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ANTIBIOTIC SUSCEPTIBILITY TESTING: A REVIEW ON CURRENT PRACTICES

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ABSTRACT

An increasing Antimicrobial resistance (AMR) has resulted in morbidity and mortality from treatment failures and increased health care costs. Appropriate antimicrobial drug use has unquestionable benefit, but physicians and the public frequently use these agents inappropriately hence, it became necessary to perform the antimicrobial susceptibility test as a routine. The aim of antimicrobial susceptibility testing is to determine the lowest concentration of existing or even new antimicrobial agents which inhibits the visible growth of the bacterium being investigated, under certain test conditions. The Disk diffusion, well diffusion, stokes and gradient diffusion methods are manual methods that provide flexibility and possible cost savings. The most commonly used testing methods include broth microdilution method using commercially available 96-well micro dilution panel. Broth dilution, tube dilution and E test provide quantitative results (e.g. Minimal Inhibitory Concentration) whereas other methods provide qualitative results which are categorized as susceptible, intermediate or resistant. Although available testing methods provide accurate detection of common antimicrobial resistance mechanisms, emerging newer mechanisms of resistance certainly attracts researcher for the development of advanced, reproducible, automated and reliable antimicrobial testing methods.

Keywords: Disk diffusion, zone of inhibition, minimum inhibitory concentration, microdilution, E-test

INTRODUCTION

Antibiotics/antimicrobial agents are the major drugs of choice of the physician's desk to treat the pathogenic infections. It has been observed that some of the clinicians prescribe the medicine based on the symptoms instead of performing diagnostic tests. This prescribing pattern may be one of the reasons for the development of resistant for the antibiotics [1]. Therefore, Antibiotics susceptibility testing (AST) plays an important role to check the effectiveness of a drug against a bacterium and select the best drug that act against the bacterium. One of the significant roles of clinical microbiology laboratory is the performance of antimicrobial susceptibility testing of various bacterial isolates. The main objectives of the testing are to find out possible drug resistance in common pathogenic microorganism and the susceptibility to drug of choice for a particular infectious microorganism can be assured.

Mechanism of antimicrobial resistance: There are number of ways by which microorganisms are resistant to antimicrobial agents. These includes: 1. Bacteria produce enzymes which destroy the antimicrobial agents before it reaches its targets e.g. Beta lactamase enzyme hydrolyses beta lactam drugs which develop resistance. 2. Impermeable cell for antimicrobial drugs e.g. Gram negative bacteria may become resistant to Beta lactam antibiotics by developing permeability barrier. 3. Mutation e.g. Ribosome methylation of ribosomal RNA develop macrolide resistant. 4. Bacterial efflux pump that expels antimicrobial drugs from cell before it can reach its targets. 5. Specific Metabolic pathways in the bacteria are genetically altered so that antibacterial agents cannot exert an effect [2, 3].

Purpose of antimicrobial susceptibility testing

a) A laboratory test which determine that how effective antibiotic therapy is against a bacterial infection. b) AST can control the use of antibiotics in clinical practice. c) AST testing will assist the clinicians in the choice of drug for the treatment of infection. d) AST can help the local pattern of antibiotics prescriptions. e) To reveal the changing trends in the local isolates.

Antimicrobial susceptibility testing methods:

*A. Qualitative Method***:** This method is used for testing of isolates from healthy patients with intact immune defenses, in less serious infections such as UTI. There are two qualitative methods.

Disk diffusion test: The disk diffusion sensitivity test also known as Kirby Bauer disk method "Figure 1", is a simple and practical which uses antibiotic-impregnated wafers (disk) to test whether particular bacteria is susceptible to specific antibiotic or otherwise [4-6]. The bacterial inoculum (approximately 1-2 X 10^8 CFU/mL) was uniformly spread using sterile cotton swab on a sterile Petri dish MH agar. The antibiotic disks were placed on top of the previously inoculated Mueller Hinton agar medium surface with the help of sterile forceps. Each disc must press down to ensure complete contact with the agar surface. The plates were incubated for 18–24 h at 35-37 ^{0}C temperature in bacteriological incubator before an interpretation of the result.

