Shree H. N. Shukla Institute of Pharmaceutical Education and Research,Rajkot

B. Pharm
Semester-5

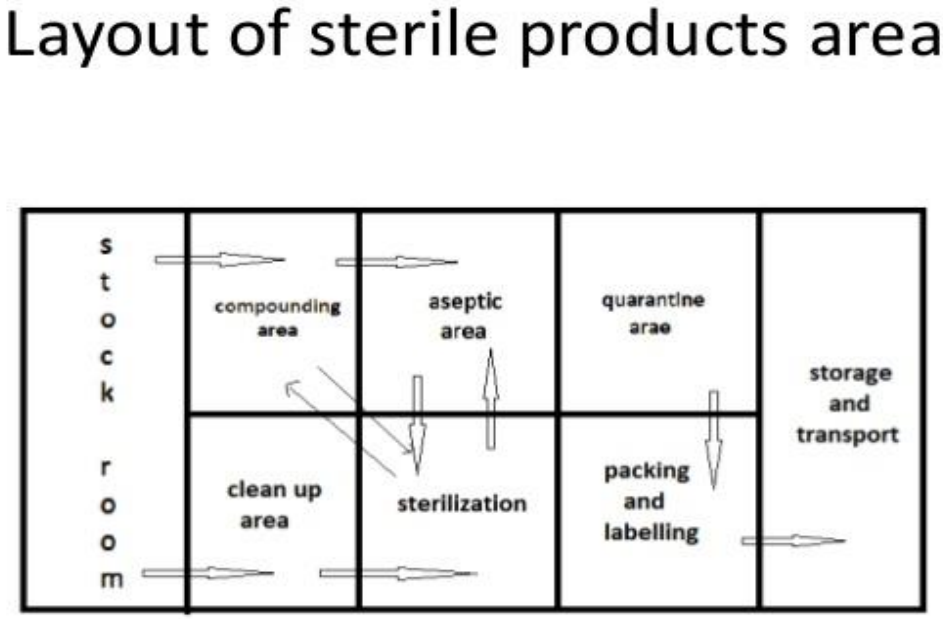
STUDY MATERIAL

Subject Name:Pharmaceutical Microbiology
Subject Code: BP504TP

CHAPTER 4 Aseptic area design and microbial assay :

CONTAINS:
Designing of aseptic area, laminar flow equipments; study of different sources of contamination in an aseptic area and methods of prevention, clean area classification.
Principles and methods of different microbiological assay. Methods for standardization of antibiotics, vitamins and amino acids. Assessment of a new antibiotic.

Designing of aseptic area



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Aseptic processing is a processing technique wherein commercially thermally sterilized liquid products (typically food or pharmaceutical) are packaged into previously sterilized containers under sterile conditions to produce shelf-stable products that do not need refrigeration.

Aseptic processing has almost completely replaced in-container sterilization of liquid foods, including milk, fruit juices and concentrates, cream, yogurt, salad dressing, liquid egg, and ice cream mix.

There has been an increasing popularity for foods that contain small discrete particles, such as cottage cheese, baby foods, tomato products, fruit and vegetables, soups, and rice desserts.

Aseptic processing involves three primary steps: thermal sterilization of the product, sterilization of the packaging material, and conservation of sterility during packaging.

To ensure commercial sterility, aseptic processing facilities are required to maintain proper documentation of production operations, showing that commercially sterile conditions were achieved and maintained in all areas of the facility.

Any breach of a scheduled process for the processing or packaging system means that the affected product must be destroyed, reprocessed or segregated and held for further evaluation In addition, the processing and packaging system must be cleaned and re-sterilized before processing and/or packaging operations can resume. Packaging equipment and packaging materials are sterilized with various medium or combination of mediums (i.e., saturated steam, superheated steam, hydrogen peroxide and heat and other treatments).

laminar flow equipment

Class II, Type C1 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.

What is a laminar flow hood?

Clean benches and biological safety cabinets are common examples of laminar flow hoods. They are laboratory enclosures designed to carefully direct HEPA filtered air. Some of these hoods protect items placed on the work surface from contamination. Others prevent exposing the user to contaminants in the work area. Laminar flow hoods are often used to work with biological samples, semiconductors or other sensitive materials.

According to the CDC, the laminar air flow principle was first developed in the early 1960s. It's still incredibly relevant for modern labs, having literally shaped the way air safely moves in many generations of laboratory enclosures. Today, many categories of laminar flow hoods exist. Although they

differ depending on the science performed within, there is one common denominator: all use this type of unidirectional airflow to aid in maintaining sterility, preventing cross-contamination and reducing turbulence.

Just what exactly is laminar air flow, why is it effective and what does it look like in labs today? Let's explore.

How is laminar air flow utilized in different types of equipment?

Class II Biosafety Cabinets, sometimes referred to as laminar flow hoods, maintain product protection through HEPA-filtered laminar downflow over the work zone. Per the NSF definition, these ventilated cabinets also feature inward airflow at the open front to protect operators and HEPA filtered exhaust air for environmental protection.

- *Class II, Type A* cabinets recirculate air back into the laboratory unless a canopy connection is warranted.
- *Class II, Type B* cabinets are hard-ducted to the outside.
- *Class II, Type C1* cabinets can function in either Type A or Type B mode. Whichever model suits your application, safe operation within biological safety cabinets is imperative to protect the integrity of your work and your personal safety.
- *PCR stations*, enclosures that are specifically designed to house polymerase chain reaction experiment, utilize vertical flow of HEPA-filtered air to maintain a particulate-free work environment. A UV light is necessary to denature genetic material (DNA, RNA, etc.) and provide secondary decontamination.
- *Clean benches* are suitable for applications that require product protection, such as media plate preparation or tissue culture maintenance. Air is drawn in through a prefilter located at the top of the clean bench before being pulled through a HEPA filter.
- In a *vertical clean bench*, laminar air is then projected vertically over the work area. In a *horizontal clean bench*, laminar air is projected horizontally towards the operator. In both instances, laminar flow provides a particulate-free work area.

