

Updating Antimicrobial Susceptibility Testing Methods

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LEARNING OBJECTIVES:

1. Explain the physician's choice of antibiotic agents according to CLSI guidelines.
2. Define and discuss MIC breakpoints.
3. Compare qualitative versus quantitative antimicrobial susceptibility testing (AST) methods.
4. Contrast the various quantitative AST methods.
5. Discuss the broth dilution reference method.
6. Discuss genotypic methods and their purposes.

ABBREVIATIONS: AST-Antimicrobial Susceptibility Testing; CLSI-Clinical and Laboratory Standards Institute; EUCAST-European Committee on Antimicrobial Susceptibility Testing; FDA-Food and Drug Administration; MIC-minimal inhibitory concentration; MH-Mueller-Hinton; CAMHB-cation-adjusted Mueller Hinton broth; ATCC-American Type Culture Collection; MRSA-methicillin-resistant *Staphylococcus aureus*; VRE-vancomycin-resistant *Enterococcus*; S, I, R-sensitive, intermediate, resistant; PCR-polymerase chain reaction; DNA-deoxyribonucleic acid; RT-reverse transcriptase; CAI-community-associated infection; HAI-hospital-associated infection; MSSA-methicillin-sensitive *Staphylococcus* sp.; PBP-penicillin-binding proteins; ESBL-extended-spectrum beta lactamase; KPC-*Klebsiella pneumoniae*, carbapenem-resistant; CFU-colony forming units; TSB-trypticase soy broth; CSF-cerebrospinal fluid; ESBL-extended-spectrum beta lactamase

INDEX TERMS: MIC breakpoints, phenotypic testing, qualitative AST methods, quantitative AST methods, genotypic testing

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The importance of antimicrobial susceptibility testing (AST) as a clinical laboratory function escalates as organism resistance to the available antimicrobial agents increases. As patient outcome is based on antimicrobial therapy, standardization of the AST methods determining therapy is essential. The Clinical and Laboratory Standards Institute (CLSI) in the US and the European Committee on Antimicrobial Testing (EUCAST) in Europe determine these standards that are updated annually.

Of concern to physicians are questions regarding the establishment of breakpoints, which are the interpretive cut-off values of minimal inhibitory concentration (MIC) in quantitative testing and inhibition zones for antimicrobial agents in qualitative testing based on geographical surveys of clinical isolates. The CLSI performance standards in the US and the EUCAST in Europe, the groups responsible for developing the breakpoints, may differ in perception and usage.^{1,2} Interpretation of qualitative results as susceptible (S), intermediate (I) or resistant (R) and quantitative measures (MICs) of antimicrobial activity are standardized by CLSI in the U.S. with breakpoint guidelines updated annually for each antibiotic tested.^{1,2,4}

Antimicrobial Agents

The clinical laboratory's list of antimicrobial agents is chosen by the infectious disease specialists and pharmacologists at the individual institution according to the institution's unique guidelines. A formulary of agents that physicians regularly prescribe is derived from the susceptibility testing of organisms typically isolated at each clinical laboratory and is guided by a monthly updated antibiogram. The number of agents tested is designated by the AST method used, e.g. 12 disks per 150 mm Mueller-Hinton agar plate or similar number

per microdilution tray/panel (e.g. automated or semi-automated systems) as well as by the site of infection (e.g. cerebrospinal fluid, blood, urine, etc.) Commercial panels may be used to test a greater number of agents thus allowing for 2-4 dilutions per agent. Appropriate agents for testing all bacteria—aerobic, fastidious and anaerobic—are listed in the CLSI annually published tables.^{3,4}

MIC Breakpoints

The MIC is the measurement on which quantitative susceptibility methods are based, levels of an antimicrobial agent in body fluids may be compared and is determined by a series of log₂ (two-fold) antimicrobial dilutions. A breakpoint or interpretive cut-off MIC value is used to establish the categories designated as susceptible (S), intermediate (I) and resistant (R). The original 2-fold broth dilution method was chosen for simplicity in preparation, while the result is an expression of the organism's statistically normal distribution plotted on a logarithmic scale. Thus, the MIC of a resistant organism that does not follow a normal distribution may be easily distinguished.

