UNIT- IV
(CHAPTER-12)
ASEPTIC TECHNIQUES

Points to be covered in this topic

1. INTRODUCTION
2. DESIGNING OF ASEPTIC AREA
3. LAMINAR AIR FLOW
4. DIFFERENT SOURCES OF CANTAMINATION IN AN ASEPTIC AREA
5. PREVENTION METHODS
6. CLEAN AREA CLASSIFICATION

❑ INTRODUCTION

❖ ASEPTIC AREA

- Aseptic techniques are employed to **provide protection to ophthalmic and parenteral products** by preventing the entry of microbial and particulate contamination.
- **Prevention of microbial contamination** is also required to **remove pyrogens and toxic bacterial products.**

with a **limit for the environmental quality** of **microbial and dust particle contamination.**

- This limit for contamination **is necessary to reduce** the product contamination.
- **The production area is normally divided into:**

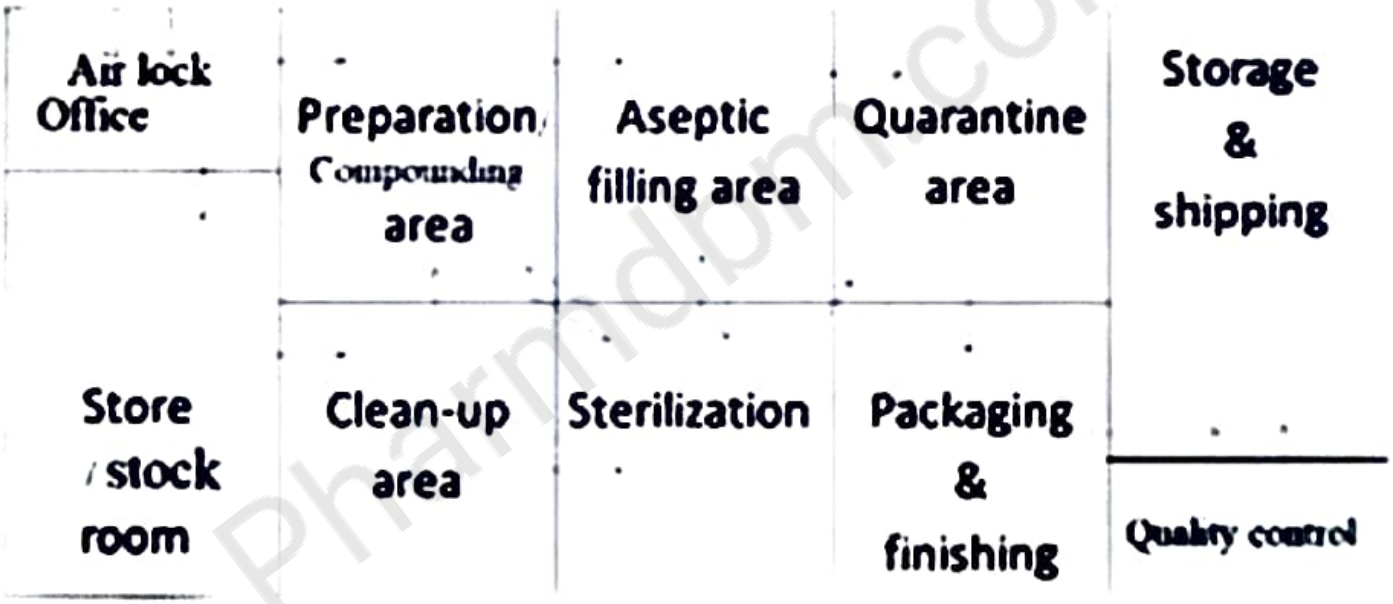
  i) The clean-up area,

  ii) The compounding area,

  iii) The aseptic area,

  iv) The quarantine area and

  v) The packaging/labelling area.



1. **Clean-up section:**

- The cleaning area **has walls and ceilings made up** of film coating materials.
- **Air inside the clean area should** be **free from dust and microorganisms.**
- This is ensured through **high efficiency (95%) filters.**
- **Air existing in the clean area** should be **frequently replace (10-15 air changes per hour).**

2. **Compounding section**

- This area **contains stainless steel cabinets** and counters and is involved

in the actual compounding.

- Unlike aseptic area, maintenance of sterile conditions is not essential, but necessary measures should be adopted to control the dust generated from raw material during weighing and compounding.

➢ DESIGNING OF ASEPTIC AREA

- All the areas should be designed and constructed for ease of cleaning, efficient operation, attractiveness and comfort of personnel.
- Clean areas for the production of sterile products are classified into grades A, B, C and D.
- These grades are categorized by the particulate quality of the environmental air when the clean area is in operating condition.
- All clean surfaces including the floor, walls and ceilings must be smooth, easy to clean, disinfected and be constructed to minimize microbial and particulate contamination.

❖ Floors:

- Flexing and non-flexing types of materials are used for construction of floors.

- Flexing floor materials are made of synthetic elastomers of which the most commonly used are polyvinylchloride (non-slip grades).
- Polyvinylchloride flooring is easily repaired, cleaned, relatively cheap and simple.
- Non-flexing floors are made of hard inorganic filler substances in a matrix material.
- When concrete is used it must be adequately sealed with a material resistant to chemicals, solvents and cleaning fluids.

❖ Walls:

- Walls must be made of non-inflammable or fire-resistant materials e.g. stainless steel, glass, enameled steel etc.
- For reduction of fungal growth, 1% of 8-hydroxy quinolone, pentachlorophenol, etc. may be added to the paint.
- Epoxy resin paints and polyurethane paints are also used to avoid cracking and peeling .

- Walls shall be flat and ledges and recesses shall be avoided.
- All pipes passing through the walls of the room should be effectively sealed and should be flush fitting and easily cleaned.

❖ Ceiling:

- Ceiling shall be solid and joints shall be sealed.
- Light-fittings and air-grills shall be flush with the walls and not hanging from the ceiling so as to prevent contamination.

❖ Doors:

- Doors and windows should fit flush with the walls
- Doors shall open towards the higher-pressure area so that they close automatically due to air pressure.
- Doors should be well fitted by maintaining the positive pressure air flow and self closing.
- Doors must be limited in number.

❖ Windows:

- Windows shall be flush with the walls. The furniture used shall be smooth, washable and made of any appropriate material other than wood.
- Windows if required, are solely to provide illumination and are not for ventilation.
- Windows should be non-openable.

