Antimicrobial Susceptibility Testing: A Review of General Principles and Contemporary Practices

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An important task of the clinical microbiology laboratory is the performance of antimicrobial susceptibility testing of significant bacterial isolates. The goals of testing are to detect possible drug resistance in common pathogens and to assure susceptibility to drugs of choice for particular infections. The most widely used testing methods include broth microdilution or rapid automated instrument methods that use commercially marketed materials and devices. Manual methods that provide flexibility and possible cost savings include the disk diffusion and gradient diffusion methods. Each method has strengths and weaknesses, including organisms that may be accurately tested by the method. Some methods provide quantitative results (eg, minimum inhibitory concentration), and all provide qualitative assessments using the categories susceptible, intermediate, or resistant. In general, current testing methods provide accurate detection of common antimicrobial resistance mechanisms. However, newer or emerging mechanisms of resistance require constant vigilance regarding the ability of each test method to accurately detect resistance.

EMERGENCE OF ANTIMICROBIAL RESISTANCE AND THE RATIONALE FOR PERFORMING SUSCEPTIBILITY TESTING

The performance of antimicrobial susceptibility testing by the clinical microbiology laboratory is important to confirm susceptibility to chosen empirical antimicrobial agents, or to detect resistance in individual bacterial isolates. Empirical therapy continues to be effective for some bacterial pathogens because resistance mechanisms have not been observed e.g., continued penicillin susceptibility of Streptococcus pyogenes. Susceptibility testing of individual isolates is important with species that may possess acquired resistance mechanisms (eg, members of the Enterobacteriaceae, Pseudomonas species, Staphylococcus species, Enterococcus species, and Streptococcus pneumoniae).

OVERVIEW OF COMMONLY USED SUSCEPTIBILITY TESTING METHODS

Broth dilution tests. One of the earliest antimicrobial susceptibility testing methods was the macrobroth or tube-dilution method [1]. This procedure involved preparing two-fold dilutions of antibiotics (eg, 1, 2, 4, 8, and 16 μg/mL) in a liquid growth medium dispensed in test tubes [1, 2]. The antibiotic-containing tubes were inoculated with a standardized bacterial suspension of 1–10^7 CFU/mL. Following overnight incubation at 35°C, the tubes were examined for visible bacterial growth as evidenced by turbidity. The lowest concentration of antibiotic that prevented growth represented the minimal inhibitory concentration (MIC). The precision of this method was considered to be plus or minus 1 two-fold concentration, due in large part to the practice of manually preparing serial dilutions of the antibiotics [3]. The advantage of this technique was the generation of a quantitative result (ie, the MIC). The principal disadvantages of the macrodilution method were the tedious, manual task of preparing the antibiotic solutions for each test, the possibility of errors in preparation of the antibiotic solutions, and the relatively large amount of reagents and space required for each test.

The miniaturization and mechanization of the test by use of small, disposable, plastic “microdilution” trays (Figure 1) has
made broth dilution testing practical and popular. Standard trays contain 96 wells, each containing a volume of 0.1 mL that allows approximately 12 antibiotics to be tested in a range of 8 two-fold dilutions in a single tray [2, 4]. Microdilution panels are typically prepared using dispensing instruments that aliquot precise volumes of preweighed and diluted antibiotics into broth into the individual wells of trays from large volume vessels. Hundreds of identical trays can be prepared from a single master set of dilutions in a relatively brief period. Few clinical microbiology laboratories prepare their own panels; instead frozen or dried microdilution panels are purchased from one of several commercial suppliers. The cost of the preprepared panels range from approximately $10 to $22 each. Inoculation of panels with the standard 5 x 10^5 CFU/mL is accomplished using a disposable device that transfers 0.01 to 0.05 mL of standardized bacterial suspension into each well of the microdilution tray or by use of a mechanized dispenser. Following incubation, MICs are determined using a manual or automated viewing device for inspection of each of the panel wells for growth [2].

The advantages of the microdilution procedure include the generation of MICs, the reproducibility and convenience of having preprepared panels, and the economy of reagents and space that occurs due to the miniaturization of the test. There is also assistance in generating computerized reports if an automated panel reader is used. The main disadvantage of the microdilution method is some inflexibility of drug selections available in standard commercial panels.

