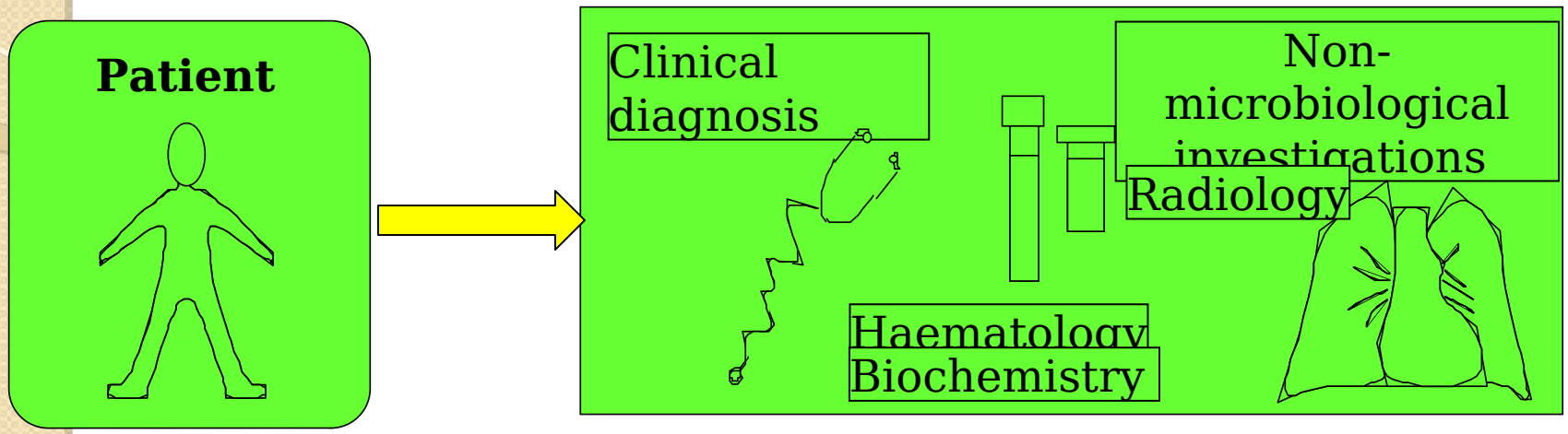
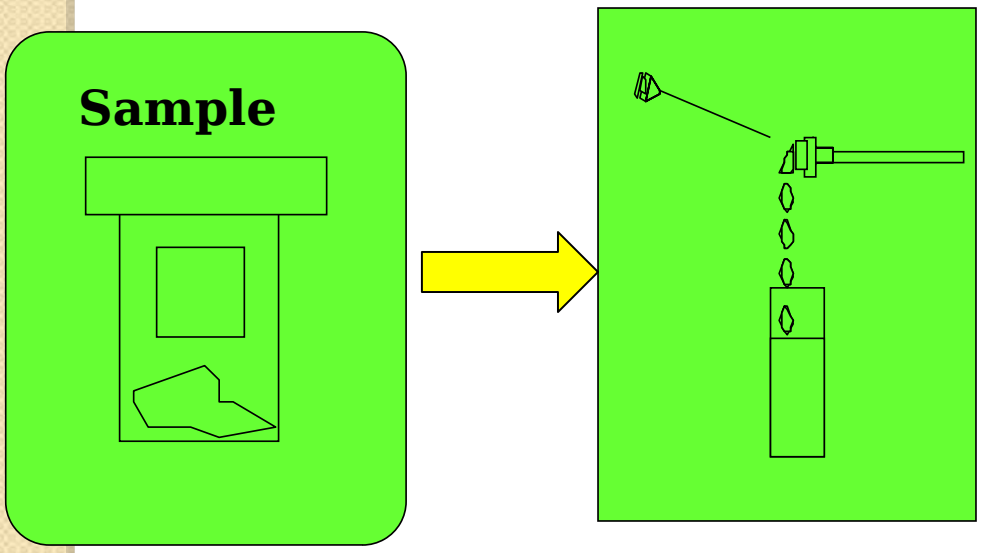
Bacterial staining, Cultivation, Culture Techniques and Antimicrobial sensitivity Testing



Diagnosis of Microbial Infection

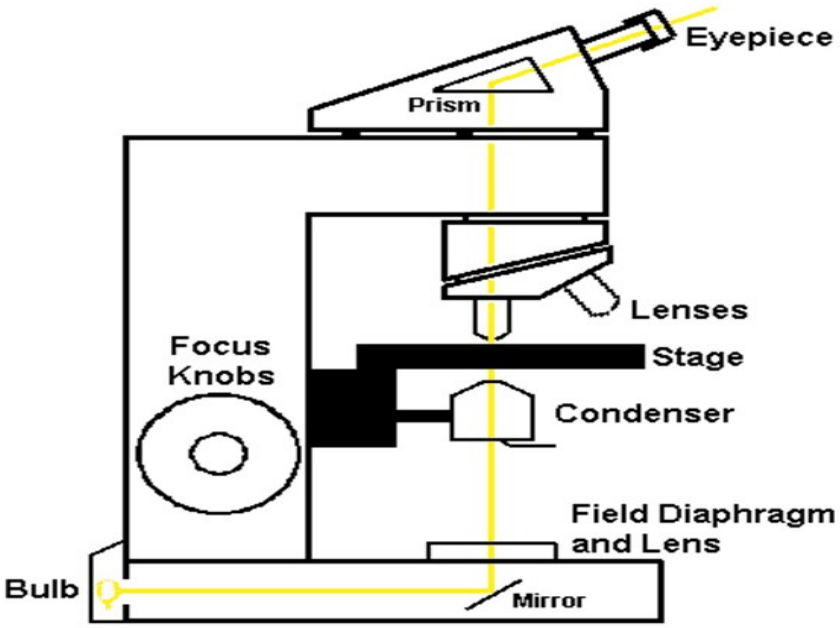




- Take the correct specimen
- Take the specimen correctly
- Label & package the specimen up correctly

The Microscope

- The combination of two words; "micro" meaning small and "scope" meaning view
- Enables us to visualize minute objects (animate and inanimate), that



TYPES OF MICROSCOPE

1. Compound light microscope (widely used in clinical laboratories)
2. Phase contrast microscope
3. Dark field microscope
4. Fluorescence microscope
5. Electron microscope