❖ Services:

- Light sources in clean rooms are fitted with the ceilings to reduce the collection of the dust and to avoid the disturbance of the air flow pattern with in the room.
- Non essential switches such as room lighting switches should be installed outside the clean area.
- Sinks and drains should not be present in areas where aseptic procedures are carried out within the clean room areas.
- Gas cylinders should be excluded and all gases should be piped from outside the area.
- They are fitted with easily cleanable traps, installed with electrically heated disinfection devices.

❖ Protective clothing:

- Is designed to prevent contamination from the body and everyday clothing.

- All protective clothing is designed to prevent the contamination from the body.
- All protective clothing must be sterilized by moist heat sterilization or ethylene oxide sterilization.
- Fresh sterile clothing should normally be provided each time the person enters the aseptic area.

❖ Personal:

- The main source of contamination of clean areas arises from skin scales which are released by the operators.
- Personnel selected to work on the preparation of a parenteral product must be neat and reliable.

- They should be in good health and free from dermatological conditions that might increase the microbial load.
- All personnel should be trained for good manufacturing practices and aseptic techniques.
- They should be in good health and free from dermatological conditions that might increase the microbial load.

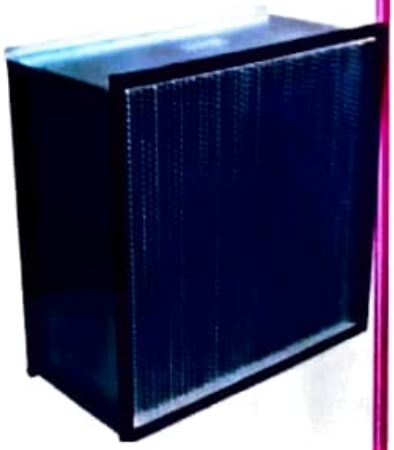
❖ **Cleaning and disinfection :**

- Cleaning and disinfection procedures are used for the removal of microbial and particulate contamination.
- Cleaning agents are the alkaline detergents, non-ionic and ionic surfactants.
- Different types of disinfectants should be employed in rotation to prevent the development of resistant strains of microorganisms.

- Different concentration of quaternary ammonium compounds, sodium hypo chloride, ethanol and formaldehyde solutions are used as disinfectants in cleaning area.
- Cetrimide or chlorhexidine in 70% alcohol are suitable for use as skin disinfectants.

❖ **Air supply :**

- The air supplied to a clean room must be filtered through High Efficiency Particulate Air (HEPA) filters.
- The HEPA filter must be positioned at the inlet of the clean room and the pre-filter may be fitted upstream of the HEPA filters to prolong the life of final filter.



- The air filtered from the HEPA filter is claimed to be 99.97% free from the microbial contamination.
- The air velocity at all parts of the filter area has to be about 0.54 m/sec or (90+20feet/min).
- Air quality is evaluated using settle plates, microbial air sampler or by particle counters.
- HEPA filters are used in the construction of vertical and horizontal laminar air flow bench.
- Removes the Dioctyphthalate (DOP) particles of size-0.3µm.

➢ LAMINAR AIR FLOW

- Laminar flow is defined as airflow in which the entire body of air within a designated space is uniform in both velocity and direction.
- It is a flow taking place along constant streamlines without turbulent.
- Laminar flow over a horizontal surface may be thought of as consisting of thin layers all parallel to each other.

- The laminar air flow principle was first developed in the early 1960s.
- All use this type of unidirectional airflow to aid in maintaining sterility, preventing cross-contamination and reducing turbulence.
- The air filtered from laminar air flow is claimed to be 99.97% free from microbial contamination.
- This level is based upon the removal of dioctylphthalate (DOP) particles of size 0.3 pm and larger.
- Air velocity at all parts of the filter should be 90 & 20 feet/min (0.54 m/sec.)

- It provides the following services during aseptic compounding
  1) Clean air to the critical sites (immediate aseptic compounding area),
  2) Constant flow of air out of the work area to prevent the entry of room air, and
  3) Outward flow of air from the hood that suspends and removes contaminants which have been introduced in the work area by personnel.
- Laminar air flow equipment can deliver clean air in a vertical, horizontal or curvilinear direction.
- They work on the laminar flow principle involving double filtration of air. Efficient in removing dust, pollen, mold, bacteria and any airborne particles having size of 0.3 micron or larger.
- It does not provide filtration to vapour or gases.
- HEPA filter is constructed of borosilicate microfibers in the form of pleated sheet.
- The efficiency of the HEPA filters is determined by aerosol tests.

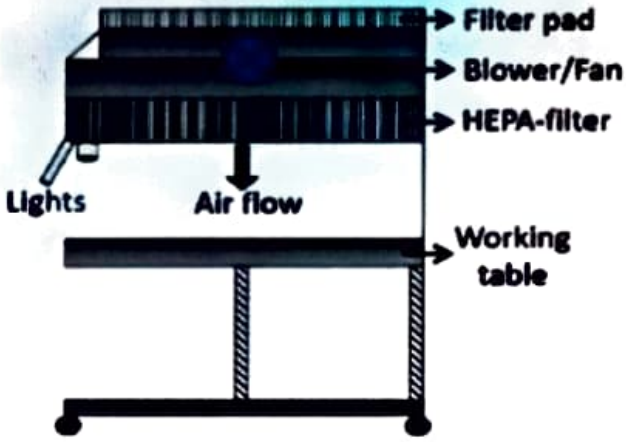
➢ **TYPES OF LAMINAR AIR FLOW:**

1. Horizontal Laminar Flow Cabinets

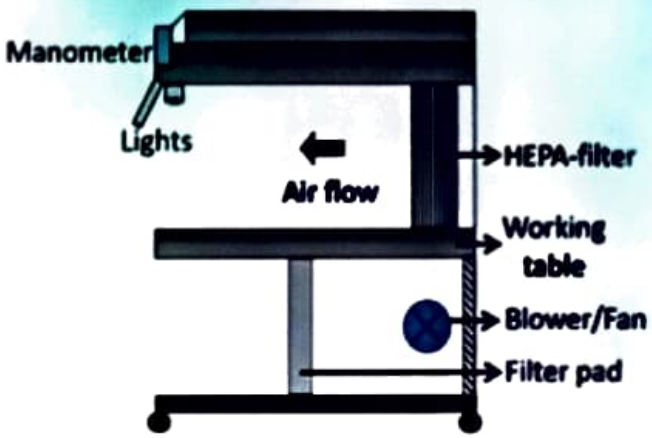
- Horizontal Laminar Flow Cabinets receive their name due to the direction of air flow which comes from above but then changes direction and is processed across the work in a horizontal direction
- The constant flow of filtered air provides material and product protection.

