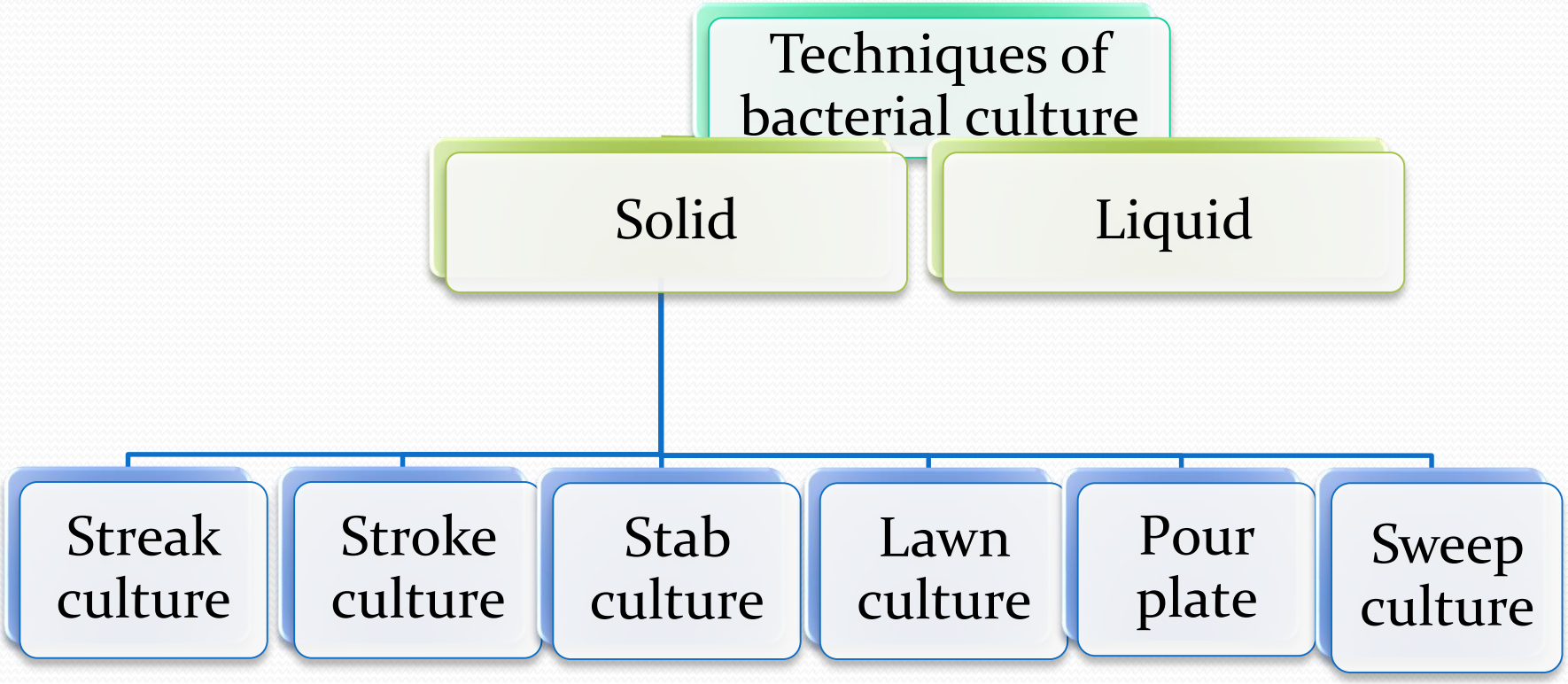
Isolation and Identification of Bacteria

Purpose:

- To isolate bacteria in pure culture.
- To demonstrate their properties.
- To obtain sufficient growth for antigen preparation.
- For typing of bacteria.
- To determine antibiotic sensitivity.

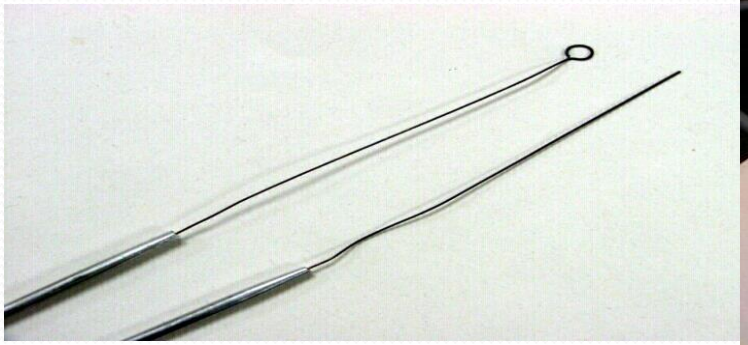
Method:

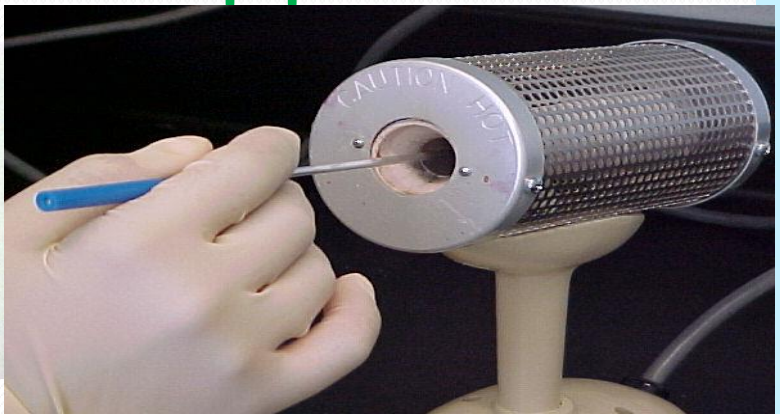
- Bacterial culture- By growing the organism on artificial culture media at suitable temperature, pH and redox potential.
- By use of laboratory animals.


Techniques of bacterial culture
Solid
Liquid
Streak culture
Stroke culture
Stab culture
Lawn culture
Pour plate
Sweep culture

Instruments used to seed culture media

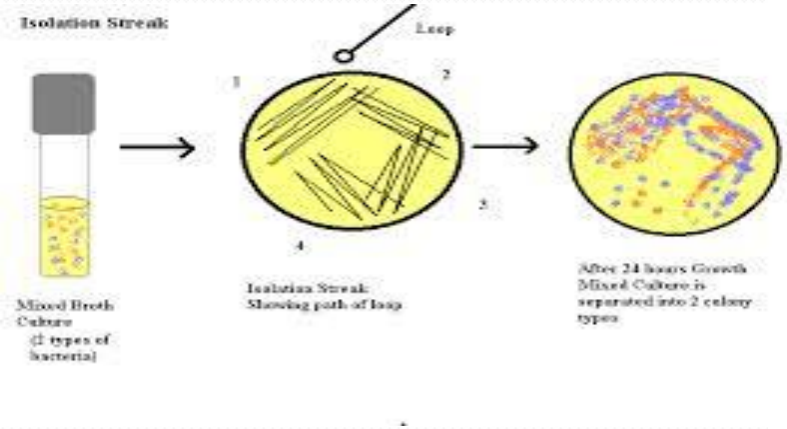
- Burner - Pilot flame is used.
- Straight wire.
- Wire loop - Made up of either platinum - iridium or nichrome or stainless steel. The internal diameter of loop is 2-4 mm while the length of the wire should be 50 mm.
- Sterile pipettes.

