Antimicrobial Susceptibility Testing: A Comprehensive Review

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ABSTRACT

Antimicrobial susceptibility testing (AST) is a widely used method to assess the antimicrobial action of an sample or a pure molecule, in order to be used in applications for public health, the economy, and the environment. Currently, there are multiple AST methods available to determine an antibiotic-resistant bacterium's susceptibility. Several methods also are employed to assess how various antibacterial drugs affect bacteria. The CLSI publications provide guidance for the most important drugs to analyze and report on in relation to such organisms, as well as value ranges to ensure precise & reproducible findings. The responsibilities are to ensure breakpoints to evaluate MICs or disc diffusion zone measurements. The disc diffusion technique is easy, useful, and works very well. The agar well diffusion methodology of Aspergillum essential oil is applied to examine the antibacterial activity of plants, bacterial extracts, or pure substances, or to examine the competition between microorganisms

Keywords: Antimicrobial Activity, Broth Dilution Method, Agar Well Diffusion Method, Agar Disk Diffusion Method, Antimicrobial Susceptibility Testing.

INTRODUCTION

To ascertain an antibiotic-resistant bacterium's susceptibility, various antimicrobial susceptibility testing methods are available. The CLSI has served as a trustworthy source of comprehensive, current guidelines and specifications for antimicrobial susceptibility evaluations used by academic laboratories for a number of years. This review is focused on novel approaches for antimicrobial activity of microorganism. An antimicrobial refers to any substance that is capable of either eliminating or inhibiting the growth of microorganisms. There are multiple Antimicrobial Susceptibility Testing (AST) techniques available to determine an antibiotic-resistant bacterium's susceptibility. While selecting a method, many factors are taken into consideration, including feasibility, flexibility, mechanization, cost, reproducibility, but also personal preferences. If data from different national or international surveillance or monitoring programs of OIE Members are to be compared, standardization and harmonization of AST procedures, which are utilized in epidemiology for microbial resistance, are essential.[1]

Clinical and laboratories institute (CLSI) antibiotic susceptibility testing guidelines

By the middle of the 20th century, medical technologists and clinical pathologists were required to maintain proficiency in all features of the commercial research lab, despite the fact that the majority lacked adequate training in diagnostic microbiology. The idea of an independent organization that could create standards that were acceptable to everyone who used them emerged from conversations among a number of interested parties in the middle of the 1960s. The National Committee for Clinical Laboratory Standards (NCCLS), currently known as the Clinical and Laboratory Standards Institute (CLSI), was created as a result. It was believed that the new name (which became official in January 2005) better accurately reflected the organization.[2] The Clinical and Laboratory Standards Institute
has lately issued a laboratory experimental guideline for antibiotic susceptibility testing of seldom seen or fussy bacteria that had not been included in previous CLSI publications. For several years, the CLSI has been a reliable source of complete, relevant guidelines and specifications for antimicrobial susceptibility testing in research laboratories. The CLSI publications provide guidance on the most important drugs to analyse and report on in relation to such species, as well as value ranges to ensure precise and reproducible findings, and responsibilities are to ensure breakpoints to evaluate MICs and disc diffusion zone measurements.\[3\]

**Antimicrobial Susceptibility Testing Methodologies:**\[4\]

The following conditions must be adhered to:

The given sample may contain pure cultures of the microorganisms that will be exposed to AST.

1. In order to consistently and accurately identify the subject bacteria to the genus and/or species level, standard reference procedures should be employed for identification.

2. Lyophilisation and freezing storage at \(-70\degree\) to \(-80\degree\)C, together with a sample of additional isolates, should also be utilized to keep microorganisms for subsequent examination.

In such a rigorous SOP, it is essential to determine, optimize, and record all essential aspects that impact AST methods:

I. Now that the bacterium was once separated in liquid culture, the necessary inoculum concentration should be calculated for precise sensitivity data. Bacteria and other such organisms must always be separated from a liquid culture before performing an AST test.

II. The formulation and processing of culture plate to stock medium (for example, pH, potassium ions, deoxythymidine and 5-methyluracil, along with the utilization of additional medium). In addition to identifying and documenting the suitable methodologies, the media lots should be assessed for performance and sterility. Antibiotics used in microtitre plates, disks, strips, and tablets have the ability to fight germs.


IV. Parameters for growth and development (time, temperature, atmosphere, e.g., Carbon-di-oxide),

V. Depth of agar,

VI. For each broth and agar dilution, the number of concentrations was assessed.

VII. The reference species to be employed as reference controls are

Due to such factors, there needs to be a specific emphasis placed on using proven, well documented methodologies and detailed procedures, as this is the only way to achieve sufficient reproducibility.

The following variables may be taken into consideration while choosing an AST methodology:

- Ease of performance,
- Adaptability,
- The capacity to adjust to automated or partially automated systems;
- Price,
- Reproducibility,
- Trustworthy,
- Perfection,
- The relevant microorganisms and antibiotics for that specific OIE member,
- Access to appropriate validation data for the numerous organisms undergoing susceptibility testing.

