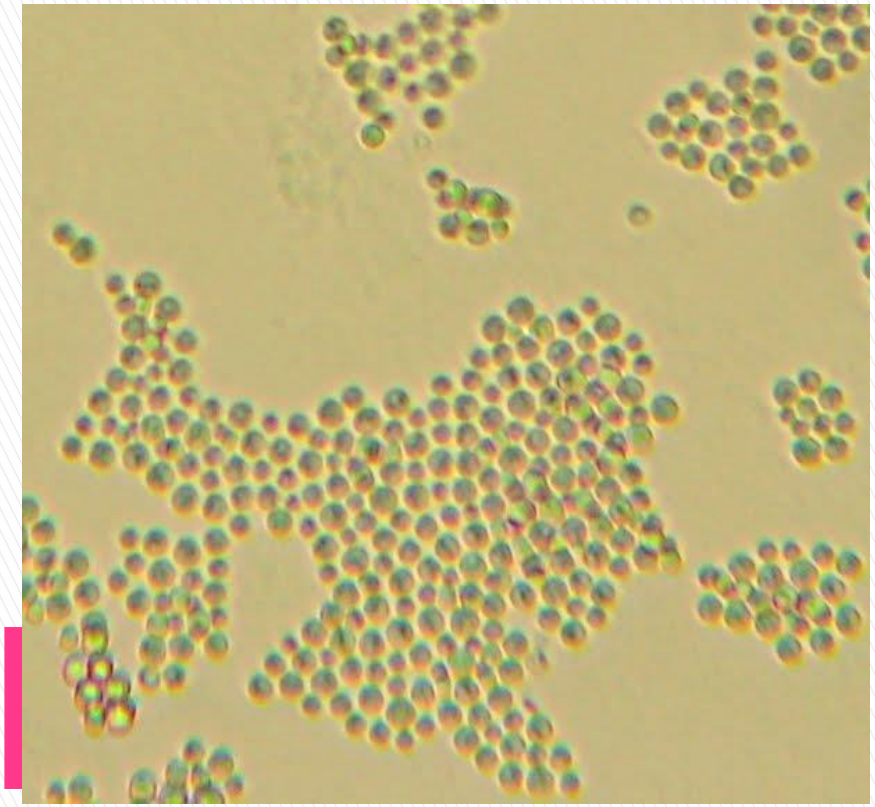
SIMPLE STAIN



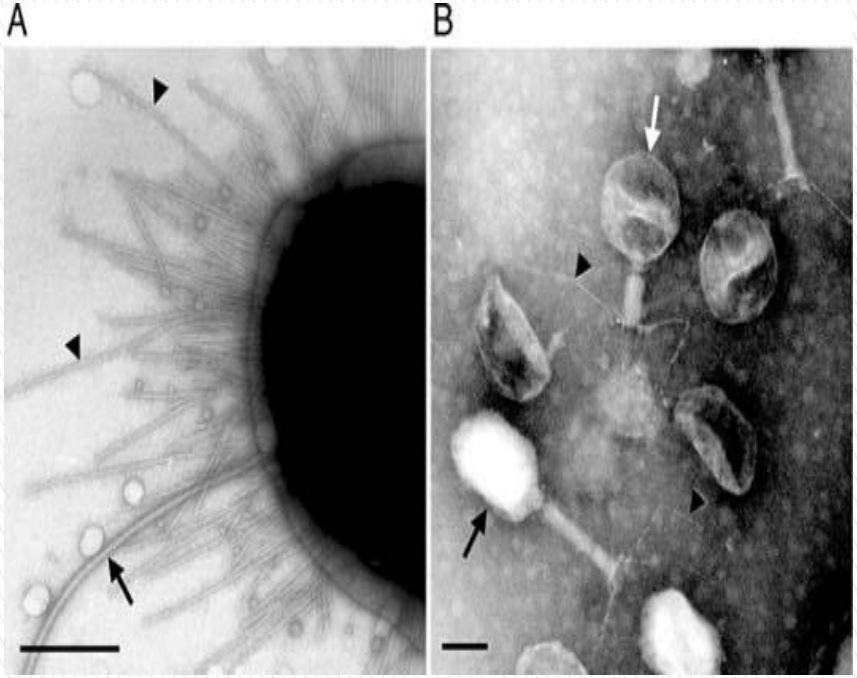


NEGATIVE STAIN

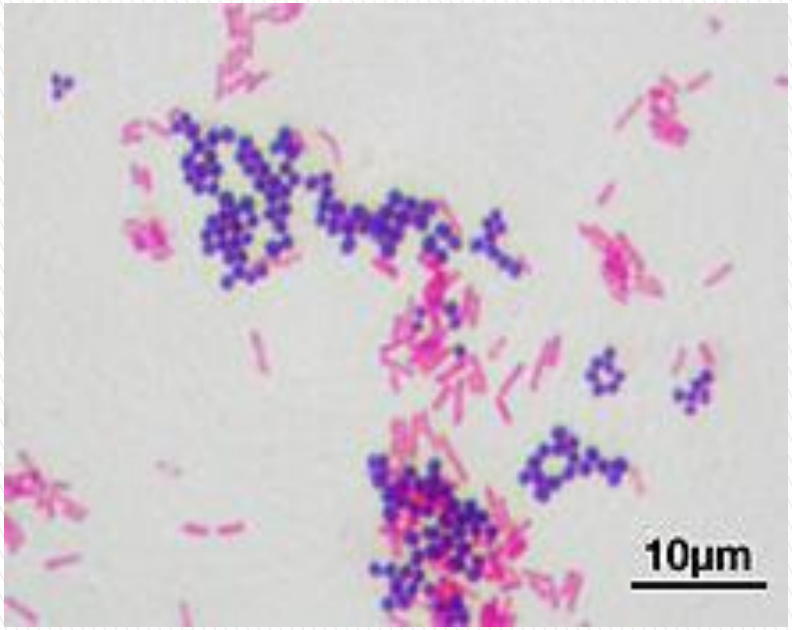




IMPREGNATION STAIN



DIFFERENTIAL STAIN



ACID FAST STAIN (ZIEHL-NEELSEN METHOD)

- It was first discovered by Ehrlich and modified by Ziehl & Neelsen in 1881.
- It is a differential staining method used to differentiate acid fast organisms like mycobacteria from non acid fast organisms.
- **Principle**:
- Organisms like mycobacteria are coated with a thick, waxy material (mycolic acid) that resist staining. So, once stained the bacterial cells resist decolorization by strong organic solvents such as acid alcohol.

- In Ziehl-Neelsen's staining, carbol fuchsin (basic fuchsin + phenol) is applied with heat. Heat and phenol facilitate penetration of the dye and act as mordant.

- Subsequently when decolourized by acid, the dye does not come out because it is soluble in phenol and phenol is more soluble in lipid substances (mycolic acid) so there is no decolorization and they retain the colour of basic fuchsin (primary stain).

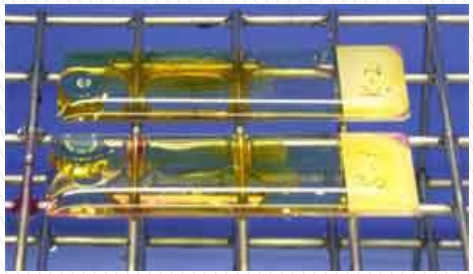
- **Procedure:**
- Cover the fixed smear with the filtered carbol fuchsin and heat until steam rises. Do not allow to dry or boil the stain. Allow to act for 5-10 minutes with intermittent heating.
- Wash with clean water.
- Decolourize with 20% sulphuric acid (H_2SO4) or with 3% acid-alcohol till the red colour of the smear changed to yellowish brown.
- Wash with clean water.
- Counterstain with methylene blue for 1-2 minutes.
- Wash with clean water and wipe the back of the slide clean and allow to air-dry.
- Examine the smear microscopically with oil immersion objective.

