EDUCATIONAL COMMENTARY – CURRENT METHODS IN ANTIMICROBIAL SUSCEPTIBILITY TESTING

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Learning Objectives

On completion of this exercise, the participant should be able to

• explain the role of standardization in antimicrobial susceptibility testing;
• describe the selection of antibiotics for antimicrobial susceptibility testing;
• compare the different methods of antimicrobial susceptibility testing, including the advantages and disadvantages of each;
• explain the process for interpretation of antimicrobial susceptibility results; and
• discuss future techniques in development for detection of antimicrobial resistance.

Introduction

Antimicrobial susceptibility testing (AST) is performed on pathogenic bacteria isolated from clinical cultures to determine which antimicrobial agent might be effective in treating the infection caused by the bacteria. Antimicrobial susceptibility testing should only be performed on a bacterial isolate that is determined to be a probable cause of the patient’s infection, and when the isolate has an unpredictable pattern of susceptibility. Susceptibility testing is important with species that may possess acquired resistance mechanisms (e.g., \textit{Staphylococcus} species, \textit{Enterococcus} species, \textit{Streptococcus pneumoniae}, \textit{Pseudomonas} species, and \textit{Enterobacteriaceae}). Antimicrobial susceptibility testing is not performed on bacteria that are predictably susceptible to the antimicrobial agents commonly used to treat infections caused by those bacteria. For example, group A \textit{Streptococcus} is not routinely tested because it is universally susceptible to penicillin. Monitoring of aggregate susceptibility testing data can also provide information on decreases in the susceptibility of bacteria to antimicrobial agents. There are several methods available for AST, all of which require a pure culture of the probable pathogen: a process that may take several days from the time of specimen collection and primary culture setup.

This educational commentary will focus on phenotypic AST methods commonly performed in the clinical microbiology laboratory. In addition to the methods discussed in this article, there are other supplemental methods to detect specific resistance mechanisms in antibiotic-resistant bacteria, including methicillin-resistant \textit{Staphylococcus aureus}, vancomycin-resistant \textit{Enterococcus} species, carbapenem-resistant
Enterobacteriaceae, extended-spectrum β-lactamase–producing gram-negative rods, organisms that induce clindamycin resistance, and β-lactamase–producing *Haemophilus* species. These supplemental methods, in addition to a number of new emerging technologies, are important for the clinical microbiologist in the detection of drug-resistant bacteria.

Standardization of Antimicrobial Susceptibility Testing

To ensure that their test results are reliable, clinical microbiology laboratories should adhere to the standards that have been developed by the Clinical and Laboratory Standards Institute (CLSI) when performing AST. Environmental and technical factors have an enormous impact on susceptibility test results. Factors such as bacterial inoculum size, growth medium, incubation conditions, and antibiotic concentrations must be standardized to minimize the impact of these variables. The goals of standardization in AST are as follows:

1. Optimize the growth conditions so test results cannot be attributed to limitations of nutrients, temperature, or atmosphere
2. Optimize antimicrobial integrity and activity so that resistance cannot be attributed to environmental drug deactivation
3. Maintain interlaboratory reproducibility and consistency of results.1,2

The CLSI standards provide information necessary to perform an AST, from media selection to quality control. Two documents that describe the standardized components of disk diffusion and dilution susceptibility testing methods are CLSI document M02, *Performance Standards for Antimicrobial Disk Susceptibility Tests*, and document M07, *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically*.3 They are to be used in conjunction with M100, *Performance Standards for Antimicrobial Susceptibility Testing*,4 which provides supplemental tables to be used with disk diffusion and minimum inhibitory concentration (MIC) tests. The MIC is the lowest concentration of an antibiotic that inhibits the visible growth of an organism. The M100 document is updated annually, and the other CLSI standards are revised frequently, so laboratories should review their procedures annually to ensure they incorporate the most current standards. These CLSI standards, resources, and other documents can be found and purchased at [http://www.clsi.org](http://www.clsi.org).