In this research, they emphasized how microbial test techniques are employed to analyse samples and purified drugs as possible antimicrobial agents in vitro.\[4\]
Today, numerous techniques are used to assess how different antimicrobial drugs affect microorganisms.\[^5\]

- **Diffusion method**
  - Disk diffusion method
  - Other diffusion method
  - Agar well diffusion method
  - Agar plug diffusion method

- **Dilution method**
  - Broth dilution method
  - Agar diffusion method

- **Antimicrobial gradient method**

- **Thin layer chromatography (TLC) Bio-autograph**
  - Agar diffusion
  - Direct bio-autography

- **Time kill test (time kill curve)**

- **Flow Cytofluorometric method**

**Diffusion Method:**

**Disk- Diffusion Method**

The disc diffusion susceptibility technique is easy, useful, and works very well.\[^6\] 1 mL of each bacterium sample was evenly distributed on a solid growth medium in a Petri dish for the disc diffusion experiment.\[^7\] The desired amount of the test material is again put into 6 mm-diameter filter discs, which are subsequently put mostly on nutrient agar plates.\[^8\] To culture the Petri plates, the right parameters are employed. Generally, an antibacterial drug added to the nutritional solution prevents both reproduction and expansion of the test microorganism, and the widths of inhibitory growth regions are measured (Fig.1). Table the culture medium, temperature incubation duration, and inocula size required to meet CLSI requirements are displayed.\[^9, 10\]

### Table 1: Culture media, microbial inoculum size and incubation condition for antimicrobial testing method.\[^4\]

<table>
<thead>
<tr>
<th>Methods</th>
<th>Organism</th>
<th>Culture Medium</th>
<th>Growth Medium</th>
<th>Incubation Temperature (°C)</th>
<th>Incubation Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Disc Diffusion Method</td>
<td>Bacteria</td>
<td>MHA</td>
<td>(0.5 McFarland) (1-2) x 10^8CFU/ml</td>
<td>35±2</td>
<td>16-18</td>
</tr>
<tr>
<td>Disc Diffusion Method</td>
<td>Yeast</td>
<td>MHA+GMa</td>
<td>(0.5McFarland) (1-5) x 10^6 CFU/ml</td>
<td>35±2</td>
<td>20-24</td>
</tr>
</tbody>
</table>

MHA: Muller Hinton Agar, MHB: Muller Hinton Broth

GMB: 0.5 mg/mL of methylene blue and 2% glucose are added to the solution

The methods mentioned are disc diffusion methods used for testing the susceptibility of organisms to antimicrobial agents. Bacteria are cultured on Mueller-Hinton agar (MHA) at a density of (1-2) x 10^8 CFU/ml, while yeast is cultured on MHA supplemented with glucose, maltose, and peptone (GMBa) at a density of (1-5) x 10^6 CFU/ml. The cultures are incubated at a temperature of 35±2°C for different durations before conducting the disc diffusion test.

**Advantages:**

1. The disc method has several advantages, including its straightforwardness, which does not require any functional apparatus, the ability to provide qualitative data that is easily understandable by physicians, and the flexibility in disc selection.

2. This technique for antimicrobial sensitivity testing is highly economical, with supplies needed for the test costing only between $2.50 and $5. This makes it the most cost-effective choice for doing such testing.\[^5\]
Fig. 1: Diffusion method: employing *C. albicans* a test organism in a microbiological extract utilizing the disk-diffusion technique

**Disadvantages:**

1. One drawback of this test is that it lacks computerization.

2. The disc test has undergone standardization to determine susceptibility of certain bacteria such as *streptococci, H. influenza, and N. meningitides*. This involves using nutrient media, specific growth conditions, and particular bacterial inhibition zone and diagnostic criteria. However, it's worth noting that some bacteria that grow slowly or require specific conditions may not be accurately assessed by this method.\[5\]

**Other Diffusion Method:**

**Agar Well Diffusion Method:**

The agar plate surface is infected in a similar way as the disk diffusion technique is, essentially distributing a quantity of such bacterial suspension across the whole plate\[11, 12\]. The process for inoculating the agar plate surface is comparable to the method used in the disk-diffusion technique. It entails distributing a specified amount of the bacterial cultures throughout the entire area of the culture medium. With a sterilized cork borer or pipette, an aseptic hole of 6–8 mm is formed, but also a quantity of the required amount (20–100 microliters), including the antimicrobial compound or isolate liquid, is put in the hole. The distribution of the antimicrobial component throughout the medium that inhibits the growth of useful microbiological organisms.\[13, 14\].

