المنهج العملي
لمادةالبكتيريا I
Bacteriology I
(3303-214)

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&

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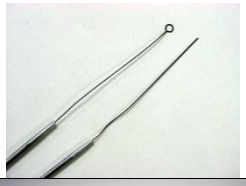
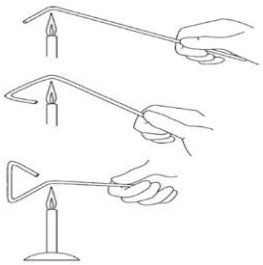
Chapter 1
Equipment and Apparatus

Equipement	Use	
Loop (wire/plastic)	Routine inoculation of agar slopes/deeps and small volumes of liquid media	
Conical flask	Large volumes of liquid media for inoculation and liquid/media for short-term storage	
Spreader (glass/plastic)	Making lawn/spread plates	
Universal bottles	Volumes of liquid and agar media/sterile solutions for inoculation or for storing sterile media or stock cultures on agar slopes	 
Test tube	Small volumes of liquid media/agar slopes/sterile solutions for inoculation	
Pipette (calibrated/ dropping; glass/ plastic)	Transfer of measured volumes/drops of culture/sterile solutions	
Petri dish (plastic/ glass)	Plastic: pre-sterilised for streak/spread/lawn/ pour plates; Glass: only for materials for sterilization by hot air oven, *e.g.* paper discs	

x

Apparatus

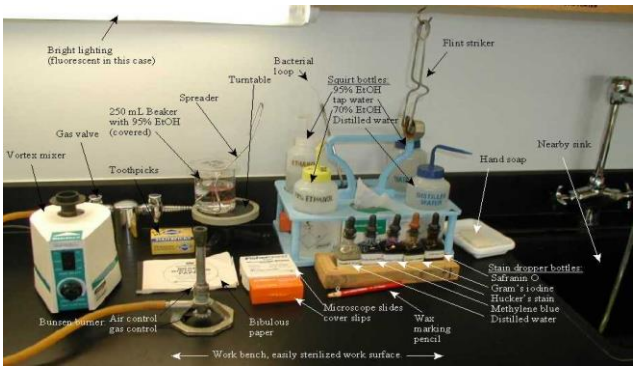
Apparatus	Use	
Bunsen burner	Sterilization of wire loops and (with alcohol) metal forceps and glass spreaders	
Autoclave/pressure cooker	Sterilization of media, solutions and equipment before use and	
Hot air oven	Sterilization of glass Petri dishes and pipettes and paper discs	
Microwave oven	Melting solidified agar media for use (but for sterilization)	
Incubator	Incubation of cultures (but many cultures will grow at room temperature in the interval between lessons)	
Water bath shake	Suitable temperature for keeping melted agar media molten for use (*ca* 50ºC); accurate temperature control	
Thermometer	Checking incubator/water bath temperatures	
pH meter	Checking and adjusting pH values of media	
Refrigerator	Storage of heat-labile materials	
Microscope, slides, cover slips, stains, staining rack, immersion oil	Microscopical observations	

Material

Culture media ingredients	Stock of a range of culture media in dehydrated form (tablets/powder)	
Disinfectants	Treatment of work surface before and after use and spillages; disposal of used pipettes and microscope slides; in soap form for hand washing	
Ethanol (70% industrial methylated spirit	Sterilization of metal forceps and glass spreaders by ignition	
Autoclave indicator tape	Changes colour in response to heat to distinguish those items that have received heat treatment	
Non absorbent cotton wool	Plugs for test tubes, flasks and pipettes	

Sterile transport swabs

Spicemen collection



Chapter 2

Culture Media

Definition:

Method of cultivating microbial organisms by letting them reproduce in predetermined culture media under controlled laboratory conditions

Important Reasons for Bacterial Culturing

- Isolate bacteria in pure cultures.
- Demonstrate their properties.
- Obtain sufficient growth for preparation of antigens & for other tests.
- Typing bacterial isolates.
- Antibiotic sensitivity.
- Estimate viable counts.
- Maintain stock cultures.

Composition of culture media:

❑ Provide similar environmental and nutritional conditions that exist in its natural habitat

❑ A culture medium contains water, a source of carbon & energy, source of nitrogen, trace elements and some growth factors

❑ The pH of the medium must be set accordingly

Classification:

Bacterial culture media can be classified in at least three ways

1.CONSISTANCY

2.NUTRITIONAL COMPONENT

3.FUNCTIONAL USE

1) <u>Classification based on consistency:</u>

A. Liquid media :

- ✖ no agar
- ✖ For inoculum preparation, Blood culture, for the isolation of pathogens from a mixture.
- ✖ Eg: Nutrient broth
- ✖ These are available for use in test-tubes, bottles or flasks.
- ✖ Liquid media are sometimes referred as **<u>"broths</u>**" . In liquid medium, bacteria grow uniformly producing general turbidity

B. Solid media:

An agar plate is a Petri dish
that contains a growth medium (typically agar plus nutrients)
used to culture microorganisms

Agar is the most commonly used solidifying agent

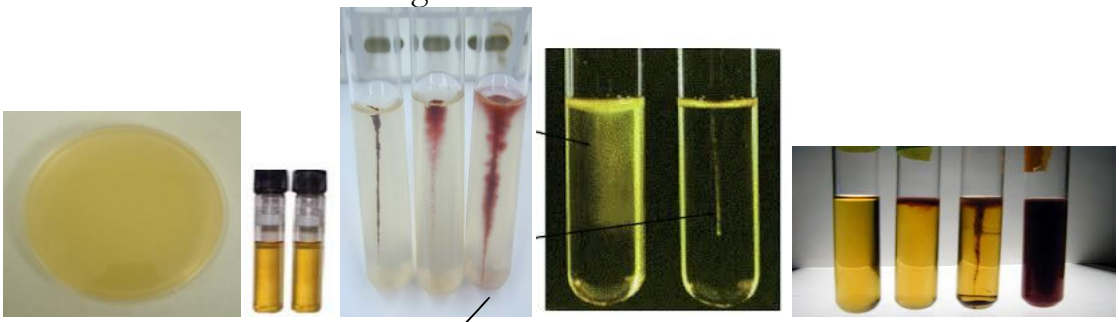
- ✖ Colony morphology, pigmentation, hemolysis can be appreciated.
- ✖ Eg: Nutrient agar, Blood agar

Agar

- Used for preparing solid medium
- Obtained from seaweeds.
- No nutritive value
- Not affected by the growth of the bacteria.
- Melts at 98°C & sets at 42°C
- 2% agar is employed in solid medium

C. Semi-solid agar

Such media are fairly soft and are useful in demonstrating bacterial motility and separating motile from non-motile strains
Semi solid medium – 0.5% agar.



2) Classification based on nutritional component

A. Simple media:
Simple media such as peptone water,
nutrient agar can support most non-fastidious bacteria

B. Complex media:
Complex media such as blood agar have ingredients whose exact components are difficult to estimate

C. Synthetic media:
specially prepared media for research purposes where the composition of every component is well known.