So, what is zoned airflow?

Zoned airflow is not truly laminar. Zoned airflow is used when equipment cannot achieve all of the protection required of a Class II biosafety cabinet with standard laminar airflow. Each zone, or column, of airflow is defined and has its own range in airspeed. This allows for higher speed barrier air columns to be utilized as an engineering solution to equipment that otherwise would have poor containment or product protection ratings.

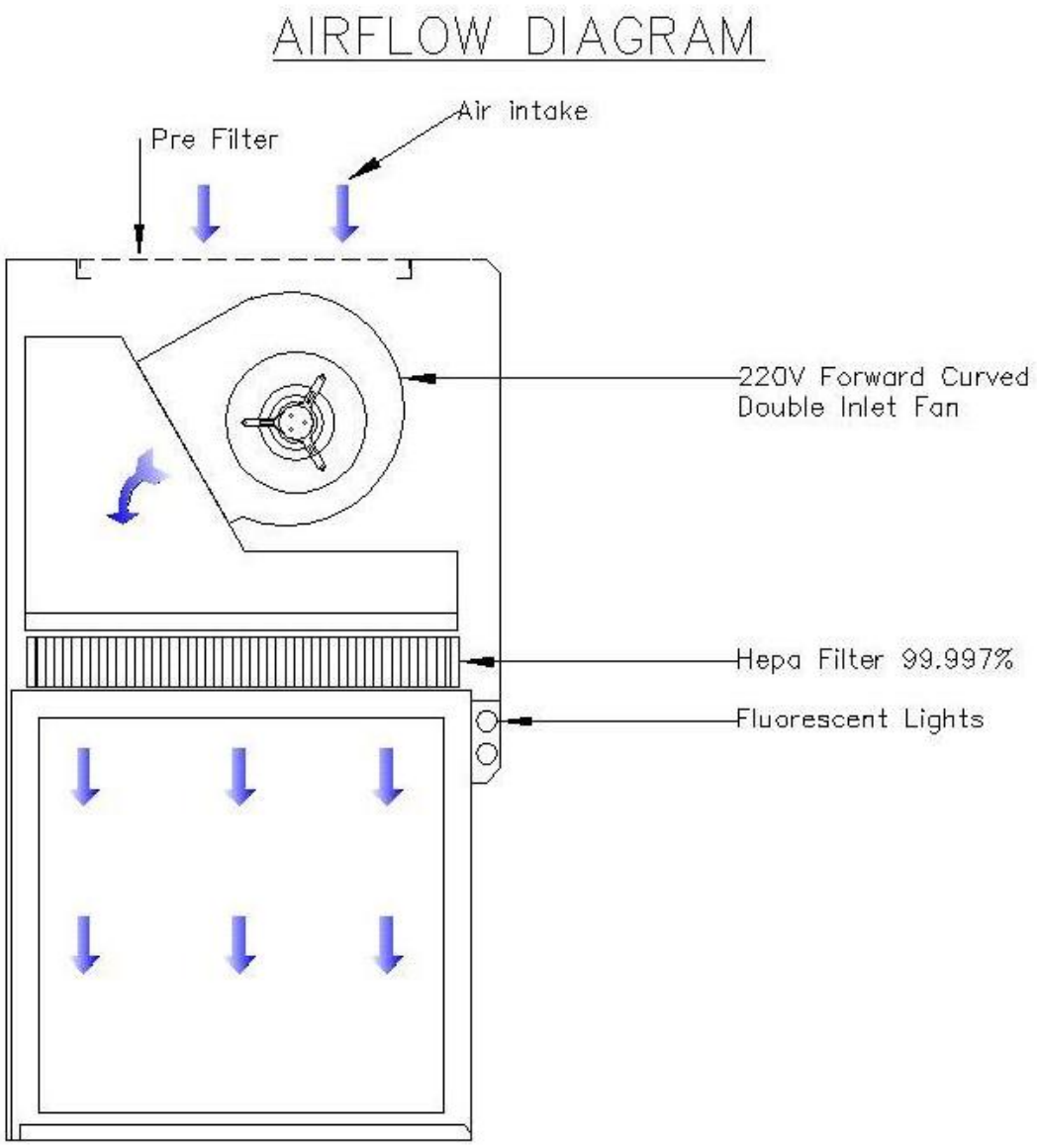
How does laminar airflow differ from dilution flow?

Dilution flow is not the same as laminar air flow. The dilution flow principle is used in equipment such as filtered glove boxes. In these instances, HEPA-filtered air mixes with and dilutes interior airborne contaminants inside the glove box, and those contaminants are removed via a filtered exhaust system.

After the contamination source has been sealed, the dilution rate—or air changes per minute—will determine how much time must lapse before materials can be removed from the main chamber.