Selection of Antibiotics for Testing

Antibiotics selected for susceptibility testing should provide information so that the physician can appropriately treat the patient’s infection. Antibiotics that are not effective against a particular pathogenic organism or within a particular body site should not be tested or reported. Those tested and reported
should be relevant to the patient’s infection and clinical condition, appropriate for the organism isolated, and follow the health care institution’s formulary, which is the group of antibiotics available for prescribing by the clinicians. The CLSI M100 document provides tables that list the antimicrobial agents appropriate for testing Enterobacteriaceae, *Pseudomonas*, other gram-negative glucose nonfermenters, enterococci, streptococci, staphylococci, *Neisseria meningitidis*, and *Haemophilus* species. The lists include recommendations for antibiotics that should be tested routinely and those that may be tested or reported selectively.

Each laboratory must consider the availability of antibiotics for testing by the laboratory’s routine testing methodology and compare that with the health care institution’s formulary. The disk diffusion and gradient diffusion methods offer the greatest flexibility in antibiotic selection, including for testing of newly available antimicrobial agents.

Methods of Antimicrobial Susceptibility Testing

To perform AST, most clinical laboratories use either disk diffusion or a broth microdilution method. In addition, many laboratories use gradient diffusion strips to perform AST on certain drug-organism combinations. All methods have been researched, and when performed correctly with the standards followed, all methods are reliable.

*Broth Microdilution*

Dilution susceptibility testing is used to quantitate the in vitro activity of an antimicrobial agent against an organism. Varying concentrations of the antimicrobial agent are tested with the organism in serial two-fold dilutions. Historically, AST dilution tests were performed using a macrobroth dilution, or tube dilution, method. Owing to the tedious preparation, large amount of space and reagents required, and possibility of errors in preparation, this method has been replaced by the automated microdilution method, which is a miniaturization of the macrodilution.

The microdilution method (Figure 1) has been standardized by use of small disposable plastic microdilution trays, cassettes, or cards that contain miniature wells. Each well contains a specific concentration of lyophilized or frozen antibiotic and a volume of 0.1 to 0.5 mL, which allows approximately 12 antibiotics to be tested in a range of 8 two-fold dilutions in a single test. The panels are fixed with specific antibiotics, contain a growth control and purity control, and are purchased from commercial manufacturers. A standardized suspension is made of pure bacterial growth and diluted so the final concentration in each well of the panel is equal to $5 \times 10^5$ colony forming units (CFU) per milliliter.
commercial systems contain automatic inoculating devices, or the microwells may be inoculated with multichannel pipettors. After incubation, MICs are determined using a manual or automated viewing device for inspection of each of the panel wells for growth. The MIC is then compared with the CLSI breakpoints in the M100 document to determine whether the organism is susceptible, susceptible–dose dependent, intermediate, or resistant.2,4

There are currently four commercial automated or semiautomated instruments cleared by the US Food and Drug Administration (FDA) available in the United States. They are the MicroScan WalkAway®, bioMérieux Vitek-2®, BD Phoenix automated system, and Thermo Scientific Sensititre™.5,7 Use of an automated instrument broth microdilution assay provides precise, reliable, and quantitative test results in a shorter period than manual readings because the sensitive optical detection system allows detection of subtle changes in bacterial growth.5

The advantages of the microdilution susceptibility test include the quantitative MIC result, streamlined workflow, convenience and reproducibility of commercially prepared panels, ability to interface with the laboratory information system to transfer results, and generation of computerized reports if an automated

Figure 1. A broth microdilution susceptibility panel containing miniature wells.
panel reader is used.\textsuperscript{1,5} Disadvantages include the inflexibility of antibiotic selection due to the commercially purchased panels, delays between the update of clinical breakpoints and clearance on commercial systems, strict regulations by the FDA concerning which antibiotic-organism combinations can be tested by commercial systems, lengthy time period between new antibiotic approval and addition to the commercial panels, and lack of performance review of commercial systems as new antimicrobial resistance emerges.\textsuperscript{7}

