CHAPTER - IV

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INTRODUCTION :

Once the drug is synthesized it should be tested for its medicinal value. It can be tested in two ways, i) In vivo and ii) in vitro,

The diseases are produced by many microorganisms in the body viz bacteria, viruses etc. The bacteria contributes major component of microorganisms. Dependingg upon their staining properties they are classified as- a) Gram positive and b) Gram negative.

"Antagonism" of one microoranism by another has been noticed from early times,[1] Cornil and Babes wrote, "If the study of mutual antagonism of bacteria were sufficiently far advanced a disease caused by one bacterium could probably be treated by another." E. Duchesne found that penicillin reduced the virulence of E. Coli and Salmonella when injected into an animal with them, and concluded : In pursuing the study and facts of bioligical antagonism between molds and bacteria one may arrive perhaps at ... other facts ... applicable to prophylactic hygiene and to therapy." Several instances of microbial antagonism were studied before the recent great development of antibiotic began. One arose from the discovery

that the damping-off fungus, Rhizoctonia solani, which kills many seedlings, is itself killed by a common soil organisms, Glioeladium fimbriatum. This led to the isolation of Gliotoxin[2], the first natural antibiotic to be prepared pure.

Penicillin is remarkable nontoxic to man and other mamals. As an antibiotic it is especially effective against cocci, both gram positive and gram negative, including pneumococci, streptococci and gonococci. Several antibiotics were discovered and they were tested for antibacterial activity using cultures of certain bacteria and inhibition to their growth.

There are many methods of assessing the antibacterial activity of antibiotics. The aim of 'in vitro' susceptibility testing is to predict as accurately as possible, the 'in vivo' activity of an agent, penicillins provide peculia difficulties in assessment because of their complex mode of action and because of the various mechanisms of resistance to them. 'In vitro' if there is a readily penetrable barrier, the antibiotic may diffuse so rapidly into the cell that the latter is killed before any penicillin present has had the opportunity to act.

But 'in vivo' the antibiotic may be delivered to the cells more slowly than in a laboratory experiment, and the penicillin may be able to inactivate the antibiotic, for example, ampicillin may inhibit penicillinase producing H.

influenzae in vitro at low concentrations when small inocula were tested because there is little barrier to penetration.[3] However, when large incula are tested, as would be present in vivo, for example in meninges, the organisms are not responsive to ampicillin.

METHODS OF ASSAY

[A] Chemical methods :

When an antibiotic is available in pure form, and its structure is at least partially known, the amount of it can be determined by chemical methods based on its reactive groups. For instance, the lactam ring of penicillin, can be hydrolysed with alkali to sodium penicilloate and the amount of base consumed can then be determined by back litrating or the penicilloic acid formed can be broken down further with acid to yield dimethylcysteine, which can be determined by the ninhydrine reaction for aminoacids.

The most specific methods, however, are those based on anntibiotic power. These of course, can be used with impure preparation at every stage from the first crude extract onward. The procedures adopted can be given as.[4]

[B] All or none growth method :

Tubes of nutrient broth are seeded with a standard small inoculum of a susceptible organism and serial

dilutions of the antibiotic added. The tubes are incubated and examined. The lowest concentration (Minimum Inhibitory concentration, MIC) necessary to inhibit growth completely is taken as end point, and the amount of antibiotic determined by comparison with similar dilutions of a known standard substance, for penicillin, M. aurens, incubated 16 hours at 37°, is used. An advantage of this method is that the volumes used can be quite small.[5]

[C] **Turbidimetric method** :

The tubes of broth are seeded with a small inoculum, but the time course of growth is followed with a turbidimeter or colorimeter. In this curve, using penicillin, the higher concentrations cause autolysis after four hours. A plot of the turbidity or optical density at a fixed time against concentration is a curve; the same plotted against log concentration usually gives a straight line from which the concentration needed for 50 per cent inhibition is easily read out.

[D] **Diffusion method** :

These methods are most particularly associated with antibiotics. A plate of solid medium, inoculated in bulk with test organisms, is treated in spots with the solution and then incubated. As the substance diffuses out into the agar, it inhibits growth throughout a zone surrounding the place of

application. The radius of the zone is a measure of the amount or concentration of the antibiotic. If the antibiotic applied is small in amount, the radius of the inhibited zone is proportional to its amount. This is true of a small drop of solution, and also, roughly, with small filter-paper circles, soaked in the solution. However, if a relatively large volume of solution is used, the radius of the zone is proportional to the concentration, or more nearly to the logarithm of the concentration, of the antibiotic in it. This is the case with the classical "Oxford cup" method[6] in which small glass cylinders, their ends warmed in the flame are placed on the agar and filled with 1 to 2 ml of the test liquid. When this method is used with penicillin, Bac. substilis is used since bulk inoculation of agar has to be done at 48 , a temperature that may damage a coccus.[7]

The original Oxford unit of penicillin was established by the cup assay. It was defined as the amount of penicillin, which under the standard conditions using "stap^{n}" aureus, gave an inhibition zone of diameter 24 mm.