Antimicrobial gradient method. The antimicrobial gradient diffusion method uses the principle of establishment of an antimicrobial concentration gradient in an agar medium as a means of determining susceptibility. The Etest (bioMérieux AB BIODISK) (Figure 2) is a commercial version available in the United States. It employs thin plastic test strips that are impregnated on the underside with a dried antibiotic concentration gradient and are marked on the upper surface with a concentration scale. As many as 5 or 6 strips may be placed in a radial fashion on the surface of an appropriate 150-mm agar plate that has been inoculated with a standardized organism suspension like that used for a disk diffusion test. After overnight incubation, the tests are read by viewing the strips from the top of the plate. The MIC is determined by the intersection of the lower part of the ellipse shaped growth inhibition area with the test strip.

The gradient diffusion method has intrinsic flexibility by being able to test the drugs the laboratory chooses. Etest strips cost approximately $2–$3 each and can represent an expensive approach if more than a few drugs are tested. This method is best suited to situations in which an MIC for only 1 or 2 drugs is needed or when a fastidious organism requiring enriched medium or special incubation atmosphere is to be tested (eg, penicillin and ceftriaxone with pneumococci) [5–7]. Generally, Etest results have correlated well with MICs generated by broth or agar dilution methods [5–9]. However, there are some systematic biases toward higher or lower MICs determined by the Etest when testing certain organism-antimicrobial agent combinations [6, 10]. This can represent a potential shortcoming when standard MIC interpretive criteria derived from broth
A Staphylococcus aureus isolate tested by the Etest gradient diffusion method with vancomycin (VA), daptomycin (DM), and linezolid (LZ) on Mueller-Hinton agar. The minimum inhibitory concentration of each agent is determined by the intersection of the organism growth with the strip as measured using the scale inscribed on the strip.

Figure 2. 

Dilution testing [10] are applied to Etest MICs that may not be identical.

Disk diffusion test. The disk diffusion susceptibility method [2, 11, 12] is simple and practical and has been well-standardized. The test is performed by applying a bacterial inoculum of approximately $1–2 \times 10^6$ CFU/mL to the surface of a large (150 mm diameter) Mueller-Hinton agar plate. Up to 12 commercially-prepared, fixed concentration, paper antibiotic disks are placed on the inoculated agar surface (Figure 3). Plates are incubated for 16–24 h at 35°C prior to determination of results. The zones of growth inhibition around each of the antibiotic disks are measured to the nearest millimeter. The diameter of the zones is related to the susceptibility of the isolate and to the diffusion rate of the drug through the agar medium. The zone diameters of each drug are interpreted using the criteria published by the Clinical and Laboratory Standards Institute (CLSI, formerly the National Committee for Clinical Laboratory Standards or NCCLS) [13] or those included in the US Food and Drug Administration (FDA)–approved product inserts for the disks. The results of the disk diffusion test are “qualitative,” in that a category of susceptibility (ie, susceptible, intermediate, or resistant) is derived from the test rather than an MIC. However, some commercially-available zone reader systems claim to calculate an approximate MIC with some organisms and antibiotics by comparing zone sizes with standard curves of that species and drug stored in an algorithm [14, 15].

The advantages of the disk method are the test simplicity that does not require any special equipment, the provision of categorical results easily interpreted by all clinicians, and flexibility in selection of disks for testing. It is the least costly of all susceptibility methods (approximately $2.50–$5 per test for materials). The disadvantages of the disk test are the lack of mechanization or automation of the test. Although not all fastidious or slow growing bacteria can be accurately tested by this method, the disk test has been standardized for testing streptococci, Haemophilus influenzae, and N. meningitidis through use of specialized media, incubation conditions, and specific zone size interpretive criteria [12].

Automated instrument systems. Use of instrumentation can standardize the reading of end points and often produce susceptibility test results in a shorter period than manual readings because sensitive optical detection systems allow detection of subtle changes in bacterial growth. There are 4 automated instruments presently cleared by the FDA for use in the United States. Three of these can generate rapid (3.5–16 h) susceptibility test results, while the fourth is an overnight system [16]. The MicroScan WalkAway (Siemens Healthcare Diagnostics) is a large self-contained incubator/reader device that can incubate and analyze 40–96 microdilution trays. The WalkAway utilizes standard size microdilution trays that are hydrated and inoculated manually and then placed in one of the incubator slots in the instrument. The instrument incubates the trays for the appropriate period, examining them periodically with either a
Figure 3. A disk diffusion test with an isolate of *Escherichia coli* from a urine culture. The diameters of all zones of inhibition are measured and those values translated to categories of susceptible, intermediate, or resistant using the latest tables published by the CLSI.

photometer or fluorometer to determine growth development. Gram-negative susceptibility test panels containing fluorogenic substrates can be read in 3.5–7 h. Separate gram-positive and gram-negative panels read using turbidimetric end points are ready in 4.5–18 hours.