**Dilution Methods:**

The best techniques for determining MIC values involve dilution because they allow you to estimate the antimicrobial agent concentration in the broth (macrodilution or microdilution) or agar (agar dilution) medium.\[4\]

**Broth Dilution Method:**

One of the first approaches used to examine the efficacy of antimicrobial medicines was one of the macrobroth or tube-dilution methods. This concern diluting the agent in a both in tubes or a microtitration plate, with decreasing concentrations (e.g. 4, 6, 8, 16, 32, and 64 mg/mL) and using a minimum of 2 mL for macrodilution or smaller amounts for microdilution. After preparing the dilutions of the antimicrobial agent, a bacterial inoculum is added to each tube or well. The inoculum is made from a standard bacterial culture that has been diluted using a 0.5 McFarland scale.\[15\]. The broth cultures are then diluted to a concentration of [(0.5- 1.0) x 106 CFU/ml]. The tubes or 96-well microtitration plate are then incubated avoiding vibration under appropriate conditions for the microorganism being tested.

The minimum inhibitory concentration is the minimum antimicrobial agent seen by the bare eye that completely stops organism development in tubes or microdilution wells. Observing devices can help with reading microdilution testing and documenting findings, with a high ability to identify development in the wells for determining the MIC endpoint. Moreover, numerous colorimetric techniques based on dye reagents have been established. Minimum inhibitory concentration (MIC) values are usually impacted by variables like inoculum size, surface area types, incubation period, and inoculum production technique.\[16\]

The MBC is characterized by the low antimicrobial agent concentrations necessary to eradicate 99.9% of the total inoculum after 24 hours of incubation, which is lower than the standard regimen specified in Article M26-A. Following broths macrodilution or microdilution, the MBC may be tested by
passaging a specimen from wells or tubes, establishing unfavorable growth of bacteria, and incubating it at 37°C on top of non-selective agar medium for 24 hours to find out the percentage of surviving cells (CFU/mL). The bactericidal endpoint (MBC) is referred to as the smallest amount of bacteria destroyed, during which 99.9% of the whole inoculum is destroyed.[17, 18]

Advantages:

- This approach has the advantage of delivering a quantifiable result (i.e., the MIC).

Fig. 2: A disposable tray inoculator and a broth micro dilution susceptibility panel with 98 reagent wells.

Disadvantage:

- This method is laborious, & preparing antibiotic solutions for each test is a laborious effort.
- The risk of failures in antibacterial solution production, as well as the relatively high volume of chemicals and space required for each test.[19]

E Test Method:

During the test, the manufacturer's instructions were observed. In addition to the disc diffusion method, direct colony inoculations were carried out on Mueller-Hinton media, but E-test strips were used in place of the antimicrobial disc. Later in incubation, a zone of inhibition was formed, and at the lower limits of the antimicrobial agent that inhibits *staphylococci* isolates from growing, the MIC was directly read from a graduated E test strip. Readings were taken according to the NCCLS guidelines (a microgram was regarded as susceptible).[20]

NOVEL METHODS:

Chemical Modification:

The resistance to antibiotics in organisms may develop through a number of mechanisms. The antibiotic is susceptible to deterioration as well as chemical modification (by acetylation, phosphorylation, ADP-ribosylation, mono-oxygenation, and glycosylation). Either the drug intake can be stopped or the efflux can be increased. A number of barriers work by changing the production of cell walls. The antibiotic may become ineffective if the target molecule undergoes additionally smaller alterations, such as an isolated mutation in the cellular protein. AST is complicated by the enormous range of antimicrobials and strategies for resistance. An isolate of bacteria is continuously exposed to a set of antimicrobials using traditional AST techniques like broth microdilution, disc diffusion, gradient tests, agar dilution, and breakpoint tests prior to growth being visually detected. The reliability and accuracy of visual systems can be further improved through the use of sophisticated optoelectronic systems, catoptrics, emulsion science, and gauges sensitive to oxidation/reduction states or pH.[21]

- The monitoring of AST cultures has been simplified as well as partially automated by a variety of commercial systems. The evaluation of turbidity for multi well liquid cultures is performed automatically by systems like Vitek and Microscan. A redox indicator is used by the BD Phoenix System TM to improve the detection of organism growth. Response times for these systems are as fast as 4 hours for identification and 6 to 8 hours for susceptibility assessment. The CE-approved Alfed 60 ASTM method (Alifax, Italy) utilizes sensitive beam-light
scattering technological advances to identify bacterial growth in a fluid culture broth and delivers resistance outcomes from the a positive sample bottles in four to six hours.\[22\]

**CONCLUSION:**

Bacterial infections are now a considerable clinical hazard, with significant morbidity and death, owing mostly to the emergence of bacteria resistant to conventional antimicrobial treatments. As a result, approaches for assessing antimicrobial susceptibility and finding new antibiotic drugs have been widely employed and are still being developed. Disc diffusion, agar dilution, broth macro- and microdilution, and concentration gradient analysis are the most often used procedures for in-vitro antimicrobial susceptibility testing. According to CLSI it’s determined to analyse, report & interpretive criteria or breakpoint of MIC & determining the zone of inhibition.

**Conflict of Interest:** The authors declare that there is no conflict of interest.

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NA

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