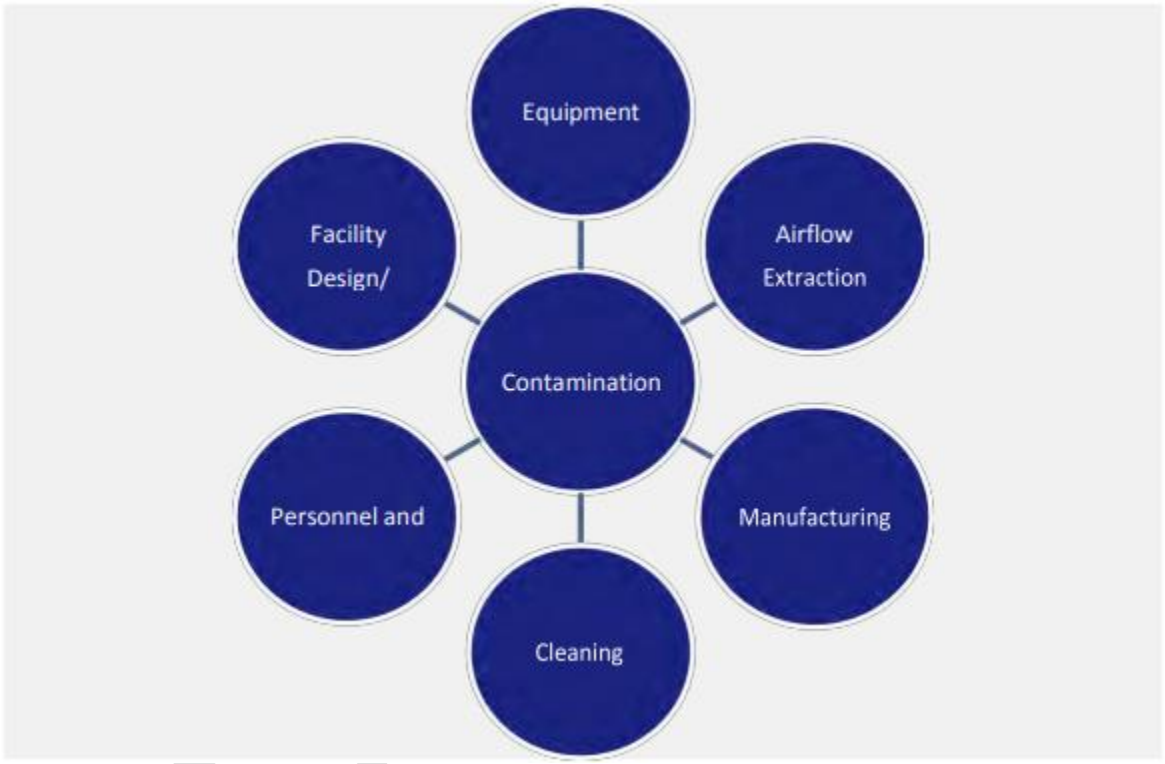
What is turbulent flow?

While laminar air flow helps to reduce turbulence, turbulent flow encourages it by creating unintentional swirls of air that place particles randomly on surfaces within an enclosure. Turbulent flow can be disruptive to work that requires a dust-free environment and can lead to contamination. Obstructions, like items left inside enclosures, can create this unwanted turbulence.



Contamination in clean rooms

Manufacturing medicinal products involves a series of processing steps using various equipment and ancillary systems within a facility. Each step/equipment/system can pose a risk of contamination. Contamination is the undesired introduction of impurities (of a chemical or microbiological nature) or of foreign matter, into or onto a starting material or intermediate or API during: ♣ production ♣ sampling ♣ packaging or repackaging ♣ storage or transport. Cross-contamination is the contamination of a starting material, intermediate or finished product with another starting material or product. Manufacturers must have processes in place, to not only avoid contamination scenarios but also provide documented evidence that contamination has not occurred. The reasons for contamination and cross-contamination can vary and be caused by technical or deficiencies within the organisation. The common sources of contamination are identified in Figure 1 below.



Sources Of Microbial Contamination In Cleanrooms

Understanding the sources of contamination can aid in developing corrective and preventive actions when excursions occur. Microorganisms are easily spread and are practically everywhere.

Microorganisms can be classified based on their mode of mobility, their shape, their atmospheric needs, their Gram stain reaction, their optimal growth temperatures, and whether they produce spores. With

regard to shape, microorganisms can be round (cocci), rod shaped (bacilli), spiral shaped, or even vibrio shaped, which looks similar to a comma.

The atmospheric needs of microorganisms will vary depending on the microorganism. Microorganisms can be aerobic, meaning they require oxygen to grow. They can be anaerobic, meaning they do not require oxygen to grow. Some microorganisms may require various other combinations of atmospheric conditions for cell growth.

Microorganisms also vary in their optimal growth temperatures. For example, psychrophiles enjoy colder temperatures, usually below 15 C.[7] Mesophiles enjoy moderate temperatures, typically 20 to 45 C.[7] Thermophiles like hotter temperatures, usually 45° to 80° C.[7] If the cleanroom is kept at a moderate room temperature, the recovered microbial flora is usually mesophilic bacteria.

A Gram stain helps to determine the structure of the outer cell wall of bacteria. Most bacteria will fall into one of two groups as a result of the reaction to the Gram stain. Gram negative cells are pink and have a thin peptidoglycan cell wall, which is surrounded by an outer membrane containing lipopolysaccharide.[7] When Gram negative rods are recovered in cleanrooms, sources of stagnant water or moisture are usually present. Gram negative bacteria can be found in water systems, on surfaces due to improper disinfection practices, in standing water, in piping or hoses with moisture, and in sinks or drains. Depending on the species, Gram negative rods may also originate from human, animal, plant, or food sources. Poor aseptic technique can contribute to spreading the contamination.

Gram negative bacteria are also of the main source of endotoxin. Endotoxins are a complex lipopolysaccharide molecule located in the outer membrane of Gram negative bacteria.[7] They are released when the bacterial cell is destroyed. This is a concern because endotoxins can trigger fevers, shock, or even death in the patient.

Gram positive cells are purple. They are surrounded by thick layers of peptidoglycan, but lack the outer cell membrane.[7] Gram positive cells are the most recovered microorganisms in cleanrooms. They are usually associated with humans and are found on skin, hair, and clothing. Poor aseptic technique, poor material transfer practices, poor disinfection practices, and improper gowning can lead to contamination with Gram positive cocci microorganisms.