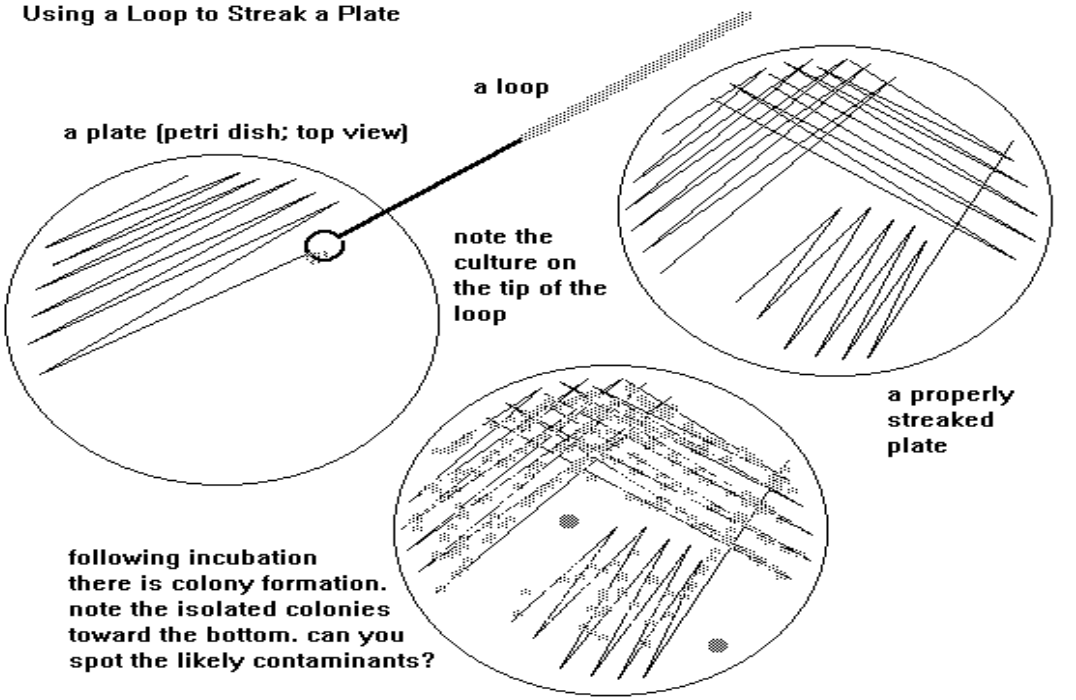




Streak culture:

It is the method routinely employed for the isolation of bacteria in pure culture from clinical specimens.

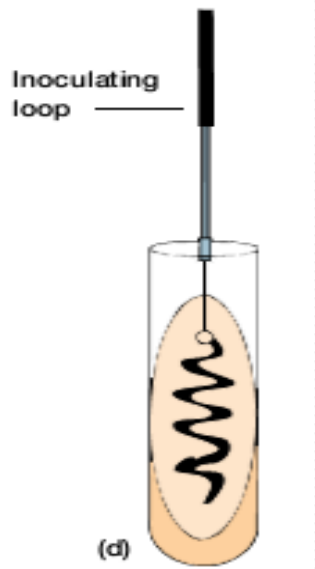




Stroke culture:

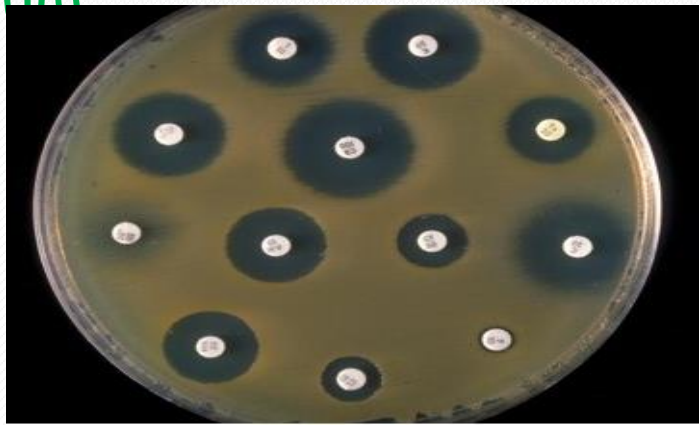
Uses: For preservation of bacterial cultures.

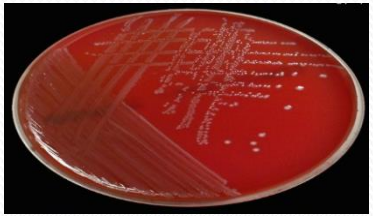
- For obtaining pure growth.
- For biochemical reaction



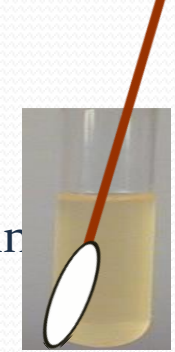
LAWN OR CARPET CULTURE

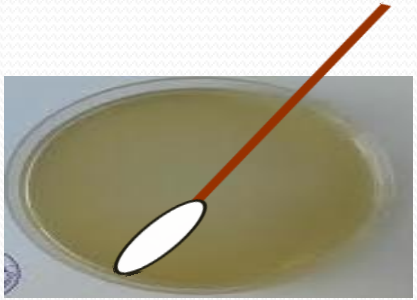
- Provides a uniform surface growth of the bacterium
- Uses-For bacteriophage typing.
  - Antibiotic sensitivity testing.
  - In the preparation of bacterial antigens and vaccines.
- Lawn cultures are prepared by flooding the
- surface of the plate with a liquid suspension of the bacterium





Suspension of organism is prepared in peptone water by similar colonies from agar plate.

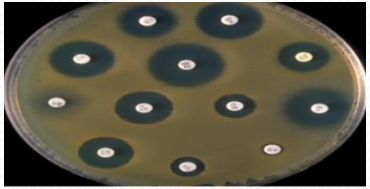




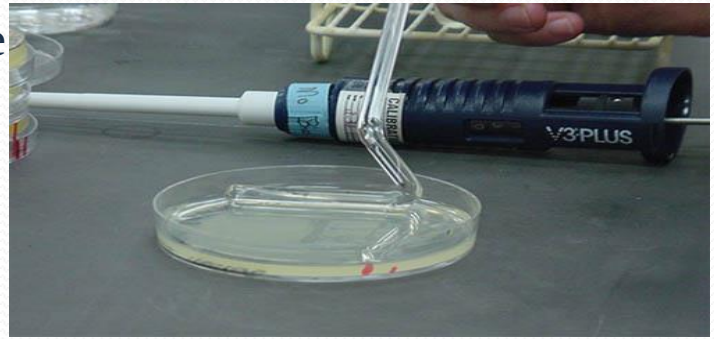
❑Suspension of organism is flooded on dried plate of agar by lawn culture method

Antibiotic discs are kept with sterile swab.