A. **Compound light microscope**

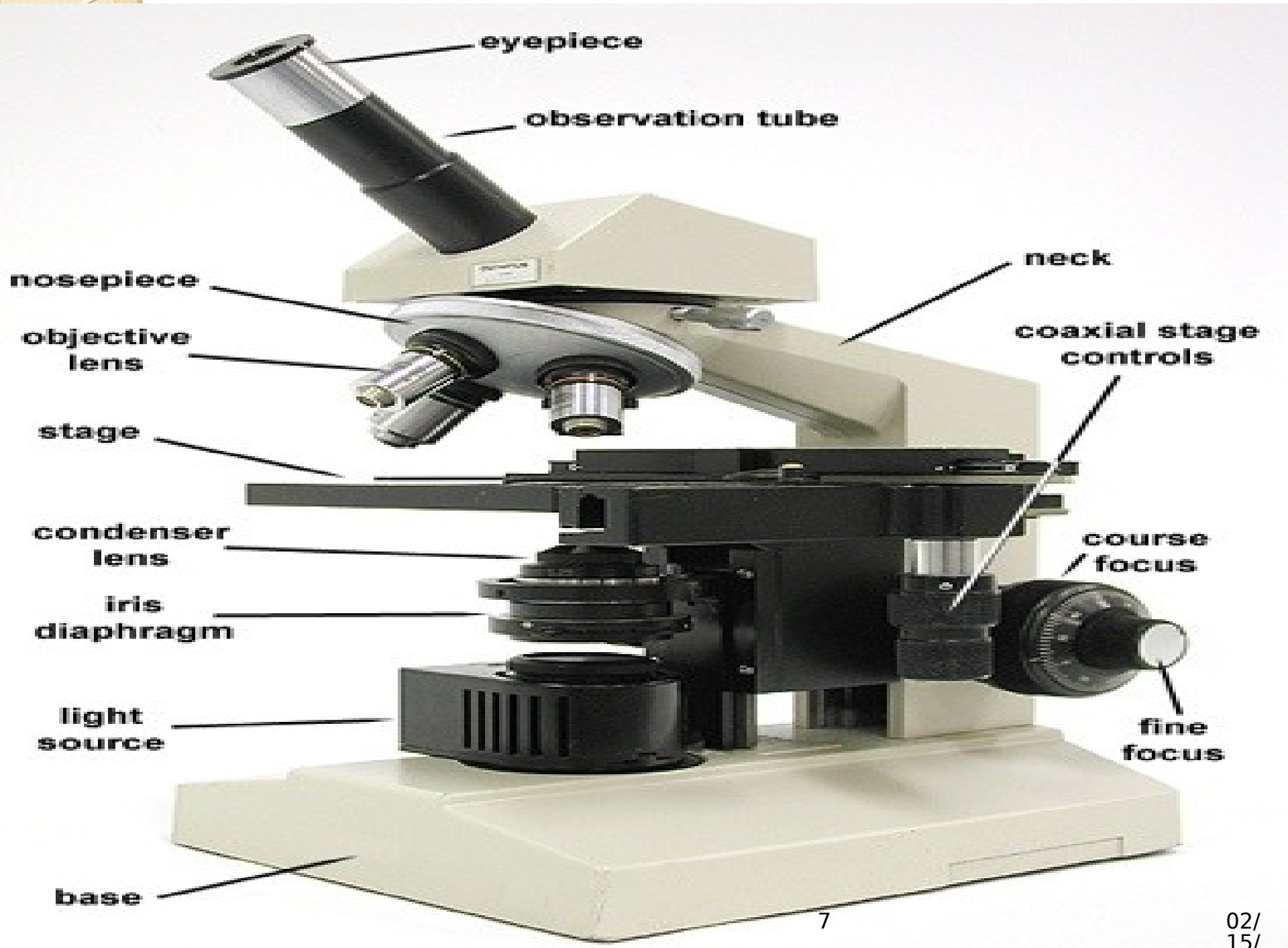
- Uses light to transmit the image to the eye
- Having more than one lens (eye &objective)
- Have a magnification power of 1000x-2000x of the original diameter of objects
- Has magnification and resolution property

Microscope Magnification

- How much the object is enlarged
- Making the diameter enlarged
  - E.g.10x magnification means the diameter of the object is magnified 10 times(not the area or object itself)
- the image that is seen is **upside down and reversed i.e the right →left ,top-→bottom** and vice versa
- Total mag=mag objective x mag eye piece

Resolution

- The amount of detail you can see in an image
- You can enlarge a photograph indefinitely using more powerful lenses, but the image will blur together and be unreadable
- The ability to see two closely placed objects as they are separate entity
- Therefore, increasing the magnification will not improve the resolution
  - human eye -→0.25mm
  - Light microscope ---→0.25um
  - Electron microscope--→0.5nm


eyepiece
observation tube
neck
nosepiece
objective lens
coaxial stage controls
stage
condenser lens
course focus
iris diaphragm
light source
fine focus
base

The microscope stand:- support for all the other parts

- **A tube**:- Which holds the objectives and the eye pieces
- **An arm**: - The basic frame of the microscope which supports the tube and body. It also provides a lifting handle for the microscope.
- **A stage**:-the table of the microscope where the slide or specimen is placed .

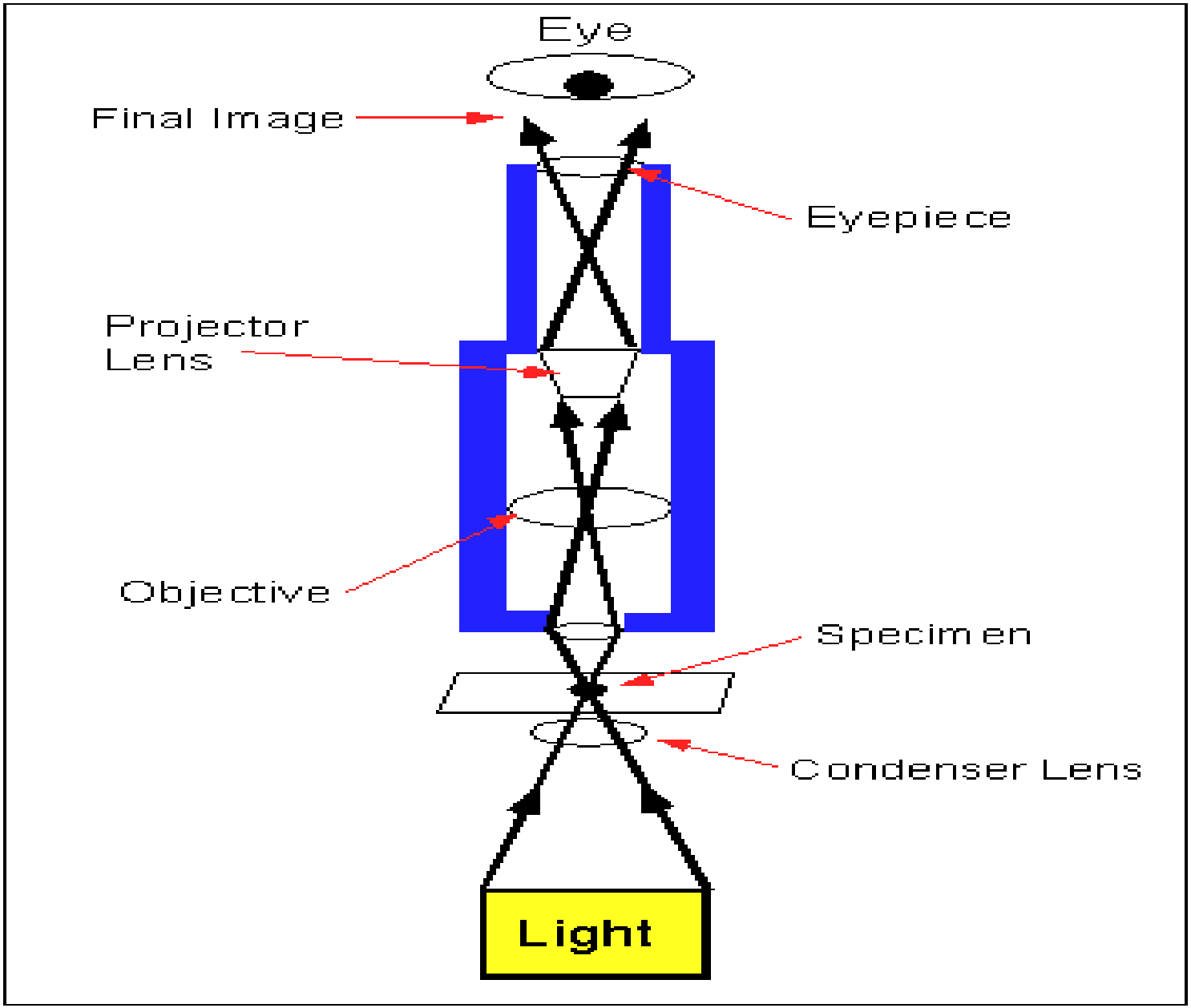
- **A foot or base**: - is the rectangular part up on which the whole instruments rests
- **Coarse and fine focusing adjustments** for the rapid and slow movement of the stage or the objectives up and down respectively.
- **Condenser adjustment** for adjustment of the condenser
- **Objectives**:-magnify the image of the specimen to form the primary image.
  - there are three types of objectives (10x,40x, 100x)
  - **low-power objective (20x**
  - **High power objective (40x**
  - **Oil immersion objective (100x**