The BD Phoenix Automated Microbiology System (BD Diagnostics) has a large incubator reader with a capacity to process 99 test panels that contain 84 wells devoted to antibiotic doubling dilutions and are inoculated manually. The Phoenix monitors each panel every 20 min using both turbidimetric and colorimetric (oxidation-reduction indicator) growth detection. Test panels for gram-negative, gram-positive, *S. pneumoniae*, β-hemolytic, and viridans group streptococci are available. MIC results are generated in 6–16 h.

The Vitek 2 System (bioMérieux) is highly automated and uses very compact plastic reagent cards (credit card size) that contain microliter quantities of antibiotics and test media in a 64-well format. The Vitek 2 employs repetitive turbidimetric monitoring of bacterial growth during an abbreviated incubation period. The instrument can be configured to accommodate 30–240 simultaneous tests. The susceptibility cards allow testing of common, rapidly growing gram-positive, and gram-negative aerobic bacteria, and *S. pneumoniae* in a period of 4–10 h. An older, less automated, Vitek 1 System is still used in some laboratories. The system is more limited with a 45-well card and does not include *S. pneumoniae*.

The Sensititre ARIS 2X (Trek Diagnostic Systems) is an automated, overnight, incubation and reading system with a 64-panel capacity. The test panels are standard 96-well microdilution plates that can be inoculated with a Sensititre Autoinculator. Growth is determined by fluorescence measurement after 18–24 h of incubation. Test panels are available for gram-positive and gram-negative bacteria, *S. pneumoniae*, *Haemophilus* species, and nonfermentative gram-negative bacilli.

The Phoenix, Sensititre ARIS 2X, Vitek 1 and 2, and WalkAway instruments have enhanced computer software used to interpret susceptibility results including “expert systems” for analyzing test results for atypical patterns and unusual resistance phenotypes [16]. Two studies [17, 18] have shown that providing rapid susceptibility test results can lead to more timely changes to appropriate antimicrobial therapy, substantial direct cost savings attributable to ordering of fewer additional laboratory tests, performance of fewer invasive procedures, and a shortened length of stay. These benefits are best realized when coupled with extended laboratory staffing schedules, and real-time, electronic transmission of verified results. One of the early shortcomings of rapid susceptibility testing methods was a lessened ability to detect some types of antimicrobial resistance including inducible β-lactamases and vancomycin resistance. However, the recently FDA-cleared instruments have made significant improvements in large part through modifications of the instruments’ computer software to either provide extended incubation for problematic organism-drug combinations, or by editing of susceptibility results using expert software to prevent unlikely results from being reported. In some cases these modifications result in prolonged incubation (ie, >10 h) of test
panels to assure accurate results, thus rendering them less “rapid.”

**SELECTION OF DRUGS FOR ROUTINE TESTING**

The laboratory must test and report the antimicrobial agents that are most appropriate for the organism isolated, for the site of the infection, and the institution’s formulary [13, 19]. The CLSI provides tables that list the antimicrobial agents appropriate for testing members of the Enterobacteriaceae, *Pseudomonas*, and other gram-negative glucose nonfermenters, staphylococci, enterococci, streptococci, *Haemophilus* species, etc. [13]. The listings include recommendations for agents that are important to test routinely, and those that may be tested or reported selectively based on the institution’s formulary.

The availability of antimicrobial agents for testing by the laboratory’s routine testing methodology must next be determined. The disk diffusion and gradient diffusion procedures offer the greatest flexibility including testing of newly available drugs. Most broth microdilution or automated test panels contain <96 wells, effectively limiting the number of agents tested or the range of dilutions of each drug that can be included. Manufacturers of commercially prepared panels have attempted to deal with this problem by offering a number of different standard panel configurations, or by including fewer dilutions of each drug in a single panel [19]. Another solution to this problem is testing antimicrobial agents that have activities that are essentially the same as the desired formulary drugs. The CLSI susceptibility testing document [13] lists groups of some antimicrobial agents with nearly identical activities that can provide practical alternatives for testing.