Plates are incubated for 18-24 hours

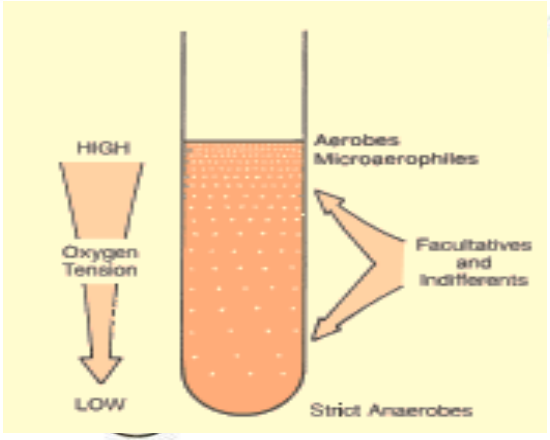


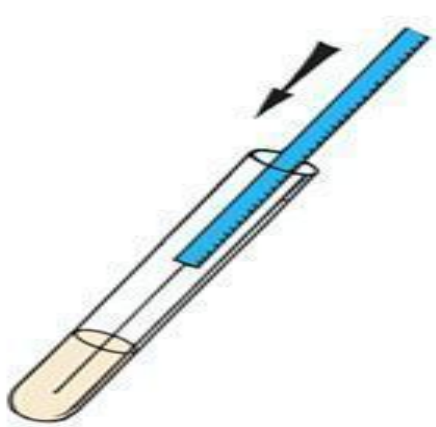
❑Zone of inhibitions around the disc is to be observed, after incubation period.

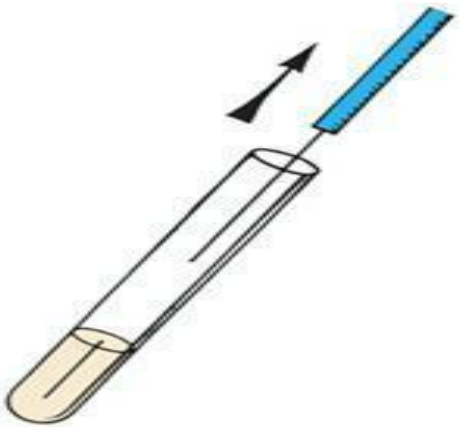


STAB CULTURE

- Prepared by puncturing a suitable medium – gelatin or glucose agar with a long, straight, charged wire.
- Uses-Demonstration of gelatin liquefaction.
  - Oxygen requirements of the bacterium under study.







1 Wire with organisms is brought into tube without touching walls of tube.

2 Wire penetrates medium to two-thirds of its depth.

3 Wire is withdrawn from medium and tube. Neck of tube is flamed and plugged.

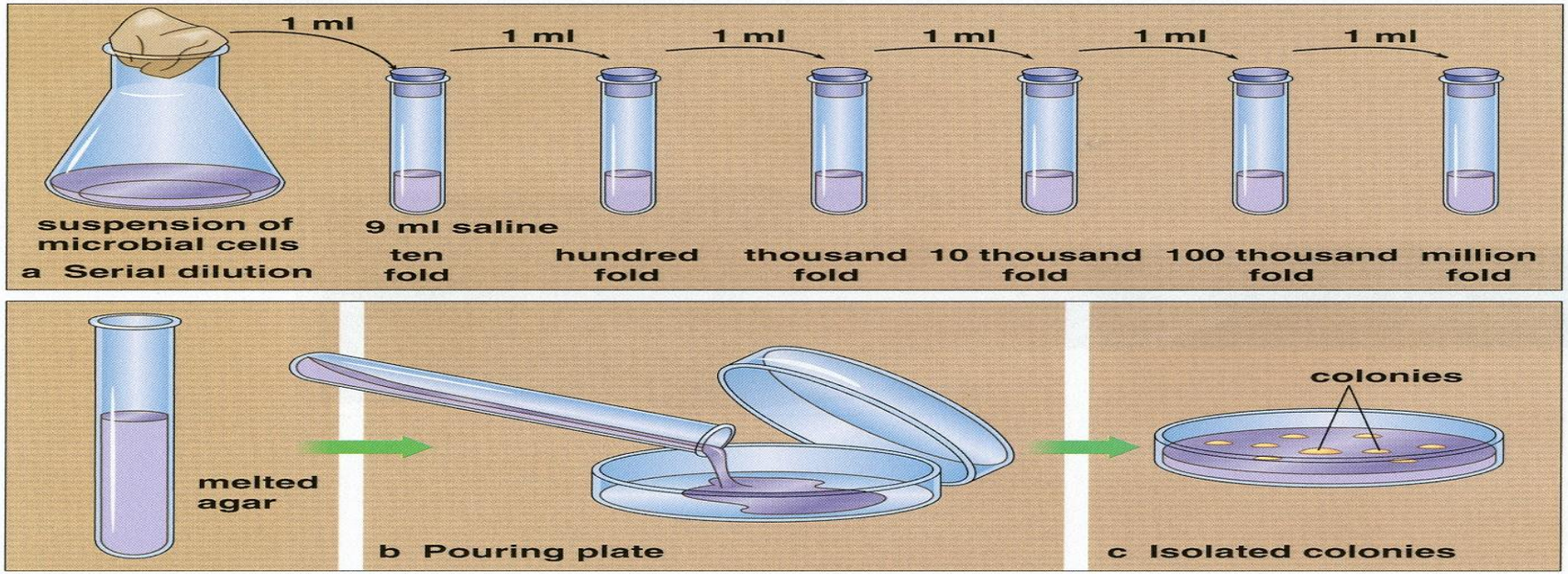
Pour plate culture

- The Inoculum to be tested is serially diluted.
- Known amount of each dilution is mixed with 15 ml of melted agar at 45-50℃ and mixed well.
- The content of the tube are pored in a sterile petridish and allowed to set.
- After overnight incubation at 37℃ , the colonies distributed throughout the depth of the media are counted.
- The number gives the viable bacterial count in a given suspension.

Pour plate culture :

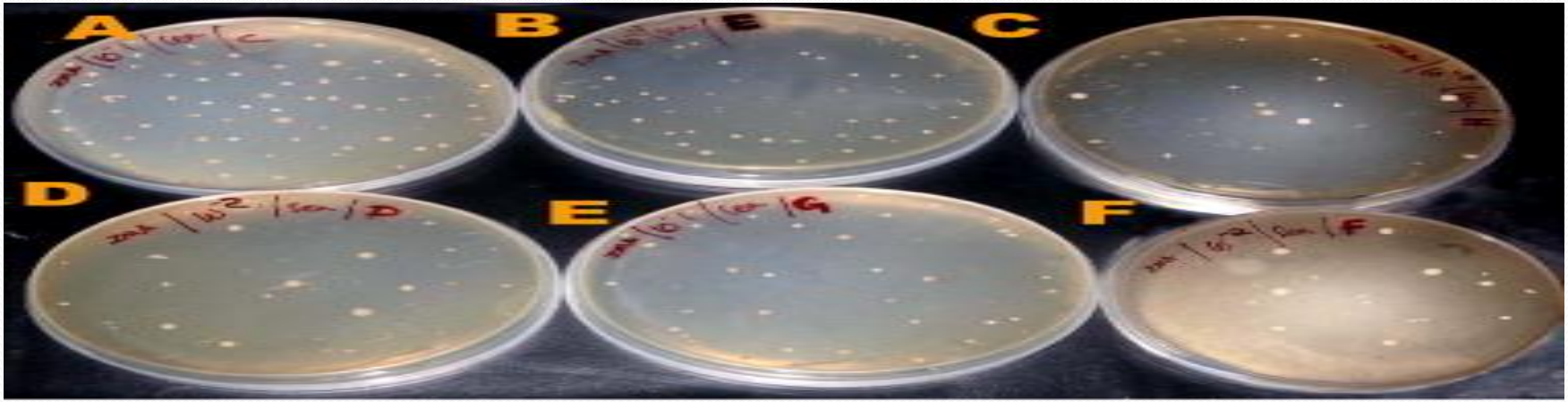
Uses : To quantify bacteria in urine culture

