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RECENT ADVANCEMENTS IN THE DETERMINATION OF MINIMUM INHIBITORY CONCENTRATION

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Abstract

Minimum inhibitory concentrations (MICs) are defined as the lowest concentration of an anti-microbial that will inhibit the visible growth of a microorganism after overnight incubation, and minimum bactericidal concentrations (MBCs) as the lowest concentration of antimicrobial that will prevent the growth of an organism after subculture on to antibiotic-free media. MICs are used by diagnostic laboratories mainly to confirm resistance, but most often as a research tool to determine the *in vitro* activity of new antimicrobials, and data from such studies have been used to determine MIC breakpoints. MBC determinations are undertaken less frequently and their major use has been reserved for isolates from the blood of patients with endocarditis. Standardized methods for determining MICs and MBCs are described in this paper. The method gives information on the storage of standard antibiotic powder, preparation of stock antibiotic solutions, media, and preparation of inocula, incubation conditions, reading and interpretation of results.

Keywords: Minimum inhibitory concentration, Minimum Bactericidal concentration, Broth dilution method, Agar dilution method, E test.

1. Introduction

The minimum inhibitory concentration (MIC) represents the concentration of antimicrobial at which there is complete inhibition of growth of organism. Dilution methods are used to determine the minimum inhibitory concentrations (MICs) of antimicrobial agents and are the reference methods for antimicrobial susceptibility testing against which other methods, such as disk diffusion, are calibrated. MIC methods are widely used in the

comparative testing of new agents. In clinical laboratories they are used to establish the susceptibility of organisms that give equivocal results in disk tests, for tests on organisms where disk tests may be unreliable, and when a more accurate result is required for clinical management.[1]

In dilution tests, microorganisms are tested for their ability to produce visible growth on a series of agar plates (agar dilution) or in micro plate wells of broth (broth micro dilution) containing dilutions of the antimicrobial agent. The lowest concentration of an antimicrobial agent that will inhibit the visible growth of a microorganism is known as the MIC.[2]

One of the popular and rapid methods for MIC evaluation, commonly used in the clinical microbiology laboratory is the antibiotic gradient diffusion (E-strip). According to the Center for Disease Control, USA, antibiotic resistance monitoring should be performed using MIC methods to determine emergence and evaluation of resistance in certain "key" pathogens.

They emphasize the role of accuracy and timeliness in detection of resistance by the laboratory. The MIC methods are advantageous for this as they give a quantitative result which can be performed for fastidious, difficult to isolate and slow-growing pathogens that cannot be tested by disc diffusion. The MIC testing by micro broth, macro broth and agar dilution are time consuming, labor intensive, require technical expertise and the pure powders of the anti-microbials from reputed firms, which tend to be expensive. The macro broth dilution method is now advocated only for research facilities.[3]

2. Methods for MIC Determination

2.1 Agar dilution method [2]

Dilution methods are used to determine the minimum inhibitory concentrations (MICs) of antimicrobial agents and are the reference methods for antimicrobial susceptibility testing against which other methods, such as disk diffusion, are calibrated. In dilution tests, microorganisms are tested for their ability to produce visible growth on a series of agar plates (agar dilution) or in micro plate wells of broth (broth micro dilution) containing dilutions of the antimicrobial agent. The lowest concentration of an antimicrobial agent that will inhibit the visible growth of a microorganism is known as the MIC.

2.1.1 Medium

Although several susceptibility testing media are available. Mueller Hinton (MH) agar shows no performance advantages over some other media but is probably the most widely used medium internationally. Supplements should not be used unless necessary for growth of the organisms. Five per cent defibrinated blood is added for fastidious organisms such as streptococci and *Moraxella catarrhalis.*

2.1.2 Antimicrobial Agents

Obtain antimicrobial powders directly from the manufacturer or from commercial sources. The agent must be supplied with a stated potency (mg or International Units per g powder, or as percentage potency), an expiry date and details of recommended storage conditions. Store powders in sealed containers in the dark at 4°°C with a desiccant unless otherwise recommended by the manufacturer.

2.1.3 Preparation of Stock Solutions

Use an analytical balance when weighing agents. Allowance for the potency of the powder can be made by use of the following formula:

Weight of powder (mg) =(Volume of solvent (mL) x Concentration (mg/L)) / Potency of powder (mg/g)

Alternatively, given a weighed amount of antimicrobial powder, the volume of diluent needed may be calculated from the formula:

Weight of solvent (mL) =(Weight of powder (mg) x Potency of powder (mg/g)) / Concentration (mg/L)

Concentrations of stock solutions should be 1000 mg/L or greater, although the solubility of some agents will be limiting. The actual concentrations of stock solutions will depend on the method of preparing working solutions. Sterilization of solutions is not usually necessary. If required, sterilization should be by membrane filtration, and samples before and after sterilization must be compared by assay to ensure that adsorption to the membrane has not occurred.

2.1.4 Preparation of Working Solutions

The range of concentrations tested will depend on the organisms and antimicrobial agent being tested, but a two-fold dilution series based on 1mg/L is conventionally used.