Eye piece

- further magnifies the primary image and brings the light rays to a focus at the eye point
- consists of two lenses mounted at the correct distance that is binocular
- Monocular microscope is with one ocular

Condenser and iris

- Condenser is a large lens with an iris diaphragm
- The condenser lens receives a beam from the light source and passes it into the objective


Eye
Final Image
Eyepiece
Projector Lens
Objective
Specimen
Condenser Lens
Light

3.Dark field microscope

- used for of lighting micro-organisms **suspended in fluid**, enabling their **structure and motility** to be seen
- The only light entering the eye comes from the micro-organisms themselves, with no light entering the eye directly from the light source
- In this way, small microorganisms are seen brightly

  - Treponema palladium (Spirochates),
    - Borreliae species in blood
    - Microfilariae in blood
    - Vibros and campylobacters



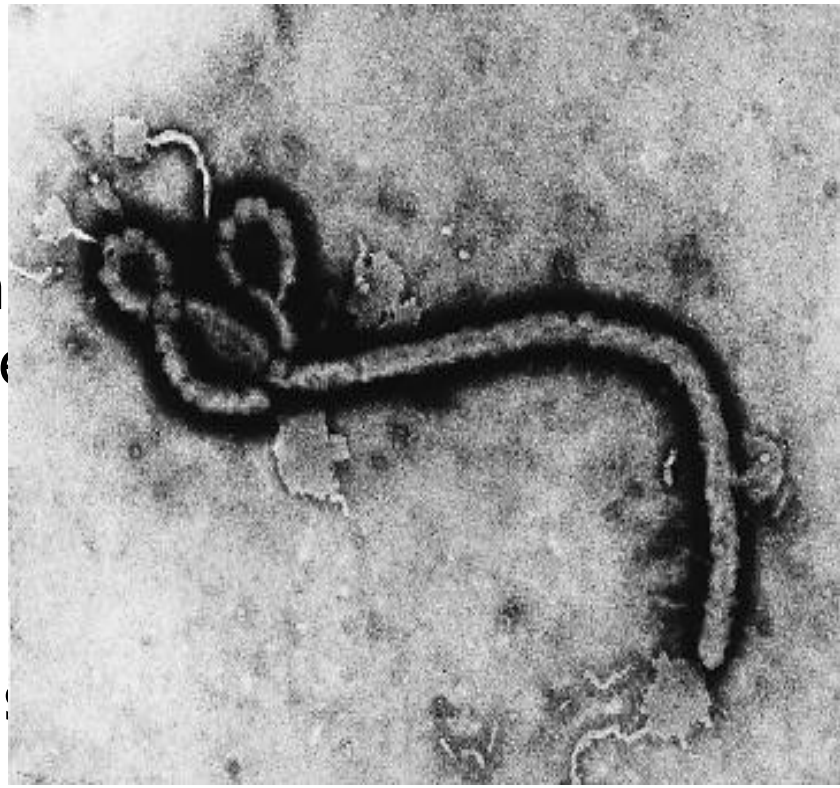
4. Fluorescence Microscopy

- widely used in **the immunodiagnosis** of important bacteriological and parasitic diseases
- **Fluorescent** molecules absorb light of a given wavelength and subsequently emit light of a longer wavelength
- A good example is a popular **fluorescent dye, fluorescein**
  - **To diagnose TB** by detecting AFB in sample
  - WHO has made recommendation for TB diagnosis



5. Electron Microscope

- 50,000 x magnification with high resolution power
- Specimen is illuminated by beam of electron produced in the electron gun
- Specimen need special preparation
- Impossible to **study livin cell** because electrons are highly damaging to living tissue
- Image on screen
- Uses **for virus particles**



Stains

- Chemically stains **are salt**
- Based on the **colouring agent**
  - **Can be basic ,acidic, neutral**

- **Basic stain:** the coloring agent is contained on the basic part of the dye

  e.g.carbol fuschin ,methylene blue, crystal violet

Acidic :the colouring agent is contained in the acidic part of the dye

e,.g**. eosin**

Neutral: the coloring agent is contained in both parts

e.g. **Giemsa**

Stains are also classified as :-

1. Simple stain
2. Differential stain

1. **Simple stain**
   - Uses only single dye
   - Monochromatic
   - No mordant chemical added
   - Do not help for the differentiation of bacteria

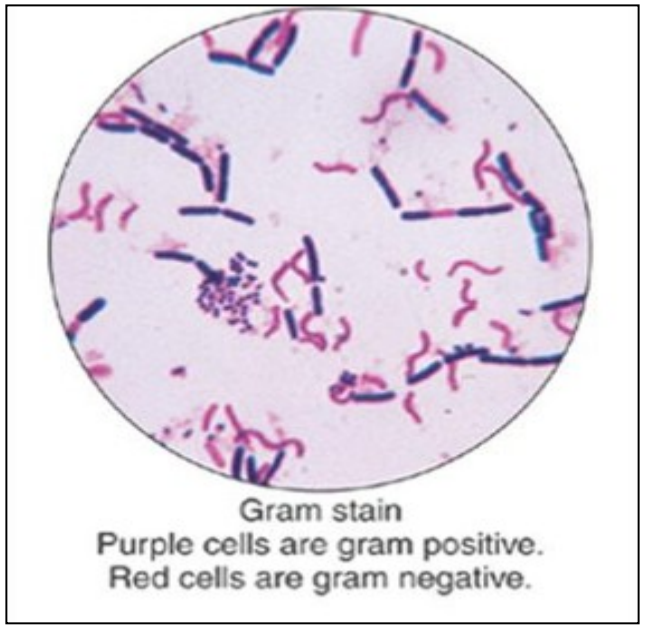
   e.g. metylene blue, carbol fuschin

II. Differential stain

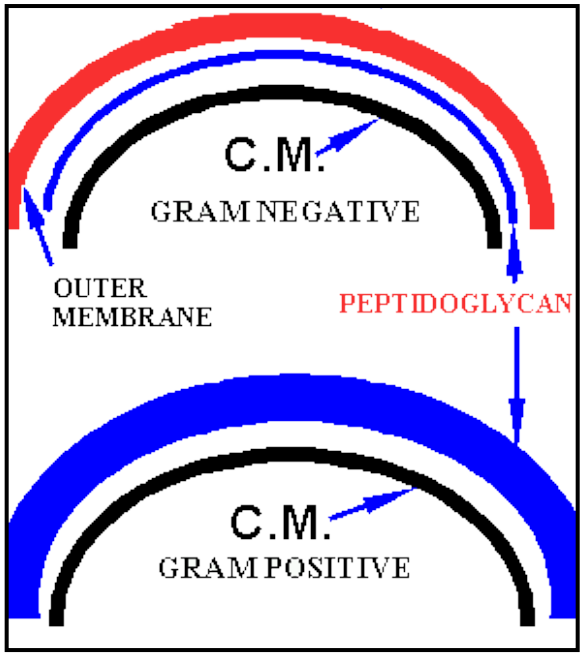
- Use more than one dye
- Used for the differentiation of bacteria
- Uses a mordant

The Gram Stain

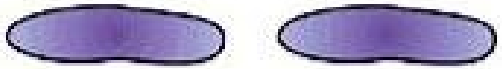
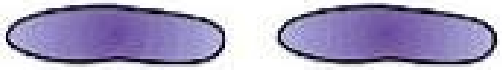
- Important basis of bacterial classification and identification
- Practical aid in diagnosing infection and guiding drug treatment
- Differential stain
  - Gram-negative
    - lose crystal violet and stain **red** from safranin counterstain
  - Gram-positive
    - retain crystal violet and stain **purple**