- Analysis of water
- For assay of antibiotics and enzymes.
-



Sweep plate :

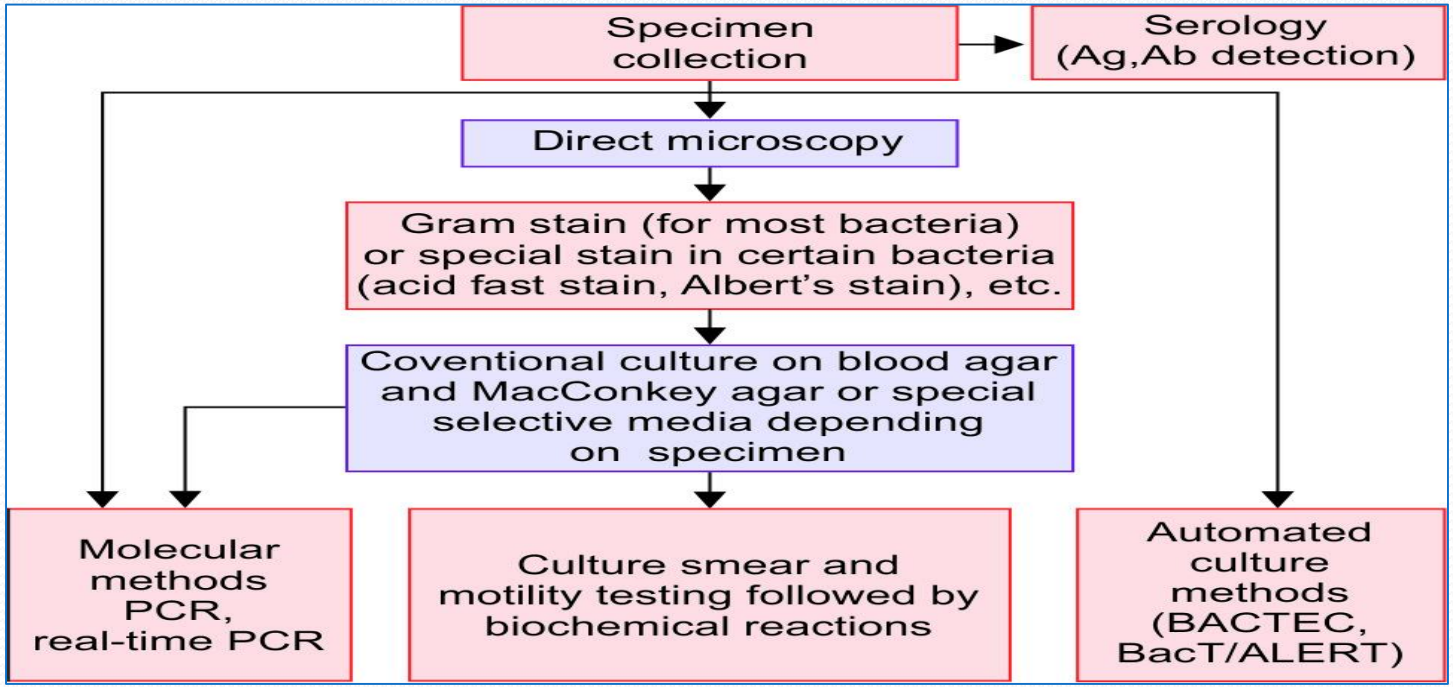
The edges of the petridish containing culture media are rubbed over the fabric with the medium facing it. The dust particles stirred up from the cloth settle on the culture medium and colonies develop on incubation .They can be counted.



Identification of bacteria is done by studying the following characteristics:

- Morphological characteristics
- Cultural characteristics
- Biochemical reactions
- Agglutination by antisera
- Typing
- Animal inoculation

Representation of microbial identification



Type of infections and various specimens collected

Type of infections	Specimens collected
Wound and soft tissue infection	Pus or exudate, swabs, aspirates for abscess
Bloodstream infection	Blood
Urinary tract infection	Midstream urine Suprapubic aspirated urine
Diarrheal diseases	Stool (mucus flakes), rectal swab
Respiratory tract infection	Sputum, throat swab, bronchoalveolar lavage or endotracheal aspirate
Eye and ear infection	Conjunctival swabs, Corneal scrapings Swabs from outer ear/eye
Infections of the sterile area	Sterile body fluids; e.g. CSF, pleural fluid, synovial fluid, peritoneal fluid, etc.

CONVENTIONAL METHOD

- **Direct Microscopy-**
  - Direct smear examination- Specimens are subjected to the following staining techniques.
  - Gram staining
  - Albert's staining
  - Ziehl-Neelsen (ZN) acid fast staining

Culture

- Depending on the type of specimen, various culture media are used.
- *Combination of blood agar and MacConkey agar* is most commonly employed for most specimens.

Culture (cont..)

- *Combination of blood agar and MacConkey agar* - pus, wound swab &other exudate specimens, sterile body fluids, urine, sputum and other respiratory specimens.
- Chocolate agar - Respiratory and sterile body fluid specimens.
- Stool specimen should be inoculated on to selective media such as-
  - Mildly selective media-MacConkey agar and
  - Highly selective media-DCA, XLD and TCBS
- Blood specimen should be directly inoculated into blood culture bottles without performing direct microscopy methods.
- CLED agar - urine specimen

Morphology of Bacterial Colony

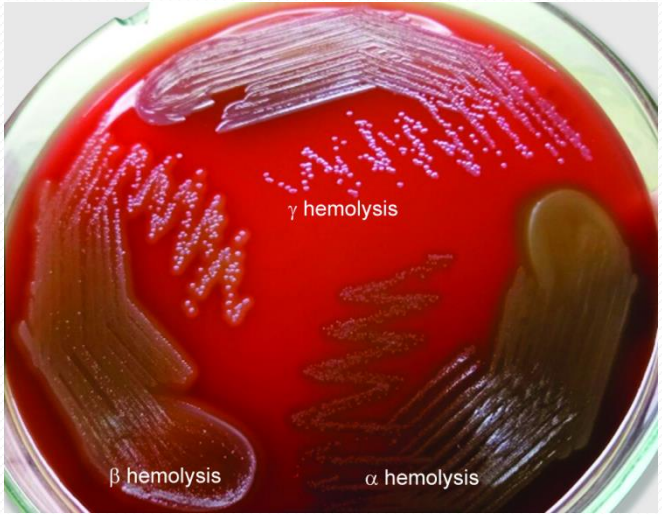
- Size-in millimetres e.g. pin head size is characteristic of staphylococcal colony & pin point size is characteristic of streptococci colony
- Shape – Circular or irregular
- Surface –glistening or dull
- Edge-Entire, crenated, lobate, undulated or filamentous
- Elevation-flat, raised, convex, umbonate, or pulvinate
- Consistency - Mucoid, friable, firm, butyrous
- Density–opaque, translucent or transparent

Morphology of Bacterial Colony

- Colour of the colony- Colonies may be colored due to properties of the media used or due to pigment production.
- **Pigment** produced by certain bacteria may also color the colony. Pigments are of two types.
  - Diffusible pigments
  - Non-diffusible pigments

Haemolysis on blood agar

- *Partial or α hemolysis*
- *Complete* or *β hemolysis*
- *No hemolysis* (γ hemolysis, a misnomer)
- *α prime hemolysis*



Culture smear and motility testing

- Colonies grown on the culture media should be subjected to Gram staining and motility testing by hanging drop method.