Twenty-milliliter volumes of agar are commonly used in 9cm Petri dishes for agar dilution MICs.

2.1.5 Preparation of Plates

Prepare agar as recommended by the manufacturer. Allow the sterilized agar to cool to 50°C in a water-bath. Prepare a dilution series of antimicrobial agents, as above, in 25-30mL containers. Include a drug-free control. Add 19mL of molten agar to each container, mix thoroughly, and pour the agar into prelabeled sterile Petri dishes on a level surface. Allow the plates to set at room temperature and dry the plates so that no drops of moisture remain on the surface of the agar. Do not over dry plates.

2.1.6 Preparation of Inoculum

Standardize the density of inoculums to give 104 colony-forming units (CFU) per spot on the agar. Use four or five colonies of a pure culture to avoid selecting an atypical variant. The inoculum may be prepared by emulsifying overnight colonies from an agar medium or by diluting a broth culture. The broth used must not be antagonistic to the agent tested. A 0.5 McFarland standard may be used for visual comparison to adjust the suspension to a density equivalent to approximately 108 CFU/mL.

2.1.7 Inoculation of Plates

Mark the plates so that the orientation is obvious. Transfer diluted bacterial suspensions to the wells of inoculums replicating apparatus. Use the apparatus to transfer the inocula to the series of agar plates, including a control plate without antimicrobial agent. Replicator pins 2.5mm in diameter will transfer about 1 mL, i.e. an inoculums of 104 CFU/spot. Alternatively, a micropipette or standard loop may be used to inoculate plates. Allow the inoculum spots to dry at room temperature before inverting the plates for incubation.

2.1.8 Incubation of Plates

Incubate plates at 35-37 ^{0}C in air for 18 h. In order to avoid uneven heating, do not stack plates more than five high. If the incubation period is extended for slow-growing organisms, the stability of the agent over the incubation period must be assessed by the inclusion of control strains with known MICs.

2.1.9 Reading Results

The MIC is the lowest concentration of the agent that completely inhibits visible growth as judged by the naked eye, disregarding a single colony or a thin haze within the area of the inoculated spot. A trailing endpoint with a

small number of colonies growing on concentrations several dilutions above that which inhibits most organisms should be investigated by subculture and retesting.

2.2 Broth Dilution Method [2]

The broth dilution method depends upon inoculation at a specific inoculum density of broth media (in tubes or microtitre plates) containing antibiotics at varying levels - usually doubling dilutions are used and after incubation, turbidity is recorded either visually or with an automated reader, and the breakpoint concentration established. Microtitre plates or ready-to-use strips are commercially available with antibiotics ready prepared in the wells. A variation on this approach is the agar dilution method where a small volume of suspension is inoculated onto agar containing a particular concentration of antibiotic, when the inoculum has dried the plate is incubated and again examined for zones of growth.

2.3 Breakpoint Testing [4]

The breakpoint testing method is a popular technique in larger laboratories because of its suitability for multiple sample testing. It is performed by adding a defined concentration of antibiotic to an agar medium. The test organism is inoculated and, following incubation is determined to be sensitive or resistant to that level of compound according to the growth (or lack of growth) observed. The ease of standardization and automation of this method makes it ideal for multiple samples testing if multipoint inoculator is used. As the antibiotic is evenly distributed throughout the agar, this method does not present a problem for poorly diffusing compounds. However, it is difficult to detect contamination or to assess inoculum density, due to the small amount of sample that is present. Problems may also be encountered with compounds that are unstable in solution and requiring small batches of plates to be prepared as required and the presence of a swarming Proteus sp. potentially can destroy multiple test results. The information provided by the breakpoint testing method is relatively limited and subtle changes in susceptibility may not be detected. Thus, other techniques are usually employed when more detailed information is required.

2.4 Disk Diffusion (The Kirby-Bauer Test) [4]

Disk diffusion or the Kirby-Bauer test is one of the classic microbiology techniques and it is still very commonly used. A suspension of the isolate is prepared to a particular McFarland standard, then spread evenly onto an

appropriate agar (such as Mueller-Hinton or for a more defined media Iso-Sensitest™ agar) in a petri dish, disks impregnated with various defined concentrations of different antibiotics are placed onto the surface of the agar. A multichannel disk dispenser can speed up placement of the disks.

After incubation, a clear circular zone of no growth in the immediate vicinity of a disk indicates susceptibility to that antimicrobial. Using reference tables the size of zone can be related to the MIC and results recorded as whether the organism is susceptible (S), intermediately susceptible (I), or resistant (R) to that antibiotic.

There are a number of critical steps in this approach, such as which medium is used; depth and moisture content of the agar in the plate; incubation conditions; accurate inoculum density; disks must be firmly placed in contact with the agar surface otherwise the diffusion rate will not be correct.

2.5 MIC Evaluators or E-Test [4]

A variation approach, of the above broth dilution and disc diffusion method, is to use a strip impregnated along its length, with a gradient of different concentrations of antimicrobial, after incubation this creates an ellipse shaped zone of no growth, where the ellipse meets the strip, the MIC can be read from the concentration markings on the strip.