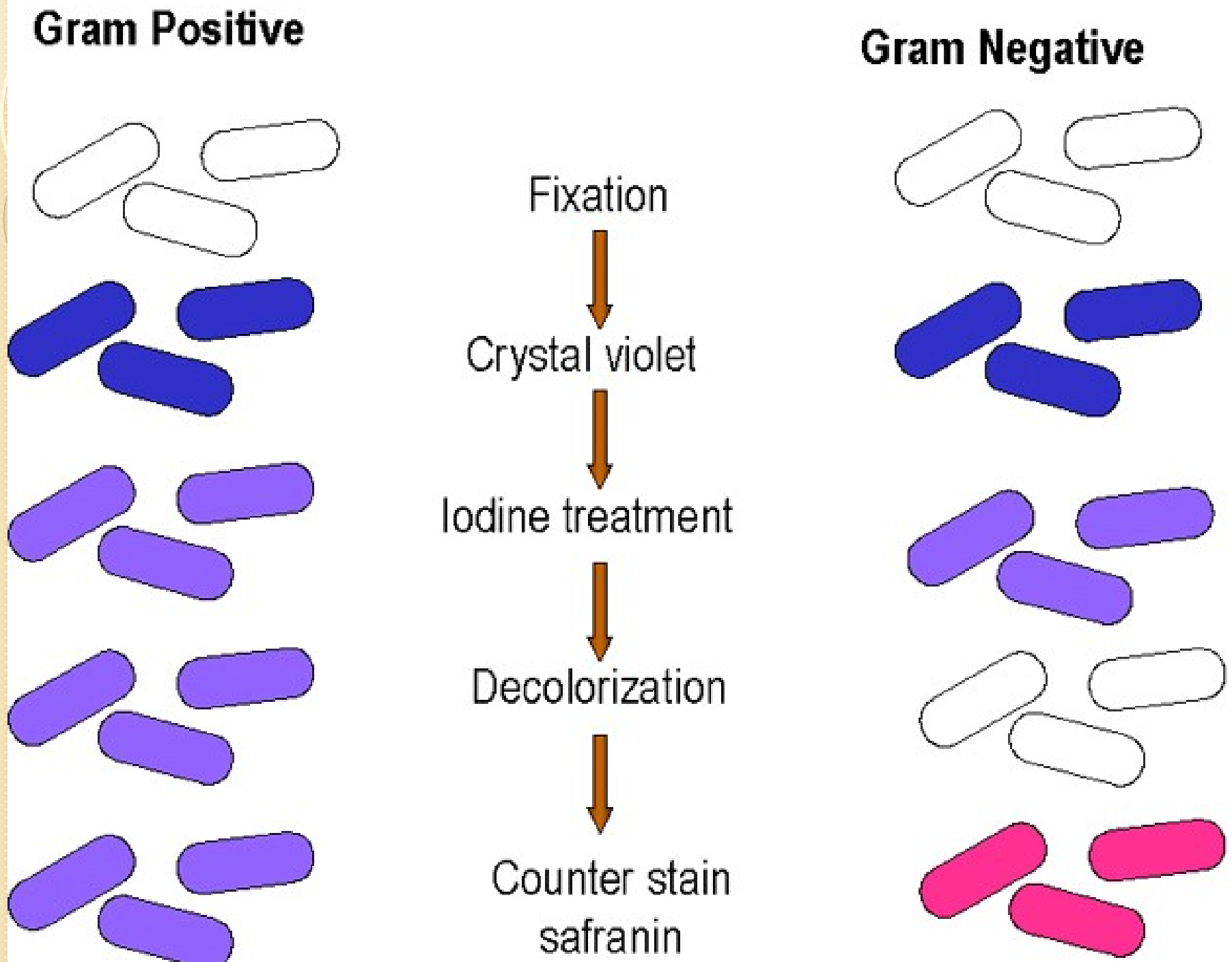


Gram stain
Purple cells are gram positive.
Red cells are gram negative.

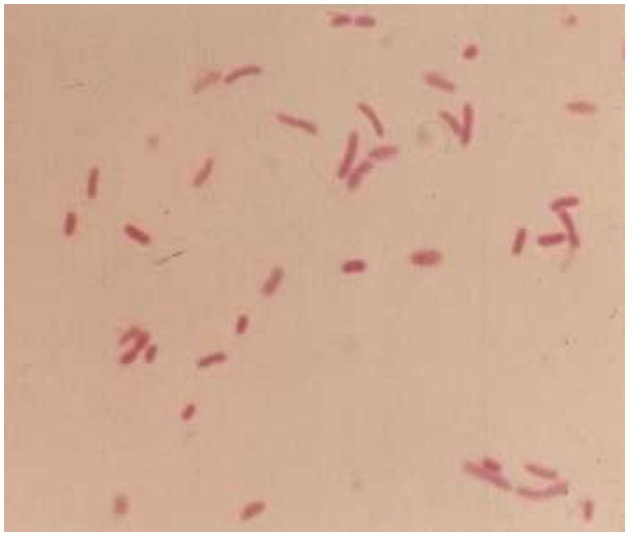


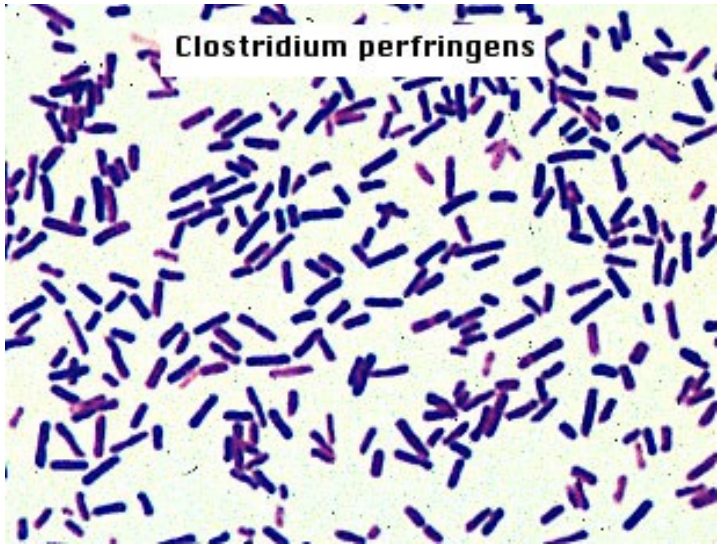
Principle of the Gram Stain

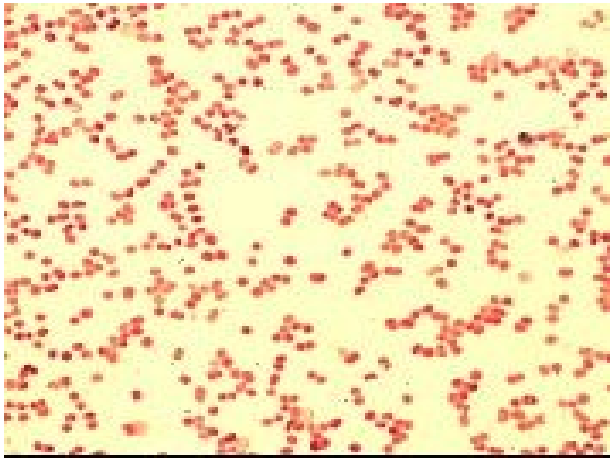
	Microscopic Appearance of Cell		Chemical Reaction in Cell Wall (very magnified view)	
Step	**Gram (+)**	**Gram (–)**	**Gram (+)**	**Gram (–)**
1. Crystal violet				
			Both cell walls affix the dye	
2. Gram's iodine				
			Dye crystals trapped in wall	No effect of iodine
3. Alcohol				
			Crystals remain in cell wall	Cell wall partially dissolved, loses dye
4. Safranin (red dye)				
			Red dye has no effect	Red dye stains the colorless cell

