

UNIT-III

Evaluation of bacteriostatic & bactericidal



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Evaluation

- Anti microbial agent as well as disinfectant both kills the microorganism or prevent their growth.
- So to check the effectiveness on microorganism, they are evaluated by following method or techniques-
- 1. Tube dilution & agar plate method
- 2. Filter paper and cup plate method
- 3. Ditch –plate method
- 4. Phenol-coefficient method
- 5. Kelsey sykes method

Tube dilution and agar plate method

- Chemical agent is incorporated in to nutrient broth as agar medium and inoculated with test microorganism.
- Those tubes are incubated at 30-35°C for 2-3 days and then the results are observed in the form of turbidity or colonies.
- The results are recorded and the activity of the given disinfectant are compared.



Clear



Slight
turbid



Moderate
turbid



More
turbid



No
Colonies



Appx
100
colonies



Approx
500
colonies



Approx.
1000
colonies

Filter paper and cup plate method

<https://www.youtube.com/watch?v=sx1uDYSfINA>

- One way is to inoculate an agar plate with a lawn of bacteria and add filter paper disks that have been moistened with the disinfectant being tested. This is known as the **filter paper disk method**, or **agar disk diffusion assay**.
- After incubation, plates are observed for the presence of a zone of **inhibition** (area around a disk where no microbial growth is detected).
- Generally speaking, the larger the zone, the more effective the disinfectant is against that particular microbe. However, other factors such as the solubility of the test agent and the molecular weight of the disinfectant molecules (which determines the diffusion rate of the disinfectant through the agar) can also affect results.

- Agar is melted and cooled at 45°C.
- Then inoculated with the test microorganism and poured in to a sterile petri plate.
- In cup plate method, when the inoculated agar has solidified, holes about 9mm diameter are created in the media using sterile cork borer.
- Antimicrobial agent is directly poured in the holes.
- In filter paper and cylinder plate method, antimicrobial agent is applied to the surface of the solidified inoculated agar by using a filter paper disc and cylinder respectively.
- The zone of inhibition is observed after incubation period.
- The diameter of zone of inhibition gives an indication of the relative activities of different antimicrobial substances against the test micro organism.

Ditch –plate method

<https://www.youtube.com/watch?v=mNBzioOudmY>

- A solution of anti microbial substance is carefully run in to the ditch which is prepared in agar plate.
- A loopful of each test microorganism is then streaked outwards from the ditch on the agar surface.
- Microorganisms resistance to the antimicrobial agent grow right up to the ditch whereas the susceptible microorganism show a zone of inhibition adjacent to the ditch or the centre of the plate.
- Width of the inhibition zone gives an indication of the relative activity of the antimicrobial agent against the respective microorganism.

Phenol-coefficient method

- In this method a test chemical is rated for its microbicidal property with reference to phenol under identical condition.
- In this test similar quantities of microorganisms are added to rising dilution of phenol and the disinfectant to be tested.
- Organism used in this test: *Salmonella typhi*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*

Phenol coefficient test includes:

- Rideal-walker test
- Chick-martin test
- United status food and drug administration test
- The US association of official agricultural chemists test
- This test is suitable for disinfectants miscible with water and which exert their antimicrobial action in a manner similar to that of phenol.

Procedure:

- Different dilution of the test disinfectant and phenol are prepared and 5ml of each dilution is inoculated with 0.5ml of the 24 hrs broth culture of the organisms.
- All tubes containing disinfection and organism with phenol and only organism are placed in a 17.5°C water bath.
- Subcultures of each reaction mixture are taken and transferred to 5ml sterile broth after 2.5, 5, 7.5 & 10 min.
- Broth tubes are incubated at 37°C for 48 hrs and examined for the presence or absence of growth.
- Then RW coefficient is calculated .
- RW coefficient = dilution of disinfectant killing in 7.5 but not in 5min / dilution of phenol killing in 7.5 but not in 5min

- If phenol coefficient or Rideal-walker coefficient of given test disinfectant is
- 1=disinfectant has the same effect as phenol
- <1 =disinfectant is less efficient than phenol
- >1 = more effective than phenol
- 20=disinfectant is 20 times more active than phenol.

Advantages:

- Inexpensive and can be performed quickly.
- Give reproducible results.

Disadvantage:

- Sampling error are large.

Kelsey-sykes method

- In this method various bacteria such as *Staphylococcus aureus*, *P. vulgaris*, *E. coli*, and *P. aeruginosa* are used.
- It can be carried out in clear or dirty conditions.
- In both cases the final concentration of bacterial cells should be about 100/ml.

Sterility testing of products (solids, liquids, ophthalmic and other sterile products) according to IP,BP and USP.

- **Definition** : The sterility of a product is defined by the absence of viable and actively multiplying microorganisms when tested in specified culture media.
- The test is applied to substance, preparations or articles which, according to the Pharmacopoeia, are required to be sterile.

- The test is used on drugs, preparations, or products that must be sterile according to the Pharmacopoeia. An acceptable result, on the other hand, just shows that no contaminating microorganisms were discovered in the sample analyzed under the test parameters.