3. Classification based on functional use or application

Basal media
Enriched media

Selective media
Enrichment media
Differential media
Transport media
Anaerobic media

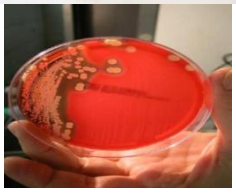
A. Basal media

Basal media are basically simple media that supports most non-fastidious bacteria
Fastidious bacteria =having complicated nutritional requirements
Exp: Peptone water, nutrient broth and nutrient agar

B. Enriched media

Addition of extra nutrients in the form blood, serum, egg yolk etc to basal medium makes them enriched media
Exp: **Chocolate agar**, blood agar

- **Chocolate agar** - is a non-selective, enriched growth medium. containing red blood cells that have been lysed by slowly heating to 80 °C. Chocolate agar is used for growing fastidious bacteria, such as *Haemophilus influenzae*
- **Blood agar**- (BAP) Contains mammalian blood (usually sheep or horse), typically at a concentration of 5–10%. BAP are enriched, differential media used to isolate fastidious organisms and detect hemolytic activity

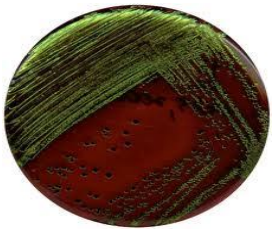


C. Selective media

- Enrichment media are designed to inhibit unwanted commensal or contaminating bacteria and help to recover pathogen from a mixture of bacteria.
- To make a medium selective include addition of inhibitory agents that don't affect the pathogen such as antibiotics, dyes, chemicals, alteration of pH or a combination of in agar

Exp:

- **Mac Conkey's** medium for gram negative bacteria
- **TCBS** – for V.cholerae
- **LJ medium** – M.tuberculosis
- **EMB** agar is selective for gram-negative bacteria. The dye **Eosin methylene blue** in the medium inhibits the growth of gram-positive bacteria; small amounts of this dye effectively inhibit the growth of most gram-positive bacteria



D. Enrichment media

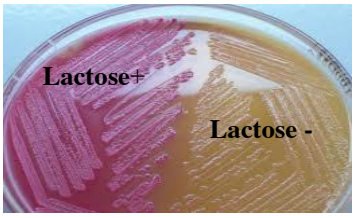
Liquid media that also serves to inhibit commensal in the clinical specimen.

Selenite F broth and alkaline peptone water are used to recover pathogens from fecal specimens.

E. Differential media

- Certain media which has substances incorporated in it enabling it to distinguish between bacteria
- are designed in such a way that different bacteria can be recognized on the basis of their colony colour

Exp: MacConkey's agar, CLED agar, TCBS agar, XLD agar etc



- **MacConkey's agar** Distinguish between lactose fermenters & non lactose fermenters
  It contains bile salts (to inhibit most Gram-positive bacteria), crystal violet dye (which also inhibits certain Gram-positive bacteria)
- **XYLOSE LYSINE DEOXYCHOLATE AGAR XLD** is used as a selective and differential medium for the recovery of Salmonella and Shigella species.

- L.J LOWEINSTON JENSON is a solid medium for MTB *Mycobacterium Tubercolysis*
- Fungal media
  Dermatophyte test medium

F. Transport media

Clinical specimens must be transported to the laboratory immediately after collection to prevent overgrowth of contaminating organisms or commensals. This can be achieved by using transport media

- e.g. Stuart's transport media
- Cary Blair medium for campylobacter species
- Alkaline peptone water medium for v. cholerae

Transport media should fulfill the following criteria:

- temporary storage of specimens being transported to the laboratory for cultivation.
- maintain the viability of all organisms in the specimen without altering their concentration.
- contain only buffers and salt.
- lack of carbon, nitrogen, and organic growth factors so as to prevent microbial multiplication.

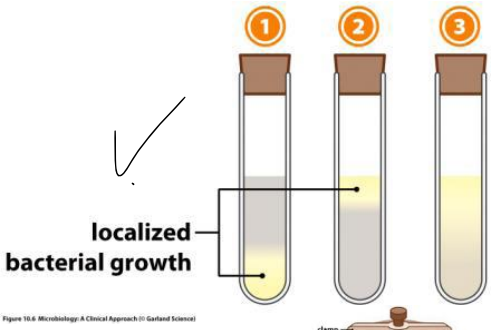
- transport media used in the isolation of anaerobes must be free of molecular oxygen.

G. Anaerobic media

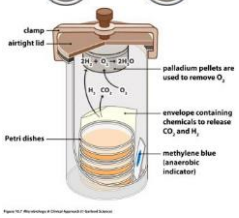
Special growth media and incubation conditions are required for anaerobic bacteria to grow because they need low oxygen content, reduced oxidation –reduction potential and extra nutrients.
Media for anaerobes may have to be supplemented with nutrients like hemin and vitamin K

The first method uses the medium **sodium thioglycolate**, which forms an oxygen gradient during growth.

1 Anaerobic organisms grow at the bottom.
2 Aerobic organisms grow at the top.
3 Facultative anaerobes grow throughout the medium.



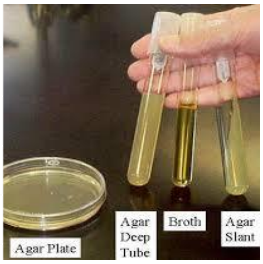
The second method for growing anaerobic organisms is in a GasPakTMjar



- This incubation container provides an oxygen-free environment.
- Only obligate and facultative anaerobes can grow via this method.

Dissolution by heat

sterilization by Autoclave



Pouring in petri dishes

Chapter 3
Sterilization
Disinfection

STERILIZATION

Definition: Sterilization means the complete destruction of all the micro-organisms including spores, from an object or environment. It is usually achieved by heat or filtration but chemicals or radiation can be used.

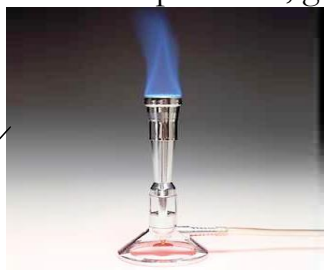
METHODS OF STERILIZATION

A. Physical methods

Heat sterilization is the most method used

Dry heat :**التعقيم بالحرارة الجافة**

- Flamming : 250^0C – 300^0C
  - Points of forceps & Inoculation loops – heat in bunsen flame till red hot
  - Slow passage through flame to destroy vegetative bacteria on surface of scalpel blade, glass slides, mouths of test tubes



- Incineration : 870^0C - 980^0C
  حرق كامل إلى رماد

Complete burning to ashes

Used for soiled dressings, animal carcasses, pathological material

تستخدم لالضمادات المتسخة، جثث الحيوانات، المواد المرضية

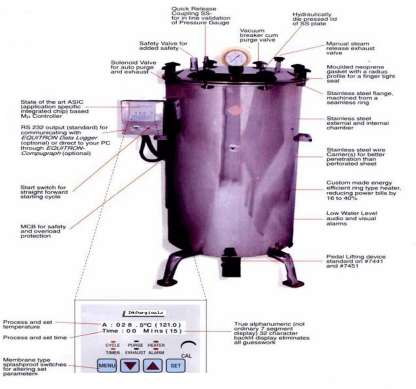
- Hot air oven 160°C 2H

  Used for Empty glassware and glass (not plastic!) pipettes and Petri dishes

Moist heat **التعقيم بالحرارة الرطبة**

Lethal effect due to denaturation & coagulation of proteins

- **استخدام جهاز الاتوكلايفAutoclave steam under pressure) - 121^0C, 15-20 min, 15 lbs**
  - The principle of sterilization in an autoclave or pressure cooker is that steam under pressure is used to produce a temperature of 121°C which if held for 15 and 20 minutes.
  - Will kill all micro-organisms including bacterial endospores.