These are easy to read, no tables need to be referenced to get an MIC value and the test requires less manipulations, as one strip will cover the whole concentration range. These again can be manually or instrument read. [5]

Figure-1: MIC Evaluator.

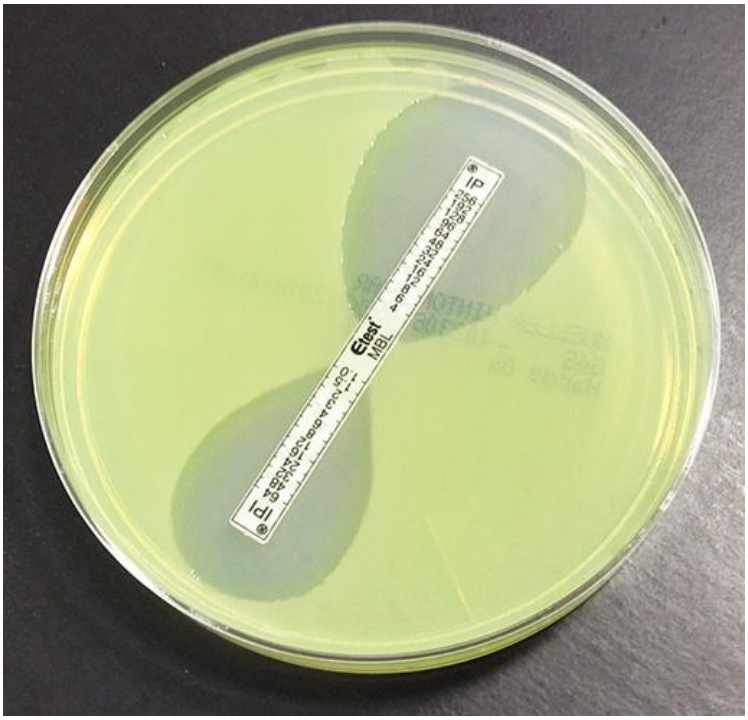

Figure-2: E Strips.

The gradient diffusion method, the E test (AB Biodisk, Solna, Sweden) is a method for quantitative anti-microbial susceptibility testing wherein the antibiotic is applied in a preformed gradient across a plastic coated strip. This diffuses into the agar medium inoculated with a lawn culture of the test organism and incubated for 24 hours. The MIC is read as the point where the ellipse touches the E-strip. This test has been validated for several pathogenic bacteria including gram positive, gram negative and mycobacteria. It combines the ease of application of disk diffusion testing, rapidity and an MIC reading. However they are much more expensive and this is a huge hurdle in a resource poor setting.

To overcome this difficulty, a modification to this test is devised in the laboratory - wherein a single E-strip was utilized to test, at one time, two different strains of the same genus, on the same Mueller Hinton agar plate. Lawn cultures of the two strains were separated by a space of approximately 3 mm and the E-strip was laid in the center of this gap. This is very similar to the Stokes' method of disk diffusion testing where the test and the control strains are separated by a 3 mm gap while applying their lawn culture. The plate was incubated overnight and the reading taken for the two strains from their respective regions where the half ellipse cuts the E-strip edge. This economizes on E-strip, culture media, labor and time.

Figure-3: Zone of inhibition observed by MICs evaluator.

This modification could not be utilized for *Mycobacterium tuberculosis* as these strains grew very slowly (three to seven days) and had varying growth phases. However, we have performed this test for a variety of pyogenic bacteria- *Staphylococcus aureus*, Group A Streptococci, Meningococci, Enterococci and *Salmonella* spp. with conclusive results. Hence, this simple modification of the E-strip method can halve the cost and give a rapid and accurate MIC results.[3]

Figure-4: Plate showing two strains of Salmonella typhi, one resistant to ciprofloxacin (>32 ìg/mL) and the other sensitive strain (0.5 ìg/mL).

4 Conclusions

In conclusion, it has been seen during recent years that there has been in the advancements in the technique of determination of minimum inhibitory concentration. As it has been seen that very labour intensive procedures (e.g. disc diffusion technique, agar dilution technique etc.) have been transformed in to very convenient and user friendly

procedures. As the advancements will comes in the techniques of MICs determination, the procedure will gradually became user friendly.

5. References

1. J.M. Andrews, Determination of minimum inhibitory concentration, J. Antimicrobial Chemotherapy 2001, 48 suppl. S1: 5-16.
2. Eucast Definitive Document E.Def 3.1. European Committee for Antimicrobial Susceptibility Testing (EUCAST) of the European Society of Clinical Microbiology and Infectious Diseases (ESCMID). Determination of minimum inhibitory concentration (MICs) of antibacterial agents by agar dilution. June 2010
3. M.R. Capoor, D. Nair, M. Deb, A. Hasan, P. Aggarwal, A simple modification of minimum inhibitory concentration determination by E-test in the clinical laboratory, Indian Journal of Medical Microbiology, 2006, 24 (4): 301.
4. http://www.pathologyinpractice.com/Story.aspx?Story=332
5. http://www.rapidmicrobiology.com/news/603h277.php

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