In-vitro testing, however, cannot encompass the multitude of *in-vivo* effects such as adverse antibiotic reactions, virulence or toxin producing strains of an organism, protein binding, or pharmacokinetic changes of drug level variation over time. Direct application of the MIC may not be possible when values for certain strains fall outside the normal range.

From the MIC breakpoints, disk diffusion parameters are chosen by plotting the inhibition zone diameter against the MIC value, determined after testing a number of different strains and a variety of species. The zone diameters are statistically calculated to correlate with MIC results, and include concern for the size of errors created by extremely resistant or susceptible organisms. CLSI and FDA discrepancies are thoroughly reviewed. Further information is available at: www.clsi.org and www.eucast.org.

Increased therapeutic complications from antimicrobial resistance make the accuracy of AST results increasingly critical. The reasons are reflected in both individual patient care and reliance on international and local epidemiologic surveillance studies, which are based on

the data accumulated by each clinical laboratory. Supervisor and director level monitoring assure that technical performance and methods are upgraded to comply with CLSI requirements. Supervision is particularly required in the use of reference quality control organisms, application of methodology and practice according to the latest testing methods, and a thorough review of patient reports. The increasing number of resistance mechanisms exhibited by microbial isolates mandates that clinical laboratories follow CLSI interpretive criteria.⁵

AST Methods

The AST methods (manual and automated) commonly performed by clinical laboratories are the conventional disk diffusion, broth dilution, agar dilution, antimicrobial gradient (e.g. the E-test, AB Biodisk) and automated instrumentation. When qualitative methods are appropriate, the standard Kirby-Bauer disk diffusion test is chosen. Quantitative results (i.e. MICs) are determined with broth or agar dilution, performed manually or by automated system, and antimicrobial gradient. The antimicrobial gradient is defined as diffusion of antibiotic drug onto the agar medium from one side of a plastic strip (e.g. E-Test). Only pathogens considered clinically problematic are tested, according to the established CLSI standards assuming that colonizing organisms should not be tested.^{1,2}

Each institution determines the testing methods according to factors that include cost, professional flexibility, size, and qualifications of the laboratory personnel. If any of these factors are in question, referral to a public health or reference laboratory is recommended.

Although molecular methods are commonly performed by researchers and epidemiologists for surveillance purposes, these methods are not part of the routine practice of clinical laboratory susceptibility testing but are briefly described in Table 1.^{3,4}

Phenotypic Susceptibility Testing Procedures

Qualitative Procedures

Disk Diffusion (Kirby-Bauer)

The disk diffusion procedure was originally standardized to test rapidly growing bacteria and should not be used in cases of unusual growth patterns, and for fastidious or anaerobic bacteria. Preparation of the ino-

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Table 1. Comparison of AST Methods

| Method | Description | Commercial Availability | Advantages | Disadvantages | Special Features ² |
|--------------------|--|---|---|---|--|
| Disk diffusion | Antibiotic impregnated discs placed on surface of MH1 agar plate; 5x 10 ⁵ CFU/ml suspension of bacterial inoculum streaked X 3 directions on 100mm MH plate | Difco, Hardy Dx., BD, et al. | Simple to perform; reproducible; less costly; allows large selection of antibiotics | No fastidious or anaerobic standardization; No quantitative result e.g. MIC | Results are determined by zone diameter: "Susceptible Intermediate or Resistant" per CLSI ² |
| Broth dilution | 2 fold serial dilution of 5 x 10 ⁵ CFU/ml bacterial inoculum & 1-3 antibiotic agent concentrations; CAMHB ³ for routine tests | Difco, Hardy Dx., BD, et al. | AST gold standard test; quantitative (MIC); fastidious and anaerobic bacteria tested | Contamination is not easily detected; time-consuming; may be costly | International reference to evaluate new methods; adapts to automated systems |
| Agar dilution | Diluted antibiotic agent added to liquid agar, poured onto Petri dishes to solidify; inoculum of 10 ⁴ CFU ⁴ per spot on agar surface | Difco, Hardy Dx., BD, et al. | Quantitative (MIC); new agent evaluation; contamination detection easier than broth; | Time-labor intensive; not validated for all antibiotics; inoculum standardization critical | Fastidious and anaerobic bacteria tested; European reference method |
| Gradient diffusion | E-test, M.I.C. AB Evaluator strips with preformed antibiotic gradient; like disk diffusion swabbed on agar surface | Biodisk, bioMerieux; Oxoid | MIC read directly from scale at point of growth inhibition; simple to perform; several strips placed on a large MH plate; agreement with broth MICs | Expensive; requires some technical expertise | Tests less-often used antibiotics; tests fastidious and anaerobic bacteria on enriched media |
| Automated System | The <i>Manual of Clinical Microbiology</i> lists manufacturers; panels for G+ ⁵ , G- ⁶ yeasts; read by turbidity or fluorometry | BD Phoenix Vitek 2, Vitek 2 XL, Microscan, et al. | Performs susceptibility and identification; labor saver; results rapid and reproducible; data management; future molecular technology application | Expensive; requires space for equipment; unable to detect some resistant phenotypes, e.g. <i>vanB</i> , <i>vanC</i> | Expert systems rapidly detect errors; CLSI & EUCAST guideline assist |