- Used for sharp instruments, infectious medical waste, culture media

استخدام جهاز ارنولد Arnold sterilizer 100^0C, 30 min, 3 days

- Nutrient media & media containing sugars or gelatin or blood .
- first day all vegetative bacteria are killed. On II & III day spores that germinate are killed

Radiations

2 types

Non- ionizing

- Infra Red radiation (rapid mass sterilization of syringes, etc)
- Ultra Violet radiation (enclosed areas)

Ionizing – Gamma, X ray, cathode ray (plastics, syringes, oil, metal foils)

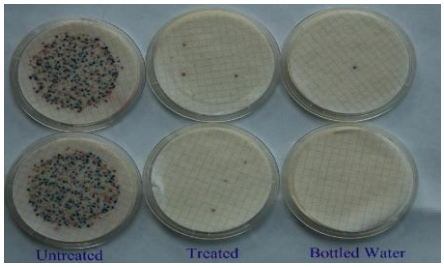
Sterilization controls

- Dosimeter – measures radiation dose
- Colored discs

B. Mechanical methods

- Types of filters

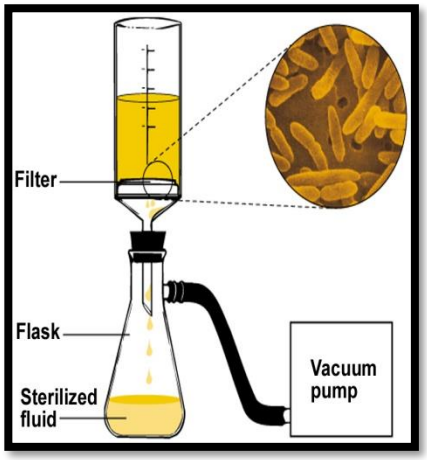
Most famous **Seitz**



Membrane filters – cellulose nitrate, cellulose acetate, polycarbonate, polyester filters

- Pore size: 0.015 – 12 μm
- HEPA filters – for large volumes of air

Sterilization control – bubble pressure test



DISINFECTANTS

Disinfection is the destruction, inhibition or removal of microbes that may cause disease or other problems e.g. spoilage but not necessarily bacterial spores. It is usually achieved by the use of chemicals.

- Strong disinfectants – for inanimate object
- Mild disinfectant (antiseptic) – superficial application on living tissue

Factors affecting DISINFECTION

- Conc of disinfectant
- Time of action
- pH of the medium
- Temperature
- Nature & number of organisms
- Presence of extraneous material
- Others – hardness of water, relative humidity

Specific disinfectants at specified working strengths are used for specific purposes

- Hypochlorite (sodium chlorate I:) Use for Discard pots for pipettes and slides

Ethanol isopropyl alcohol: use for Skin disinfection

  - Skin antiseptics at 70%
  - Denature bacterial proteins

- Aldehyde: Formaldehyde - 10% used
  - In aq. soln is, bactericidal
  - Used to fumigate wards, sick rooms, labs
  - Toxic when inhaled

- Ethylene oxide
  - Especially for heart lung machines, respirators, sutures, syringes, dental equipments, plastic petri dishes

- Dyes combined with nucleic acids exp: crystal violet
  - Skin & wound antiseptics
  - Bacteriostatic, more active against Gram Positive bacteria

- Phenolics : Carbolic acid – 2-5%

Powerful microbicidal, very corrosive

General purpose disinfectant in hospital

- Surface active agents

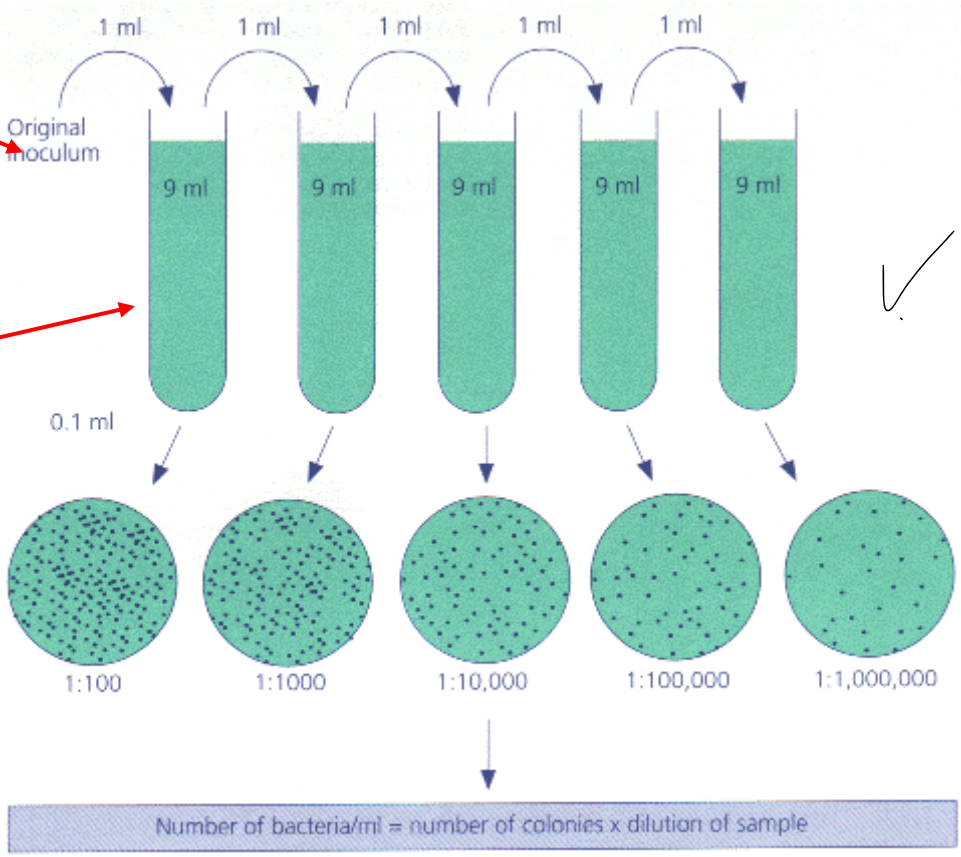
Chapter 4
Isolation ,
Methods of streak
Quantification of bacteria

