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Current Concepts in Laboratory Testing to Guide Antimicrobial Therapy

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CME Activity

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Abstract

Antimicrobial susceptibility testing (AST) is indicated for pathogens contributing to an infectious process that warrants antimicrobial therapy if susceptibility to antimicrobials cannot be predicted reliably based on knowledge of their identity. Such tests are most frequently used when the etiologic agents are members of species capable of demonstrating resistance to commonly prescribed antibiotics. Some organisms have predictable susceptibility to antimicrobial agents (ie, *Streptococcus pyogenes* to penicillin), and empirical therapy for these organisms is typically used. Therefore, AST for such pathogens is seldom required or performed. In addition, AST is valuable in evaluating the activity of new and experimental compounds and investigating the epidemiology of antimicrobial resistant pathogens. Several laboratory methods are available to characterize the in vitro susceptibility of bacteria to antimicrobial agents. When the nature of the infection is unclear and the culture yields mixed growth or usual microbiota (wherein the isolates usually bear little relationship to the actual infectious process), AST is usually unnecessary and results may, in fact, be dangerously misleading. Phenotypic methods for detection of specific antimicrobial resistance mechanisms are increasingly being used to complement AST (ie, inducible clindamycin resistance among several gram-positive bacteria) and to provide clinicians with preliminary direction for antibiotic selection pending results generated from standardized AST (ie, β-lactamase tests). In addition, molecular methods are being developed and incorporated by microbiology laboratories into resistance detection algorithms for rapid, sensitive assessment of carriage states of epidemiologically and clinically important pathogens, often directly from clinical specimens (ie, presence of vancomycin-resistant enterococci in fecal specimens).

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Assessment of the antimicrobial susceptibility patterns of significant bacterial isolates is among the primary responsibilities of the clinical microbiology laboratory. From a practical perspective, clinicians often perceive such test results to be at least as important as determination of the etiologic agents of patients' infections. In the face of ever-escalating antimi-

crobial resistance and the frequent need for treatment with newer, often more expensive antibiotics, antibacterial susceptibility testing (AST) results take on an increasingly important role.[1]

DILUTION METHODS FOR AST

Agar and broth dilution methods may be used to determine the minimum concentrations of antibiotics that are required to inhibit or kill microorganisms. Drugs under study are typically tested at 2-fold doubling ($\log_2$) serial dilutions (eg, 4, 8, 16 μg/mL, and so on), with the lowest concentration of each antibiotic that inhibits visible growth of organisms designated as the minimum inhibitory concentration (MIC). In the United States, results are usually reported in micrograms per milliliter, whereas in some other parts of the world results may be reported in milligrams per liter. The concentration ranges tested vary with the antimicrobial agent, the pathogen under study, and the infection site. Ranges should encompass the concentrations used to define the interpretive categories (susceptible, intermediate, and resistant) of the antimicrobial agent and the ranges of expected MICs for reference quality control organisms. Alternative dilution methods may include only a single or a selected few concentrations of antibiotics, such as breakpoint AST and single-drug concentration screening. Dilution methods offer flexibility in that the standard Mueller-Hinton medium used for the testing of frequently encountered pathogens (eg, members of the family Enterobacteriaceae, staphylococci, enterococci, and some nonfermentative gram-negative bacilli, such as *Acinetobacter baumannii* and *Pseudomonas aeruginosa*) may be supplemented or, in some cases, replaced by another medium, allowing for accurate testing of many fastidious organisms for which standardized methods are not available for reliable disk diffusion testing. Dilution methods are also amenable for use in automated antibiotic susceptibility testing systems.

Breakpoints derived by regulatory bodies and professional groups are frequently similar. For example, there are relatively small numbers of discordant breakpoints between the US Food and Drug Administration (FDA) and the Clinical and Laboratory Standards Institute (CLSI), and those discrepancies are under active review by both organizations. By comparison, there are sometimes sizable differences in the interpretive criteria used in different countries or regions of the world for the same antibiotics. Such disparities are sometimes a function of the fact that different dosages and/or administration intervals are used for the same antimicrobial agents. In addition, some breakpoint-setting organizations are more conservative than others in assessing susceptibility to anti-infectives, placing more emphasis on detection of emerging resistance based on examination of microorganism population distributions. Technical factors, including incubation temperature and atmosphere, inoculum size, and test medium formulation, can also affect zone diameters and MICs, justifying different breakpoints.

Agar Dilution Method

Mueller-Hinton agar is the medium recommended for routine testing of most rapidly growing aerobic and facultatively anaerobic bacterial pathogens. The solvents and diluents that are required to prepare stock solutions of antibiotics and the methods used to perform such testing are defined in the CLSI standard on dilution AST.[2] The agar dilution approach to susceptibility testing is both well standardized and reproducible and may be used as a reference method in the evaluation of other dilution assays. This method facilitates the concomitant and efficient testing of large numbers of organisms. In addition, population heterogeneity (ie, resistant subpopulations of organisms) and inoculum contamination (ie, "mixed" cultures) are more easily detected by agar than by broth testing. The primary disadvantages of this testing approach are the labor-intensive, time-consuming steps required to prepare testing plates, particularly when the number of compounds to be tested is high or when only a limited number of bacteria are to be studied, or both. For these reasons, most clinical microbiology laboratories do not use this approach for routine AST.