EXPERIMENTAL TECHNIQUES :

To study the biological activity of prepared penicillin derivatives, the methods used can be summarised as :

A) For sensitivity test :

To test whether the antibiotic is sensitive to inhibit growth of bacteria were tested by "Agar diffusion method" (Pour-plate method).

B) For MIC values :

If the antibiotic was capable of inhibition of bacterial growth then the Minimum Inhibitory Concentrations (MIC values) were obtained by "Ten fold dilution method".

A) Sensitivity Test :

The steps involved can be summerised as follows

1] Sterilization : It is that complete distruction of all living organisms by physical or chemical agents.

Plugged glassware and petri dishes must be sterilzed before use to destroy all living organisms adhering to the inner surfaces. Likewise, culture media must be sterilized prior to use to destroy all contaminating organisms presents. When once sterilized, glassware may be kept in a sterile condition indefinitely if protected from outside contamination. The same applies to culture media if in addition, evaporation can be prevented.

Three types of sterilizers are generally used in bacteriology.

i) The hot air sterilizer..

ii) The arnold sterilizer.

iii) The antoclave.

The sterilization method adopted can be given as :

The petri plates and glass wares were washed thoroughly and covered with papers and placed in an oven at high temperature till the paper becomes black.

The nutrient agar used for plate culture and plant culture is sterilized by placing them in glass test tubes having cottonn stopper in presence cooker, after one whistle the bumbers were simmed off and further heating for 20 minutes.

2] Method for inoculation of media :

The pure culture can be prepared by :

a) Agar deep culture.

b) Agar slant culture.

c) Broth culture.

Agar slant culture : This technique is used for preparation of pure culture for "Sensitivity test' as well as "MIC test." Sterlized the wire loop in the flame. Allow the loop to cool for a few moments. Remove the cotton stopper from the culture, by grasping it with small finger of the right hand, and flame the neck of the tube. Hold the tube slanted, not upright, to minimize aerial contamination. Remove a loopful of the growth with the loop. Again flame the neck of the culture, replace the cotton stopper, and set the tube aside

in test tube block. Remove the cotton stopper from the agar slant to be inoculated by grasping it with the small finger of the right hand, flame the neck of the tube. Spread the inoculum over the surface of the agar slant by making streaks back and forth a few milimeters apart. Withdraw the loop from the tube. Again flame the neck of the tube, and replace the cotton stopper. Flame the loop before setting it down on the table. Mark the tube with the name of the organism and the date. Incubate the culture in slant position in incubator at 30° for 24 hours to allow proper growth of organism for the experiment.

The transfer of culture from Agar slant culture to sensitivity test in petriplates can be done as :

Microorganisms from culture were transfer to tube containing 1 ml sterile saline solution (NaCl solution), in the similar way as explained in Agar slant culture taking precaution that it does not get contaminated with outside atmosphere.

The sensitivity tests were done and by transfer of culture in petri-plates. Two methods are generally followed.

a) Streak - plate method.

b) Pour - plate method.

Pour - plate method : Take a sterile Agar solution tube of 20 ml volume. Remove the cotton stopper and flame the neck of the tube, hold the tube in up-right position in between the

flame of the two burners and mixe the sterile saline solution (1 ml) tube having culture of specific organism, again flame the neck of tube and replace the cotton stopper. Stirr the tube holding in between two palms of hands so as to make uniform susspension of organism in Agar medium. Place the tube aside. To avoid coagulation of Agar medium in tube it should be transfered to sterile petri-plate while it is in liquid form. Take a sterile petri-plate and raise the liquid of petri plate just high enough to transfer Agar medium from tube, by holding the petriplate in between the flames of two burners, transfer the Agar medium from tube and cover the lid of petri-plate. Place the petri-plate on the table having uniform surface and allow to solidty the agar medium along with culture.

Take metal borer having the diameter of 2 mm sterile it by flame and allow to cool, take a petri-plate in one hand and 'raise the lid make a 4 wells with the help of burner in such a way that each well should occupy corner of regular square. Cover the lid of petri-plate and place it aside.

3] <u>Preparation of Antibiotic solution to study "Sensitivity" and "MIC value"</u> :

The solution of antibiotic can be prepared in buffer. These are the salts of weak acids which have the power of preventing pronounced changes in the reaction of

solutions on the addition of acids and alkali.

Bacteriological peptones contain proteoses, peptones, peptides and amino acids all of which are buffers. They posses both acidic and basic properties. The acidity of solution up to certain concentration prevents further multiplication of the organisms. In absence of buffer, the activity of the organism may cease after a few hours. Clark and Lubs[8] proposed a series of buffer standards covering the range from pH 1.2 to 10.

Taking the advantage of this fact the buffer solution is used for the preparation of anitbiotic solution.

A known weight of antibiotic is dissolved in a known volume of buffer solution and stoppered with cotton.