Isolation of bacteria from soil, water, environment ……

Decimal dilution

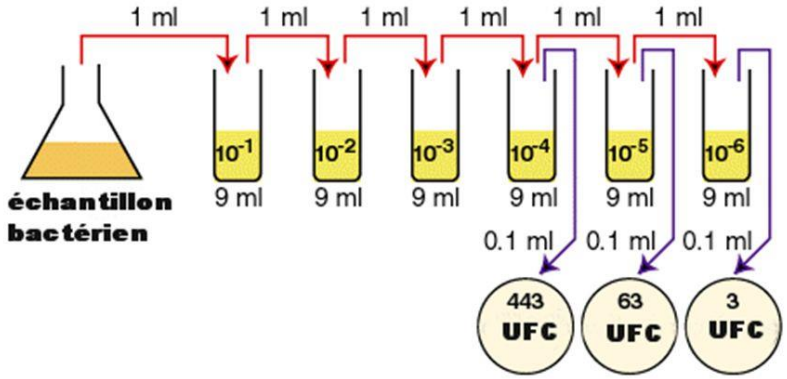
1g in 10ml Of medium or distilled water: bacterial suspension

Distilled water



>250 colonies : Non countable

Number of bacteries in UFC ; unit formant colonies

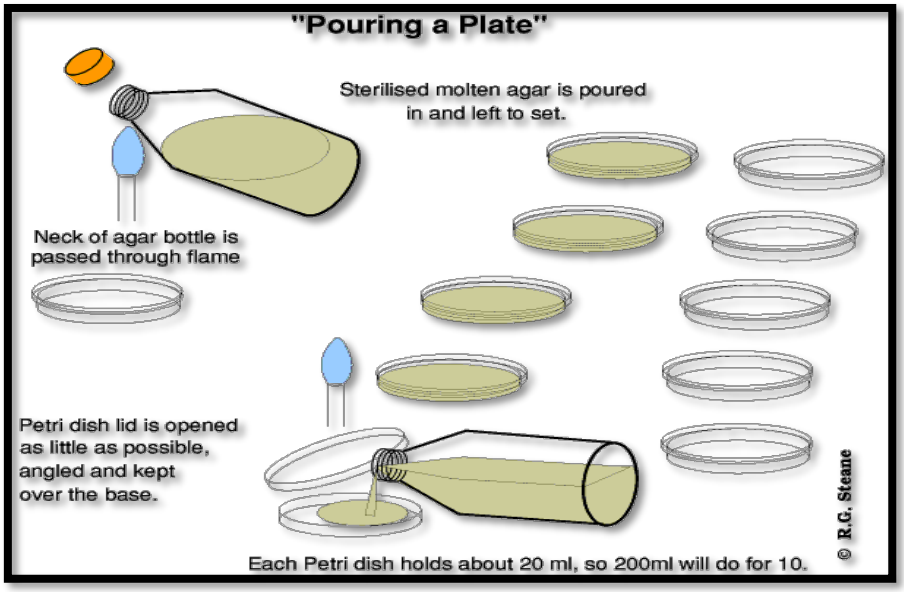


63 UFC/0.1 ml de 10^{-5}

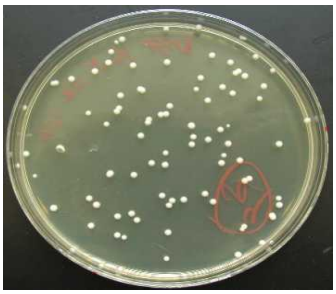
630 UFC/1.0 ml de 10^{-5}

630 UFC/ml X 10^5 = 6.3 x 10^7/ml dans l'échantillon original

Pouring plate



Isolation of bacteria in pure cultures



Pure culture contain one type of colonies: **individuals colonies**

Mixture culture contain different types of colonies

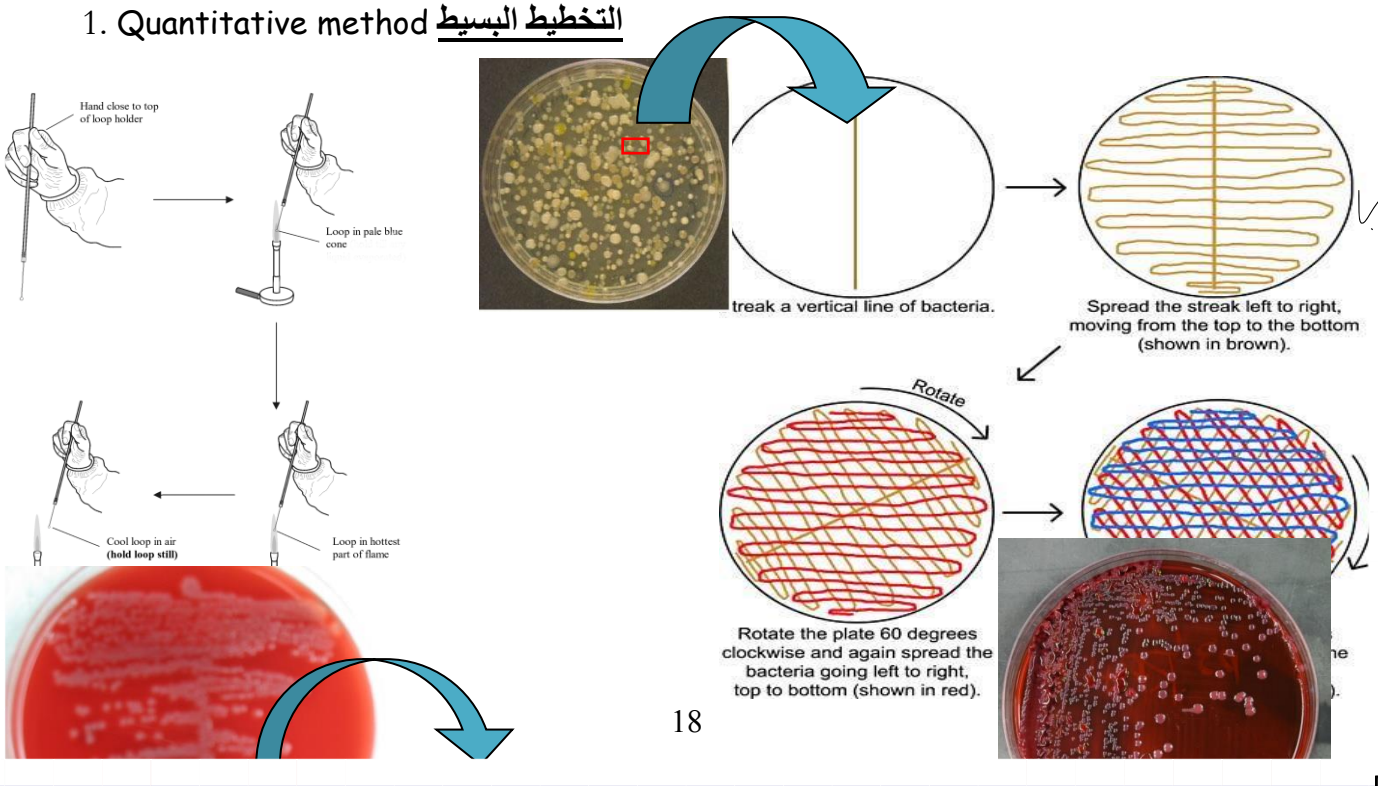
For obtaining pure cultures of bacteria, you should provide:

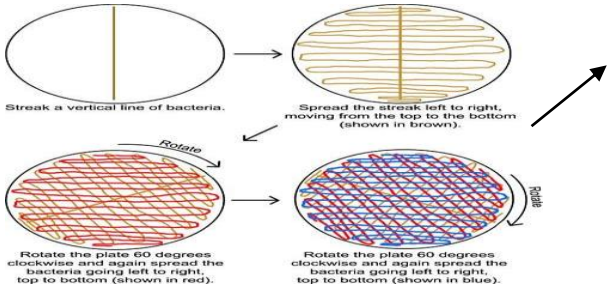
Single colonies separated and solid culture media

Based on the streak plate methods

Streak plate methods

1. Quantitative method **التخطيط البسيط**

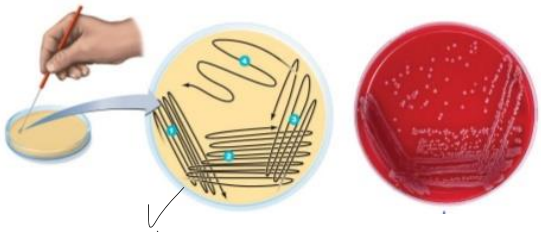


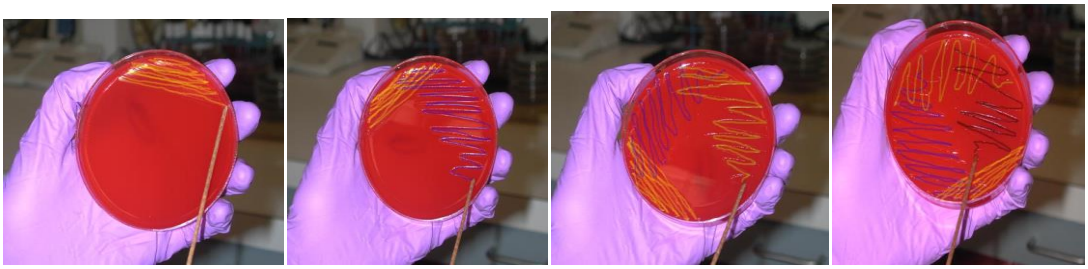


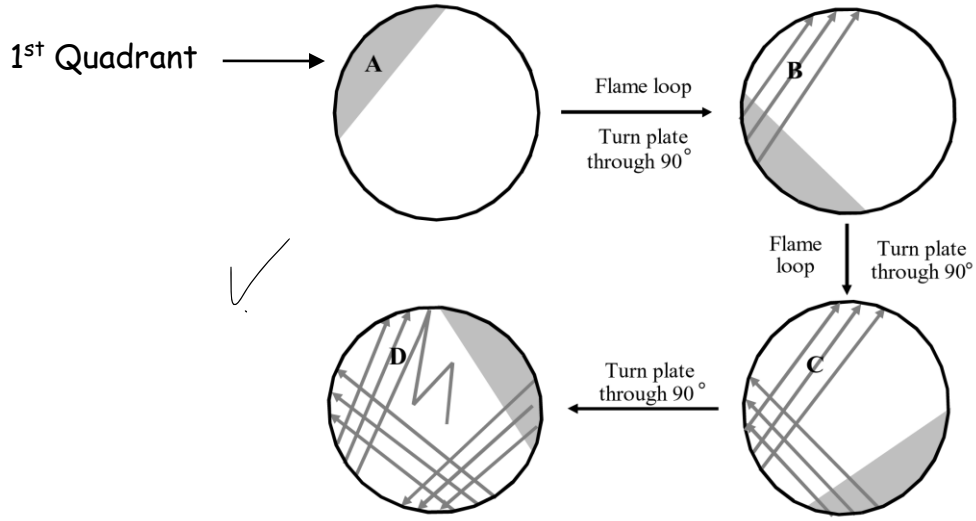
2. Semi-quantitative method التخطيط المتعامد

Techniques to isolate microorganisms in pure cultures or axenic cultures

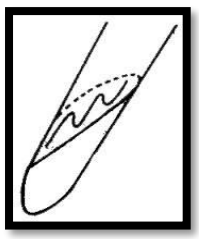
- **Streak-plate technique of isolation**







- **Streak on agar slant plate طريقة التخطيط على الآجار المائل**



لتخطيط المتعرج أو بعمل خط مستقيم على سطح البيئة من الأسفل إلى الأعلى
Zigzag streak or a straight line on the surface from the bottom to the top

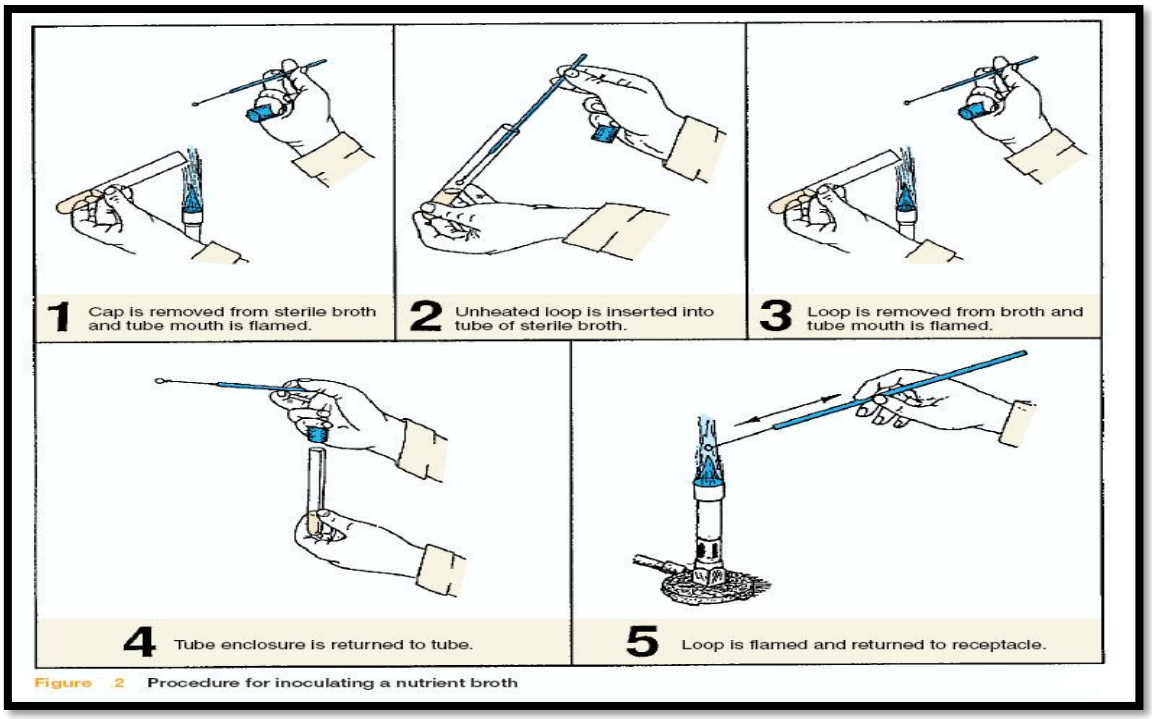
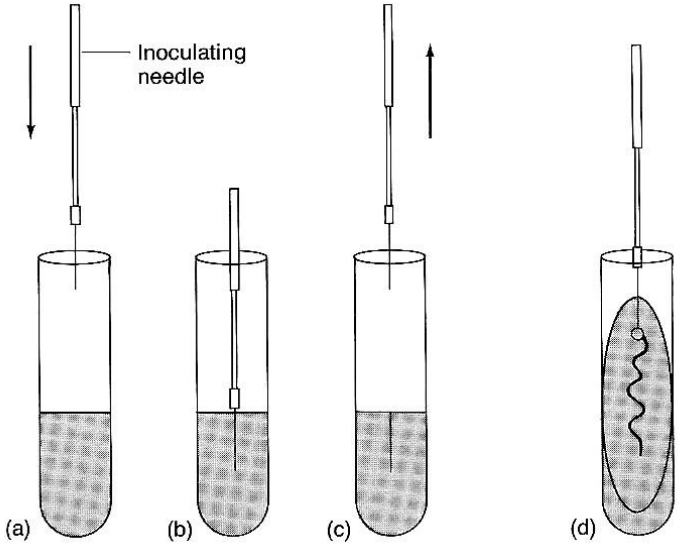