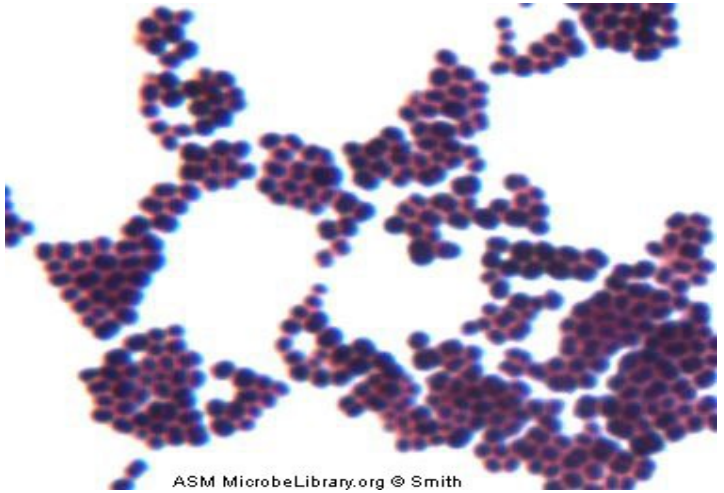

Gram Positive
Gram Negative
Fixation
Crystal violet
Iodine treatment
Decolorization
Counter stain
safranin

Gram stains Pictures









Ziehl Neelsen staining

- For the detection of **acid alcohol fast bacteria (AFB) e.g. TB, MOTB**,
- Uses the **waxy coat** of mycobacteria to trap stain.
- Not as sensitive as **auromine-phenol** from direct smears
- Recommended use of confirmation of cultures

Ziehl Neelsen staining

- Mycobacterium do not stained by gram staining technique do to the **mycolic acid** found in the cell wall
- Mycobacteria retain the basic dye **carbolfuschin** combined with phenol
- Not decoloured by acid
- The acid removes the **carbol fuschin** from the background and cells other than **acid fast bacilli**
- The counter stain **malachite green or methylene blue** will stain the background

Procedure ZN staining

Materials required

- **Carbol fuschin**
- **Acid alcohol(3%)**
- **Malachite green /methylene blue**

1. Fix the dried smear by **70% ethanol** or methanol (2-3min)
2. Cover the smear with **carbol fuschin** and heat the stain until vapour begins and leave - 5 min.
3. Wash the stain with clean water
4. Cover the smear with 3% **acid alcohol** -5min
5. Wash off with clean water
6. Cover the smear with **malachite green /methylen** blue -2 min
7. Wash of the stain with water
8. Eaxmine the smear by oil immersion to look **AFB**

RESULTS

Acid fast bacilli(AFB)----------red bacilli

Cells ----------green/blue

Background ----------green /blue

If any red bacilli seen report as positive

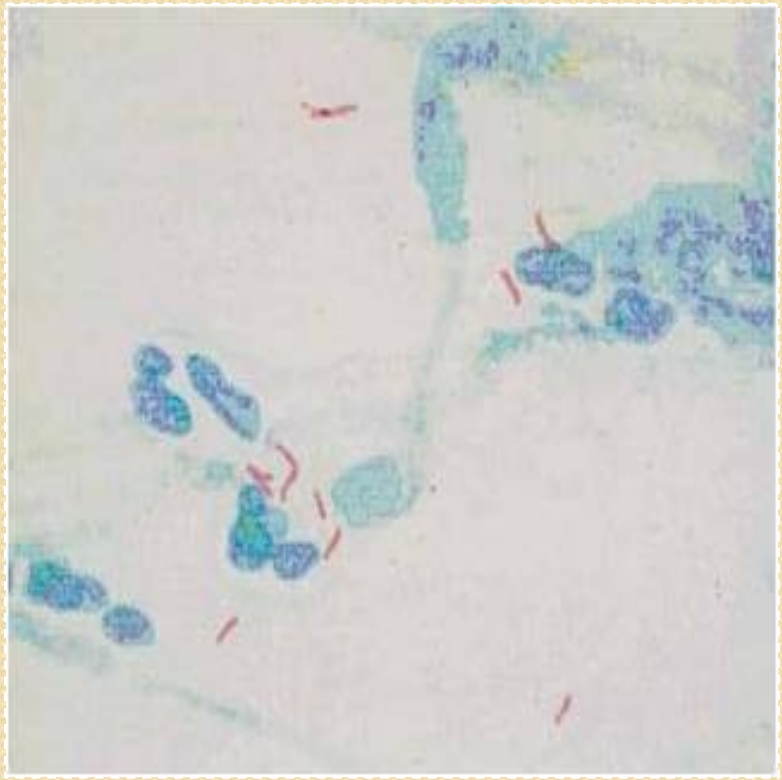
>10AFB/F----------+3

1-10AFB/F----------+2

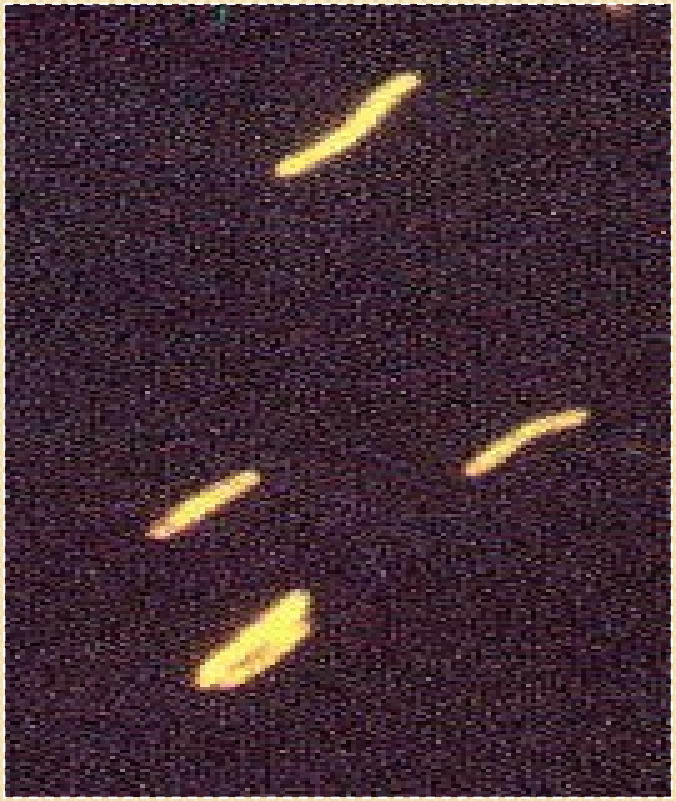
10-100AFB/F----------+1

1-9AFB/100F----------exact number

ZN stain



Auromine-phenol stain



Culture of Microorganism

- **Culture media** –artificial prepared environment used for the growth of bacteria,

- Used to isolate pathogens in pure culture
- To perform antimicrobial sensitivity testing
- Most bacteria can be cultured provided
  - ✓Medium contain required nutrients correct amount ,PH,and osmotic pressure
  - ✓Incubated in correct atmosphere and temperature

Growth Requirements of Bacteria

- Water (80% of living weight of bacteria)
- **Nitrogen ,carbon , oxygen & hydrogen**

  CHONP
- vitamins
- Phosphorous
- Mineral

Common Ingredients of Culture Media

- **Peptone**-water soluble product which is obtained from hydrolysis of animal and plant proteins
  -provide nitrogen ,CHO, minerals & vitamins
- **Meat extract** – provide aminoacids, vitamines, phosphates, sulphates
- **Mineral salt** –used fore bacterial enzyme activity
  Mg,K,Fe,Ca,NaCl
- **Carbohydrate** –simple & complex sugar which is important source of carbon and energy
  -differentiation of bacteria
- **Agar**-inret polysaccharide extracted from marine algae
  -solidify culture media
- **Water**-for growth any microorganism
  -should be free from any chemicals