**Disk Diffusion**

Disk diffusion susceptibility testing, or the Kirby-Bauer disk diffusion test, is performed by inoculating a standardized suspension of pure bacterial growth equal to $1.5 \times 10^5$/mL CFU, or equivalent to a 0.5 McFarland turbidity standard, to the surface of a Mueller-Hinton agar plate, creating a lawn of growth of the bacteria.\textsuperscript{1,2,6} If the organism tested is fastidious and requires an enriched medium to grow, the Mueller-Hinton agar may be supplemented with additional growth ingredients such as sheep’s blood (e.g., *Streptococcus pneumoniae*) or hemin and nicotinamide adenine dinucleotide (NAD) (e.g., *Haemophilus* species). Commercially prepared paper disks with specific concentrations of antibiotics are then placed on the inoculated agar surface. The plates are incubated in ambient air or CO$_2$ at 35°C for 16 to 24 hours depending on the suspected organism and antibiotic. After incubation, the plates are examined for growth inhibition which creates a zone around the antibiotic disk (Figure 2). The diameter of the zone of inhibition is measured to the nearest millimeter. The zone diameter is an indicator of the relative susceptibility of the organism to the antibiotic. Disk diffusion AST is a qualitative susceptibility test, that is, results are reported only as susceptible, intermediate, or resistant rather than including a quantitative MIC.\textsuperscript{1,2,6}

There are several advantages of the disk diffusion AST, including that it is technically easy, does not require special equipment, is inexpensive, provides flexible antibiotic choices, and results are easily interpreted. The disadvantages of the disk diffusion test are the lack of automation, making it time-consuming to perform and interpret, and the lack of quantitative MIC results, which are necessary for treatment in some infections.\textsuperscript{1,2,5,6}
Gradient Diffusion

Gradient diffusion tests are a variation of the disk diffusion test which provides a quantitative MIC value. The bioMérieux ETEST® is a commonly used gradient diffusion AST available in the United States. This susceptibility test consists of a predefined gradient of antibiotic concentrations on one side of a thin plastic strip, with an interpretation scale printed on the other (top) side of the strip. The agar plate is inoculated the same as in disk diffusion, and the gradient strips are placed on the agar surface with the antibiotic side on the agar. After 24-hour incubation, an inhibition ellipse is visible (Figure 3). The quantitative MIC corresponds to the point on the strip where the antimicrobial concentration is no longer inhibiting bacterial growth, or where the elliptical zone of inhibition intersects the strip.¹ ² ⁵ ⁶
Gradient diffusion susceptibility tests are flexible in their ability to test specific antibiotics chosen by the laboratory. However, the strips are expensive, especially if more than a few drugs are tested. This method is best applicable to situations where only one or two antibiotics are needed, or when a fastidious organism requiring an enriched medium or special incubation atmosphere is to be tested.

Figure 3. A *Streptococcus pneumoniae* isolate tested by gradient diffusion method on Mueller-Hinton agar supplemented with sheep’s blood. The minimum inhibitory ellipse concentration of each antibiotic is determined by the intersection of the organism growth with the strip as measured using the scale printed on the strip.

**Interpretation of Antimicrobial Susceptibility Test Results**

The zone diameters or MIC of each antibiotic are interpreted using the breakpoints published by CLSI or those included in the inserts of FDA-approved products. A breakpoint is defined as the concentration of
an antibiotic that enables interpretation of AST to define isolates as susceptible, susceptible–dose dependent, intermediate, or resistant.\textsuperscript{2,4,7}

Laboratories can use either FDA or CLSI breakpoints if performing AST by noncommercial methods such as disk diffusion or gradient diffusion. If using an automated commercial system, US law requires that the devices use FDA breakpoints. Owing to the challenge of commercial system manufacturers’ lag in updating the breakpoints on a yearly basis, laboratories may make CLSI updates to the breakpoints on commercial systems by performing in-house verification studies to ensure analytic performance of the modification before reporting patient results.\textsuperscript{7}

The results of a susceptibility test are compared with the table of values in the CLSI M100 document that relate to proven clinical efficacy of each antibiotic for various bacterial species.\textsuperscript{2,6} \textit{Susceptible} indicates that the antibiotic used in the recommended dosage to treat the site of infection is clinically effective for that patient’s isolate. \textit{Intermediate} indicates clinical efficacy in body sites where the antimicrobial agent is physiologically concentrated or when a higher than normal dosage of antimicrobial agent can be used. \textit{Resistant} indicates that the isolate is not inhibited by the usually achievable concentrations, likely owing to resistance mechanisms, and clinical efficacy has not been shown in treatment studies. \textit{Susceptible–dose dependent} indicates that the susceptibility of the patient’s isolate depends on the dose and regimen of the antimicrobial agent and an altered administration regimen - usually higher dosage, more frequent administration, or both - is needed to reach clinical efficacy.