Figure 2 Procedure for inoculating a nutrient broth

inoculation of agar deep tube تلقيح الآجار العميق (المستوى)

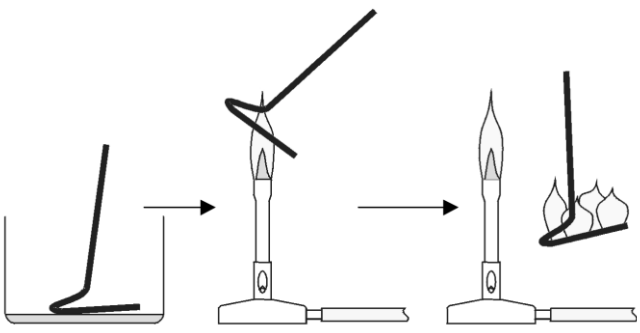
- Provide anaerobic conditions for the growth of bacteria

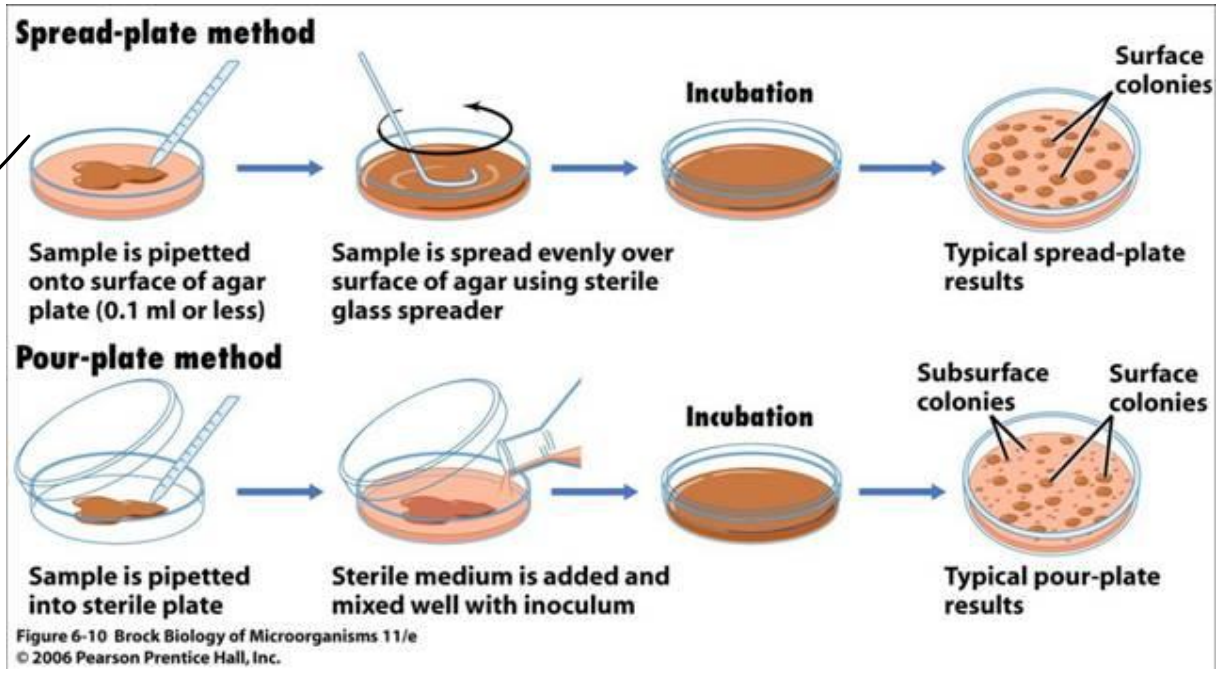
- Test the motility of bacteria



Spread plate

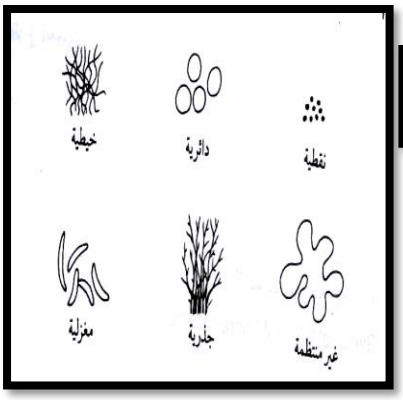
Flaming a glass spreader

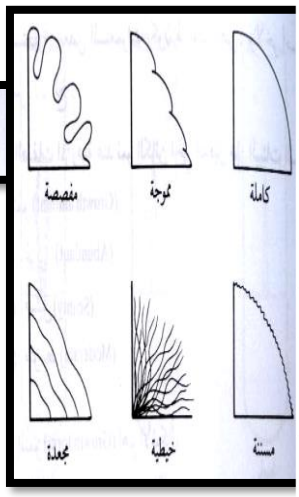




Bacterial Cultural Characteristic

Shape	Circular	Rhizoid	Irregular	Filamentous	Spindle	
Margin	Entire	Undulate	Lobate	Curled	Rhizoid	Filamentous
Elevation	Flat	Raised	Convex	Pulvinate	Umbonate	
Size	Punctiform	Small	Moderate	Large		
Texture	Smooth or rough					
Appearance	Glistening (shiny) or dull					
Pigmentation	Nonpigmented (e.g., cream, tan, white) Pigmented (e.g., purple, red, yellow)					
Optical property	Opaque, translucent, transparent					





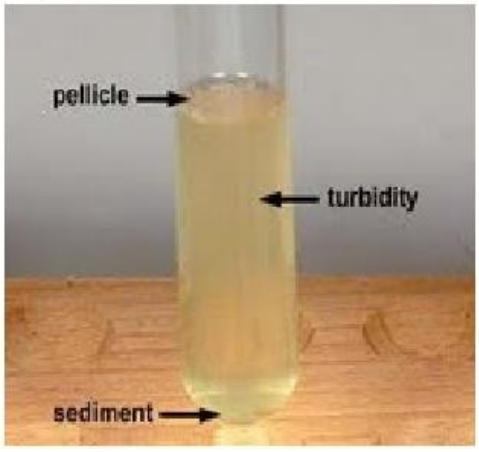
Bacterial growth and propagation

Phenomena of bacterial growth in liquid medium

Broth (a common liquid medium) cultures can exhibit: (i) forming cloudiness in broth (growth with uniform turbid pattern), or (ii) forming a ring at the top of broth (growth with suspension pattern), or (iii) forming sediment at the bottom of broth (growth with sedimentary pattern).