**INTERPRETATION OF SUSCEPTIBILITY TEST RESULTS**

The results of a susceptibility test must be interpreted by the laboratory prior to communicating a report to a patient’s physician. Optimal interpretation of MICs requires knowledge of the pharmacokinetics of the drug in humans, and information on the likely success of a particular drug in eradicating bacteria at various body sites [20]. This is best accomplished by referring to an expert source such as the CLSI, which publishes interpretive criteria for MICs of all relevant antibiotics for most bacterial genera [13]. Indeed, both MIC values and disk diffusion zone diameters must be interpreted using a table of values that relate to proven clinical efficacy of each antibiotic and for various bacterial species [12]. The CLSI zone size and MIC interpretive criteria are established by analysis of 3 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 mechanisms of resistance that have been defined either phenotypically or genotypically; (2) pharmacokinetic and pharmacodynamic data; and (3) clinical studies results (including comparisons of MIC and zone diameter with microbiological eradication and clinical efficacy) obtained during studies prior to FDA approval and marketing of an antibiotic [20].

A “susceptible” result indicates that the patient’s organism should respond to therapy with that antibiotic using the dosage recommended normally for that type of infection and species [13, 20]. Conversely, an organism with a MIC or zone size interpreted as “resistant” should not be inhibited by the concentrations of the antibiotic achieved with the dosages normally used with that drug [13, 20]. An “intermediate” result indicates that a microorganism falls into a range of susceptibility in which the MIC approaches or exceeds the level of antibiotic that can ordinarily be achieved and for which clinical response is likely to be less than with a susceptible strain. Exceptions can occur if the antibiotic is highly concentrated in a body fluid such as urine, or if higher than normal dosages of the antibiotic can be safely administered (eg, some penicillins and cephalosporins). At times, the “intermediate” result can also mean that certain variables in the susceptibility test may not have been properly controlled, and that the values have fallen into a “buffer zone” separating susceptible from resistant strains [13, 20]. Generally, reporting of a category result of susceptible, intermediate, or resistant provides the clinician with the information necessary to select appropriate therapy. Reporting of MICs could aid a physician is selecting from among a group of similar drugs for therapy of infective endocarditis or osteomyelitis, in which therapy is likely to be protracted.

It is important that the tables used for susceptibility test interpretations represent the most current criteria. Indeed, the CLSI documents are reviewed and updated frequently, usually once per year. Use of old or outdated information from the original editions of FDA-approved drug labels or older CLSI tables could represent a serious shortcoming in the reporting of patients’ results.

**WHAT IS THE ACCEPTABLE ACCURACY OF A SUSCEPTIBILITY TEST METHOD?**

When assessing the accuracy of various susceptibility testing methods as compared to standard reference methods, the terms very major and major errors have been used to describe false-susceptible or false-resistant results, respectively. In evaluations of new susceptibility testing methods it is important to examine a representative number of strains that are resistant to various drugs to verify the ability of the new test to detect resistance and to test a number of susceptible strains to determine the rate of major errors that might be expected in a typical clinical laboratory setting [16, 21]. To be cleared for marketing in the United States, the FDA requires that very major errors attributable to a test device should be <1.5% for individual species/drug comparisons, major errors should not exceed 3%, and an
overall essential MIC agreement of >90% of device MICs within one doubling dilution of a CLSI reference MIC [22]. A recent, international standard on susceptibility test device evaluation proposes similar but not identical criteria for acceptable accuracy [23]. The emergence of new antimicrobial resistance mechanisms, including some that may be difficult to detect (eg, vancomycin intermediate susceptibility in S. aureus and carbapenemase production in some gram-negative organisms) requires that the performance of susceptibility devices be constantly reassessed and updated when needed. In some cases, it has been necessary to employ special ancillary testing methods (eg, single concentration screening agars, modified Hodge test for carbapenemase production) [13] to supplement routine testing by a commercial instrument system.

CURRENT TEST METHODS AND FUTURE DIRECTIONS

The antimicrobial susceptibility testing methods described in this article provide reliable results when used according to the procedures 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. To accomplish this, it will likely be necessary to explore different methodologic approaches for detection of bacterial growth. The direct detection of resistance genes by polymerase chain reaction or similar techniques has limited utility, because only a few resistance genes are firmly associated with phenotypic resistance (eg, meca, vanA, and vanB) [24]. There are hundreds of β-lactamases, and numerous mutations, acquisitions, and expression mechanisms that result in fluoroquinolone, aminoglycoside, and macrolide resistance [25]; too many to be easily detected by current molecular techniques. Thus, it seems likely that phenotypic measures of the level of susceptibility of bacterial isolates to antimicrobial agents will continue to be clinically relevant for years to come.

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References


