ANTIMICROBIAL SUSCEPTIBILITY TESTING: REVIEW ON COMMONLY USED TECHNIQUES IN LABORATORIES

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Abstract- *Antimicrobial resistance has significant concern to public health. Controlling of antimicrobial resistance requires accurate and timely detection of drug resistance, followed by appropriate antimicrobial treatment and management. Microbial resistance is detected by antimicrobial susceptibility testing (AST) of various techniques such as disk diffusion, agar dilution broth macro dilution, broth micro dilution and a concentration gradient method. This article reviews various AST techniques, their benefits and drawbacks for better utilization in microbiological laboratories.*

Keywords: Antimicrobial Resistance, Susceptibility, Drug, Dilution method, Diffusion, Broth.

I. INTRODUCTION

Antimicrobial agents are employed against disease-causing micro-organism such as bacteria, fungi and viruses due to their antimicrobial activity to eradicate or restrain from infections. In recent era, various antimicrobial agents have been developed and studied for their sensitivity and resistance against microorganisms in a suitable culture medium. [1, 2]

To ascertain susceptibility of antimicrobial agents, its resistance, antimicrobial susceptibility testing (AST) is used. The assessment of interaction between antimicrobial agent and microbes is determined by different kinds of in-vitro methods are disk-diffusion, agar gradient method, well diffusion, and agar or broth dilution methods. Further, Bioluminescent and Cytofluorometric method, Time kill test, poison food methods are also used. Typically, most of the AST procedures produce qualitative (susceptible, intermediate or resistance) ae well as quantitative outcomes of Minimum Inhibitory Concentration (MIC). [2]

In AST at least 24 hours for producing bacterial colonies, and another 24 hours to characterize the acquired isolate need, while for some slow fastidious bacteria and anaerobes takes prolonged time. Guidelines and standards of AST techniques are routinely reviewed and published by European Committee on Antimicrobial Susceptibility Testing EUCAST, Clinical and Laboratory Standards Institute (CLSI) in USA. [3]

This review provides an overview of a variety of AST techniques which are commonly used in laboratories, its ability to determine the resistance and susceptibility level, to compute susceptibility level of microbes to agent, merits and demerits.

II. DILUTION METHODS

Broth dilution and agar dilution tests of both are dilution methods of AST used to study Minimum Inhibitory Concentrations (MICs) of antimicrobial agents can against filamentous fungus, fastidious or nonfastidious bacteria, and yeasts. [2, 4]

2.1. Broth dilution

The process of "broth dilution" involves inoculating containers with a known quantity of bacteria while using equal quantities of broth with antimicrobial solution in gradually increasing concentrations [5]. There are two ways to carry out broth dilution.

1. Micro dilution: performed in a micro titre tray and uses broth volume of about 0.1ml
2. Macro dilution: performed in a well standardized tubes containing broth volume of 1ml in each tube [6].

2.1.1. Broth micro dilution

The term "broth microdilution" describes how the broth dilution test is conducted in microscopic plates with a capacity of ≤500 µl per well [5].

The broth micro dilution (BMD) method is really now regarded as the standard international susceptibility testing method [7]. This approach is accomplished through the usage of small sterile disposable polystyrene micro titration plates,

generally containing 96 wells each properly contains an extent among 0.1and 0.2 ml and for this reason it permits checking out of approximately 12 antibiotics over a number eight twofold dilutions on one plate. The antimicrobial agents are loaded in the trays and then inoculated the plate with microbial suspension diluted. After well mixed the panels are incubated under suitable conditions depending on the test organisms following it measurement of MICs are done to determine the susceptibility [8].

Micro dilution of aerobic and anaerobic bacteria is standardized by CLSI [9].

The main benefits of the method is reduced time, reduced reagents [10], cost of the method is low and easier and efficient. The main drawback is limited selection of drug/agent, contaminants detection is difficult.

ComASP Colistin (Liofilhem, Roseto degli Abruzzi, Italy), MBD Sensititre System (Thermo Fisher Scientific, Waltham, MA, USA), formerly SensiTest Colistin are now commercially available BMD systems.