The antibiotic diffuses from the disc into the agar in decreasing amounts the further it is away from the disk. If the organisms were killed or inhibited by the concentration of the antibiotic, there will be no growth in the immediate area around the disks represented as zone of growth inhibition. The diameter of the zone of inhibition is directly proportional to the sensibility of the isolate and to the diffusion rate of antibiotics through the agar medium. A zone of inhibition was measured in millimeters by either measuring: (A) Radius: Measure half the distance of the zone and then multiply by 2. This method was used when part of the zone is not clear or has grown into another zone. (B) Diameter: Measure the entire length of the zone and subtract the disk diameter (Standard disk size 5-6mm). The result of the test can be interpreted by using the criteria published by Clinical and Laboratory Standard Institute (CLSA formerly the National Committee for the Clinical Laboratory Standard or NCCLS) [7].

The results of the disk diffusion test are "qualitative" and will be reported out as:

Susceptible: 'The term "susceptible" represent that isolates are inhibited by the usually recommended dosage of an antimicrobial agents. However, this term doesn't assure clinical success; in fact predicting clinical outcome based on susceptibility testing and the use of drugs shown to be in the susceptible category is very imprecise. This imprecision is due to the effect of host responses, site of infection, toxin production by bacteria that is independent of antimicrobial susceptibility, the presence of biofilm, drug pharmacodynamics and other factors.

Intermediate: 'The "intermediate" category includes isolates with antimicrobial MICs that approach usually attainable blood and tissue levels and for which response rates may be lower than for susceptible isolates. The intermediate category implies clinical efficacy in body sites where the drugs are physiologically concentrated (e.g. quinolones and beta-lactams in urine) or when a higher than normal dosage of a drug can be used (e.g. beta lactams).

Resistant: 'The category indicates that isolates are not inhibited by the usually achievable concentrations of the antibiotics with normal dosage schedules, which demonstrate an existence of the specific microbial resistance mechanisms (e.g. beta-lactamases).

The merits of the disk diffusion methods are simplicity in test, most economic, flexibility in disk selection, and the result can be easily interpreted by clinicians. However, the demerits include manual test, lack of automation and all fastidious or slow growing bacteria cannot be accurately tested by this method. The limitation of this testing show that the microbiologist and clinician both should not forget that the response therapy in vivo may not always reflects the result of testing the sensitivity of patient's pathogen in vitro. Rakesh Kumar exploited this method to study antimicrobial sensitivity pattern of *Escherichia coli* from urine samples of UTI patients [8].

Well diffusion method: In this agar well diffusion method, a suitable agar medium was prepared, once the agar is solidified the medium was inoculated and swabbed with bacterial suspension of approximately 1-2 X 10^8 CFU/mL using cotton swab. The wells were prepared by punching with a six millimeters diameter standard sterile cork borer made up of stainless still "Figure 2". These wells were filled up with 25 – 50 µL of the antimicrobial solution/s. to be tested. Well diffusion test has been used for susceptibility testing of antifungals like fluconazole, itraconazole [9, 10]. The plates were incubated at 35 ±

2°C for 18 – 24 h. The antimicrobial activity is calculated in millimeter by using the expression: ZOI = Total Diameter of growth inhibited zone minus diameter of the well, where, ZOI is Zone of inhibition.

The factors which may affect the result of AST included Density of an inoculum, Disk application time, Temperature of incubation, Potency of drug, inappropriate storage conditions, pH the agar medium, Moisture on the surface of the medium and effects of Thymidine or Thymine containing agar medium [1].

Stokes method: Stokes Disc Diffusion Technique varies from Kirby Bauer disc diffusion in the use of both control and test strain on a same plate. Stokes disc diffusion technique is not as highly standardized as the Kirby-Bauer technique and is used in laboratories particularly when the exact amount of antimicrobial in a disc cannot be guaranteed due to difficulties in obtaining discs and storing them correctly or when the other conditions required for the Kirby-Bauer technique cannot be met. Comparative disc diffusion techniques based on Stokes method is still in wide use in majority of laboratories in UK, to determine antibiotic susceptibility. The stokes' method allows each individual isolate to be compared with a sensitive control of the same or similar species which is subjected to the same technical conditions of medium, incubation time, atmosphere, temperature and disc content. As control and test organisms are adjacent on the same plate the difference between respective zone sizes can be measured directly.