i *ii* *iii*

- **Transformation of a clear broth medium to a turbid suspension.**

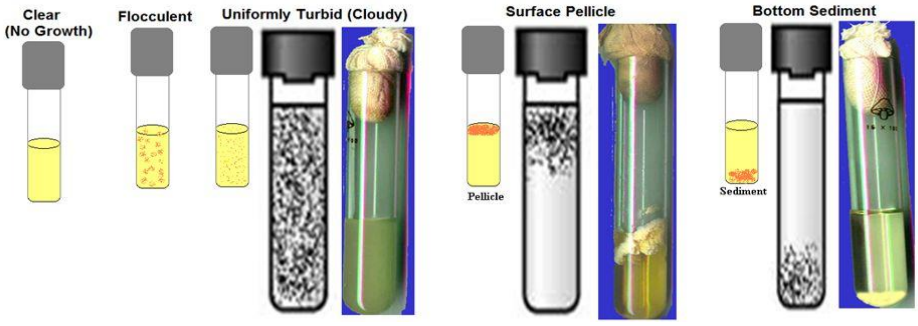


Pellicle are the thin layer of organic substances and dead bacterial cells which float at the top surface of the liquid broth culture.

Turbidity in the broth culture occurs due to increase in the bacterial population after growth.

Sediment is formed at the bottom of a broth-culture tube due to accumulation of mature bacterial cells

Bacterial growth in liquid media Patterns



How to Graph Bacterial Growth (i)

(I) **Measuring the numbers of bacteria.**

Common methods include:

a) Turbidity: to measure the total bacteria (live and dead) in liquid cultures. This is usually quantitated with a spectrophotometer, the absorption wavelength at 600 nm will be measured.

b) Colony counting method: that means counting the colony numbers on a medium plate after inoculated with a known volume of bacterial liquid culture.

Chapter 5
Preparation of bacterial smear

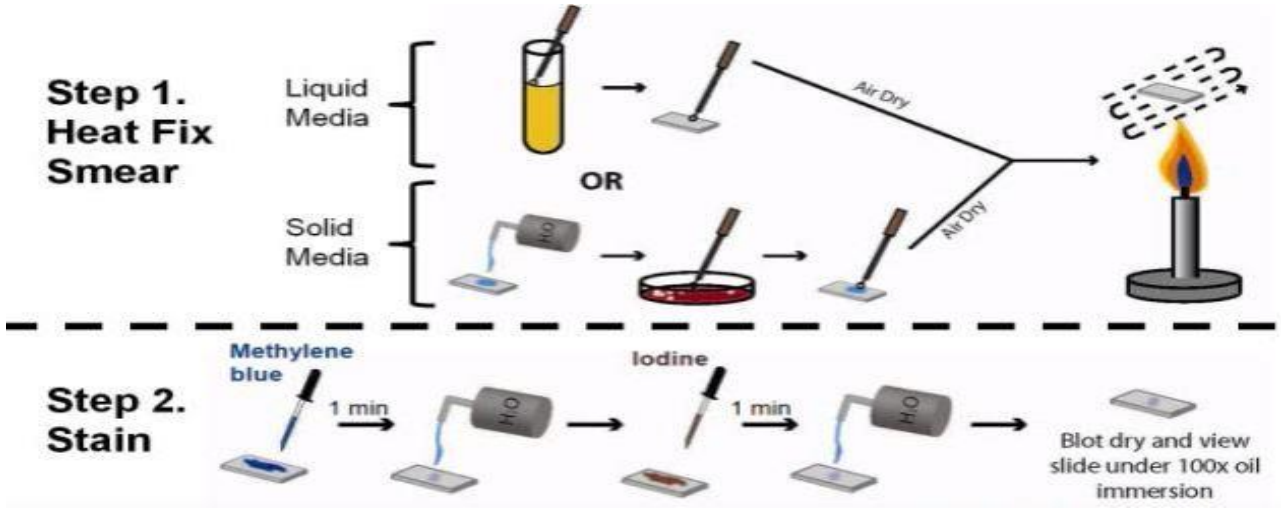
Smears

- Performing quality smears takes practice. There are many steps in the preparation of a smear that must be performed correctly. (Think of Goldilocks that wants her porridge "just right")

- 1. Smear bacteria onto slide from broth or agar
  - Too thick and you can't see through specimen, too thin and you can't find any bacteria

- 2. Air dry
  - Again, you do not want too large a smear or it will take a long tin for the smear to completely air dry.

- 3. Heat Fix (see next slide)



Smear Preparation for Staining:

- ✓ **For the broth culture**, shake the culture tube and, with an inoculating loop, aseptically transfer 1 to 2 loopful of bacteria to the center of the slide.

  Spread this out to about a 1/2-inch area.

- ✓ **When preparing** a smear from a agar slant or agar plate, place a loopful of distilled water in the center of the slide.

  With the inoculating needle, aseptically pick up a very small amount of culture and mix into the drop of water and spread it out.

- From liquid medium

A- draw a target circle on the bottom of the slide

B-Place 2 loopfuls of culture in the center of target circle and spread over the entire circle

C-Let it air dry

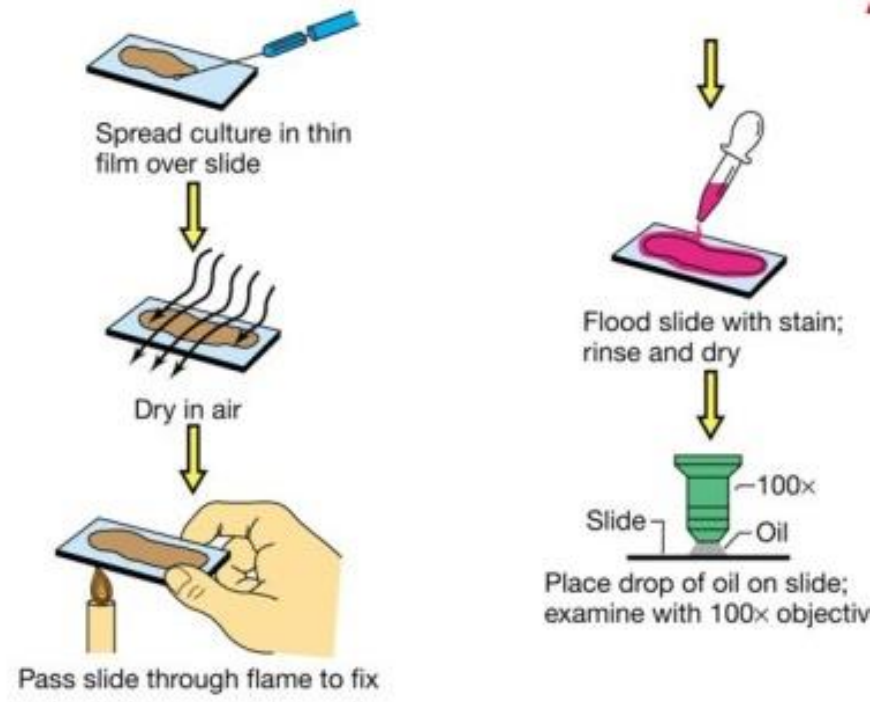
D-Using a clothespin, grab slide and pass it several times over the flame to heat kill and fix the organisms.