In cleanrooms, the phrase “Gram positive rod” typically sparks thoughts of contamination from dirt or soil. Gram positive rods are often isolated from dirt, soil, dust, air, cardboard, paper, water sources, mops, humans, food, and clothing. Gram positive rod microorganisms can either be spore formers or non-spore formers. Spores make bacteria harder to kill by allowing the bacteria to survive extreme habitat fluctuations. For example, the bacterium *Geobacillus stearothermophilus* is commonly used in autoclaves and isolator qualifications because of its resistance to heat and vaporized hydrogen peroxide. Poor aseptic technique, poor material transfer practices, poor disinfection practices, and improper gowning can lead to contamination with Gram positive rod microorganisms.

Yeasts usually appear to be very large oval cells under the microscope. When isolated from clean rooms, common sources of contamination are humans or food items. Poor aseptic technique, poor material transfer practices, poor disinfection practices, improper cleaning, and improper gowning can lead to contamination with yeast.

Molds can grow almost anywhere and are typically found in damp, dark, and humid areas. When molds are isolated in cleanrooms, paper, cardboard, wet drywall, heating, ventilation, and air conditioning (HVAC) systems, or wooden pallets are the typical sources of contamination.[8] They are also associated with the building, walls, ceilings, dirt, and even dust. Improper disinfection practices, improper cleaning, poor material transfer practices, poor gowning practices, or improper aseptic technique can lead to contamination with mold.

Testing of clean and aseptic rooms

Designed to limit airborne particulates and other environmental conditions, cleanrooms are certified to a specific class, based on the standard ISO 14644-1, or similar standards. The room must perform, according to the standards, to meet or exceed the ISO parameters to maintain compliance. Cleanroom testing and certification involves checking that the cleanroom is functioning to the specific parameters and ISO classification.

Typically the room is initially certified when construction is completed to ensure it was built to the client's specifications. The room is also then routinely retested (annually or semiannually) to ensure the quality has not changed during operations. The standards which cleanrooms are tested to are ISO14644-1, ISO 144644-2 and ISO 14644-3.

The certification should include which occupancy state the room was tested: as-built, which is the initial, empty facility, at-rest: which is the room with all production equipment running but no personnel present, or operational, which is the room in full operation. It is also a common practice to test and certify a cleanroom has been renovated or a contamination incident. Selecting which tests to perform are based on many variables, mainly standard requirements, auditing agency or client requirements, or client's preference. The typical tests are listed below:

PRIMARY CLEANROOM TESTS
(Annually or Semi Annual based upon specification)

Airflow volume / Velocity Readings	Assures that both unidirectional (velocity preferred) and non-unidirectional flow (airflow volume preferred) areas are properly balanced and unidirectional zones are maintaining proper air patterns.
HEPA Filter Integrity Testing	Test HEPA filters and system for leakage through the filtration system. This test is not required by ISO standards, but many auditing agencies such as the FDA will require it.
Non-Viable Particle Counting	Reports the amount of airborne particulate of a specified size in the clean zone. This test determines cleanliness class.

Room Pressurization Testing	Verifies that room differential pressures are operating according to the design.

OPTIONAL CLEANROOM TESTS:

Optional tests are based upon the organization's need for monitoring, industry requirements and internal QA/SOPs.

Air Balancing	Adjusts airflow in the air handling systems to achieve design airflow, room exchange rates and pressure cascade.
Airflow Visualization Testing/ Smoke Testing	Verifies the airflow direction using a source of visible fog.
Viable Environmental Monitoring (EM)	Air and surface sampling for microbe enumerations.
Room Air Exchange Rates	States if the area is meeting its design airflow.
Temperature/Relative Humidity Testing	Examines whether the air HVAC controls are functioning uniformly and properly.
Lighting, Vibration, and Sound Testing	Assures workers comfort.

Any and all acceptance criteria should be clearly spelled out for the certification company. For instance: the room must meet ISO Class 7 in the operational mode at 0.5 and 5.0 micron. The pressure must cascade from the main cleanroom (highest pressure), to the gown room, to ambient. Or, the airborne sound pressure levels cannot exceed 72 dB "A" scale for worker comfort.

Microbiological assay

Two general methods are usually employed, the cylinder-plate (or cup-plate) method and the turbidimetric (or tube assay) method.
The cylinder-plate method (Method A) depends upon diffusion of the antibiotic from a vertical cylinder through a solidified agar layer in a Petri dish or plate to an extent such that growth of the added micro-organism is prevented entirely in a zone around the cylinder containing a solution of the antibiotic. The turbidimetric method (Method B) depends upon the inhibition of growth of a microbial culture in a uniform solution of the antibiotic in a fluid medium that is favourable to its rapid growth in the absence of the antibiotic.

The assay is designed in such a way that the mathematical model on which the potency equation is based can be proved to be valid. If a parallel-line model is chosen, the two log doseresponse lines of the preparation under examination and the standard preparation should be parallel; they should be rectilinear over the range of doses used in the calculation. These conditions should be verified by validity tests for a given probability. Other mathematical models, such as the slope ratio method, may be used provided that proof of validity is demonstrated.

MEDIA:

Prepare the media required for the preparation of test organism inocula from the ingredients listed in Table 1. Minor modifications of the individual ingredients may be made, or reconstituted dehydrated media may be used provided the resulting media have equal or better growth-promoting properties and give a similar standard curve response.

Dissolve the ingredients in sufficient waterto produce 1000 ml and add sufficient 1 M sodium hydroxideor 1 M hydrochloric acid, as required so that after sterilization the pH

STANDARD PREPARATION AND UNITS OF ACTIVITY

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 µg per mg of the pure antibiotic.

The Standard Preparations for India are certified by the laboratory of the Indian Pharmacopoeia Commission or by any other notified laboratory(ies) and are maintained and distributed by the agency(ies) notified for the purpose.