In this method a control sensitive bacterial culture were inoculated partly on the surface of Mueller Hinton agar plate and a bacterial suspension to be tested was inoculated on the remainder part of the agar plate. The antibiotic disks were placed exactly at the interface "Figure 3". The plates were incubated at 35 -37 ^{0}C temperature for 18 – 24 h. The control and the test results for zone of inhibition were compared. The use of a sensitive control shows that the antibiotic was active and if the growth was observed on test area it may safely be assumed that the test organism was resistant to that drug. The bacterial culture was susceptible to drug "x" but resistant to drug "y". However, the disc containing drug "y" represent that an active antibiotic is present in the disk as shown by the zone of inhibition it causes in the control bacterium [11].

The Advantages of Stokes method includes: the control strain and test strain can be checked on the same plate. More reliable for the quality testing of discs. The effect of variation of environmental condition like temperature, time affect both simultaneously thus minimizing error. Errors due to using too heavy or light inoculums will be detected.

B. Quantitative method: This method is applied in the treatment of severe infections such as endocarditis or osteomyelitis. The principle of this method is based on the dilution and diffusion and dilution together. Dilution susceptibility testing methods are used to determine the minimal concentration of antimicrobial to inhibit or kill the microorganism. This can be achieved by dilution of antimicrobial agents in either agar or broth media. The aim of the broth and agar dilution methods is to determine the lowest concentration of the antimicrobial that inhibits the visible growth of the bacterium being tested (MIC, usually expressed in µg/ml or mg/liter). However, the MIC does not always represent an absolute value. The 'true' MIC is a point between the lowest test concentration that inhibits the growth of the bacterium and the next lower test concentration. Hence, MIC determinations performed using a dilution series may be considered to have an inherent variation of one dilution.

Tube or Macro broth dilution test: In the broth dilution method antibiotic solutions are prepared by two fold dilutions (1, 2, 4, 8, 16, 32 and 64 µg/mL) in the liquid growth medium dispensed in the test tubes. The standardised bacterial suspension of 1-5 X 10^5 CFU/mL was inoculated in the antibiotics containing tubes "Figure 4". These tubes were incubated for 16–20 h at 35–37 ^{o}C temperature and observed for visible bacterial growth as judged by turbidity. Minimum inhibitory concentration (MIC) is the lowest concentration of an antimicrobial that will inhibit the visible growth of microorganisms after overnight incubation [12, 13]. Standard strain of known MIC value run with the test is used as the control to check the reagents and conditions. The main advantage of this technique is the generation of quantitative result. The disadvantage includes the possible errors in preparation of antibiotics solutions [13].

Preparation of Stock solution: Stock solution can be prepared using the formula
1000/P x V x C= W where P=Potency given by the manufacturer in relation to the base, V= Volume in ml required, C=Final concentration of solution (multiples of 1000), W= Weight of the antimicrobial to be dissolved in the volume V. Example: For making 10 ml solution of the strength 10mg/ml from powder base whose potency is 500 mg/g, the

quantities of the antimicrobials required is W = 1000/500 x 10 x 10 = 200 mg.

Micro broth dilution test: This is a miniaturization and mechanization of the macro broth dilution test. In this test a small, disposable, polystyrene micro – dilution panel "Figure 5", is used. The standard panel contain 96 wells (8X12) having volume of 100 µL each. Approximately 12 antibiotics can be tested in a range of 8 two fold dilutions in a single panel. The procedure followed in this method is same as mentioned in macro broth dilution test [13 - 16]. The MIC is expressed as the highest dilution which inhibited growth judged by lack of turbidity in the well. Minimum inhibitory concentrations can be determined by reading manually or by using automated turbidity readers [13]. The reproducibility and availability of the preprepared panel are the advantages of this method.