Biochemical reactions

- Based on the type of organisms detected in culture smear, the appropriate biochemical tests are employed.
- Initially, catalase and oxidase tests are done on all types of colonies grown on the media.
- For Gram negative bacilli- Common biochemical tests done routinely are-
  - Indole test
  - Citrate utilization test
  - Urea hydrolysis test
  - Triple sugar iron test (TSI)

Biochemical reactions (cont)

- If there is any doubt in correct identification of bacteria, then further biochemical tests are put such as-
  - Sugar fermentation test
  - MR (methyl red) test
  - VP (Voges Proskauer) test
  - OF test (oxidation –fermentation test)
  - Nitrate reduction test
  - Decarboxylase test
  - PPA test (phenyl pyruvic acid test)

For Gram positive cocci; certain useful biochemical tests are

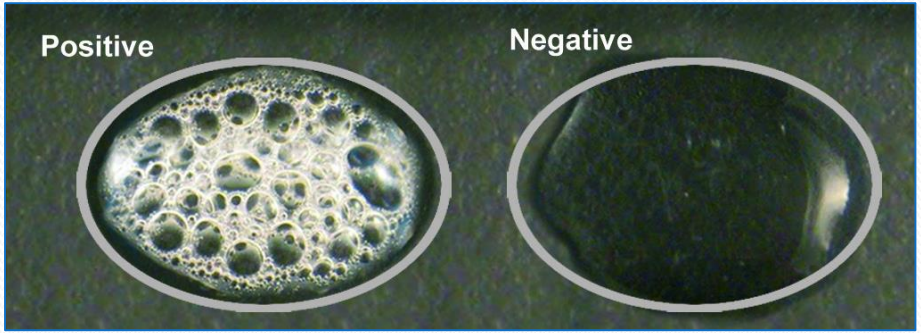
- Coagulase test (for *Staphylococcus aureus*)
- DNase test (for *Staphylococcus aureus*)
- CAMP (Christie Atkins Munch-Petersen) test for Group B *Streptococcus.*
- Bile esculin hydrolysis test (for *Enterococcus*)
- Heat tolerance test (for *Enterococcus*)
- Sugar fermentation test
- PYR test (for *Streptococcus pyogenes* and *Enterococcus*)
- Bile solubility test (for pneumococcus)

Antimicrobial susceptibility tests done for bacterial identification

1. Novobiocin susceptibility test- done for *Staphylococcus saprophyticus*
2. Optochin susceptibility test (for pneumococcus)
3. Bacitracin susceptibility test-done to differentiate group A and group B *Streptococcus*)

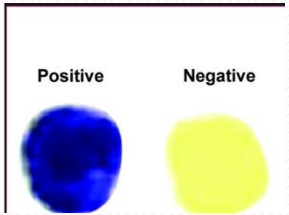
Catalase test

- When a drop of hydrogen peroxide (3% H_2O_2) is added to a colony of any catalase producing bacteria, effervescence or bubbles appear due to breakdown of H_2O_2 by catalase to produce oxygen.



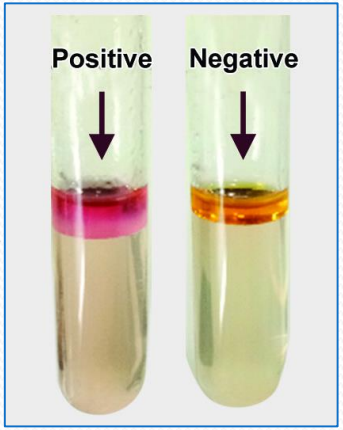
Oxidase tests

- Detects the presence of cytochrome oxidase enzyme in bacteria which catalyses the oxidation of reduced cytochrome by atmospheric oxygen.
- Oxidase positive (deep purple)- Examples include *Pseudomonas* , *Vibrio, Neisseria, Bacillus* etc.
- Oxidase negative (no colour change)-Examples include; members of family Enterobacteriaceae, *Stenotrophomonas*, etc.



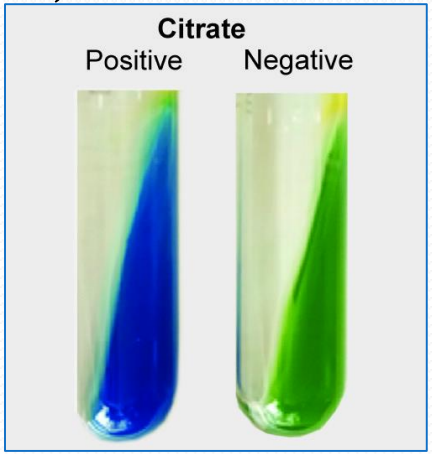
Indole test

- Detects the ability of certain bacteria to produce enzyme tryptophanase that breaks down amino acid tryptophan present in the medium into indole.
- Indole positive *Escherichia coli, Proteus vulgaris, Vibrio cholerae* etc.
- Indole negative - Examples include- *Klebsiella pneumoniae, Proteus mirabilis, Pseudomonas, Sh Salmonella,* etc.



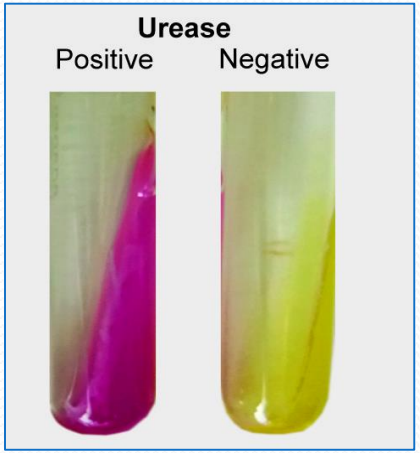
Citrate utilization test

- Dtects the ability of a few bacteria to utilize citrate as the sole source of carbon for its growth, with production of alkaline metabolic products.
- Citrate test is positive for *Klebsiella pneumoniae, Citrobacter, Enterobacter* etc.
- Test is negative for *Escherichia coli, Shigella, Salmonella* Typhi, etc.



Urea hydrolysis test

- Urease producing bacteria can split urea present in the medium to produce ammonia that makes the medium alkaline.
- Urease test is positive for-*Klebsiella pneumoniae*, *Proteus* species, *Helicobacter pylori, Brucella,* etc.
- Urease negative - *Escherichia coli, Shigella, Salmonella,* etc.