Broth Dilution Methods

General approaches to broth dilution testing include both macrodilution, wherein volumes of broth in test tubes for each dilution typically equal or exceed 1 mL, and broth microdilution (BMD), in which antimicrobial concentrations are most frequently of smaller volumes in 96-well microtiter plates. The broth macrodilution approach is both reliable and well standardized and is of particular utility in research studies and in testing of a single antimicrobial agent for 1 bacterial isolate. The method is, however, both laborious and time intensive and, because of the ready commercial availability of convenient microdilution systems, is not generally considered practical for routine use in clinical microbiology laboratories.

The convenience afforded by BMD has led to its widespread use in both clinical and reference laboratories. This approach is, in fact, now considered the reference international testing method.[3] Plastic, disposable plates containing a panel of several antibiotics to be tested concomitantly may be prepared

within the laboratory or, alternatively, purchased from commercial vendors either as freeze-dried or frozen trays. The BMD technique is also well standardized and reliable. The inoculation and reading procedures readily lend themselves to the simultaneous testing of several antibiotics with single bacterial isolates. Although most commercially available systems use multipoint inoculating devices or automated inoculation instruments, plates may also be inoculated with multichannel pipettors. Testing results may be determined either visually or through the use of semiautomated or automated instruments. An example of a commercially available manual BMD is Sensititre (TREK Diagnostic Systems, Cleveland, OH). Examples of automated BMD platforms include the BD Phoenix (Becton Dickinson, Franklin Lakes, NJ), Microscan (Siemens Healthcare Diagnostics, Deerfield, IL), and Vitek (bioMérieux, Marcy l'Etoile, France).

AGAR DISK DIFFUSION TESTING

In many clinical microbiology laboratories an agar disk diffusion method is routinely used for the testing of common, rapidly growing, and some fastidious bacterial pathogens, allowing categorization of most such isolates as susceptible, intermediate, or resistant to a wide range of antimicrobial agents. This approach is particularly common in resource-limited settings and when performed according to standardized methods, such as those published by the CLSI, provides accurate direction to clinicians making therapeutic antibiotic decisions.[4] With this testing approach, commercially prepared filter paper disks impregnated with specified predetermined concentrations of the antibiotics to be assessed are applied to the surface of a defined agar medium previously inoculated with the challenge bacterial pathogen. The antimicrobial agents then diffuse from the disks through the agar, and as the distance from the disks increases, the drug concentrations decrease in a logarithmic fashion, creating gradients of drug concentrations in the medium around the disks. Simultaneously with the diffusion of the drugs, the bacteria inoculated to the agar surface not inhibited by the concentrations of the antibiotics in the agar multiply, creating a visible lawn of growth. In areas where the test organism is inhibited by the antimicrobial agents, growth fails to occur, resulting in zones of inhibition around each active drug. The inhibitory zone diameters are influenced by the diffusion rates of the various antimicrobial agents through the agar, a function of the molecular sizes and hydrophilicities of the compounds. The zone sizes are inversely proportional to the logarithms of the antibiotic MICs. After incubation at recommended temperatures, atmospheric conditions, and times, depending on the pathogen under study, the diameters of the zones of inhibition are measured in millimeters and interpreted based on published standards. The most recent criteria for interpreting zone diameters of inhibition for antibiotics approved for use by the FDA are listed in Table 3 of a document updated annually by the CLSI.[5]

Such disk diffusion interpretive criteria (breakpoints) are chosen after the establishment of MIC breakpoints, which is accomplished by plotting the inhibition zone diameters against the MICs derived from the testing of a large number of strains of various species. A statistical approach using a linear regression formula may be used to calculate the appropriate zone diameter intercepts for previously determined MIC breakpoints. An alternative, practical approach to deriving disk diffusion interpretive criteria is the use of the error rate–bounded method by which the zone diameter breakpoints are selected based on the minimization of disk interpretive errors, particularly very major errors.[6,7] This most recent CLSI approach focuses on the rate of interpretive errors near the proposed breakpoint vs error rates for MICs greater than a single $\log_2$ dilution from the MIC breakpoints.[8] The concept of this approach is that errors occurring with organisms for which MICs closely approximate the MIC breakpoints are less of a clinical concern than errors for more highly susceptible or resistant isolates.