A Standard Preparation may be replaced by a working standard prepared by any laboratory which should be compared at definite intervals under varying conditions with the standard.

Buffer solution

Prepare by dissolving the following quantities given in Table 2 of dipotassiumhydrogen phosphate and potassium dihydrogen phosphatein sufficient waterto produce 1000 ml after sterilisation, adjusting the pH with 8 M phosphoric acidor 10 M potassium hydroxide.

Table 2 – Buffer Solutions

Buffer No.	Dipotassium Hydrogen Phosphate, K_2HPO_4(g)	Potassium Dihydrogen phosphate, KH_2PO_4(g)	pH adjusted after sterilization to
1	2.0	8.0	6.0±0.1
2	16.73	0.523	8.0±0.1
3	-	13.61	4.5±0.1
4	20.0	80.00	6.0±0.1
5	35.0	-	10.5±0.1*
6	13.6	4.0	7.0±0.2

* After addition of 2 ml of 10M potassium hydroxide

Preparation of the standard solution

To prepare a stock solution, dissolve a quantity of the Standard Preparation of a given antibiotic, accurately weighed and previously dried where so indicated in Table 3, in the solvent specified in the table, and then dilute to the required concentration as indicated. Store in a refrigerator and use within the period indicated. On the day of assay, prepare from the stock solution five or more test dilutions, the successive solutions increasing stepwise in concentration, usually in the ratio 1:1.25 for Method A or smaller for Method B. Use the final diluent specified and a sequence such that the middle or median has the concentration specified in Table 3

Preparation of the sample solution

From the information available for the substance under examination (the “unknown”), assign to it an assumed potency per unit weight or volume, and on this assumption prepare on the day of

the assay a stock solution and test dilution as specified for each antibiotic in Table 3 but with the same final diluent as used for the Standard Preparation. The assay with 5 levels of the Standard requires only one level of the unknown at a concentration assumed equal to the median level of the standard.

Test organisms

The test organism for each antibiotic is listed in Table 4, together with its identification number in the American Type Culture Collection (ATCC). Maintain a culture on slants of the medium and under the incubation conditions specified in Table 5, and transfer weekly to fresh slants.

Table 4 - Test Organisms for Microbiological Assay of Antibiotics

Antibiotic	Test Organism	ATCC1 No.
Amikacin	*Staphylococcus aureus*	29737
Amphotericin B	*Saccharomyces cerevisiae*	9763
Bacitracin	*Micrococcus luteus*	10240
Bleomycin	*Mycobacterium smegmatis*	607
Carbenicillin	*Pseudomonas aeruginosa*	25619
Chlortetracycline	*Bacillus pumilus*	14884
Erythromycin	*Micrococcus luteus*	9341
Framycetin	*Bacillus pumilus*	14884
	Bacillus subtilis	6633
Gentamicin	*Staphylococcus epidermidis*	12228
Kanamycin sulphate	*Bacillus pumilus*	14884
	Staphylococcus aureus	29737
Neomycin	*Staphylococcus epidermidis*	12228
Novobiocin	*Staphylococcus epidermidis*	12228

Nystatin	*Saccharomyces cerevisiae*	2601
Oxytetracycline	*Bacillus cereus var, mycoides*	11778
	Staphylococcus aureus	29737
Polymyxin B	*Bordetella bronchiseptica*	4617
Spiramycin	*Bacillus pumilus*	6633
Streptomycin	*Bacillus subtilis*	6633
	Klebsiella pnumoniae	10031
Tetracycline	*Bacillus cereus*	11778
	Staphylococcus aureus	29737
Tobramycin	*Staphylococcus aureus*	29737
Tylosin	*Staphylococcus aureus*	9144

Techniques for microbial assay

The microbiological assay may be carried out by the following two methods

1 cup- plate method or cylindrical plate method

2 turbidimetric method or tube assay method

1 cup- plate method or cylindrical plate method

Official Circular 198 (Ruehle and Brewer, 1931) gives directions for performing the agar cup-plate test. However, in the limited literature dealing with the subject, no mention is made of the influence of varying the volume of agar, the amount of inoculum, the hydrogen ion concentration or other factors. In the course of other studies with the agar cup-plate method (Rose and Miller, 1939; Miller and Rose, 1939) it was learned that a slight modification in procedure caused a considerable difference in the experimental results. It therefore became necessary to study the influence of variations in technique in greater detail. The object was to standardize the method so that more constant data could be obtained. The present communication deals with this study.

TECHNIQUE

A simple device is used to remove the disc of agar from the medium. This instrument' consists of a thin-walled stainless steel cylindrical chamber measuring 2.5 cm. in length and having a diameter of 1.5 cm.; the cutting edge is bevelled on the inside. A capillary metal tube, about 10 cm. long, is attached to the bottom of the cylindrical chamber. The chamber is sterilized by dipping in alcohol and flaming. Placing the open end of the chamber on the surface of a poured agar plate, the disc is cut easily with slight pressure. A finger is then placed over the tip of the capillary tube, as in pipetting; the disc of agar is gently pulled slightly to one side and removed. With practice, no cracks are made in the walls of the cup.