Precautions to avoid microbial contamination

- The **sterility test is performed under aseptic circumstances**. To attain such circumstances, the test environment must be tailored to the manner in which the sterility test is carried out. In terms of microorganisms exposed in the test, the steps made to prevent contamination do not affect them. The working conditions under which the tests are carried out are checked on a regular basis by suitable sampling of the working area and the implementation of relevant controls.

Methods are defined in Pharmacopoeia:

- **Membrane Filtration Method (open or a closed system)**
- **Direct Inoculation Method**
- When the preparation to be tested has an antimicrobial effects, these effects must be reduced or neutralized by adding an appropriate substance to the specified test media, to diluents or solvents, or to the preparation prior to testing.

Media types

- ▣ Fluid Thioglycollate medium (FTM)
- ▣ Soybean Casein Digest Medium (SCD or TSB)

Prior to test, make sure that:

- ▣ Media is sterile
- ▣ Media supports growth of microorganisms

2 components in Media validation :

- ▣ Media sterility Test
- ▣ Growth Promotion Test

Media sterility

- ▣ Negative Control - may be used to identify a “false positive” test result
- ▣ Incubate for 14 days prior to use, may be conducted concurrently with test
 - 30 - 35°C for Fluid Thioglycollate medium (FTM)
 - 20 - 25°C for Soybean Casein Digest Medium (SCD/TSB)

Acceptance criteria:

- ▣ Should be sterile, no growth observed

- Membrane Filtration Method (Open Funnel Method)

Sample been filtered and rinsed



Membrane filter is cut into half



Membrane into medium



Incubate



- Membrane Filtration Method (Closed System Method)

Sample been filtered and rinsed



Adding medium (FTM/SCD) into apparatus



Incubate



Growth Promotion Test

- ❑ To test the ability of media to support the growth of micro-organisms
- ❑ The media should be inoculated with <100 cfu of challenge organisms. The challenge inoculum should be verified by concurrent viable plate counts
- ❑ Growth promotion challenge organisms should show clearly visible growth in the test media within 3 days for bacteria and 5 days for fungi.

Direct Inoculation of the culture medium

- ▣ Transfer the preparation directly into the culture medium
- ▣ Volume of the product is not more than 10% of the volume of the medium.

Table 2.6.1.-2 – *Minimum quantity to be used for each medium*

Quantity per container	Minimum quantity to be used for each medium unless otherwise justified and authorised
<p><i>Liquids</i></p> <ul style="list-style-type: none"> – less than 1 ml – 1-40 ml – greater than 40 ml and not greater than 100 ml – greater than 100 ml <p><i>Antibiotic liquids</i></p> <p><i>Other preparations soluble in water or in isopropyl myristate</i></p>	<p>The whole contents of each container</p> <p>Half the contents of each container but not less than 1 ml</p> <p>20 ml</p> <p>10 per cent of the contents of the container but not less than 20 ml</p> <p>1 ml</p> <p>The whole contents of each container to provide not less than 200 mg</p>
<p><i>Insoluble preparations, creams and ointments to be suspended or emulsified</i></p>	<p>The whole contents of each container to provide not less than 200 mg</p>
<p><i>Solids</i></p> <ul style="list-style-type: none"> – less than 50 mg – 50 mg or more but less than 300 mg – 300 mg to 5 g – greater than 5 g 	<p>The whole contents of each container</p> <p>Half the contents of each container but not less than 50 mg</p> <p>150 mg</p> <p>500 mg</p>
<p><i>Catgut and other surgical sutures for veterinary use</i></p>	<p>3 sections of a strand (each 30 cm long)</p>

Incubation and Examination

- ❑ All test & sterility control containers – incubated for at least 14 days (unless microbial contamination detected earlier)
- ❑ Examine for evidence growth
- ❑ Preparation not readily seen (turbid/cloudy due to its nature) – after 14 days of incubation ⇒ transfer a suitable portion (2-5% of contents) to fresh, same medium ⇒ incubate for 7 days



- No evidence of microbial growth is found.

If turbidity or other evidence of growth is seen:

- ❑ Streak on solid media
- ❑ Examine the suspected growth microscopically by Gram stain
- ❑ Identify the isolates, as far as the genus and preferably species level

Validation (bacteriostasis & fungistasis) Test

- The test should be validated by inoculation with <100 cfu of challenge organism strains to the media/product container at the beginning of the test incubation period.
- The challenge inoculum should be verified by concurrent viable plate counts.



Validation (bacteriostasis & fungistasis) Test

- The challenge organisms, preferably, should be added directly to the product prior to membrane filtration or direct inoculation. If this is not practicable, the challenge organisms should be added to the last rinse solution (membrane filtration) or directly to media containing the product (direct inoculation).
- Validation done should mimic the test proper in every detail.
- Perform a growth promotion test as a positive control. Incubate all the containers containing medium for not more than 5 days.

Interpretation of results

- Challenge organisms should clearly show visible growth of bacteria within 3 days, and fungi within 5 days in the test media containing product.
- Visually comparable to that in the control vessel without product.