Triple sugar iron agar test (TSI)

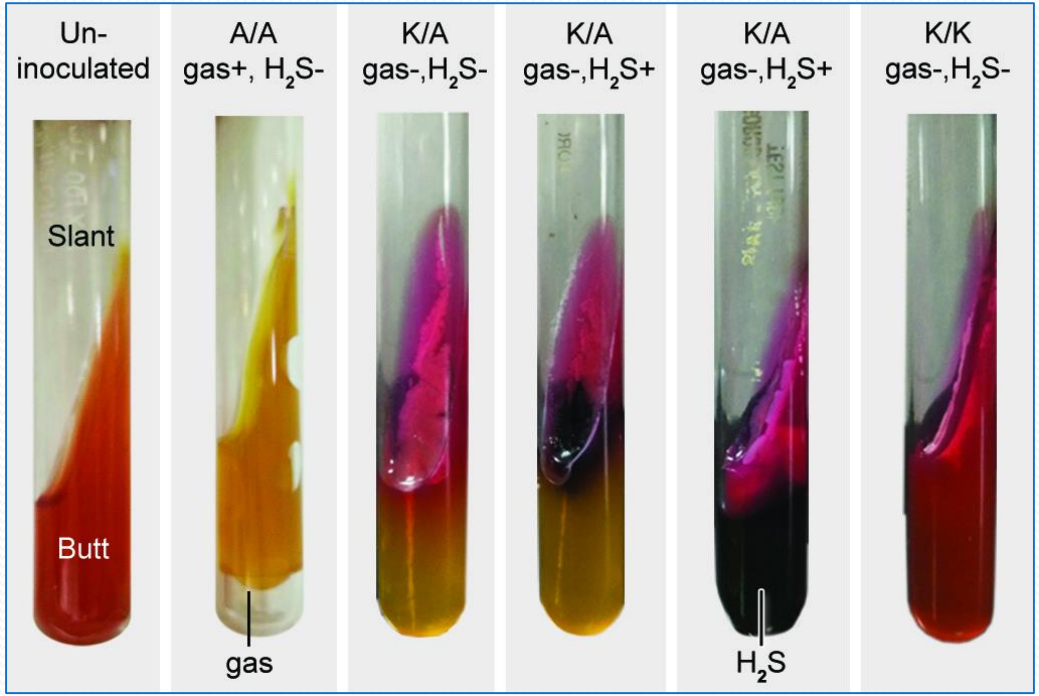
- **Composition**- It is a composite solid agar medium in tube having a butt & a slant. Its constituents include-
- Three sugars- glucose, sucrose and lactose in the ratio of 1:10:10 parts
- Phenol red as an indicator of acid production
- Ferric salts as an indicator of hydrogen sulphide (H_2S).

TSI (cont..)

Interpretation

- *Ability to ferment sugars to produce acid*
- *Ability to produce gas*
- *Ability to produce H2S*

TSI


Un-inoculated
A/A gas+, H_2S-
K/A gas-,H_2S-
K/A gas-,H_2S+
K/A gas-,H_2S+
K/K gas-,H_2S-
Slant
Butt
gas
H_2S

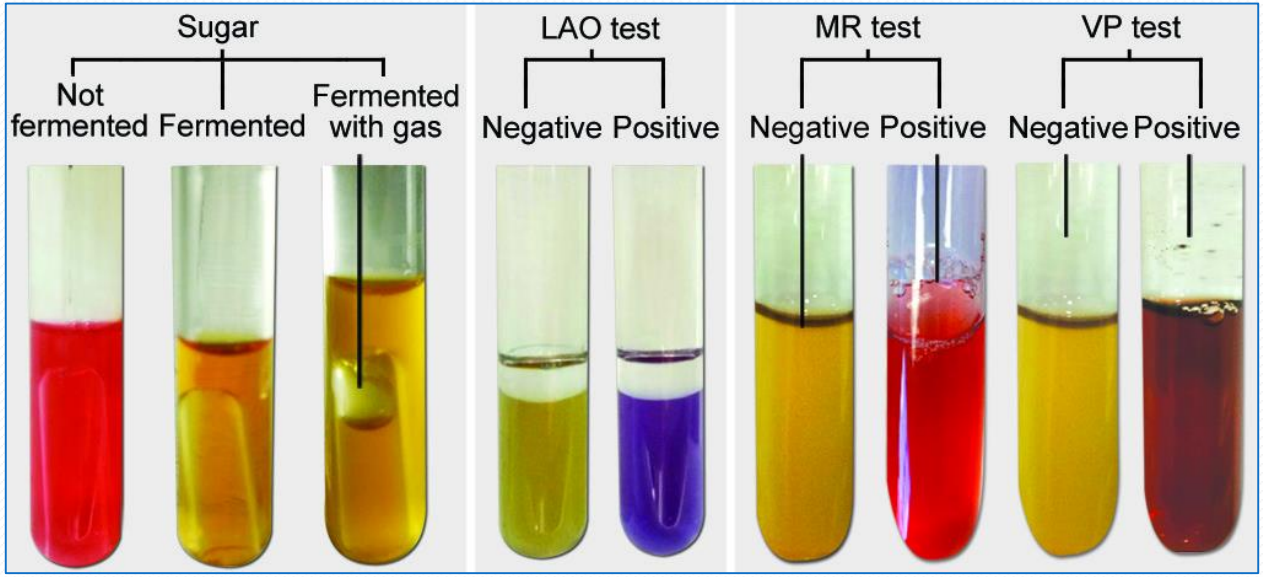
Various reactions in TSI

Reactions in TSI		Examples
Acidic slant/acidic butt		≥ 2 sugars fermented • Glucose fermented & • Lactose and/or sucrose fermented
	A/A, gas produced, no H_2S (fig 6B)	Escherichia coli Klebsiella pneumoniae
Alkaline slant/acidic butt		Only glucose fermenter group
	K/A, no gas, no H_2S (fig 6C)	Shigella
	K/A, no gas, H_2S produced (small amount), fig 6D	S.Typhi
	K/A, no gas, H_2S produced (abundant), fig 6E	Proteus vulgaris
	K/A, gas produced, H_2S produced (abundant)	S.Paratyphi B
	K/A, gas produced, no H_2S	S.Paratyphi A
Alkaline slant/alkaline butt		Non fermenters group
	K/K, no gas, no H_2S (fig 6F)	Pseudomonas Acinetobacter

Sugar fermentation test

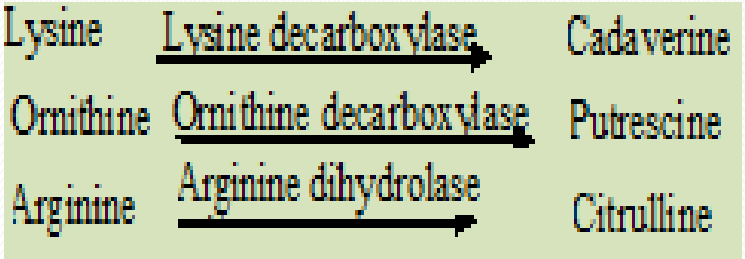
- Detects the ability of an organism to ferment
- a specific carbohydrate (sugar) incorporated in a medium producing acid with/without gas. Glucose, lactose, sucrose and mannitol are widely used for sugar fermentation.
- Acid production is detected by using indicators such as-
  - Andrade's indicator
  - Phenol red indicator
  - Gas production is detected by using an inverted Durham's tube

Biochemical tests



Decarboxylase test

- Detects the presence of substrate specific decarboxylase enzyme in the bacteria that break down amino acids such as lysine, arginine and ornithine to produce alkaline by-products.