References: The Manual of Clinical Microbiology, 2011 and The Clinical Laboratory Standards Institute, 2009

¹ MH = Mueller-Hinton, ² CLSI = Clinical and Laboratory Standards Institute, ³ CAMHB = cation-adjusted MH broth, ⁴ CFU = colony-forming units, ⁵ G+ = gram positive, ⁶ G- = gram negative

culum may be made directly from bacterial growth on an agar plate or from overnight growth in broth. A dilution in sterile saline or trypticase soy broth (TSB) is made with the turbidity adjusted to approximately 10⁸ colony forming units (CFU)/mL to match a 0.5 McFarland standard. The inoculum is again diluted 1:100 with broth or saline. When this dilution is

applied to an agar plate or when 1 mL is added to 1 mL of antimicrobial drug diluted in cation-adjusted Mueller Hinton broth (CAMHB) for broth dilution the final inoculum will be 5 X 10⁵ CFU/mL.⁶

A refrigerated 150 mm Mueller-Hinton (MH) agar plate is brought to room-temperature and the prepared

inoculum is streaked evenly across the plate with a cotton swab. The plate is then rotated and swabbed two more times so that three planes are streaked.

The amount of antimicrobial agent impregnated in the disks used for this method is standardized, sold commercially in separate containers, and protected from moisture by storage with a desiccant. A mechanical disk dispenser can be used to distribute 12 disks onto the agar plate or the disks may be placed separately with sterile forceps, allowing at least 24 mm between each. All materials are tightly covered and stored at 2° to 8° C, or frozen at -20° C or below, then warmed to room temperature prior to use. Inoculated plates are incubated overnight (12-16 hours) in a non-CO₂ incubator at 35°C, zones of inhibition of test organisms as well as American Type Culture Collection (ATCC) reference strains for quality control are measured and recorded. Some organisms, e.g. MRSA, VRE, etc. require an extended period of incubation to allow for inducible resistance Figure 1.⁶

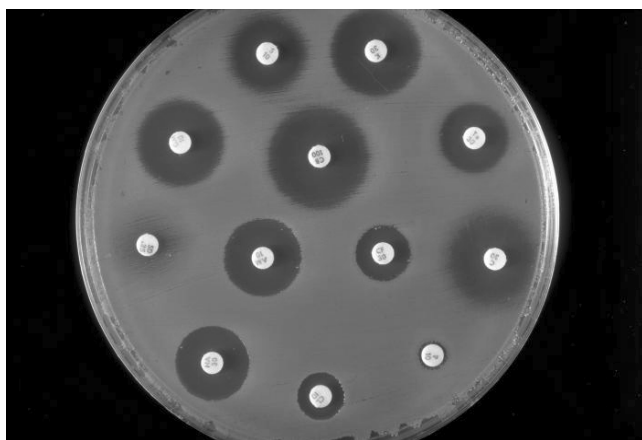


Figure 1. PHIL 3031 GNB Susceptibility Test by Disk Diffusion on Mueller-Hinton Agar (<http://phil.cdc.gov/phil/details.asp>).