Take a sterile micropipette or a dropper and take small quantity of above solution by holding the tube and dropper in flames of two burners. Place the micropipette in such a way that its mouth should be between the burners to avoid atmospheric contamination.

Take a petriplate having wells in it, lift the lid of plate and allow one drop of above solution to fill the single well. In similar manner, allow four wells to fill by solutions of 4 different antibiotics, cover the lid and mark each well by its antibiotic on the bottom with sketh pen or marker.

Place the petriplates in an incubator at 35° for 24 hours to have proper growth of organims and their inhibition by antibiotics.

Observe the plates after 24 hours. There appears an inhibition zones on culture media which looks like a transperent while other surface is full of growth of microorganism. Measure the diameter of the inhibition zone which is "sensitivity test" of perticular organism for particular antibiotics.

[B] MINIMUM INHIBITORY CONCENTRATION (MIC) VALUES :

After sensitivity test, depending upon size of "Inhibition zone" it is possible to select perticular concentration of that antibiotic for further study of M.I.C. value. It is calculated by "Ten fold dilution method" which can be summarised as follows :

The steps involved are as give as :

1) Sterilization : The sterilization of micropipette, test tubes, loops etc can be done before start of experiment as given in "sensitivity test".

The sterilization of Agar Nutrient is also done in the similar way.

2) Inoculation of media : The inoculation of media as well as transfer of microorganissms were done in the similar way as that of "sensitivity test".

3) <u>MIC values</u> : MIC values can be determined as follows :

Place 9 ml of the Nutrient Agar in each test tube for one organism ten test tubes were required which forms ten different concentrations of antibiotics under study. Stopper the tubes with cotton stopper lightly and sterilize them in pressure cooker as explained in first step.

The solution of known quantity (5 mg) of antibiotic is prepared in 1 ml of Buffer solution which gives the concentration as 5000 mg/ml.

The test tubes of Nutrient Agars after sterilisation were numbered from 1 to 10. The saline solution of microorganism is taken in micropipette and one drop of it is added to above test tubes. One ml of above antibiotic solution is added to test tube number and it is stirred well. 1 ml of Agar Nutrient from test tube No.1 is transfered to test tube No.2 and test tube No.1 is corked tightly and place apart. The similar procedure was repeated for test tubes No.2 to 10. Now 1 ml of Nutrient agar from, test tube No.10 is taken out and placed apart. All the test tubes are stirred well and placed in an incubator at 37° for over night.

The concentration of antibiotic in each tube can be given as shown in table No. 5.

Table No. 5

Test tube No.	1	2	3	4	5	6	7	8	9	10
Concentration of Antibiotic, mcg/ml	5×10^{2}	5×10	5×10^{0}	5×10^{-1}	5×10^{-2}	5×10^{-3}	5×10^{-4}	5×10^{-5}	5×10^{-6}	5×10^{-7}

All the test tubes were observed after over night. The growth of the microorganisms occurs in the form of turbidity in test tubes. If the antibiotic is effective against perticular organism for specific concentration, then from that concentration, there is no growth of microorganisms and the Nutrient Agar appears as clear (no turbidity). The concentration at which no turbidity appears in test tube is the "Minimum Inhibitory Concentration" (MIC) of that antibiotic for that microorganism.

Observations :

The penicillin derivatives thus prepared were further tested for their antibacterial activity as explain above. The antibacterial activity of the compounds prepared, at concentration 500 µg/ml is given in Table No.6.

Table No. 6

Antibacterial Activity of Penicillin Derivatives

Microorganism	Compound		
	4b	4c	4d
S. aureus	+	-	+
E. coli	-	-	+
S. typhi	-	-	+
K. pneumonae	+	-	+
P. aeruginosa	-	-	+

NOTE : +ve sign indicates that the compounds is active.

-ve sign indicates that the compound is inactive.

-: R E F E R E N C E S :-

1. Bryson, V. Antibiotics and Experimental Aspects 1962, In, Suwey Biol, Prog. IV : 345-440.
2. Weindling, R. and Emerson, O.H. 1936, Phytopathol, 26 : 1068-1070.
3. Medeiors, A.A. and O'Brien T.F. (1975), Ampicillin-resistant Haemoph, influenzae type possessing a TEM-type beta-lactamase but little permeability barrier to ampicillin. Lancet, 1, 716.
4. Thimann, K.V., (1963), The life of Bacteria, Their growth, Metabolism and relationship, pp 803.
5. Thimann, K.V. (1963). The life of Bacteria Their growth, Metabolism and relationships, pp 804.
6. Beadle, G.W., Mitchell, H.K. and Bonner, D.J. Bact. 49 : (1945) 101-104.
7. Foster, H. Wand Woodruff, H.B. 1944-1946, J. Bact., 47 : 43-58, 51 : 363-369 (1946).
8. Clark, W.M., "The determination of Hydrogen Ions". Baltimore, The Williams and Wilkins Company, (1928) and Lubs, H.A., The colorimetric determination of hydrogenio concentration, J. Bact. 2:1, 109, 191, (1917).