2. Vertical Laminar Flow Cabinets

- Vertical Laminar Flow Cabinets function equally well as horizontal Laminar Flow Cabinets with the laminar air directed vertically downwards onto the working area.



Vertical Laminar Flow



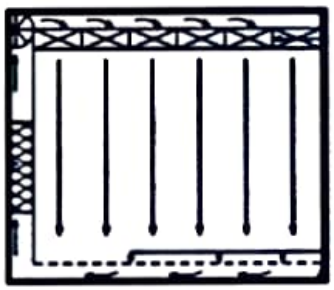
Horizontal Laminar Flow

➢ Air Supply

- **The air supplied to a clean room must be filtered through high-efficiency particulate air (HEPA) filters.**
- **In these HEPA filters, a pleated fibre glass paper is used as the filter medium.**
- **The filter consists of a continuous sheet of filtration material, pleated with a corrugated separator placed between each pleat and sealed into a rigid metal frame.**

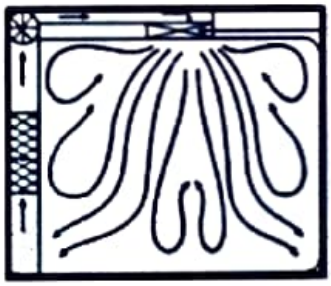
➢ Airflow Pattern

The general airflow patterns in clean rooms are unidirectional airflow, non-unidirectional airflow and combination airflow.



❖ Unidirectional air flow:

- **The air within the room moves with uniform velocity along parallel flow lines.**
- **Because of operating costs, unidirectional airflow clean rooms are not often used in pharmaceuticals.**

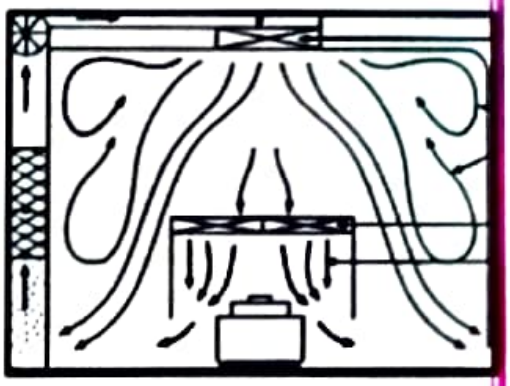


❖ Non- Unidirectional air flow:

- **The air enters the clean rooms through filters and exits through outlet ducts, positioned low down on**

the wall or in the floor at sites remote from the air inlet.

❖ Combined air flow:



- In many pharmaceutical clean rooms, the background area is ventilated by a non-unidirectional airflow and critical areas are supplied with unidirectional airflow.
- Various vertical unidirectional airflow systems are used in combination clean rooms.
- There has been a trend towards protecting the critical procedures within combination clean rooms by using isolator cabinets.

➢ Uses

1) It is required for smaller items, e.g., particle sensitive electronic devices.
2) It is used for special operations in laboratories.
3) It can be tailor made to the specific requirements of laboratories and are also used for general lab work, especially in medical, pharmaceutical, electronic, and industrial sectors.

➢ Advantages

1) The sterile area is spacious.
2) Since the sterile area does not become hot, the plant material can stay for a longer duration.
3) Requires no moving parts or no wear and has a simple design.
4) Bigger flasks having wide lids can be used.

❑ DIFFERENT SOURCES OF CONTAMINATION IN AN ASEPTIC AREA

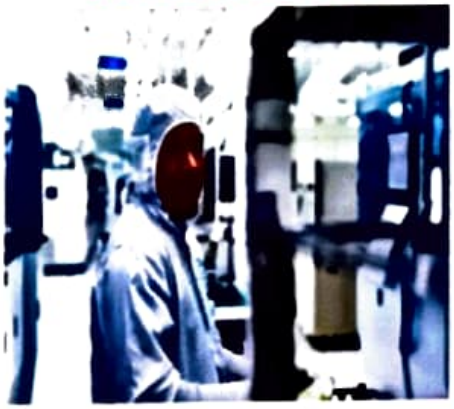
- There are various contamination sources in an aseptic area :

1. Atmosphere:

- The atmosphere is unable to support microbial growth but dust particles in outside air (originate from soil) may carry soil microorganisms. e.g. *Bacillus spp., Clostridium* spp., yeasts and molds.
- Microbes also occur in droplets expelled into the atmosphere from the mouth and respiratory tract by talking, sneezing and coughing.
- A damp atmosphere usually contains less microorganisms than a dry one as the contaminants are carried down by the droplets of moisture. Thus, the air in a cold store is usually free from microorganisms.
- The microbial count of air or atmosphere may be reduced by chemical disinfection, ultraviolet light, filtration and gaseous agents.

2. Operator:

- The skin, hair and clothing of the operator are potent sources of microbial contamination.

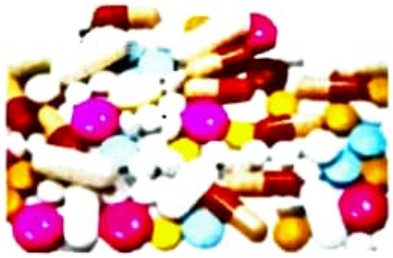


- Inadequate training
- Microorganisms mainly present on the skin include *Staphylococcus*, lipophilic yeasts and dermatophytic fungi.
- Poor personal hygiene can result in the presence of skin coliforms and other intestinal bacteria.
- Open wounds are a source of saprophytic and pathogenic microorganisms.

- Direct contact between the hands and starting materials, primary packaging materials, and intermediate or bulk products.
- Improper hygiene.
- Unauthorized personnel entering the production, storage, and product control areas.
- Insufficient gowning and protective equipment.

3. Raw materials:

- Improper storage and handling, which leads to mix-ups or selection errors.
- Microbial or chemical contamination.
- Drugs which are prepared from animal, plant or other natural sources are frequently contaminated with bacteria, yeasts and molds.
- Degradation due to extreme environmental conditions (like heat, cold, sunlight, moisture, etc.
- Water is also a prime source of microbial and particulate contamination, Deionized water, distilled water and water for injections are commonly used for preparation of different pharmaceuticals.
- Wrong labelling.
- Incorrect sampling and testing.