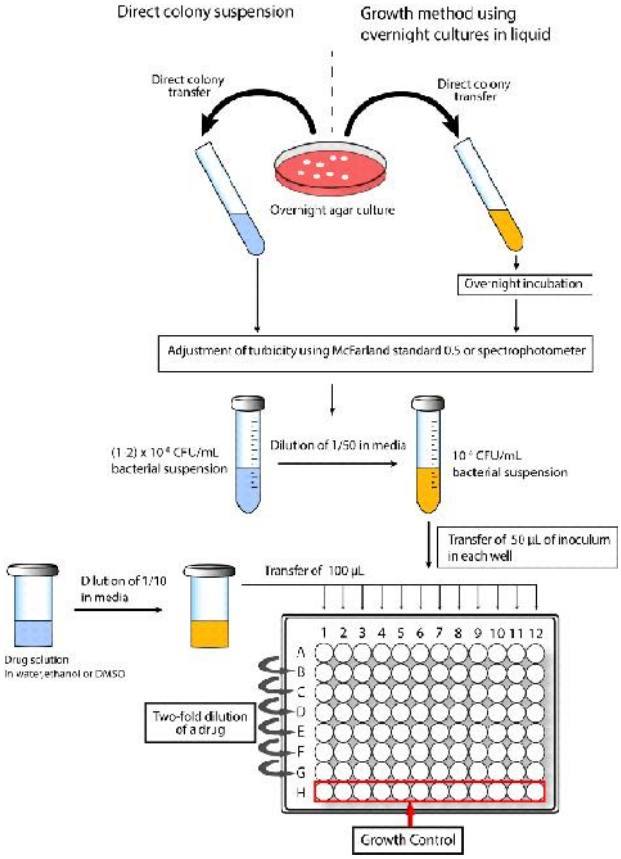

Fig.1. Determination of minimum inhibitory concentration by broth microdilution method.

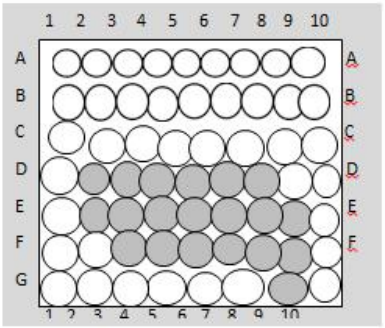

Fig .2. Broth dilution panel

2.1.2. Broth macro dilution

Broth macro dilution, also called in-tube test, in which the broth volume for each antimicrobial concentration is ≥1.0 ml (commonly 2ml) contained in test tubes. Cation adjusted Mueller-Hinton broth (CAMHB) is usually used for the procedure [11].

The process is completed by preparing twofold dilutions of the antimicrobial agent in a liquid growth medium distributed in test tubes containing a minimal volume (2 ml) of the standardized microbial suspensions. Following incubation at 37°C for 24 h (bacterial strains) or at 25°C for four–10 days (fungal strains), the tube is examined for the presence of microbial growth through turbidity. The lowest concentration of the antimicrobial agent in which the growth become completely inhibited (no turbidity) represents the MIC. The precision of broth macro dilution was taken into consideration to be plus/minus one twofold dilution concentration, because of the manual preparation of the dilutions. MICs measured are interpreted based on the CLSI guidelines.

The quantitative results of MICs value and MBCs (minimum bactericidal concentration) were obtained is the main advantage of this method and the disadvantage is the time-consuming and a substantial number of reagents are required for every dilution [10].

2.2. Agar dilution method

Agar dilution (AD) method was manual approach standardized by Clinical and Laboratory Standard Institute (CLSI). The AD method uses serial two-fold dilution of antimicrobial agent of different concentration integrated into molten agar medium. After then, the standardized inoculum was inoculated on the agar plate surface and incubated overnight to measure the MICs obtained [12]. It uses Mueller - Hinton broth as growth medium for testing.

Fosfomycin 0.25-256 (Liofilchem, Inc.) is now commercially available ready-to-use AD kit for performing AST. As a standardized procedure for fastidious organisms, agar dilution is frequently suggested technique [13]. Both testing for antibacterial and antifungal sensitivity can be done using this method. Additionally, it has been employed in antifungal agent-drug combinations against dermatophytes, Aspergillus, and Candida species.

III. DIFFUSION METHODS

3.1. Agar Disk diffusion method

In 1940 disk diffusion method (DDM) was developed [4]. It is a simple and most routinely practiced AST method. DDM is used for certain fastidious bacteria such as Neisseria gonorrhoea, Streptococci, Lactobacilli etc., and also for fast growing bacteria. This method was performed as per the standard guidelines of clinical and laboratory standards institute (CLSI) [14]. This could be achieved by Kirby Bauer technique or stokes method [15]. In routine susceptibility test Mueller Hinton agar was used to perform the test because which has good reproducibility, low sulphonamides and trimethoprim [16].

In Kirby Bauer method Mueller Hinton agar plate is inoculated with standardized inoculums of test microorganisms. Filter paper discs of 6mm diameter containing required concentration of agents on the surface of agar plate. The plates are then incubated at 37ºc for 24 hours. Then zone of inhibition is formed around the disc after incubation. Thereby, measuring the zone of inhibition diameter determines the susceptibility of the agent.