ZN STAINING PROCEDURE

Carbol fuschin

Intermittent heating

Decolorizer

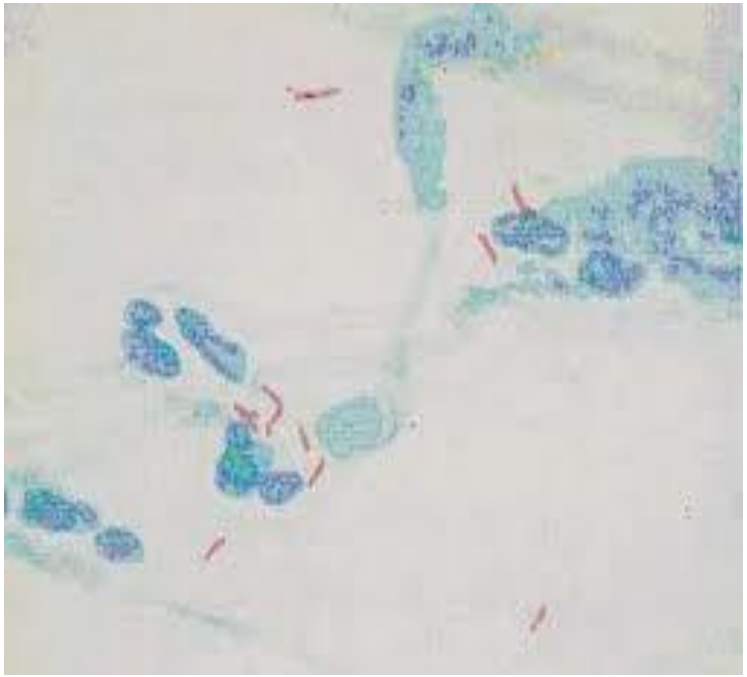


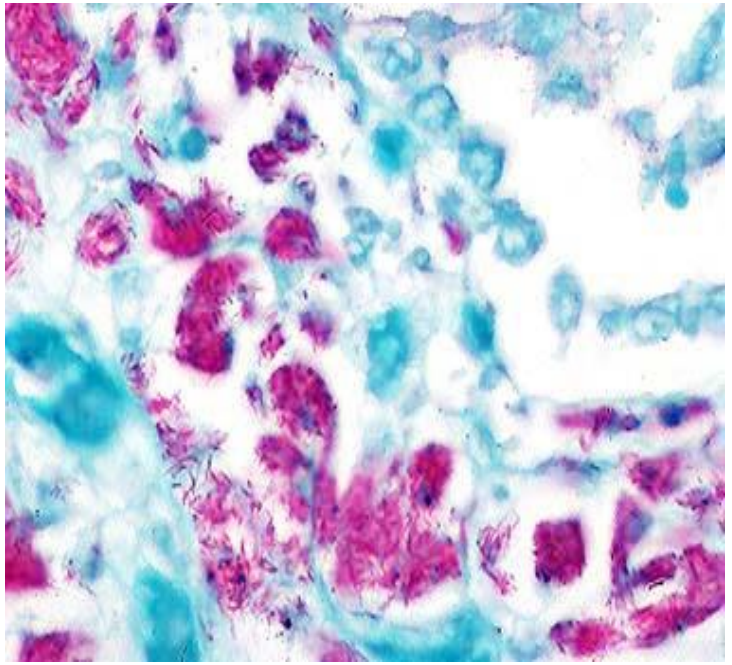
counterstain

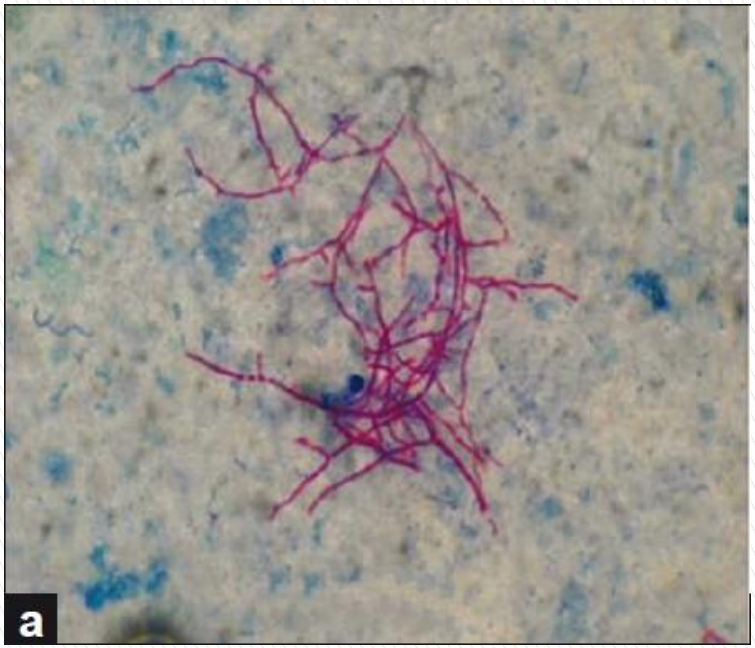
Acid fast

Non acid fast

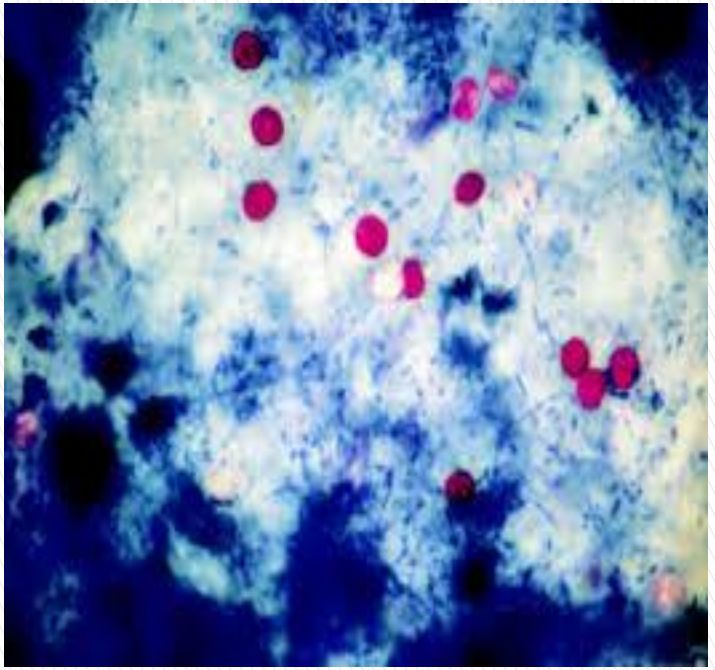
- **Observation:**
- Acid fast bacilli appear bright red
- Pus cells, epithelial cells and other organisms appear blue