Quantitative Procedures

Broth Dilution

The established reference method for antimicrobial susceptibility testing is a log₂ (two-fold) serial dilution of antibiotic agents where the lowest concentration inhibiting bacterial growth is defined as the MIC. Stock antibiotic solutions are prepared according to manufacturer and CLSI standard instructions and may be kept at 4–8° C for 5 days. The range of concentration varies with each antibiotic, usually encompassing 2-3 or more concentrations necessary to

determine the breakpoint categories: susceptible, intermediate, and resistant. The diluted drugs are then distributed by 0.1 mL volumes to tubes containing ≥ 1.0 mL CAMHB. One mL of standardized suspension is added to each tube of diluted antibiotic to produce a final inoculum of 5 X 10⁵ CFU/mL. One tube without antibiotics is used as a growth control.⁶

Quality Control

Reference strains are procured from the ATCC, which stores the genetically stable bacterial strains required for the purpose of quality control. Approved by CLSI for broth dilution and disk diffusion are *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Enterococcus faecalis* ATCC 29213, and *Staphylococcus aureus*. Their MIC ranges are published and updated annually in the CLSI documents. Weekly testing of quality control strains is acceptable after the clinical laboratory has satisfied the initial daily testing for accuracy required by CLSI. Unless testing results fall within the acceptable range of MIC values, patient results may not be reported.⁶

Agar Dilution

The commercially prepared agar base is melted and then distributed in aliquots to test tubes as necessary to dilute the antibiotics by 10-fold. The tubes then are sterilized and equilibrated at 48° to 50°C in a water bath. To attain the correct drug concentration, an appropriate, exact volume of antibiotic is added to each tube. The tubes are inverted and poured into 100 mm round or square Petri dishes on a flat surface and left to solidify. The plates then are sealed in plastic bags and stored at 4° to 8°C for use within 5 days.

Bacterial inoculum is prepared and diluted as for the broth dilution method. The suspension is adjusted to reach a final dilution of 10⁴ CFU per spot, which will appear on the solid agar surface. A pipette, calibrated loop or inoculum-replicating device delivers the suspension to the plate, which has been prepared with a series of drug concentrations (low to high) for each antibiotic tested and to a growth control plate. When the inoculum appears dry, the plates are inverted and incubated at 35°C without CO₂ for 16 to 20 hours. Vancomycin and oxacillin plates require 24 hours of incubation. Control strains are tested with each run.

Plates may be placed on a dark background for reading

and interpretation where appearance of any haze, or a single colony is interpreted as no visible growth. The MIC, in $\mu\text{g/mL}$, is recorded as the lowest concentration that inhibits visible growth. The corresponding interpretive categories S, I, & R are reported. CLSI standards must be consulted to assure all standards for testing are met and updated and that interpretive criteria are followed.

Prior to the broth dilution reference method, agar dilution was the method of reference, particularly in Europe. Its use to evaluate other testing methods and easy detection of bacterial contamination made it a reliable method. Disadvantages include labor-intensive plate preparation and performance time, as well as lack of validation for certain drugs (e.g. daptomycin).⁶

Gradient Diffusion

The E-Test (AB Biodisk, bioMérieux) and the M.I.C. Evaluator (Oxoid) are commercially available strips used for the quantitative testing of a preformed antimicrobial agent that is applied to one side of a plastic strip allowing diffusion of the drug into an agar medium. Similar to the disk diffusion method, the inoculum is a 0.5 McFarland suspension of the organism to be tested swabbed in three directions across the surface of a large MH plate. The strip contains a gradation of antibiotic from lowest to the highest.

After incubation at 35°C without CO₂ for 16 to 20 hours, the MIC can be read directly from a scale on the strip located at the point where inhibition of the inoculum growth intercepts the strip (Figure 2). Strips with different antimicrobial agents to be tested may be placed on the surface of a single MH plate. Agreement of the gradient strip with the MIC determined by the broth dilution method is acceptable and standardized by CLSI. The accuracy and ease of performance, application for fastidious or anaerobic organisms with special media and incubation requirements make this a reliable method for clinical and reference laboratories.⁶

Automated System Testing

Commercially prepared trays of frozen or freeze-dried antimicrobial agent dilutions stored at -20°C or room temperature, respectively, are available for inoculation with a bacterial inoculum suspension. These systems also include organism identification, data management software to interface with a laboratory information

system and the proficiency of epidemiologic analyses. Regulatory oversight by the FDA is required where AST systems are classified as medical devices which require FDA clearance before they can be marketed.