In stokes method control strain is inoculated on the agar plate adjacent to the test microorganisms in which the agar plate is splitted into three parts horizontally. In these divided parts, the upper and lower is inoculated with control strain and test strain inoculated in the middle part. In modified stokes method the steps are vice versa to stokes method [15].

The results are interpreted by comparing the obtained zone diameters. The diameter of growth inhibition zone is measured using Vernier caliper in millimeters [17]. The results obtained are categorized in accordance with the suggested clinical breakpoints as qualitative results of susceptibility i.e., susceptible(S), intermediate (I) or resistance(R) and not suitable for determination of MIC value [18].

The main advantage of this technique is simplicity, interprets results easily, low cost, specialized equipment is not required. The disadvantages are lack of automation of the test, lack of ability to determine MIC [19].

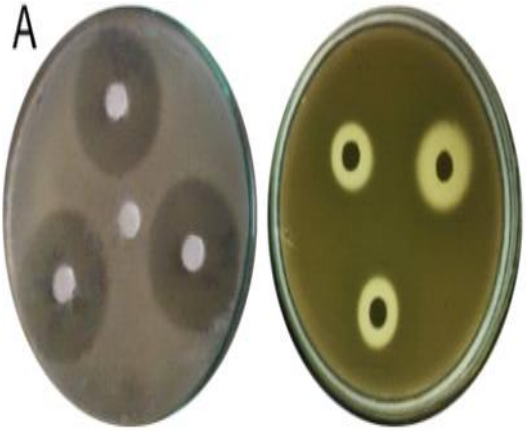

Fig.3. disk-diffusion method of microbial extract using C. albicans as test microorganism

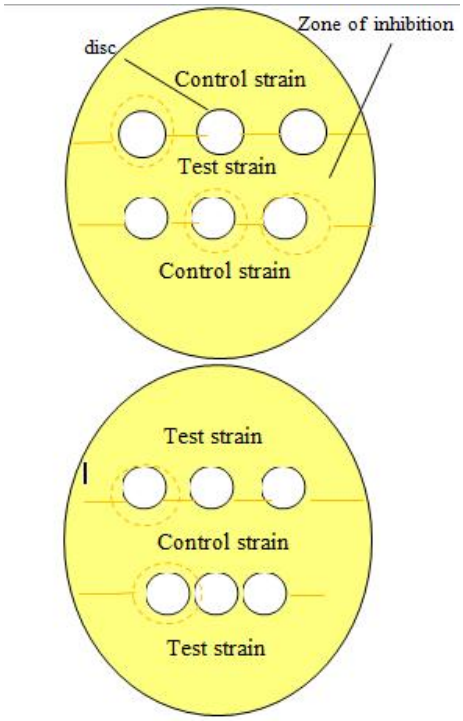

Fig.4. stokes disc diffusion and modified stokes disc diffusion method.

3.2. Antimicrobial gradient method

The dilution and diffusion concepts are combined in the antimicrobial gradient strip approach. Commercially procurable versions of this approach include the Etest (bioMerieux, Marcy-L'Etoile), MIC Test Strip (Lio-filchem Inc., Waltham, MA), M.I.C. Evaluator (Oxoid, Basingstoke, UK), and Ezy MIC Strip (Hi Media Laboratories Pvt. Ltd, India) in which etest bioMerieux commonly used one [17].

The method was mainly used to determine the MIC of the antimicrobial agent [20].

In accordance with the instructions provided by the manufacturer, a gradient strip test is conducted [21]. Tests are set up similarly to disc diffusion tests, with the exception of the disc being swapped out for the strip [15]. The procedure involves a plastic or paper strips were used. In this the agar is previously inoculated with the tested microorganisms. Then the strips are saturated with the dried antimicrobial agent from one end to the other and deposited on the surface of agar medium which is placed in a Petri dish. After incubation period, by measuring the intersection of the strip with an ellipzed zone of inhibitions [17]. Two strips may be placed on a standard sized 100mm Petri plate while up to six strips drenched with antibiotics can be tested concurrently on the larger Petri plate of 150 mm [4].

In earlier investigations e-test MIC values have shown good correlation with broth or agar dilution and DD methods [20, 22]. Carolyn N Baker et al. 1991 concluded that e-test showed high agreement results when compared to the disc diffusion (95.1%), broth micro dilution (95.1%), and agar dilution (95.2%) tests with a chosen group of challenge strains [23]. A. C. Nicodemo et al 2004 stated that e-test is suitable.