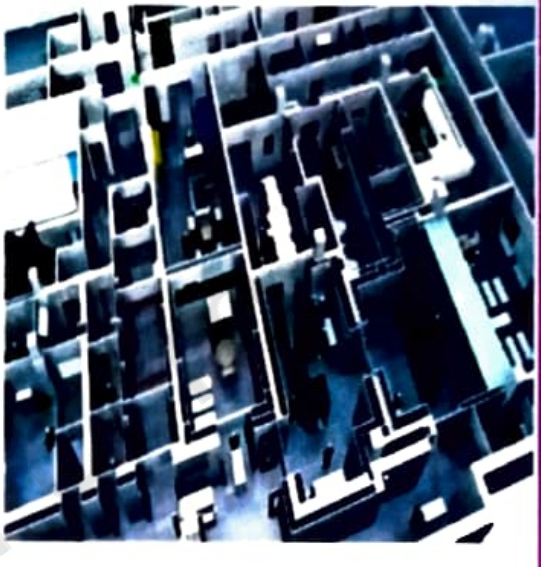


4. Equipments:

- Unsuitable design, size, corrosion-causing materials, static material accumulation, and/or adulteration with lubricants, coolants, dirt, and sanitizing agents.
- Working surfaces and external surfaces of equipment are also potential sources of contamination due to sedimentation of particles and droplets from the atmosphere.

- Inadequate cleaning and sanitization due to their designing.
- Inappropriate calibration and irregular service, and using defective equipment.

5. Buildings and facilities;



- Inadequate size and organization of the space, which lead to selection errors (such as mix-ups or cross-contamination between consumables, raw materials, in-process materials, and finished products).
- Poor filth and pest controls.
- Rough floors, walls, and ceilings.
- Absence of air filtration systems.
- Inadequate lighting and ventilation systems, poorly located vents, ledges, and drains.
- Non-directional airflow within production or primary packing areas.

6. Manufacturing process:

- Absence of facilities required for manufacturing of a single product.
- Improper cleaning between batches for minimizing the amount of product changeovers.
- Use of an open manufacturing system for exposing the product to the room environment.
- Improper zoning.
- Lacking an area line clearance (as per the approved procedures) after each cleaning process and between each batch.
- Lack of cleaning status labelling on all equipment and materials used within the manufacturing facility.

❑ METHODS OF PREVENTION OF CONTAMINATION :

1. Equipments:

- Equipment's may be sterilized or disinfected by heat, gaseous agents or chemicals.
- Many tanks, containers, small equipment's and reaction vessels are sterilized by steam under pressure (autoclave).

2. Raw materials:

- Water may be treated by ultraviolet light, filtration or stored at elevated temperature to discourage microbial growth.
- Heat treatment, filtration, recrystallisation, irradiation or any other sterilization processes are mainly applied for reduction of the microbial load in raw materials.

3. Personal:

- Access to production areas by the unauthorized personnel should be restricted, and only trained ones should be allowed to enter.
- Adequate personnel hygiene should be maintained.
- The personnel should be given proper and regular training with respect to hygiene as well to ensure that their activities do not hamper the product quality.
- Personnel entering the manufacturing area should wear protective clothing (over-garments, hair cover, beard or moustache cover, and overshoes).
- Personnel should avoid touching with naked hands the exposed products or any part of equipment in contact with the product.

4. Access to areas

- Access to production, packaging, and QC areas by unauthorized personnel should be restricted.
- Personnel should gain access to these areas only via changing rooms.
- Materials should be accessed via specific routes (generally air locks).

5. Building requirements

- Smooth, crack-free, and easily cleanable floors, walls, and ceilings should be used as they facilitate easy and effective cleaning.
- Windows or viewing panels should be closed (non-opening), fixed with wall panels, and sealed to prevent accumulation of dust and microbial contaminants.
- The designing of pipe work, ventilation, and light points should be such that Creation of recesses which are not easily cleanable is avoided.
- Sinks of stainless steel should be present within the production areas.

6. Cleaning and disinfection

- In aseptic rooms, the differential air pressures should be higher than the adjacent controlled areas.
- Air filtration and air change rates should be set to attain the defined clean room class.
- Unidirectional (laminar flow) air flow should be maintained over critical areas at sufficient velocity to sweep particles away from filling/closing area.
- Ambient temperature and humidity should not be very high.
- The ventilated cabinets, RABS (Restricted Access Barrier System), isolators systems, etc. should be used depending on the facility/product risk assessment results in order to achieve an absolute or partial barrier to contain microorganisms at their point of use.

❑ CLEAN AREA CLASSIFICATION:

- It can be seen that the British Standard Class 2 for environmental cleanliness closely approximates to US Federal Standard Class 10,000 and is the standard applicable to clean areas.
- Class 10,000 conditions mean that not more than 10,000 particles per cubic foot of size 0.5 pm or greater, shall be found in the measured area.
- The more stringent British Standard Class 1 approximates to US Federal Standard Class 100 and is the standard applicable to aseptic areas.
- The operation of clean and aseptic rooms are recognized by three main areas as black or dirty areas, grey or semi-clean areas and white or clean areas.
- Black areas are those where particle and microbial control is almost impossible.
- Grey areas are those where high standards of hygiene and cleanliness operate and reduction of microbial contamination is possible.
- In white areas where full environmental control is operational and special protective clothing is worn by the operators.
- The area may be a Class-1 (aseptic) room or a Class - II (clean) room possibly with laminar flow units installed at terminal operation points such as filling lines.

Grade	Viable microorganisms / m^3 of air	Settle plate (9 cm)/ 4 hours	Contact plate (5.5 cm)	Glove point (5 fingers)
A	<1	<1	<1	<1
B	10	5	5	5
C	100	50	25	Not applicable
D	200	100	50	Not applicable

- The sterile production unit must be separated from the general manufacturing area within the hospital pharmacy or factory.
- This sterile production unit must not be accessible to unauthorized personnel.
- The clean room should be remote from main corridors, stairways or lifts which provide airways for bacterial and particulate transmission. It is important to design the clean room to be the smallest size, bearing in mind the operations to be undertaken and the number of people likely to be employed in the area.