Type of culture media

The main type of culture media are :-

- Basic
- Enriched /enrichment
- Selective
- Differential
- Transport

1. Basic media

- For growth of microorganism which do not need special growth requirements
- Preparation of enriched media
- subculturing from differential and selective media before performing biochemical test

  e.g. nutrient agar, nutrient broth

2.Enriched/Enrichement Media

- For growth of pathogens which need special growth requirement
- For growth of **Haemophilus Influenzae, Neisseria**
- Enriched with:-
  - whole blood
  - lyzed blood
  - serum
  - Extera peptone
  - Vitamines

  e.g. Blood agar

  selenite F broth



3.Selective media

- Contain substances which prevent the growth of microorganisms rather than pathogen which the media intended
- E.g.XLD (xylose lysine deoxycholate)- salmonella & shigela
- MNYC(Modified New York city)- Neisseria

4.Differential /indicator/media

- Contain dyes or substances which are important for the differentiation of microorganism
- Most media are selective &differential
  - ✓MacConkey agar
  - ✓XLD agar
  - ✓TCBS agar
  - ✓DCA agar

5.Transport media

- **Semisolid** media which contain ingredients that prevent growth of commensals bacteria
- Support the growth of pathogenic bacteria
- For the transportation of specimens

e.g. **Carry –Blair** media-for **enteric** bacteria

Amies transport media-for **gonnocci**

Choice of culture media

- Pathogens to be isolated,growth requirements ,features of recognition
- From where the sample collected/sterile or not
- **Cost ,availability ,satiability** of differential media
- Trained man power

Forms of culture

1. Solid –used in petri-dishes as plate culutur and can also used in tube

   E,g. Blood agar ,klinger iron agar, XLD

2. Semi-solid-for transport media

   Ameis transport medium, caryblair transport

3. Fluid –for enrichement media, biochemical test

   Alkaline peptone water ,selinte F broth

Biochemical test for microorganism

- Used for differentiation of bacteria by break down of substance

1 Catalase -To differentiate **staphylococci** from streptococci



H202 Catalase H2O+ O2(bubling)

2.Citrate-to differentiate **entrobacetrcea** from other Bacteria that use citrate as only source of energy

3.Coagulase –to differentiate **S.aures ,clots plasma**

4.Urease to differentiate urease producing organisms

5.Indole –to differentiate gram negative rods especially

E.coli

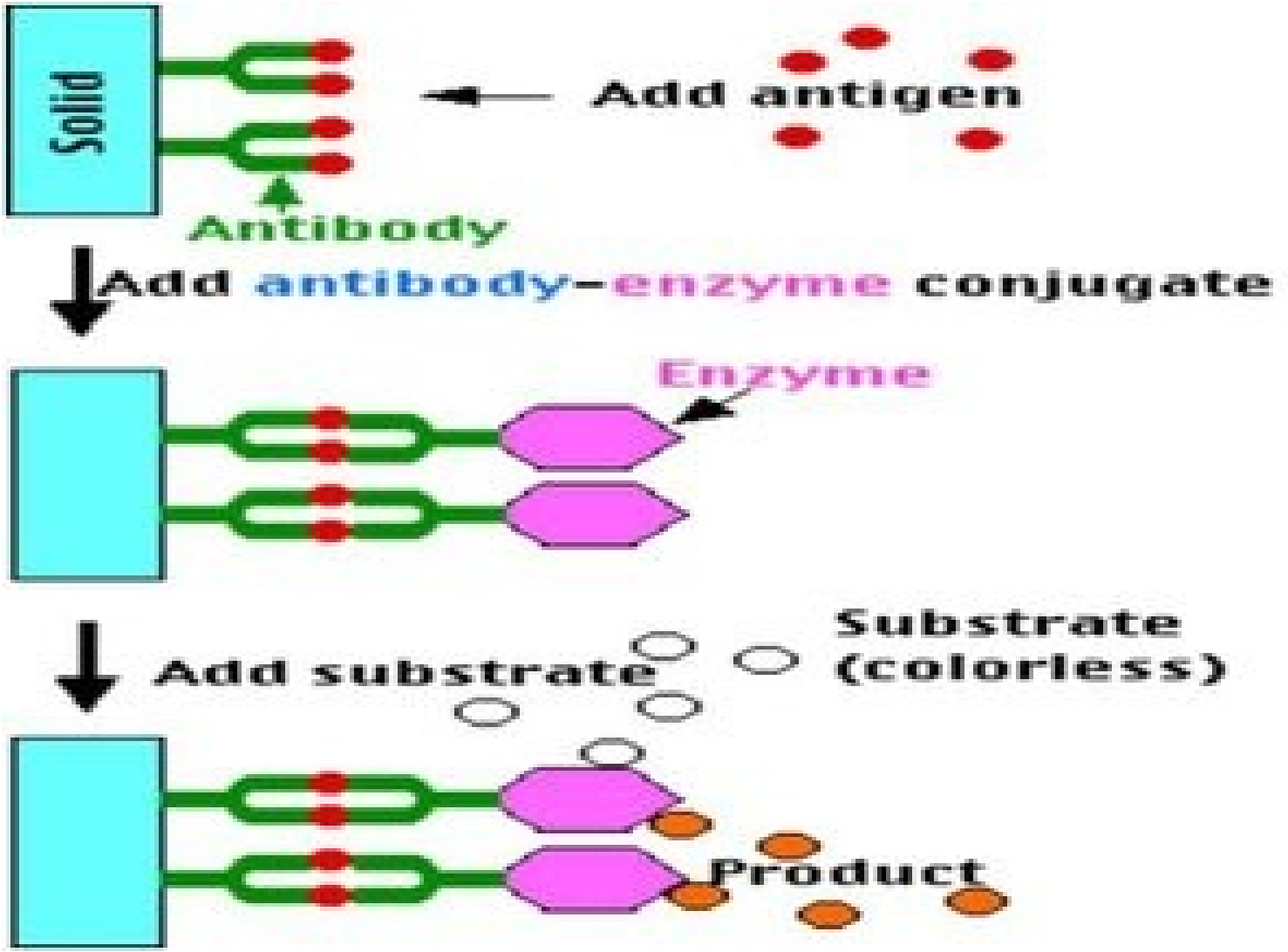
Bacterial serology & antigen detection

- Why?
- Diagnose disease when organism cannot be grown, easily isolated, or not present
  - **Syphilus, brucellosis, viral disease**
- Early/ presumptive diagnosis of disease
  - **Meningitis**
- ID & or serotype a pathogen
  - **Streptococci, *Salmonella*** *sp*
- Screening
  - Rising **antistreptolysin** O levels in **BHS gpA** infection
- Measure community Ab levels for spread, control & prevalence of a disease

Serological techniques

- Divided into two main groups
- ***Antibody test***
  - Known Ag detect &/or measure Ab in patients serum.
  - Rise in Ab titre required to establish a diagnosis
- ***Antigen test***
  - Uses specific antisera to detect presence of organisms Ag in specimens/ isolates

ELISA 2


Solid
Add antigen
Antibody
Add antibody–enzyme conjugate
Enzyme
Add substrate
Substrate
(colorless)
Product

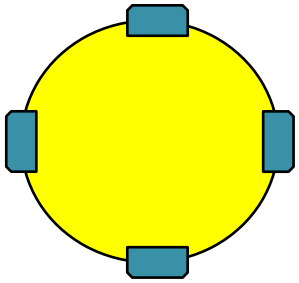
Elisa uses

- Regular uses in **serological screening** (**ELISA1**)
  - **Syphilus**
  - **Rubella**
  - **VZ**
- Regular use in **Ag detection** (**ELISA2**)
  - **Giardia/ Cryptosporidia**
  - **GDH (*Clostridium difficle* toxin)**