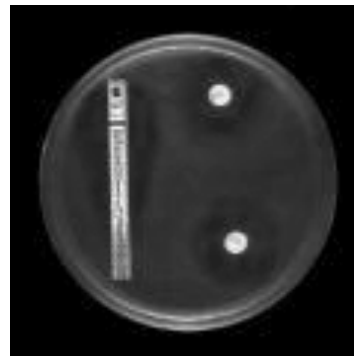


Figure 2. PHIL 10852 Antimicrobial Susceptibility E-Test and Disk Diffusion (<http://phil.cdc.gov/phil/details.asp>)

Brief descriptions of the available testing systems, which use turbidity or fluorometry to determine organism inhibition, report both MIC and organism identification are provided in Table 2.

Genotypic Susceptibility Testing

Molecular Detection

AST continues to be the most functional and globally accepted reference method in guiding antimicrobial therapy. Genotypic PCR-based assays are revolutionary to clinical laboratory testing and epidemiologic monitoring because of the rapid detection of resistant organisms with greater accuracy than phenotypic testing. Detecting mutations associated with fluoroquinolone and penicillin resistance is more successful with genetic tests. In the future, treating resistant strains of *Mycobacterium tuberculosis* by detecting similar mutations will be available to guide initial therapy, especially in developing countries.

Earlier PCR assays that used agarose gel to visualize the amplified products are being replaced by real-time PCR, DNA sequencing, reverse-transcriptase (RT-PCR) and simpler methods that produce results in a few hours. Some earlier DNA probe tests for resistance genes have been replaced by novel PCR assays. RT-PCR detects amplification products with techniques using molecular beacons, peptide nucleic acid fluorescent in-situ hybridization probes, microarrays and pyrosequencing. Rapid detection (< 1 hour) from clinical specimens allows efficacious treatment, reduced transmission of in-

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Table 2. Automated Testing Systems Methods Comparison

| Manufacturer | Capacity | Reading/Incubation | Analysis/Features |
|---------------------------------------|---|--|---|
| Becton-Dickinson BD Phoenix | 100, 2- sided panels for ID1 & AST2; 14-22 antimicrobial agents, full range of concentrations | Direct (not calculated) MICs reads every 20 minutes. Results in 6 -16 hours | Turbidometric; reads growth/no growth per well |
| BioMerieux Vitek 2 | 64-well cards, 9-20 agents, 1-6 concentrations. ID cards for G+ ³ and G- ⁴ run together | Reads for light transmission proportional to growth every 15 minutes. Report at 4-6 hours | Linear regression analysis, algorithm-derived MICs |
| Siemens Microscan Walkaway Plus | 96 microwell trays as MIC panels or panels for full range MICs | 4 Models with different reading times. Incubation 3.5 – 15 hours | Turbidometric or fluorometric |
| TREK Sensititre ARIS 2X | 96 microwell trays as MIC panels or ID plates | Incubation time 16-24 hours | Fluorometric; includes epidemiology for trends and antibiograms |

¹ ID = Identification, ² AST = Antimicrobial Susceptibility Testing, ³ G+ = gram positive bacteria, ⁴ G- = gram negative bacteria

fection and greater accuracy in the epidemiologic monitoring of both hospital and community associated-infections.