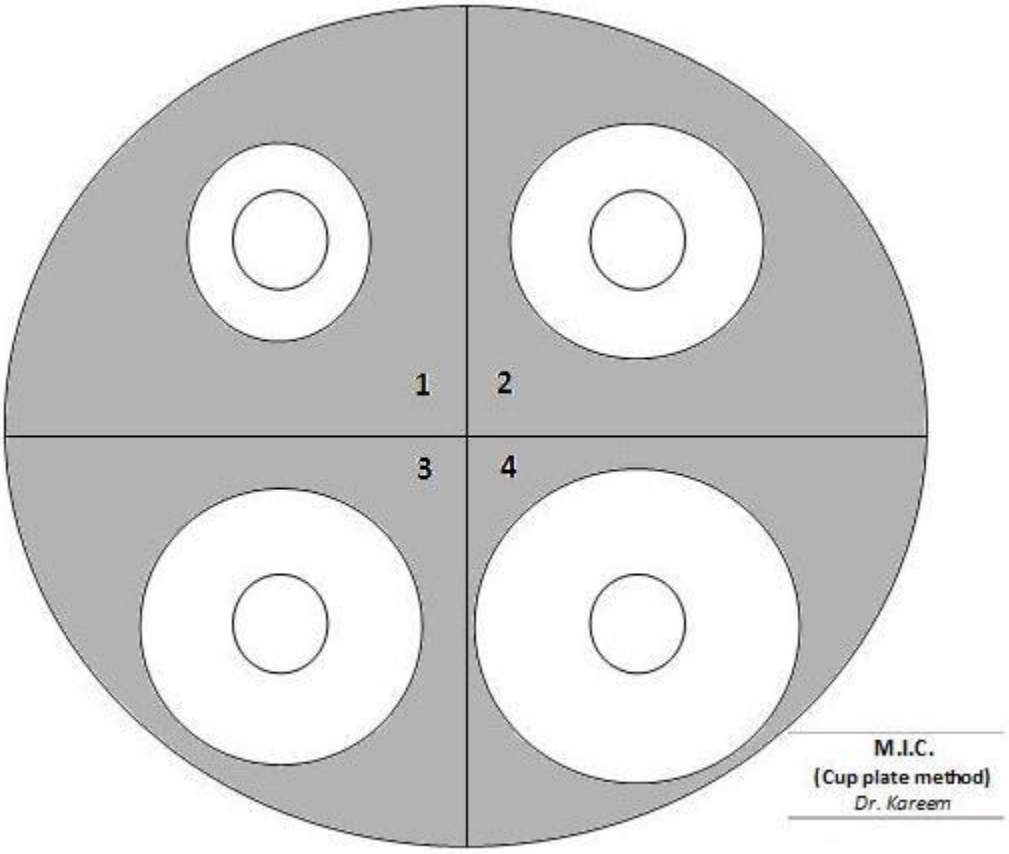
The ordinary inoculating loop is not entirely satisfactory for removing small pieces of agar for subculture. A micro-spoon2 is convenient for such purposes. The spoon is formed at the end of a piece of 10 per cent iridium-platinum No. 19 B. & S. gauge wire, 4 cm. long; it measures 2 mm. in diameter and has a shallow concavity approximately 0.75 mm. deep. The handle is mounted on a standard needle holder. About 30 seconds are required to cool the micro-spoon after sterilization in an open flame.

When it is necessary to determine whether the zone of inhibition of bacterial growth is bactericidal or bacteriostatic, a small piece of agar is removed with the micro-spoon, transferred to 10 ml. of broth, and incubated at 370C. Readings are made at the end of 48 hours. Growth in a subculture tube indicates inhibitory rather than germicidal action of the antiseptic.

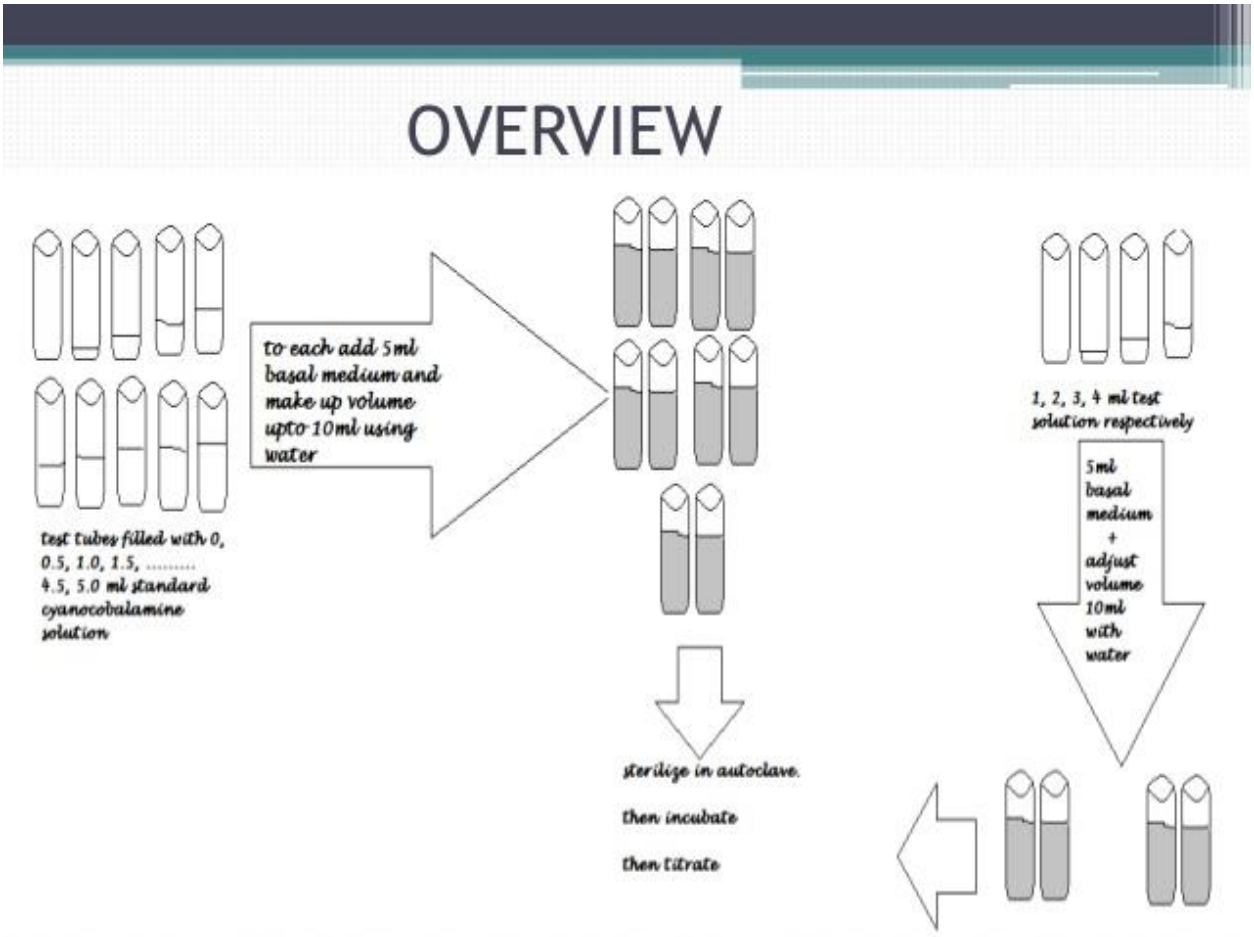
The antiseptics used in this study were limited to a single member of each of three groups. Carbolic acid was chosen for the phenol group because it has long been used as a standard. The dilution of 1: 20 was employed because this was the weakest dilution which produced a zone free of bacterial growth of approximately 3 mm.; this size was found to be a convenient minimal one for accurate measurement. Mercury bichloride and crystal violet,3 each in 1:1,000 dilution, were taken as representatives of the heavy metals and antiseptic dyes respectively. In one instance, neutral acriflavines was added, to illustrate further the effect of the hydrogen ion concentration of the medium on the size of the zone. All hydrogen-ion-concentration determinations were made colorimetrically.