Latex agglutination uses

- Regular uses – commercial kits
  - ß haemolytic streptococcal grouping
  - CSF Ag detection
    - *S.pneumoniae, N.meningitidis*
  - Blood Culture Ag detection
    - *S.pneumoniae, N.meningitidis*
  - Rapid diagnosis of infection
  - Rapid ID

Agglutination Test

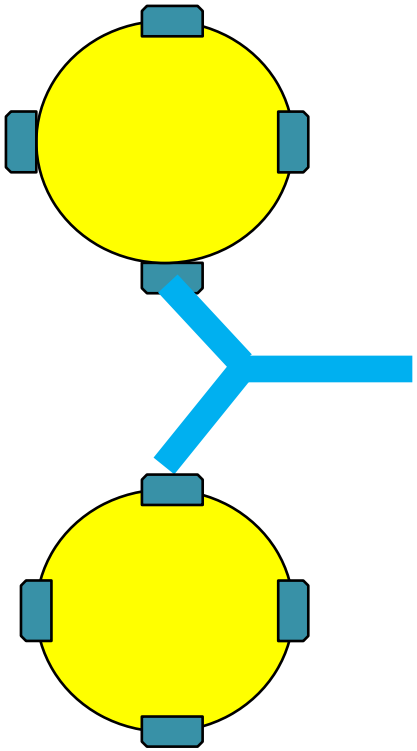


Cell with surface Ag





Specific Ab



Ab combines with its Ag &
Agglutinates cells

ANTIMICROBIAL SENSITIVITY (SUSCEPTIBILITY) TESTING

- The test is used to measure the ability of the drug to inhibit or kill pathogens in vitro.
- it is used to select effective antimicrobial drugs
- Sensitivity test is performed:
  - For organisms with variable antibiotic sensitivity (un predictable sensitivity) E.g. **Shigella**
  - For non responding patients after taking adequate therapy.
  - For patients whose immune system is depressed
  - For relapsing cases (reappearance of disease)

Anti-Microbial sensitivity testing

- Antimicrobials are drug that have bacteristatic or bactericidal activity
- The quantitative measures of the in vitro activity of antibiotics
- Uses
  - Decreases drug resistance
  - For the selection of the correct drug
  - For the identification of microorganisms

Method of Antimicrobial Susceptibility Testing

1. Disc diffusion method

2. Dilution method

1. Disc diffusion method

- bacterial isolate is inoculated uniformly onto the surface **Muller Hinton Agar plate**
- A filter disk impregnated with a standard amount of an antibiotic is applied to the surface of the plate and the antibiotic is allowed to diffuse into the adjacent medium
- Following incubation Zones of inhibition of bacterial growth may be present around the antibiotic disk

- The size of the zone of inhibition is dependent on the diffusion rate of the antibiotic, the degree of sensitivity of the microorganism, and the growth rate of the bacterium
- The zone of inhibition in the disk diffusion test is inversely related to the MIC
- Determines MIC the lowest conc. of the antibiotic that results in inhibition of visible growth (i.e. colonies on a plate or turbidity in broth
- The MBC is the lowest concentration of the antibiotic that kills 99.9% of the original inoculums in a given time

Required materials

Muller Hinton agar

Antibiotics disc

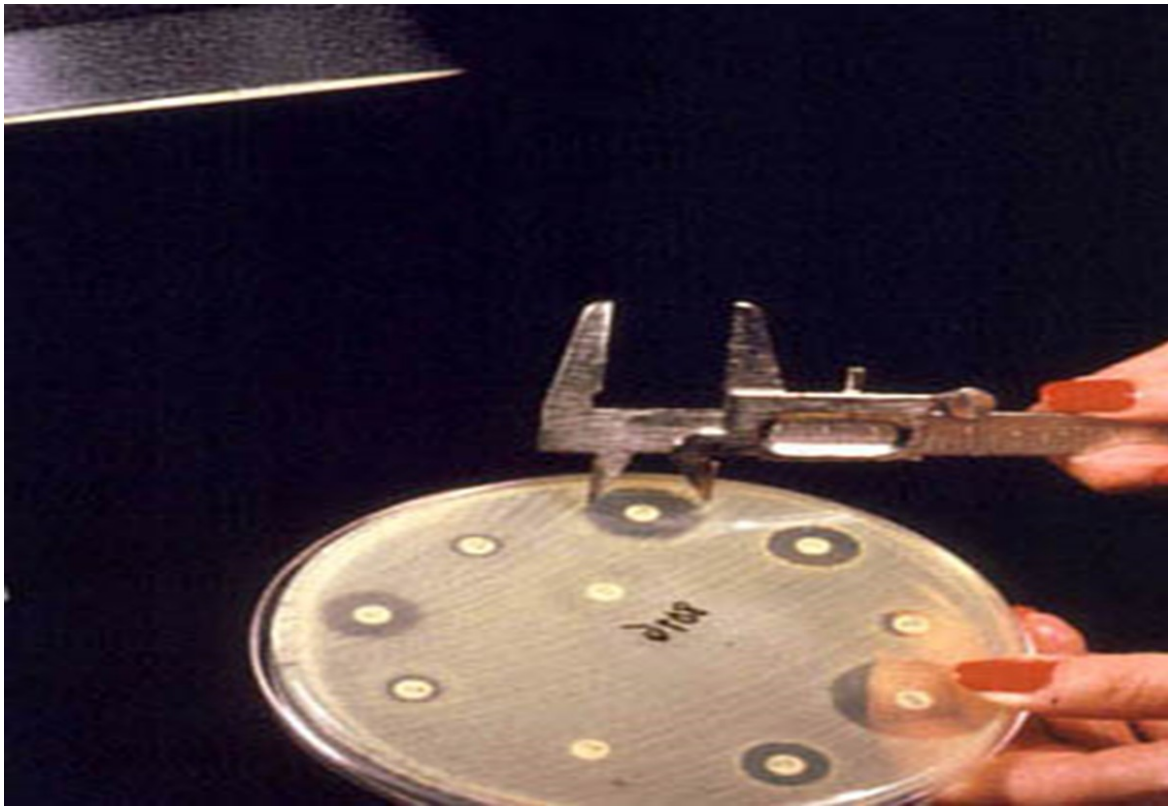
turbidity standard



Interpretation

- After 24hrs of incubation examine the culture
- Measure the zone of inhibition including the diameter of the disc using sliding calipers