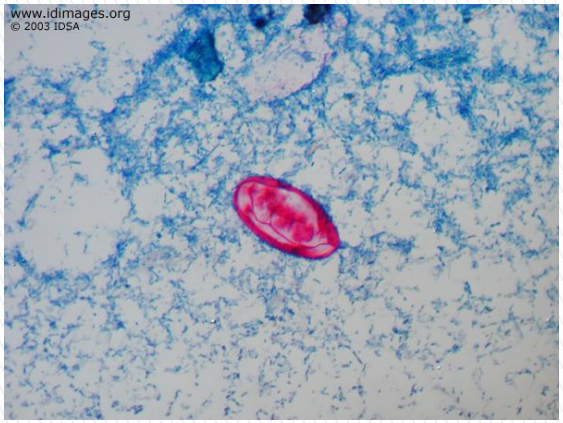




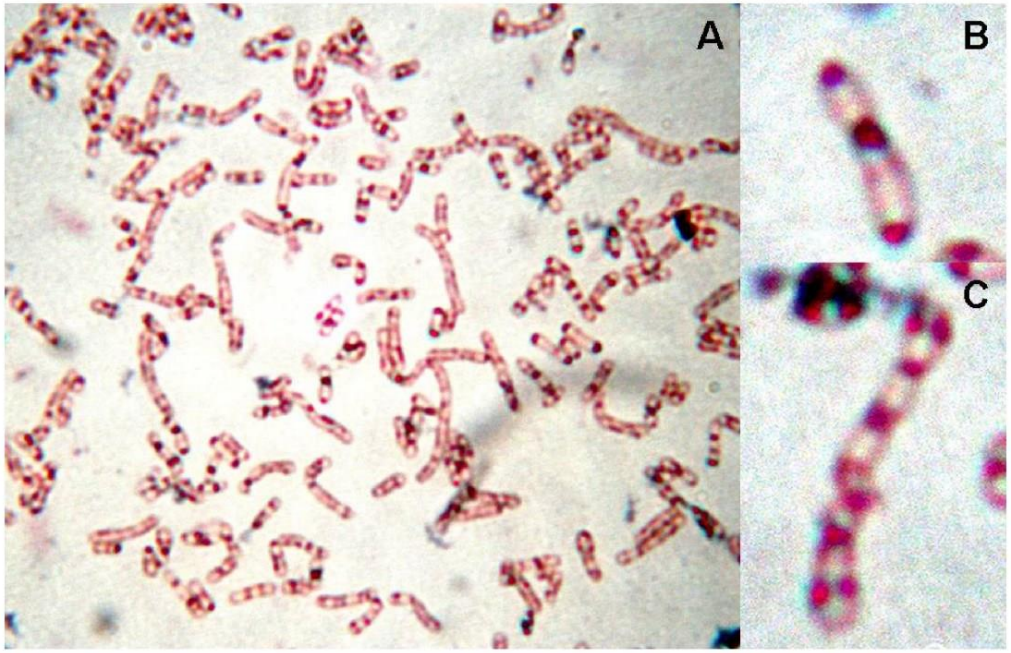
Nocardia



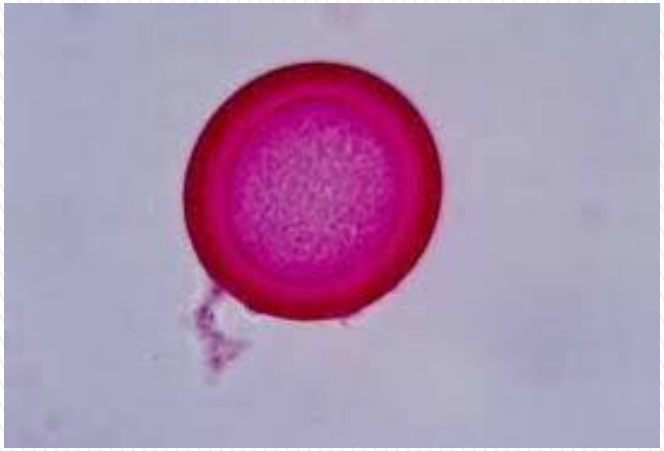
Cryptosporidium



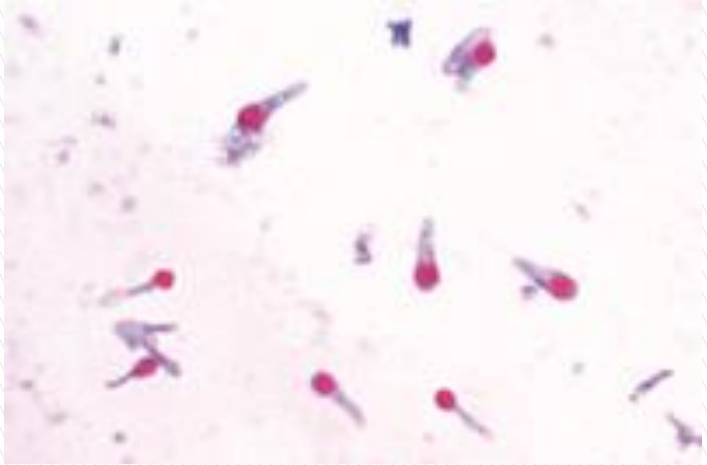
Isospora belli



Rhodococcus species



Taenia saginata



Clostridium tetani spore

Modification of Ziehl-Neelsen's method:

- 5% sulphuric acid (H_2SO4) is used for lepra bacilli.
- 1% sulphuric acid is used for Nocardia, Parasites like Cryptosporidium,isospora and cyclospora, Egg of Taenia saginata
- 0.25-0.5% sulphuric acid is used for spores.
- Cold staining: In this method heating is not required but concentration of phenol is increased and a wetting agent tergitol is added for rapid penetration of the stain.

- **Enumerate the acid fast organisms**
  a) Mycobacterium
     - –M. tuberculosis
     - –M. leprae
     - – M.bovis
     - – Atypical mycobacteria
  b) Bacterial spores
  c) Nocardia
  d) Cryptosporidium parvum
  e) Isospora belli and cyclospora
  f) Egg of Taenia saginata

- **Which are other staining techniques to see acid fast organisms? What are the advantages?**
- Kinyoun's cold staining
- Flouroscent staining
  - Auramine O
  - Auramine Rhodamine

Advantage- More no. of smears can be seen in less time.
- skilled personnel are not required.

- **What are substitutes of methylene blue?**
- 0.2% Malachite green
- 1% Picric acid

- **How will you report a positive result of Acid fast stain?**

No. of bacilli per oil immersion field	No. Of field examined	Grading
> 10 AFB / oil immersion field	20	+3
1-9 AFB / oil immersion field	50	+2
10-99 AFB / 100 oil immersion field	100	+1
1-9 AFB / 100 oil immersion field	100	SCANTY- _____ Record Actual No. of AFB
No AFB /100 oil immersion field	100	Acid fast organisms not seen

THANK YOU