Agar dilution method: Agar dilution involved an incorporation of different concentrations of the antimicrobial agent into a nutrient agar medium followed by swabbing of the standardized number of microbial cells with the sterile cotton swab on to the surface of the agar plate [13 - 16]. The plates were incubated for 18 – 24 h at 35 – 37 ^{0}C and examined for the growth inhibited zones. The MIC is expressed as the highest dilution which inhibited growth by measuring the zone of inhibition. Agar dilutions are most often prepared in petri dishes and have advantage that it is possible to test several organisms on each plate. The dilutions are made in a small volume of water and added to agar which has been melted and cooled to not more than 60^{o}C. Blood may be added and if 'chocolate agar' is required, the medium must be heated before the antibiotic is added. The pH of the agar must be between 7.2 and 7.4 at room temperature. Supplemental cations must not be added to the agar. It may be supplemented with 5% defibrinated sheep blood or lysed horse blood. The reproducibility of the results and satisfactory growth of most nonfastidious organisms can be expected advantages from agar dilution method. However, its disadvantages include the labor required to prepare the agar dilution plates and their relatively short shelf life.

E Test (Diffusion and dilution): The principle of E test (also known as Epsilmeter test) method is based on antimicrobial concentration gradient in an agar plate. An 'E' in E test refers to the Greek symbol epsilon (ε).The E test (bioMerieux AB Biodisk) is a quantitative method for antimicrobial susceptibility testing applies both the dilution and diffusion of antibiotic into the medium. A predefined stable antimicrobial gradient is present on a thin plastic inert carrier strip. These strips are impregnated on the underside with a dried antibiotic concentration gradient and are labeled on upper surface with a concentration scale "Figure 6". When this E test strip was placed onto an inoculated agar plate, there was an immediate release of the drug. Following overnight incubation, a symmetrical inhibition ellipse was produced. The MIC value over a wide concentration range (> 10 dilutions) is determined by intersection of the lower part of the ellipse shaped growth inhibition area with the test strip. Some investigators have reported an excellent correlation between E-test results and broth dilution or agar dilution methods [16].

E test have been used to determine MIC for fastidious organisms like *S. pneumoniae*, ß-hemolytic streptococci, *N.gonorrhoeae*, *Haemophilus* sp. and anaerobes. It can also be used for Nonfermenting Gram Negative bacilli (NFGNB) for eg-*Pseudomonas* sp. and *Burkholderia pseudomallei* [17 - 19]. The cost the E test is little more when compared to disk diffusion method. However the E test is simple, accurate, and reliable and is also used to determine the Minimum Inhibitory Concentration (MIC) of antifungal agents and antimycobacterial agents [20].

Current test methods and future perspective

The antibiotic susceptibility testing methods discusses here in this article provide reliable results when procedures are followed as defined by the CLSI or by the manufacturers of the commercial products. However, there is considerable opportunity for improvement in the area of rapid and accurate recognition of bacterial resistance to antibiotics. There is a need for development of new automated instruments that could provide faster results and also save money by virtue of lower reagent costs and reduced labor requirements [21].

Conflict of interests: The author declared no conflict of interest.