Except where experimental conditions required a change in procedure, the following technique was employed. Beef extract agar (Ruehle and Brewer, 1931) was kept melted at 480C. in constant temperature incubator. Each 30 ml. amount of 1.5 per cent extract agar at pH 6.8 was inoculated with 0.1 ml. of an 18-hour growth of the organism being studied. The cultures were standardized to a density of 1 billion organisms per milliliter with a barium sulphate nephelometer. Unless otherwise specified, our WP3 strain of Staphylococcus aureus was employed. As soon as the bacteria were added, the container was rotated to insure uniform distribution of organisms. Approximately 30 ml. amounts were poured into standard 100 x 15 mm. petri dish bottoms. The dishes were covered with Coors porcelain lids glazed on the outside. The porcelain lids absorbed water of condensation and prevented the deposition of moisture on the test medium. When the agar solidified, one disc of agar was usually cut from each plate. At times it was desirable for comparative purposes to cut three or four agar cups in a single dish. The standard volume of antiseptic employed was 0.2 ml. of a given dilution. The agar cup-

plates were incubated in an upright position at 370C. for 24 hours and readings were then made. All tests were performed in duplicate.



2 Turbidimetric method



The method has the advantage of a shorter incubation period for the growth of the test organism (usually 3 to 4 hours) but the presence of solvent residues or other inhibitory substances affects this assay more than the cylinder plates assay and care should be taken to ensure freedom from such substances in the final test solutions. This method is not recommended for cloudy or turbid preparations.

Prepare five different concentrations of the standard solution for preparing the standard curve by diluting the stock solution of the Standard Preparation of the antibiotic (Table 3) and increasing stepwise in the ration 4:5. Select the median concentration (Table 3) and dilute the solution of the substance being examined (unknown) to obtain approximately this concentration. Place 1 ml of each concentration of the standard solution and of the sample solution in each of the tubes in duplicate. To each tube add 9 ml of nutrient medium previously seeded with the appropriate test organism.

At the same time prepare three control tubes, one containing the inoculated culture medium (culture control), another identical with it but treated immediately with 0.5 ml of *diluteformaldehyde solution* (blank) and a third containing uninoculated culture medium.

Place all the tubes, randomly distributed or in a randomized block arrangement, in an incubator or water-bath and maintain them at the specified temperature for 3 to 4 hours. After incubation add 0.5 ml of *dilute formaldehyde solution* to each tube. Measure the growth of the test organism by determining the absorbance at about 530 nm of each of the solutions in the tubes against the blank

Estimation of potency. Plot the average absorbances for each absorbances on the arithmetic scale and concentrations on the logarithmic scale. Construct the best straight response line through the points either by inspection

Microbiological assay of vitamins

is a type of biological assay performed with the aid of microorganisms. • Many therapeutic agents, which either inhibit the growth of microorganisms or are essential for their growth are standardized by microbial assay.

WHAT IS THE UNDERLYING 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.

HOW DO WE DO IT?

The Indian pharmacopoeia gives two methods:

• The cylinder-plate (cup-plate) method

• The turbidimetric (tube assay) method

A QUICK REVIEW OF VIT. B12

Also known as cyanocobalamin. It's a water soluble vitamin.Structure is similar to that of heme where the iron is replaced with cobalt as a centre of molecule. Its main sources are liver, eggs, milk, meat & fish. VitB12 deficiency causes Macrolyticanemia, pernicious anemia. National Research Council, USA recommends a daily intake of about 5mg of vit B12

PREREQUISITES FOR THE ASSAY:

Test organism selected must be able to utilize free cyanocobalamin LACTOBACILLUS LEICHMANNII satisfies this requirement It is easily available, non pathogenic and easily culturable Isolated from milk, cheese and other dairy products

PRECAUTIONS

Great care must be taken to avoid contamination .All the glasswares must be free from detergents and other chemicals. lasswares must be heated to 2500C for at least 1 hr before use. The whole experiment must be carried out under proper aseptic condition

REAGENTS

STANDARD B12 STOCK SOLUTION A solution of cyanocobalamin of concentration 1.0 microgram per ml is made using 25% ethanol

STANDARD B12 SOLUTION dilute stock solution to prepare a solution of conc. 0.01-0.04 microgram/ml . Prepare freshly

TEST SOLUTION TO BE ASSAYED Accurate amount of material to be assayed is taken & dissolved in water, Dil HCl or NaOH is added to adjust ph at 6.0.