MR (methyl red) test

- In glucose phosphate broth, certain bacteria ferment glucose to produce stronger acids that maintain the pH below 4.4 which turns methyl red indicator from yellow to red color
  - MR Positive (red color)-*Escherichia coli*
  - MR negative(yellow, i.e. no change in color)- *Klebsiella pneumoniae*

VP (Voges Proskauer) test

- In the presence of alkali and atmospheric oxygen, acetoin is oxidised to diacetyl which reacts with α-naphthol to give red colour
- VP positive- *Klebsiella pneumoniae, Enterobacter,* El Tor vibrios, *Staphylococcus,* etc.
- VP negative - *Escherichia coli, Shigella, Salmonella,* etc.

Nitrate reduction test

- Detects the presence of an enzyme nitrate reductase in the organism, which reduces nitrate present in the medium (nitrate broth) to nitrite or free nitrogen gas.
- Nitrate test positive- All members of family Enterobacteriaceae.

PPA test (phenyl pyruvic acid test)

- Specific test done for members of tribe Proteeae; which includes *Proteus*, *Morganella* and *Providencia*.
- They possess a specific enzyme that deaminates phenylalanine present in the medium to phenyl pyruvic acid (PPA).
- PPA reacts with few drops of 10% ferric chloride solution to to produce green color.

Automated blood culture techniques

Advantages:

- *Continuous automated monitoring*
- *More sensitive*- increase in the yield of positive cultures from clinical specimens.
- *Rapid*- takes much less time than conventional methods.
- *Less labor intensive*- saves man power

Automated blood culture techniques

Disadvantages

i) high cost of the instrument and culture bottles

ii) inability to observe the colony morphology as liquid medium is used

iii) no separate detection in mixed cultures

iv) ↑overgrowth by contaminants

v) v) for techniques based on radiometric detection- there is need for disposal of radioactive materials.

Common automated blood culture systems- BacT/ALERT automated blood culture system

- Principle of BacT/ALERT is based on colorimetric detection of growth in contrast to fluorometric detection by BACTEC.

Common automated blood culture systems- BacT/ALERT VIRTUO automated blood culture

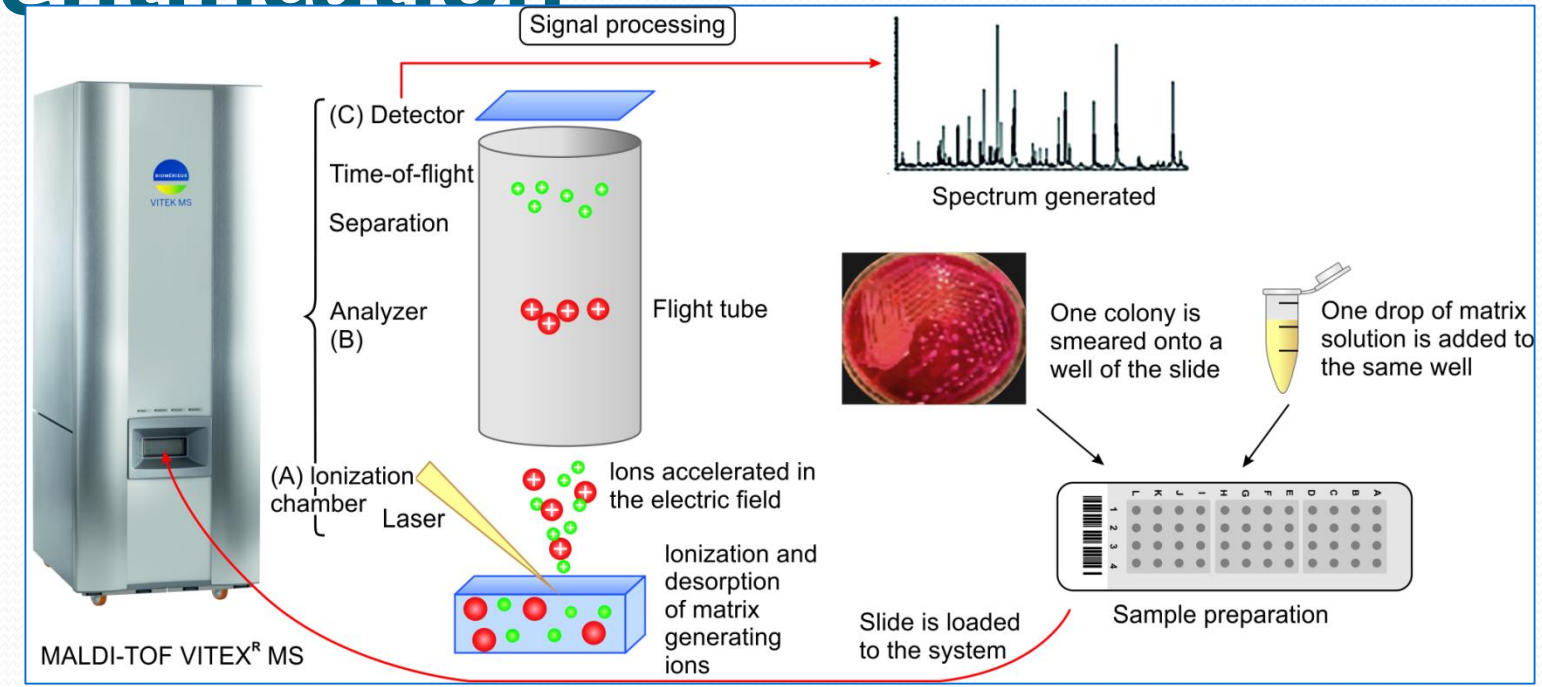
BacT/ALERT VIRTUO (bioMieux)

- Advanced form of BacT/ALERT which offers several advantages such as:

(i) automatic loading and unloading of bottles,
(ii) faster detection of growth
(iii) can determine the volume of blood present