The method also can be used to study the combined effect and interaction of two antimicrobial agents [2]. For up to 20 hours the gradient stays stable, it is appropriate for a range of pathogens including fast- growing aerobic and anaerobic bacteria and slow growing fastidious bacteria [10]. However, it has a disadvantage that a greater number of drugs cannot be tested because the strip cost about 2-3 dollars each which is expensive one [2].

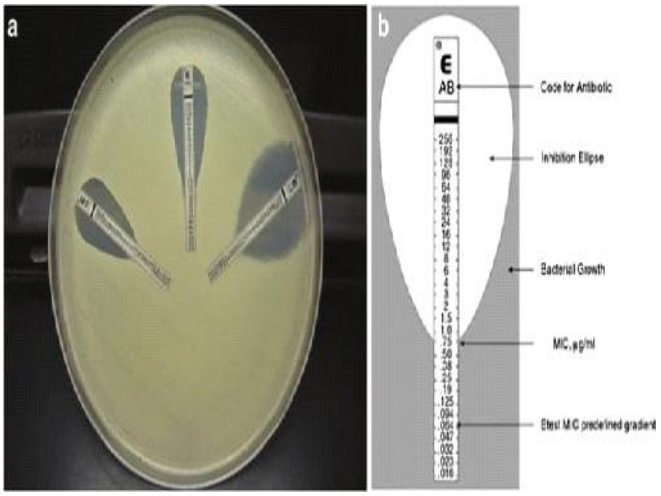

Fig.5. Etest Strip

3.3. Agar well diffusion method

The antibacterial and antifungal properties of various solvent extracts were screened using the agar well diffusion method [24]. Magaldi developed the well diffusion approach in 1997 as a refinement to the disc diffusion technique [25].

The procedure is similar to the discs; an appropriate agar medium was prepared. Once the agar had solidified, it was inoculated and swabbed with a bacterial suspension. A standard sterile, stainless steel corn borer with a 6mm diameter was used to punch the wells. 25 to 50 µl of the antimicrobial solution or solution to be evaluated were placed in these wells. After the plates were incubated at 35± 2ºc for 18-24hr. The antimicrobial agent diffuses in the agar medium and inhibits the growth of the microbial strain tested and the zone of inhibition is measured in millimeters [26]. Antifungal agents like fluconazole, posaconazole, itraconazole have been tested for susceptibility using the well diffusion technique [25].

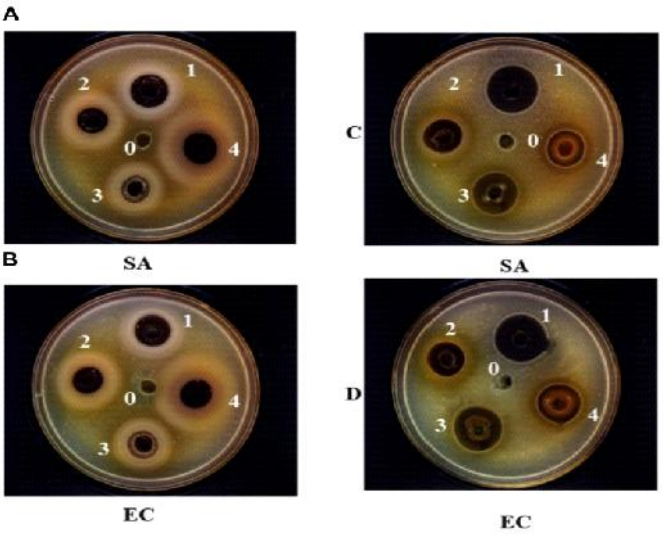

Fig .6. Agar well diffusion method.

IV. TIME KILL TEST

The bactericidal efficacy of one or more antimicrobial agents against a specific bacterial strain is evaluated using the time-kill method. This is accomplished by assessing the viability of several bacterial strains throughout time. The 96-well microtiter plate would be used to conduct the experiment [27]. More information is given by the in vitro time-kill assay, which shows the relation between antimicrobial concentration and the stage of microbial development. Time-kill analysis can also assess the effectiveness of antifungal agents and predict the dose of these drugs [28].

Beatriz E et al reported that time kill assay showed that when compared to cefoxitin and clarithromycin, amikacin was more active against M.abscessus [29].

V. CONCLUSION

In this review, we discussed about the commonly used AST techniques in laboratories. Although there are some demerits for traditional AST techniques (DDM, BDM, AD, etc.) but they are required to acquire a correct result and to compare with results of new techniques. These methods are old and time consuming, however these are still now used in the laboratories. But furthermore, improvement in the methods used for the detection of the antimicrobial agent's potency.

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