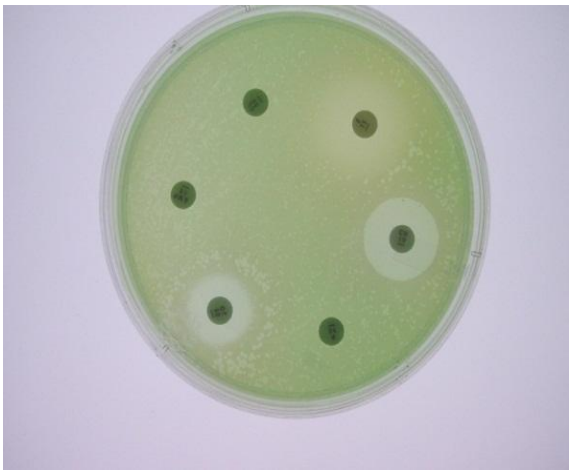

Figure 1: Kirby Bauer disk disc diffusion method

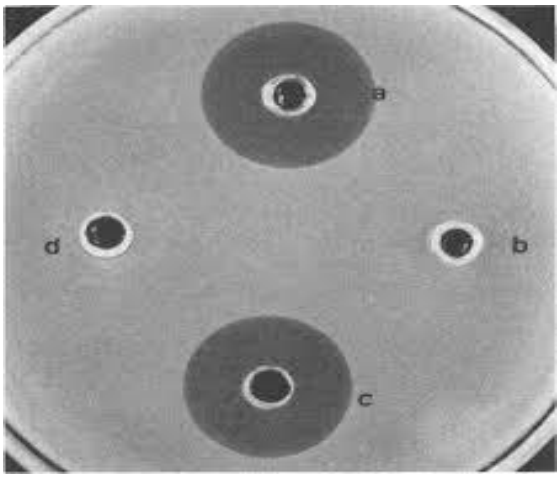

Figure 2: Agar well diffusion method

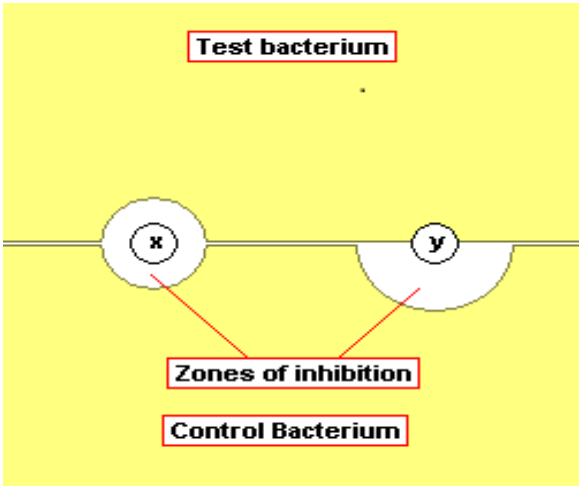

Figure 3: Stokes Disc Diffusion method

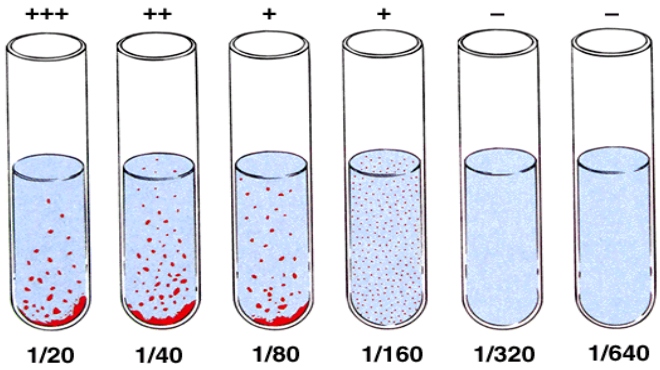

Figure 4: Tube or Macro broth dilution test

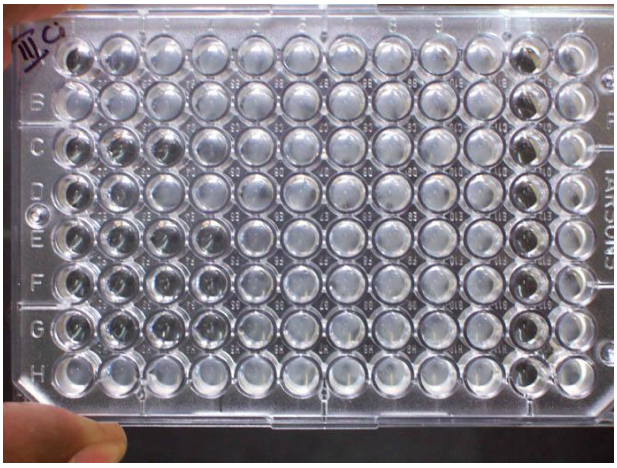

Figure 5: A broth microdilution panel containing 96 wells

Figure 6: E Test gradient diffusion method

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