- in bottle

Automated Systems for Bacterial Identification

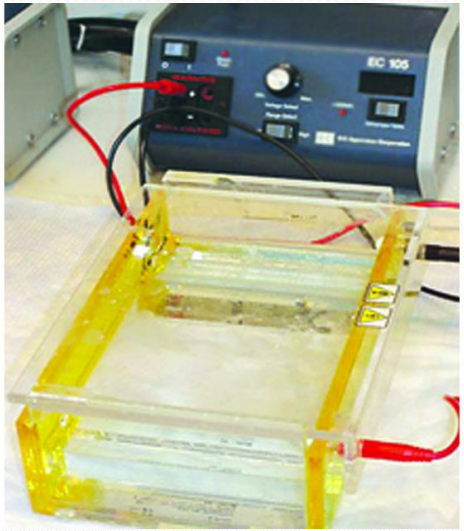


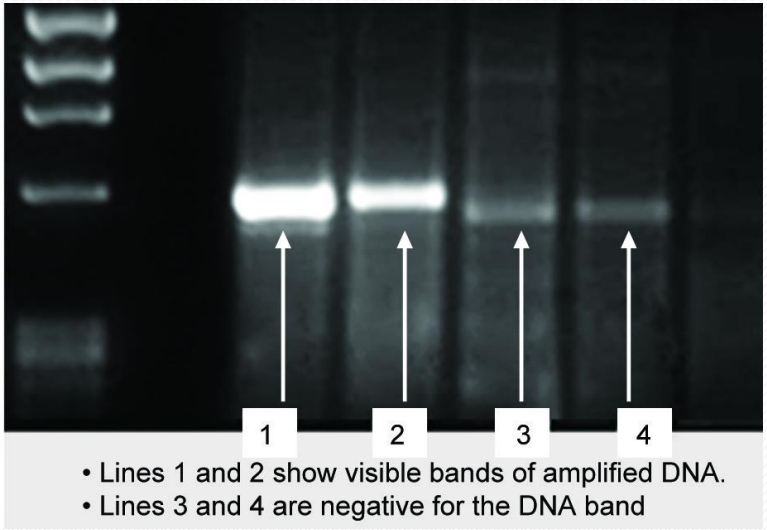
VITEK 2 automated system

The VITEK 2 is an automated system used for
identification and antimicrobial susceptibility testing (AST) of bacteria and yeast.

- uses colorimetric reagent card containing 64 wells; each well contains an individual test substrate.
- Separate cards are available for gram-negative, gram-positive bacteria, fastidious fastidious bacteria and yeasts

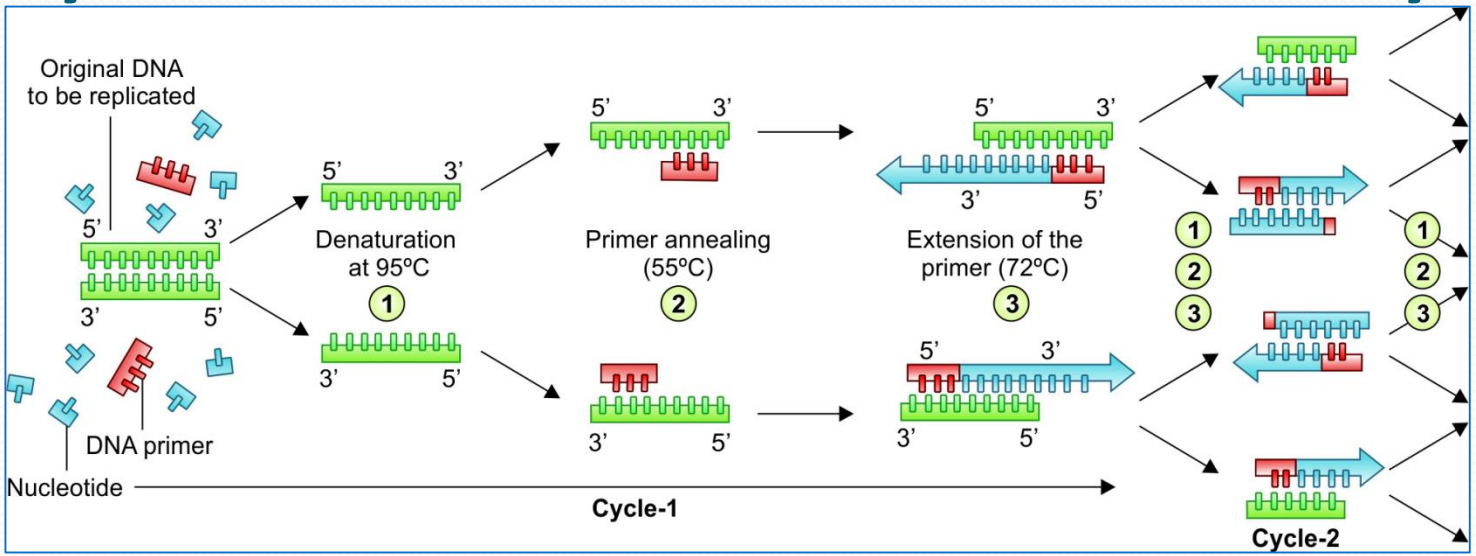
MOLECULAR METHODS





A. Thermocycler machine (Eppendorf); B. Gel electrophoresis of amplified product; C. Visualization of amplified DNA under UV light

Polymerase chain reaction cycle

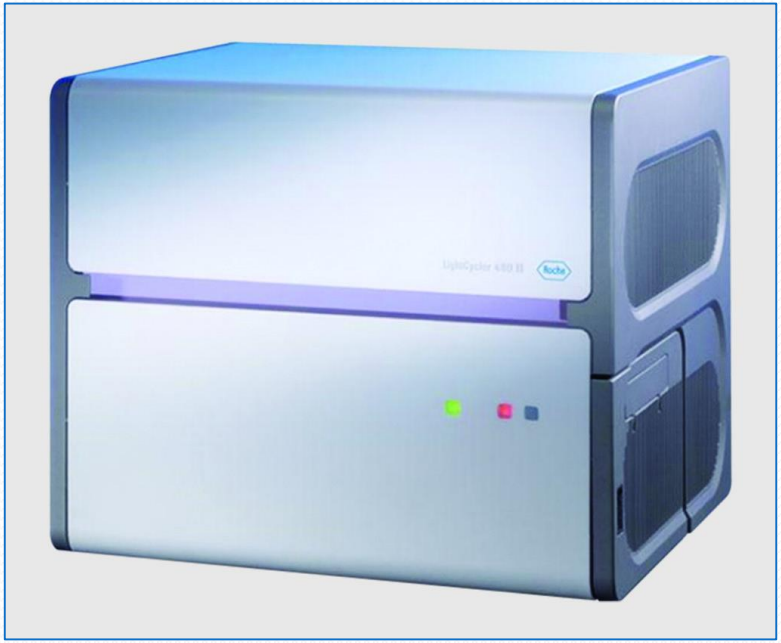


Biofire FilmArray

- Multiplex nested PCR system
- Four panels are available such as respiratory, gastrointestinal, meningitis-encephalitis and blood culture identification panels

Real-time PCR (rt-PCR)

- PCR technology, which is used to amplify and simultaneously detect or quantify a targeted DNA molecule on real-time basis.



Loop Mediated Isothermal Amplification (LAMP)

- LAMP is an isothermal nucleic acid amplification technique

MICROBIAL TYPING

- Microbial typing refers to characterization of an organism beyond its species level.

Phenotypic methods	Genotypic methods
Bacteriophage typing	**Non-amplification-based methods**
Bacteriocin typing	Plasmid profile analysis
Biotyping	Chromosomal DNA analysis
Antibiogram typing	Restricted fragment length polymorphism (RFLP)
Auxotyping	Ribotyping (RFLP of ribosomal DNA)
Morphotyping	Pulse field gel electrophoresis (PFGE)
Serotyping	**Amplification-based methods**
	PCR-RFLP
	Amplified fragment length polymorphism (AFLP)
	Sequencing-based methods
	Microarrays