Real time-PCR assays of hospital-associated infections (HAIs), e.g. MRSA and VRE, have abrogated the need for isolation and treatment of carrier patients entering the hospital by detecting resistance at the time of patient admission, saving the exorbitant cost of laboratory tests and hospitalization. Implementation of genetic technology for problematic community-associated infections (CAI) (e.g. *Salmonella* spp., pneumococci and extensively-resistant *Mycobacterium tuberculosis*) also promises to deter the HAI overload.⁸

β-Lactam Resistance

Available commercially to clinical laboratories, real-time PCR assays for both MRSA and methicillin-sensitive *Staphylococcus aureus* (MSSA) allow detection directly from growth in blood cultures. However, penicillin and extended-spectrum cephalosporin resistance in *S. pneumoniae* via penicillin-binding proteins (PBPs), requiring identification of *pbp* genes—2x, 2b and 1a—is only somewhat successful for 2b because of the structural remodeling that occurs. Amplification-based

PCR assays cannot be used to detect gram-negative beta lactamases of the Enterobacteriaceae (TEM or SHV) arising through point mutation and resulting in amino acid substitution. Multiplex PCR assays are available for genes encoding TEM, SHV, CTX-M extended-spectrum beta lactamases (ESBLs). These enzymes can be identified by primers for their genes (e.g. *bla*_{TEM}, *bla*_{SHV}, *bla*_{CTX-M}) using a monoplex or multiplex PCR format. A multiplex PCR format with primers targeting six families of plasmid-mediated AmpC enzymes has also been described.

In another example of success, the carbapenemases found in *Klebsiella pneumoniae*, carbapenem-resistant (KPC) have been identified in other species of the Enterobacteriaceae family, *Pseudomonas* spp. and *Acinetobacter* spp. Real-time PCR rapidly detects all *bla*_{KPC} (presently, 9 genes), thus alleviating the interpretive ambiguity resulting from automated system testing.⁸

When clinical laboratories attempt to determine accurate MICs for some antimicrobial agents, e.g. carbapenems, the automated testing systems are known to generate unreliable reports. Arbitration of MIC results near the breakpoint for KPC-producing isolates,

but not in the resistant range for carbapenems, is **Resistance to Other Antibiotics**

Chlorophenicol acetyltransferases found in gram-positive and gram-negative organisms that are encoded by genes *catA1*, *cmlA* in *Salmonella* sp. and by *cfi* in some *S. aureus* isolates can be detected with PCR primers. Vancomycin and other glycopeptide resistance first exemplified by the *vanA*, *vanBs*, *vanCs* and the *vanDs*, *vanE* and *vanG* are detected by PCR assays and multiplex PCR for simultaneous results. Macrolide and lincosamide resistance, quinolone resistance—*gyrA*, *gyrB*, *parC*, *parE*—all are detectable with genotyping.

Despite obvious benefits, PCR assays are hampered by disadvantages including false positive results from non-specific products such as CSF, blood specimens, contamination with genes from extraneous sources, reliability issues with gene detection versus gene expression, and quality control measures, which is particularly crucial for direct detection from clinical specimens. Real-time PCR is less prone to some of these problems, but false positive results do occur when non-specific products are primed. The importance of quality control accuracy in identifying these products cannot be overstated. However, the outlook for the future is bright for PCR assays and other molecular techniques that reduce the need for empiric therapy, leading to the early, successful treatment of infection.⁸

Limitations and Future direction

The AST methods presented represent those commonly practiced by clinical laboratories in the United States. Table 1 compares these methods with a brief description of their advantages, disadvantages and unique features. For more detailed information, refer to *The Clinical Manual of Microbiology* and the CLSI performance standards. Other less commonly performed methods are described in the Manual with references to their commercial sources. These methods include macro-broth dilution, semi-automated instrumentation for disk diffusion and broth microdilution and manual broth microdilution systems. Also not detailed are a plethora of PCR assays, other amplification methods and an array of product detection techniques such as electrophoresis and mass spectrometry, with some capable of producing quantitative results. Manufacturers Gen-Probe (the

another advantage of PCR based assays.⁸ TIGRIS system), Roche (AmpliPrep-COBAS TaqMan system), Abbott (m2000 system) and BD Diagnostics (Viper System) have developed completely automated instruments that perform sample processing, nucleic acid extraction, amplification and product detection.^{8,9}

Future genetic technology will incorporate advanced methods such as ultra-deep sequencing that allows correction of treatment failure associated with HIV, Hepatitis B and failed therapy resulting from biofilm conditions (e.g. chronic diabetes foot ulcers or pressure ulcers). Genetic techniques will provide essential additions, if not replacements, for some phenotypic methods; thereby decreasing the present reliability on empiric therapy.⁹

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