1. Sensitive
2. Intermediate
3. Resistant

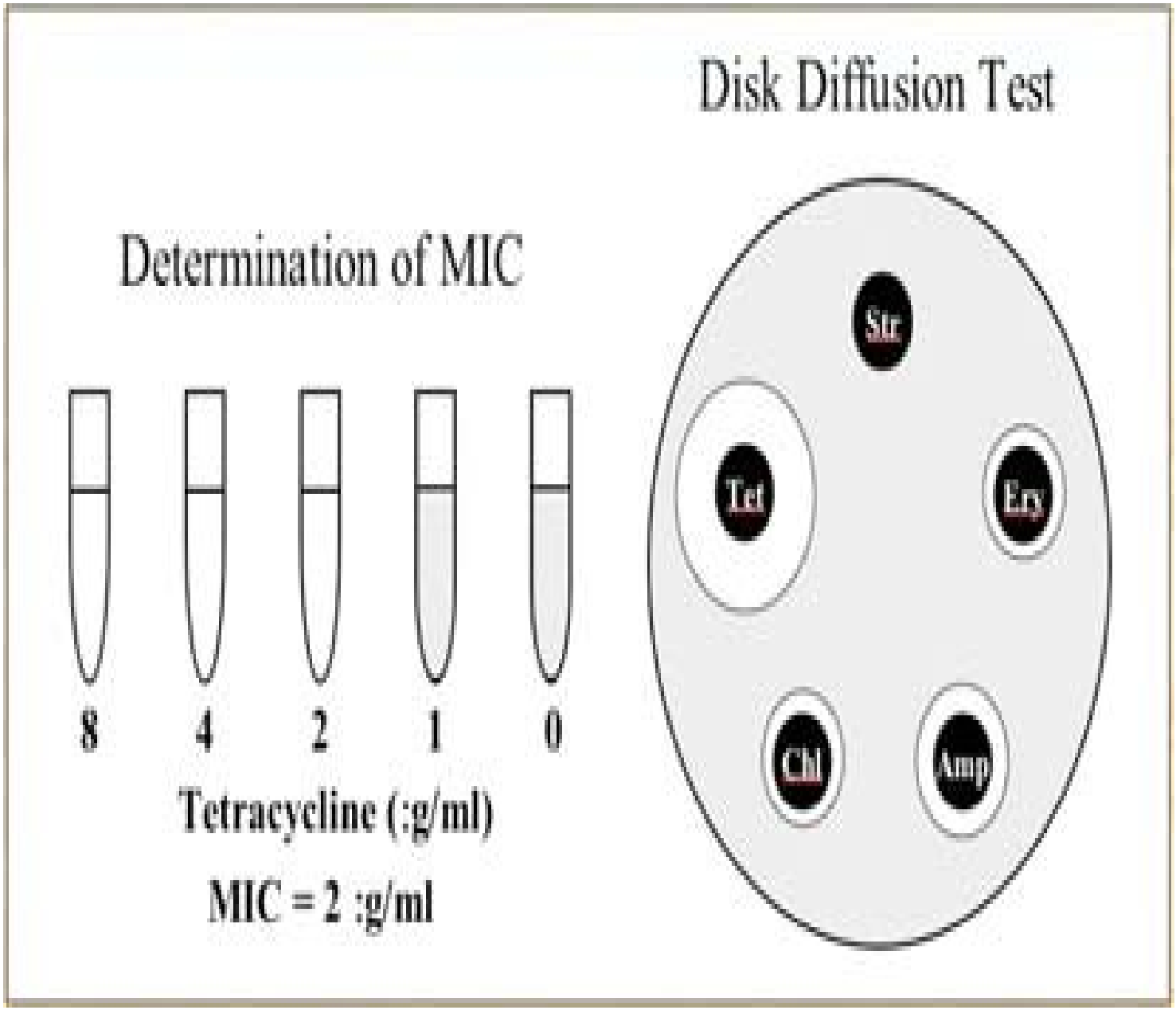


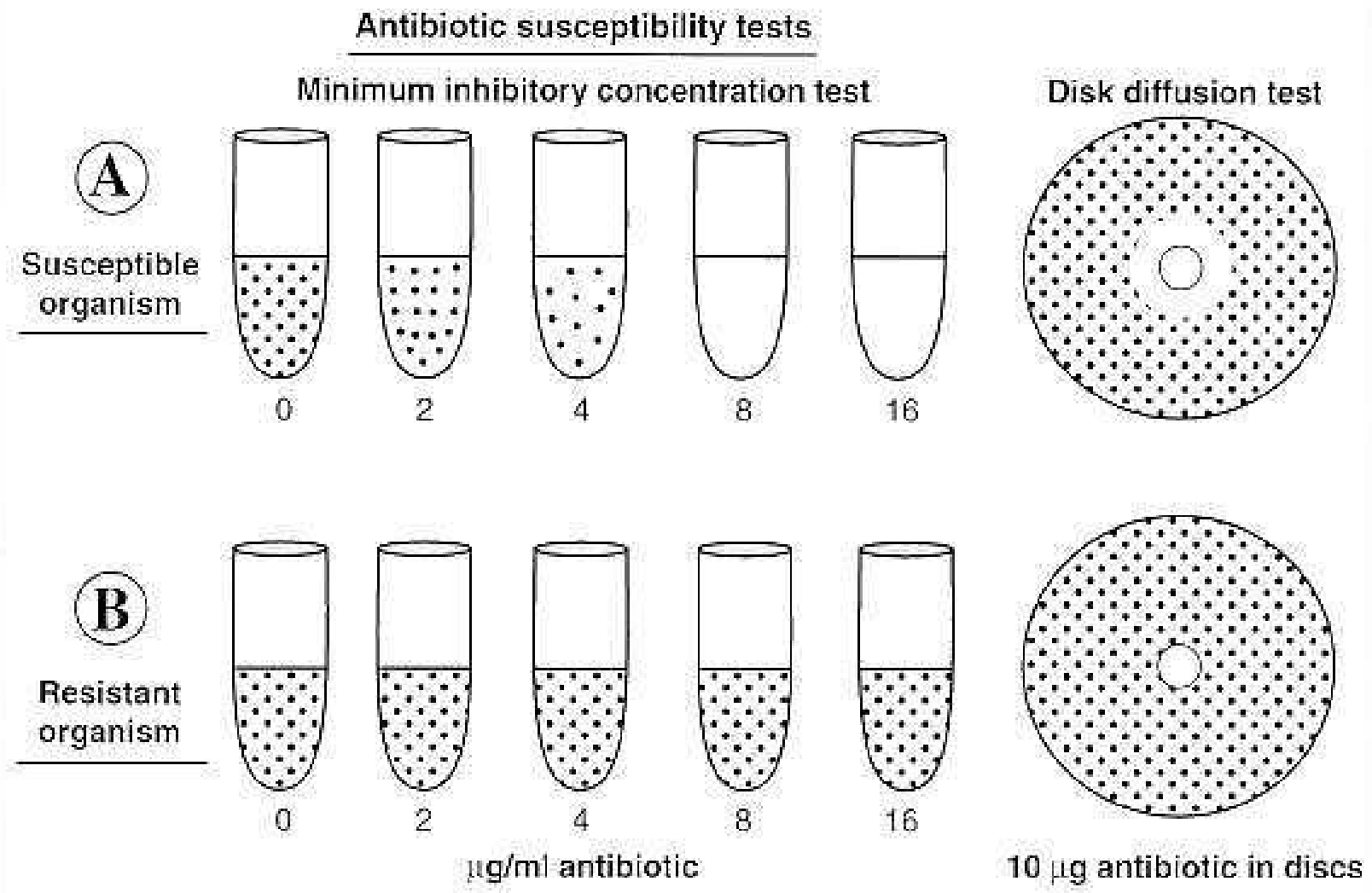
Interpretation of Zone Size

- ‘Resistant’, Intermediate (moderately sensitive) or ‘Sensitive’ (susceptible).
- ***Resistant***: A pathogen reported as ‘resistant’ implies that the infection it has caused will not respond to treatment with the drug to which it is resistant irrespective of dose or site of infection.
- ***Intermediate sensitive***: A pathogen reported as intermediately sensitive suggests that the infection it has caused is likely to respond to treatment when the drug is used in larger doses than normal
- ***Sensitive (Susceptible)***:- A pathogen reported as sensitive suggests that the infection it has caused is likely to respond to treatment when the drug is used
- **NB**: It is necessary to report the first and second choice of antibiotics for a patient’s infection unless the strain is resistant

2.Dilution Method

- A dilution test is Carried out by adding dilution of antimicrobial to a **broth or agar** medium
- A standardized inoculums of the test organism is then added
- After overnight MIC reported the last tube that does not show visible growth
- MIC is expressed as the lowest dilution, which inhibited growth by lack of turbidity in the tube
- MBC determined by subculturing the last tube to show visible growth and all the tubes in which there is no growth


Disk Diffusion Test
Determination of MIC
Str
Tet
Ery
Chl
Amp
8
4
2
1
0
Tetracycline (:g/ml)
MIC = 2 :g/ml


Antibiotic susceptibility tests
Minimum inhibitory concentration test
Disk diffusion test
A
Susceptible organism
0
2
4
8
16
B
Resistant organism
0
2
4
8
16
μg/ml antibiotic
10 μg antibiotic in discs

Any Last questions ???