- 3. Heat Fix
  - Pass slide through flame quickly 3-4 times
  - Heat fix too little and organisms may wash off slide
  - Heat fix too much and organisms may be distorted
  - Heat fixation:
    - 1. kills organisms
    - 2. adheres specimen to slide
    - 3. promotes stainability of specimen

2. Chemically fix:

- **by flooding the slide with methanol and allow to evaporate (Denatures bacterial enzymes and enhances adherence of bacteria to the slide)**

Preparing a smear (pg. 31-32)



SIMPLE STAINING:

- **Simple to perform- only one basic stain used.**

E.g. Crystal violet, Methylene blue, Basic fuschin etc.,

- **Principle:**

- All bacteria in smear takes stain and appears in colour of stain.

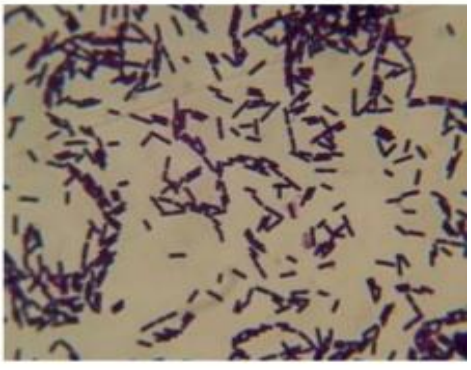
- Basic stain more affinity towards bacterial surface & stains the bacteria.

- **Uses:**

To study morphology and arrangement of bacteria.

PROCEDURE:

- **A bacterial smear is prepared, air-dried and heat-fixed.**
- **A Heat-fixed smear is flooded with either one of the basic stain and allowed to react for 1-2 minutes and then washed under running tap water.**
- **Air dried and focused with 10x,45x & 100x.**



Chapter 6
Gram staining

- Gather all equipment and supplies
  - Gram stain
  - Microscope slides
  - Heat source
  - Sterile swab or loop
  - Culture or specimen
  - Microscope

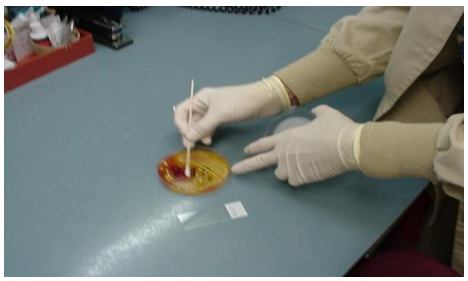
Reagents used in gram staining

- Gram Crystal Violet 0.5%
- Gram Iodine
  - Potassium Iodide 2%
  - Resublimed Iodine 1%
- Gram Decolorizer
  - Methanol 80%
  - Acetone 20%
- Gram Safranine 1%

1. CRYSTAL VIOLET
   - Primary stain
   - Violet colored, stains all micro-org
2. GRAM IODINE
   - Mordant
   - Forms Crystal violet iodine complexes
3. DECOLORIZER
   - Acetone + Methanol
   - Removes Crystal violet iodine complex from thin peptidoglycan layers
   - Dissolves outer layer of Gram negative org
4. GRAM SAFRANINE
   - Counter stain
   - Red colored

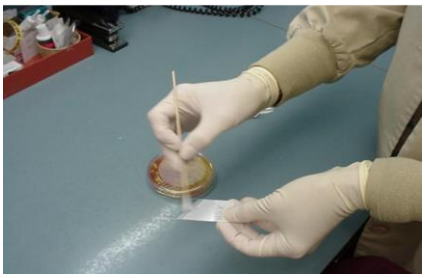
Steps

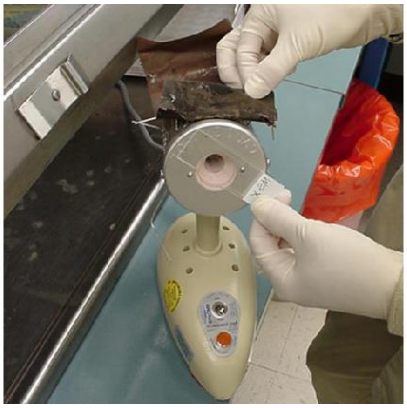
Step 1 - Prepare a Smear



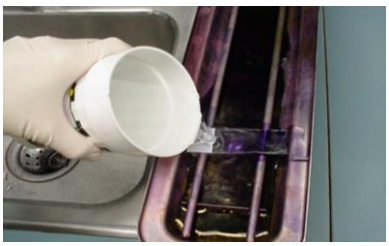
- Label the slide with the patient's initials or the specimen number.
- Choose an isolated colony off of the agar plate and obtain bacteria with a sterile swab.
- Place the swab on the microscope slide and spread the colonies in a circular motion.

- Heat fix the microorganisms to the slide by placing the bottom of the slide to heat for approximately 30 seconds

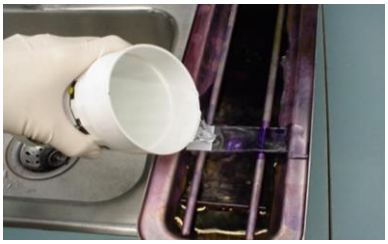




Step 2. **Apply the Primary Stain**
Flood the surface of the slide with Crystal Violet stain and let sit for one minute.
Rinse the slide with distilled water.

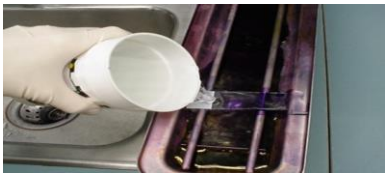


Step 3. Apply the mordant: **iodine**
Flood the slide with Gram's Iodine and time for one minute.
Rinse the slide with distilled water



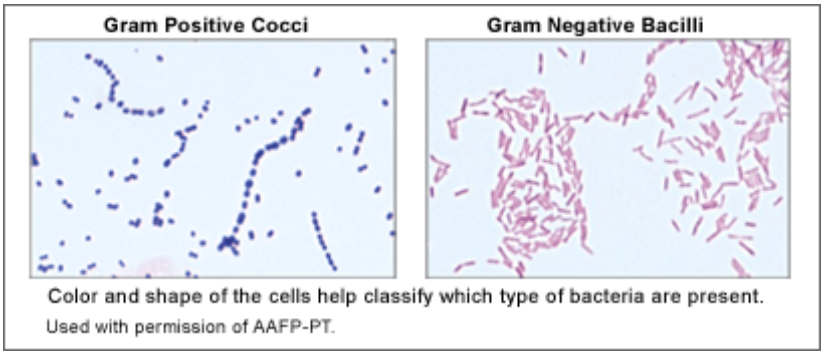
Step 4. Flood the slide with Gram's **decolorizer** and time for 30 seconds

Step 5. Counterstain



Flood the slide with the counterstain, Safranin, and let sit for one minute
Rinse the slide with distilled water

Step 6. Rinse, Dry and Observe



Rinse with water to remove excess stain
Blot dry
Observe under oil immersion