BASAL MEDIUM STOCK SOLUTION

Ingredients	Quantity
L–Cystine	0.1 gm
L-Tryptophane	0.05 gm
1N HydrochloricAcid	10 ml
Adenine-Guanine-Uracil Solution	5 ml
Xanthine Solution	5ml
Vitamin Solution I	10ml
Vitamin Solution II	10ml
Salt Solution A	5 ml
Salt Solution B	5ml
Asparagine Solution	5ml
Acid-hydrolyzed Casein Solution	25ml
Dextrose, Anhydrous	10 gm
Sodium Acetate, Anhydrous	5 gm
Ascorbic Acid	1 gm
Polysorbate 80 Solution	5 ml

DISSOLVE IN 100 ml WATER

SUSPENSION MEDIUM

Prepare a 100ml solution by mixing equal volumes of BASAL MEDIUM STOCK SOLUTION and distilled water

PREPARATION OF INOCULUM

1.Transfer a loopfull of Lactobacillus liechmannii from a recent subculture into two tubes each containing 10ml of sterile culture medium. 2. Incubate the tubes for 18-24hrs @ 37oC. 3. Centrifuge. Make a suspension, of the cells that settle down, using 10ml of sterile suspension medium. 4. Centrifuge again and once again suspend the cells in 10 ml sterile suspension medium. 5. Aseptically transfer 1ml of the so prepared suspension to 10ml sterile suspension medium. 6. This suspension is used as innoculum.

COMPOSITION OF CULTURE MEDIUM : (PH = 6.8)

• Yeast extract -0.75gm • Peptone -0.75gm • Dextrose -1gm • Potassium dihydrogen phosphate -0.2gm • Tomato juice filtrate -10ml • Sorbitan monooleate solution-1ml • Water upto-100ml

WHAT IS THE PROCEDURE? Assay of vitamin B12 can be carried out by two methods 1. Titrimetric method 2. Turbidimetric method

TITRIMETRIC METHOD

- Clean ten test tubes & add to it 0, 0.5, 1.0, 2.0, 2.5, 2.5, 3.0, 3.5, 4.0, 4.5, & 5ml of standard cyanocobalamin solution.
- To each tube add 5ml of Basal medium solution
- Volume of each is adjusted to 10ml by water
- In another 4 test tubes add 1, 2, 3, 4ml of test solution which is to be assayed .
- To each of this also add 5ml of Basal medium stock solution & adjust volume to 10ml with water.

- Sterilize all test tubes in autoclave at 121°C for 15mins.
- Cool the test tubes at room temperature.
- Inoculate a drop of inoculum prepared of lactobacillus liechmannii.
- Incubate the test tubes for 64 to 72hrs at temperature range of 30 to 37°C.
- After incubation period titrate contents of each test tube with 0.05N NaOH using bromothymol blue as indicator until green colour.
- Record all the titre readings clearly.

INTERPRETATION OF THE RESULTS

- Determine the average of titration values of each level of both standard & test solutions.
- Plot a graph considering average titration values(in ml) of 0.05N NaOH against concentration of standard cyanocobalamin solution.
- A linear graph is obtained.
- By interpolating the standard curve determine the concentration as activity per ml of vit B_{12}.
- From the graph the concentration of test solution of cyanocobalamin is found & reported.

Table 26.5 Some examples of microorganisms used as bioassays for vitamins

Assay microorganism	Vitamin
Lactobacillus casei	Biotin
L. arabinosus	Calcium pantothenate
L. leichmannii	Cyanocobalamin
L. casei	Folic acid
Saccharomyces uvarum	Inositol
L. arabinosus	Nicotinic acid
Acetobacter suboxydans	Pantothenol
L. casei	Pyridoxal
Neurospora crassa or S. carlsbergiensis	Pyridoxine
L. casei	Riboflavine
L. viridans	Thiamine

TURBIDIMETRIC METHOD

• Incubate all the test tubes at 30-37oC for 16- 24hrs • Set the wavelength of the specrtophotometer at 640nm • Take readings

Microbial assay of amino acids

There are 20 amino acids present in proteins. Many microorganisms are used for assay od amino acids.

Assay microorganism	Amino acid assayed	Assay response	Detection method	Reference
Streptococcus faecalis	Valine, Leucine Threonine	Cell growth Acid production	OD Titration	Blackmore and Parry [22] Cardinal and Hedrick [21]
Streptococcus zymogenes	Tryptophan, Methionine	Cell growth	OD	Wells *et al.* [23]
Leuconostoc mesenteroides	Methionine Lysine, Arginine, Proline, Phehylalanine, Methionine, Cystine, Serine, Alanine, Aspartic acid	Cell growth Acid production	OD Titration	Blackmore and Parry [22] Cardinal and Hedrick [21]
Lactobacillus arabinosus	Leucine, Isoleucine, Valine, Glutamic acid	Acid production	Titration	Cardinal and Hedrick [21]
Pediococcus acidilactici	Lysine, Methionine	Cell growth Acid production	OD Titration	Odunfa *et al.* [24]
Tetrahymena pyriformis	Lysine	Cell growth	Cell count	Stott and Smith [19]; Bos *et al.* [20]
Escherichia coli	Cysteine, Glutamine, Methionine, Lysine, Threonine, Tryptophan	Variable	Variable	Table 2 this review