UNIT- IV
(CHAPTER-13)
MICROBIOLOGICAL ASSAY

Points to be covered in this topic

1. INTRODUCTION
2. STANDARDIZATION OF ANTIBIOTIC
3. STANDARDIZATION OF VITAMINS
4. STANDARDIZATION OF AMINO ACID
5. ASSESSMENT OF ANTIBIOTIC

❑ INTRODUCTION

➢ MICROBIOLOGICAL ASSAY

- The **microbiological or microbial assay** is a **type of biological assay performed with microorganisms e.g. bacteria, yeast and molds.**
- **In a typical microbial assay, evaluation is performed** with a **culture of micro organisms and the measurement represents the average response** of an **extremely large population of test organisms.**

Uses of assay in the performance of bioassays for:

- Determining the concentration of certain compounds (e.g., amino-acids, vitamins and some antibiotics) in complex chemical mixtures or in body fluids.
- Diagnosing certain diseases.
- Testing chemicals for potential mutagenicity or carcinogenicity.
- Monitoring purposes involving the use of immobilized enzymes.
- Sterility testing of antibiotics.
- Microbiological assays are used during production to determine the potency and quality control.
- These are used to determine the pharmacokinetics of drugs in animal and human.
- In antimicrobial chemotherapy to monitor, in managing, controlling the chemotherapeutic agents.

➢ Principle

A microbiological assay relies on the principle that when certain compounds are present in limited amounts, the amount of microbial growth corresponds to the amount of these compounds.

The microbiological assay is based upon a comparison of the inhibition of growth of micro-organisms by measured concentration of the antibiotics to be examined with that produced by known concentrations of a standard preparation of the antibiotic having a known activity.

➢ Preparation of standard solution

- A standard preparation is an authentic sample of the appropriate antibiotic for which the potency has been precisely determined by reference to the appropriate international standard.
- The potency of the standard preparation may be expressed in International Units or in ug per mg of the pure antibiotic.

- Dissolve a quantity of the standard preparation of a given antibiotic in the solvent.
- Dilute the preparation to get the required concentration as stated and store in a refrigerator.
- On the day of assay, prepare from stock solution five or more test dilutions, the successive solutions increasing stepwise in concentration, usually in the ratio 1:1.

➢ Preparation of sample solution :

From the information available for the substance under examination (test sample), assign to it an assumed potency per unit weight or volume, and on this assumption prepare on the day of assay a stock solution and test dilution as specified for each antibiotic.

➢ Preparation of buffer solution :

Prepare the buffer solutions of given quantities of dipotassium hydrogen phosphate and potassium dihydrogen phosphate in sufficient water to produce 1000 ml after sterilization, adjusting the ph. with 8 M phosphoric acid or 10 M potassium hydroxide.

Two general method are usually employed:-

1. The cylinder-plate (or cup-plate) method.
2. The turbidimetric (or tube assay) method

1. The cylinder-plate (or cup-plate) method :

- This method depends on the diffusion of an antibiotic from a vertical cavity or a cylinder, through the solidified agar laver in a Petri plate.

⬇

- The growth of test microorganisms is inhibited entirely in a circular area or zone around the cavity or cylinder containing a solution of the antibiotic A.

⬇

- liquefied assay medium (43 to 45°C) is inoculated by suspension of test microorganisms and the inoculated medium is poured immediately into sterile Petri plate or

per-prepared agar plates by using an assay medium and then spread the test culture or microorganisms on the surface of plates/spread plate technique).

- Solutions from known concentrations of the standard preparation and the test antibiotic are prepared in appropriate solutions.

- These solutions are added in sterile cavities or cylinders prepared in a solid medium. When paper discs are used, these discs should be sterile first and then dipped in the standard solutions or the test solutions and placed on the surface of the medium.
- The plates are left standing for 1 to 2 hours at room temperature or 4°C
- All plates are then incubated for about 18 to 24 hours at the temperature indicated.
- The diameters or areas of the circular inhibition zones produced by standard and test antibiotic solutions are accurately measured.
- The graph which relates zone diameter to the logarithm of the concentration of antibiotic is plotted and the unknown concentration of test antibiotics is calculated.

2. The turbidimetric (or tube assay) method :

- This method depends upon the growth of a microbial culture in a uniform solution of the antibiotic in a fluid medium that is favorable to its rapid growth in the absence of the antibiotic.
- Five different concentrations of the standard solution are prepared by diluting the stock solution
- A medium concentration is selected and the test sample of the antibiotic solution is adapted by dilution.

- One ml of each concentration of the standard solution and of the sample solution are placed in each of the tubes in duplicate.

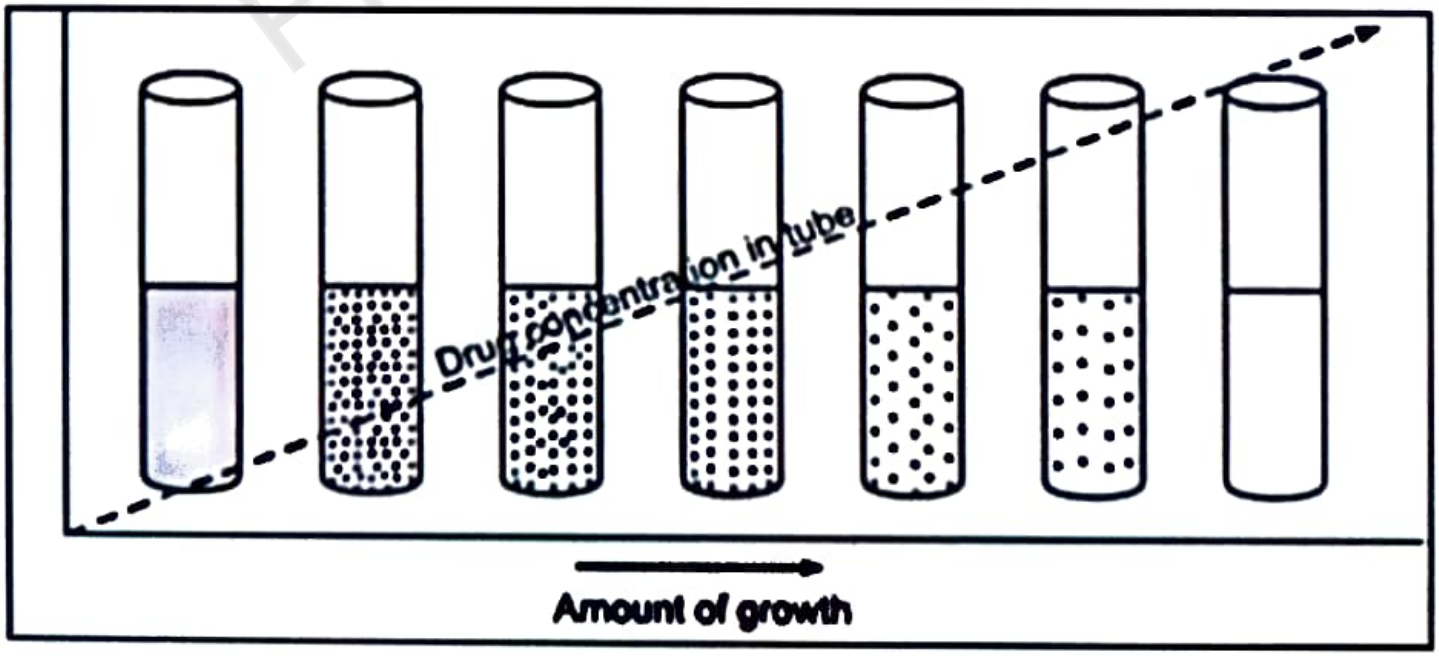
- To each tube, ml of nutrient medium previously seeded with the appropriate test microorganisms added.