The CLSI zone size and MIC breakpoints are established by analysis of three kinds of data:

1. Microbiologic data, including a comparison of MICs and zone sizes on a large number of bacterial strains, including those with known resistance mechanisms
2. Pharmacokinetic and pharmacodynamic data
3. Clinical studies results obtained before FDA approval and marketing of an antibiotic.\textsuperscript{8}

The results of AST on the patient’s report for the clinician should include the category result of susceptible, susceptible–dose dependent, intermediate, or resistant. This information is needed for the clinician to prescribe appropriate targeted antimicrobial therapy. If an MIC is available it can be reported to aid the clinician in certain infections, such as infective endocarditis or osteomyelitis, because the MIC aids in selection of the antibiotic in similar groups. Generally, the reporting category of susceptible, intermediate, susceptible–dose dependent, or resistant will suffice, because the MIC is primarily used by infectious disease clinicians and pharmacists.\textsuperscript{2,4,5}
Future Directions

The ASTs described above are commonly performed phenotypic susceptibility tests that have been used by clinical microbiology laboratories for many years. In an effort to provide rapid results for the detection of infectious pathogens and antimicrobial resistance, newer methods have been developed and approved by the FDA and are being adopted by clinical laboratories. The direct detection of resistance genes by molecular methods such as polymerase chain reaction (PCR) or similar techniques is widely used for the detection of specific targets that are associated with phenotypic resistance (e.g., mecA, vanA, vanB and KPC). Depending on the method, these tests can be performed directly on patient specimens or colonial growth. Although these molecular methods have had a significant effect in the rapid detection of specific antibiotic resistance, there are hundreds of other mechanisms of resistance, such as β-lactamase enzyme production, alteration of antibiotic binding sites, and alteration of cell permeability, that result in antibiotic resistance to many different classes of antimicrobial agents. There are too many resistance mechanisms to be easily detected by current molecular techniques. This is why phenotypic AST, which measures the level of susceptibility of bacterial isolates to antimicrobial agents, is still performed today.

There is a need for the development of new automated instruments that can provide faster results by direct specimen testing because the current culture-based AST tools are time-consuming and require isolation of the pathogen for susceptibility testing, which results in several days’ delay before the MIC values are determined and reported. Different methodologic approaches for the detection of bacterial growth have been explored and are currently being researched by commercial entities for clinical translation. New AST techniques based on optical imaging, microchannel resonators, and other biosensors have been pursued. There is currently a multiplexed automated digital microscopy imaging-based test available that has been approved by the FDA for blood and urine specimens that utilizes an image of single bacteria growing into colonies with antibiotics and quantifies the growth rate to produce an MIC result within 3 to 5 hours. The emerging technologies for AST promise results within a few hours, and some can be directly applied to patient samples without any sample pretreatment.

Conclusion

Antimicrobial susceptibility testing is used to determine antibiotic resistance profiles of bacterial isolates, to guide antibiotic treatment decisions, and to predict therapeutic outcome. The phenotypic AST methods currently used in the clinical microbiology laboratory include disk diffusion, gradient diffusion, and microdilution. Disk diffusion AST is easy to perform, low cost, and allows for flexibility in selection of antibiotics. Gradient diffusion also offers flexibility in selection of antibiotics but is expensive. It is usually reserved for situations in which an antibiotic is not yet available for testing on commercial systems, or
there is a fastidious organism that requires an enriched medium or special incubation. Microdilution is the most commonly performed AST method because it can be automated, can be interfaced with the laboratory information system and generate computerized reports, and many antibiotics can be tested on a single panel. In the future, new innovative technologies will enable an AST within a few hours, which will have the potential to enable accurate antibiotic treatment at disease onset, improving antimicrobial stewardship and clinical outcomes.

References


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