- At the same time, three control tubes containing the inoculated culture medium(culture control), another identical with it but treated immediately with 05ml of dilute formaldehyde solution (black) and a third containing uninoculated citrus medium are prepared.

- All the tubes are placed in an incubator at the specified temperature few hours.

- After incubation add 5 mt at dilate formaldehyde solution to each tube.

- The growth of the test microorganisms is measured by determining the absorbance at about 530nm of each of the solutions in the tubes against the blank.



➢ METHODS FOR STANDARDIZATION OF ANTIBIOTICS :

- The microbiological assay of an antibiotic is based upon a comparison of the inhibition of growth of micro-organisms by measured concentrations of the antibiotics under examination with that produced by known concentrations of a standard preparation of the antibiotic having a known activity.
- Two general methods are usually employed, the cylinder-plate (or cup plate) method and the turbidimetric (or tube assay) method.

➢ Principle

- The basis of this assay is to measure the ability of test organism to utilize the substance being assayed under a proper nutritional condition.
- The response (growth of test organism) is proportional to the dose (amount of factor) added to medium.

❖ Preparation of inoculums

- Inoculums is the mixture of microbes along with the culture media in which it is growing.

➢ Steps involved:

- Maintain the test microbes on slant of medium A and transfer to a fresh slant once a week.
- Incubate the slant at the specified temperature for 1day Using 3ml of slant solution, wash the microbes from agar slant on to a large surface of medium A such as a Roux bottle containing 250ml of agar media Incubate for 1day at the required temperature.
- Wash the growth from the nutrient surface using 50ml of saline solution. Store the test microbes under refrigerator

❖ The turbidimetric (or tube assay) method PROCEDURE :

- Five tubes containing the inoculated culture medium with standard drug with a specific dose and test organism.
- Five tubes containing culture medium with test organism and the test sample with different dosages.
- Another one treated immediately with 0.5 ml of dilute formaldehyde solution(blank)
- All the tubes are placed in an incubator and maintain at the specified temperature- 37°C for 3 to 4 hour.
- The growth of the test organism is measured by determining the absorbance at 530 nm of its against the blank.
- The standard calibration card is prepared and the absorbance obtained for the sample is plotted on it to obtain the concentration of the test antibiotic.

❑ Estimation of Potency

- Average absorbance for each concentration of the standard are plot sed on semi-logarithmic paper with the absorbance on the arithmetic scale and concentration on the logarithmic scale. Best straight response line is plotted through the points either by inspection or by means of following expressions:

$$L = \frac{3a + 2b + c - e}{5}$$

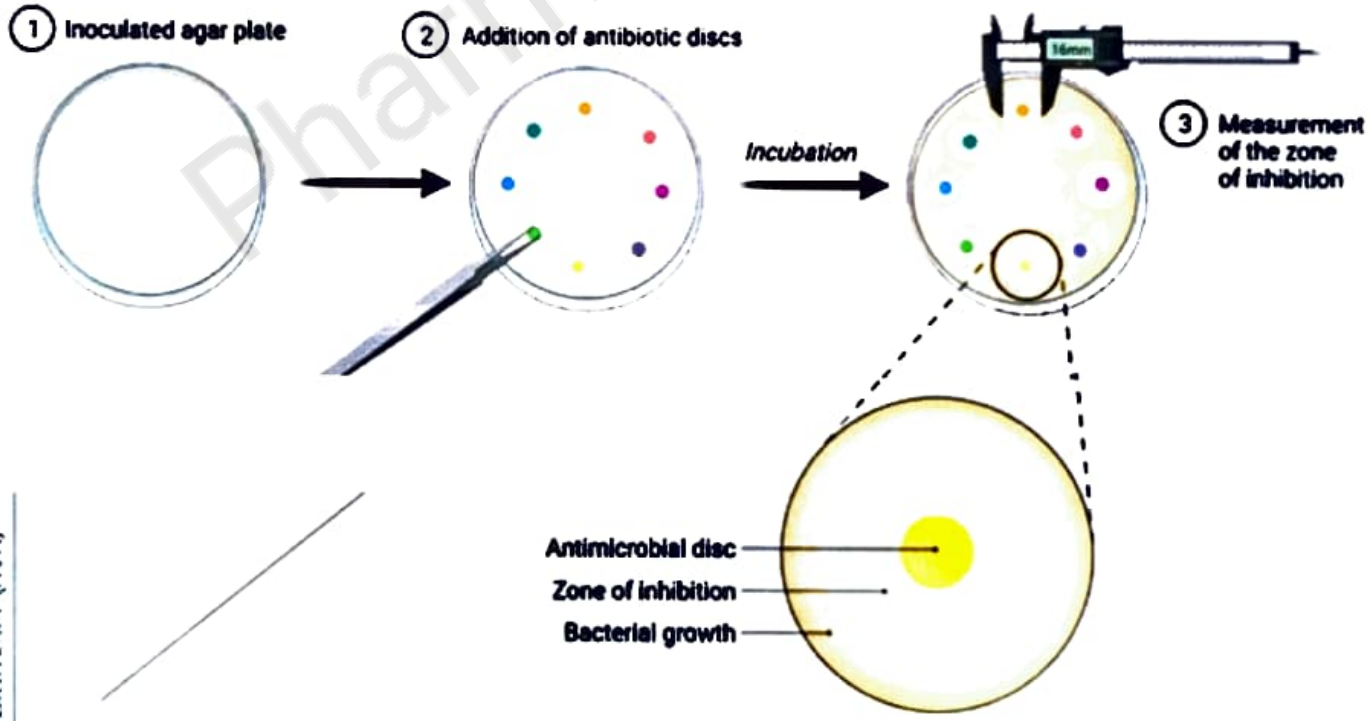
$$H = \frac{3e + 2d + c - a}{5}$$

- Where, L= calculated absorbance for lowest concentration of standard response line,
- H= calculated absorbance for highest concentration of standard response line,
- a, b, c, d, e = average response values for each concentration of standard response line lowest to highest, respectively.

- Values obtained for L and H are plotted and the points are connected. Absorbance for the sample are read and antibiotic concentration computed from standard response line.
- The antibiotic content of the sample is obtained by multiplying the concentration by the appropriate dilution factors.

❑ The cylinder-plate (or cup-plate) method

- A previously liquefied medium with the required quantity of microbial suspension is inoculated.
- The suspension is added to the medium at a temperature between 40-50 degree and inoculated medium is immediately poured.
- The solution are applied to the surface of the solider medium in sterile cylinder or in ager cavities
- They are incubated for about 18 hours at the temperature indicated.
- The quantities estimation of antibiotic is done by accurately measuring the diameter or areas of the circular inhibition zones .



METHODS FOR STANDARDIZATION OF VITAMINS :

- A microbiological vitamin assay uses microorganisms to conduct the test. Growth of microorganisms depends on the presence of vitamins and amino acids.
- Under proper nutritional conditions, the aim of this essay is to measure the ability of an organism to utilize a substance. The following table provides some examples of microorganisms used as bioassays for vitamins

MICROORGANISMS	VITAMIN
Lactobacillus casei	Biotin, folic acid, riboflavin
L. Leichmannii	Cyanocobalamin
L. Arabinosus	Nicotinic acid
L. viridans	thiamine

Material required :

- Inoculum media, stock solutions, and assay media are needed. We then generate standard curves.

Assay of vitamin B12:

- Vitamin B12 is also known as cyanocobalamin. Water-soluble vitamins are essential for healthy living. Water-soluble vitamins are essential for healthy living. Macrocytic anemia and Pernicious anemia caused by B12 deficiency.

Principle:

- *Lactobacillus liechmannii* is selected as the test organism because it can use free cyanocobalamin. Assays are conducted either by titrimetric or turbidimetry.

Preparation of standard stock solution :

- A precise weight of Cyanocobalamin reference standard should be weighed and the amount of cyanocobalamin added to enough 25% ethanol (resulting in a solution containing 1.0 g of cyanocobalamin per mL) needs to be added

- before the solution is stored in a refrigerator. In addition to this stock solution, you can dilute it by adding 1 ml to 99 ml purified water (1 ml is equal to 10 ng) and another I ml to 199 ml purified water (1 ml is equal to 0.05 ng).

➢ Test solution :

- To measure vitamins, an accurate amount of each is dissolved in water. Using diluted HCl or NaOH, the pH is adjusted to 6.0, followed by adding water to reach the desired ph.

➢ Inoculum preparation:

- Put 10 ml of sterile culture medium in each of two tubes with a loop of Lactobacillus leichmannii derived from a recent sub-culture.

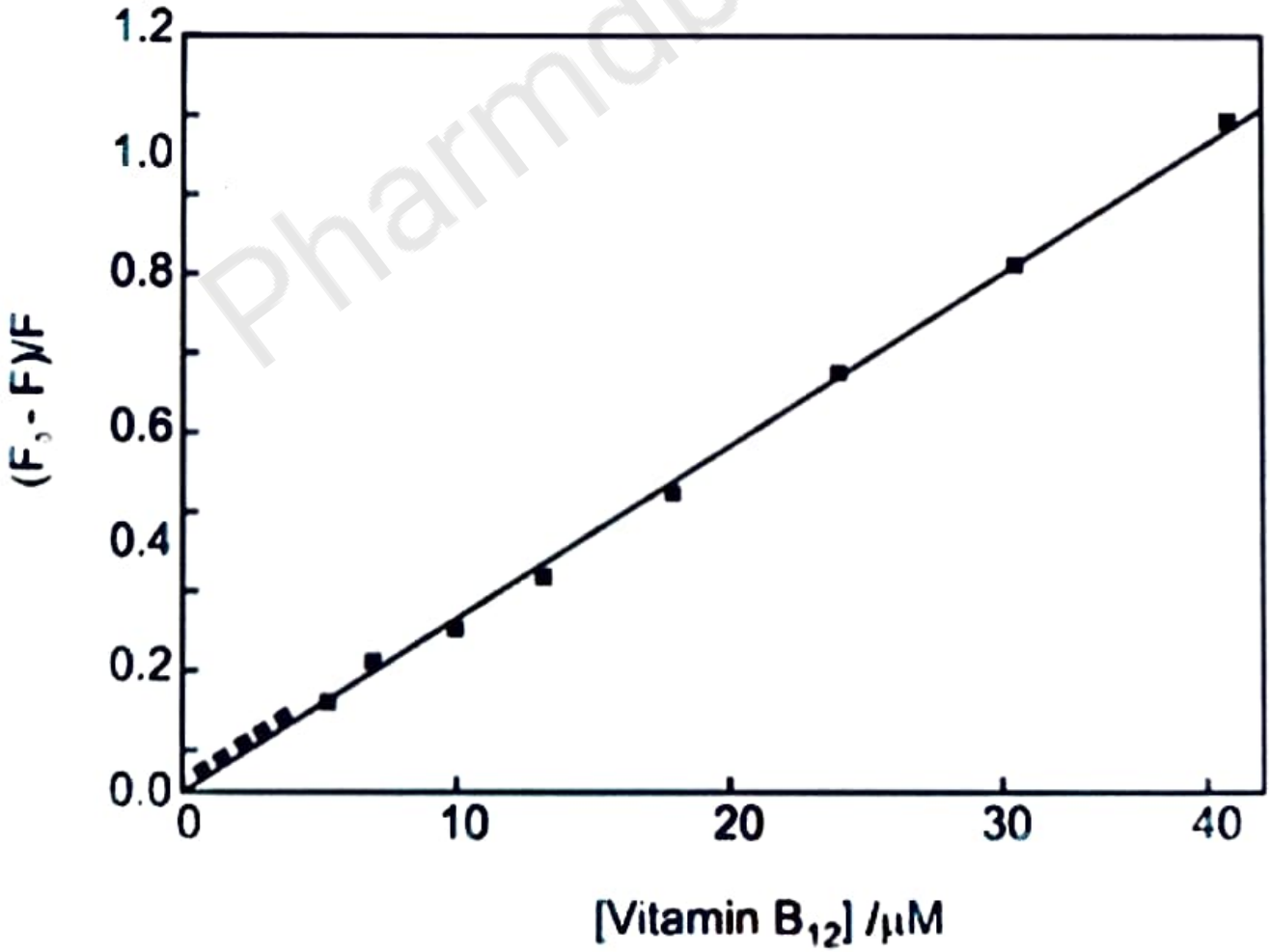
➢ Composition of culture media:

- For 18 to 24 hours, the culture is incubated at 37 degrees Celsius.
- A centrifuge is used to separate the supernatant fluid from the incubated culture under aseptic conditions.
- To suspend the cultured cells in Basal medium stock solution for 10 ml, centrifuge and decant off the supernatant fluid, then centrifuge again.
- One ml of the uniform cell suspension is transferred to sterile medium and mixed aseptically with 10 ml of the medium. Inoculums are prepared by taking this resulting cell suspension.

➢ Assay procedure

- The following ten test tubes should be cleaned, and cyanocobalamin solutions added in 0, 0.5, 1.0, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, and 5 ml.
- Water is added to the 5 ml of Basal medium solution in each tube to make the volume equal to 10 ml.
- Four more test tubes should be filled with 1, 2, 3, 4 ml of the test solution to be tested. For each of these, add 5 ml of Basal medium stock solution, add 10 ml of water, and mix.

- For all test tubes, an autoclave at 121 °C for 15 minutes is used to sterilize them, and then the tubes are cooled at room temperature.
- The test tubes were incubated for 64 to 72 hours at 30 to 37° C with an inoculum prepared with Lactobacillus liechmannii.
- Using bromothymol blue as an indicator, titrated each test tube with 0.05 N NaOH until a green color appeared.
- Take the readings and calculate them.0.05 N NaOH titration values (in ml) of cyanocobalamin standard solution are used to construct a graph plotting the average titration values against the concentration of standard solution at each level.
- To calculate the concentration of vitamin B12 as activity per ml, the standard curve is interpolated to produce a line graph.
- A graph can provide information about cyanocobalamin concentrations.



❖ METHODS FOR STANDARDIZATION OF AMINO ACID

- Standard conditions are required for a microbiological assay to determine the degree of activity of a compound based on the amount required to develop the predicted effect.
- Microbiological analyses take a lot of time and are not applicable to all amino acids.
- Some microorganisms need amino acids to grow and reproduce.
- These microbes rely on specific amino acids in many strains. The growth of such microorganisms will be limited if a small amount of an amino acid is supplied as measured by turbidimetry, or if only a small amount of pH levels are increased by microtitration.
- Guithne and Susi developed a modified version of the microbiological test using diffusion in gels to screen blood samples for the presence of elevated phenylalanine levels in clinical biochemistry laboratories.
- It is named after its inventors as the Guthrie test, and it is one of the most commonly used microbiological methods to evaluate amino acids.
- In the assay, phenylalanine is evaluated by comparing its ability to inhibit Bacillus subtilis growth against the chemical competitor 8-2-theienylalanine.
- This test makes use of an agar plate, onto which suspensions of B subtilis spores are added, along with growth nutrients and an additional amount of 3-2 thienyl alanine for supplementation.
- In addition to the filter paper soaked in blood, phenylalanine standards are also soaked in blood and placed on the agar surface. They are then incubated overnight at 37°C.
- When phenylalanine concentrations in blood discs are high enough 0-2 thienyl alanine can't prevent bacterial growth.
- The discs themselves show growth zones around them.

- We measure the circumference of each zone of growth the next day, and correlate this with phenylalanine levels.

➢ ASSESSMENT OF NEW ANTIBIOTIC:

- Minimum Inhibitory Concentration (MIC) is the lowest concentration of antimicrobial compound found to inhibit the growth of a particular test microorganism. It may be applied to assess new disinfectants, antiseptics, preservatives and antibiotics. MIC values are usually expressed in terms of µg/ml or units/ml.
- Minimum inhibitory concentration of different anti- microbial compounds may be determined by the liquid dilution method or the solid dilution method.

➢ Liquid dilution method or test tube method:

- Use a series of test tubes which contain a double-strength medium and are labelled.
- In the first tube (un-inoculated), inoculum is not added which is used for checking the sterility of the medium.
- All other in a eleven test tubes, inoculum (3 to 4 drops) is added to reach the final concentration of microorganisms is 10 to 10 cells/ml.
- In all test tubes, test chemical is added ranging from 0.5 to 5 ml except in the uninoculated and control tube.
- The second tube (control) is used to check the suitability of the medium for growth of the test microorganism and the viability of the inoculum.
- The final volume (10 ml) in all test tubes is adjusted by using sterile water. The contents of all test tubes are properly mixed and incubated at 37°C for 2 to 3 days.
- After incubation, all test tubes are examined for the growth in the form of turbidity and the results are recorded and minimum inhibitory concentration is calculated.

- It is also necessary to conduct a **preliminary experiment** to determine the **approximate range (test solution)** which would be **suitable for the test.**

Solid dilution method:

In this method, **test chemical is first mixed** into **molten agar** and then poured into Petri plates. After solidification, the **Inoculums is spread on the surface of agar medium.** All plates are incubated **at 37°C for 2 to 3 days.** After incubation, all plates **are observed for growth of inoculum** and the **minimum inhibitory concentration of the** test chemical is calculated.

The advantages of this method are:

- **Several microorganisms can be tested** at the same **time by use of multi point inoculator.**
- **Contaminations are easily detected**, because colony features on solid media are more distinctive **than turbidity differences in fluid media.**