The disk diffusion approach for AST has been standardized primarily for commonly encountered, rapidly growing bacterial pathogens and is applicable to neither anaerobes nor fastidious species that demonstrate marked variability in growth rate from strain to strain.[9] The disk diffusion test approach has been modified, though, to allow for reliable testing of several species of fastidious bacteria, including *Haemophilus influenzae* and *Neisseria gonorrhoeae*. There are several advantages to the disk diffusion approach to AST, including the following: (1) it is technically easy to perform and results are reproducible, (2) the reagents and supplies are inexpensive, (3) it does not require the use of expensive equipment, (4) it generates categorical interpretive results well understood by clinicians, and (5) it allows for considerable flexibility in the selection of antibiotics for testing. However, this method also has a number of drawbacks. For example, only a limited number of bacterial species can be tested using this approach. In addition, the disk diffusion test is inadequate for detection of vancomycin-intermediate *Staphylococcus aureus*. Of importance, it provides only a qualitative result, whereas a quantitative MIC result that indicates the degree of susceptibility may in some cases be required (eg, when prolonged or continuous infusion of specific antimicrobial agents is being considered for treatment of infections caused by relatively resistant bacteria).

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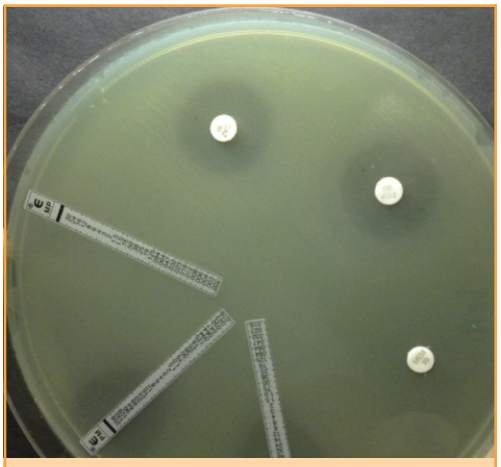

FIGURE. Manual susceptibility testing of *Pseudomonas aeruginosa* by Etest and disk diffusion. Various patterns of susceptibility and resistance are seen.

GRADIENT DIFFUSION METHODS

The M.I.C.Evaluator (Oxoid, Cambridge, UK) and Etest (bioMérieux, Durham, NC), are commercially available gradient diffusion systems for quantitative AST. Both systems use preformed antimicrobial gradients applied to 1 face of a plastic strip to generate diffusion of drug into an agar-based medium. The assays are performed in a manner similar to that for disk diffusion using a suspension of test organism equivalent in turbidity to that of a 0.5 McFarland standard to inoculate the surface of an agar plate. Following recommendations for incubation temperatures, times, and atmospheric conditions, the MIC is read directly from a preprinted scale on the top of the strip at the point at which the ellipse of organism growth inhibition intercepts the strip. An example of an isolate tested for antimicrobial susceptibility by both Etest and disk diffusion is shown in the Figure. Several strips containing different antimicrobial agents may be applied in a radial arrangement to the surface of large round plates, or they may be placed in opposite directions on large rectangular plates. Minimum inhibitory concentrations generated by these methods generally agree well with those obtained using standard agar and broth dilution methods.[10-12] Such gradient methods are similar in flexibility and simplicity to the disk diffusion approach, but they enable one to determine quantitative MICs. Significant advantages of the agar gradient diffusion systems include the ability to generate quantitative MIC results for infrequently tested antimicrobial agents and the option to test fastidious and anaerobic organisms, for which reliable disk diffusion methods and/or commercial systems are not available, through the use of specific enriched media. Gradient diffusion strips are, however, considerably more expensive than the paper disks used for diffusion testing.

METHODS FOR FASTIDIOUS BACTERIA

Many fastidious bacterial species do not grow satisfactorily using standard in vitro susceptibility testing approaches with unsupplemented media. For several of the more frequently encountered pathogens (eg, *Streptococcus pneumoniae* and *Streptococcus* spp other than *S pneumoniae*, *N gonorrhoeae*, and *Neisseria meningitidis*, and *H influenzae* and *Haemophilus parainfluenzae*), modifications have been made to the standard CLSI MIC and disk diffusion methods to allow laboratories to perform reliable AST. Such modifications typically involve the use of test media with supplemental nutrients, prolonged incubation times, and/or incubation in an atmosphere with an increased concentration of carbon dioxide. Specific MIC and zone diameter breakpoints have been established by the CLSI for such organisms, as have recommended acceptable ranges for the testing of applicable quality control strains. The CLSI has also published guidelines for AST of the fastidious and/or infrequently recovered bacteria listed in the Table.[13] Methods for the standardized testing of potential agents of bioterrorism (eg, *Bacillus anthracis, Francisella tularensis, Brucella* spp, *Yersinia pestis*, and *Burkholderia pseudomallei*) have also been developed, and specific conditions for their testing are defined in Table 2 of the CLSI M45-A2 document.[13] Currently, specific recommendations for several other fastidious bacteria, including *Legionella* and *Bordetella* spp, do not exist, partially because infections caused by these species typically respond well to the recommended drugs of choice, they are relatively uncommon isolates in clinical laboratories, and they require special complex media for recovery in vitro, presenting unique problems in the development of AST assays.

For the most part, breakpoints for these bacteria were predicated on interpretive criteria established for other organisms as published in CLSI standards and adapted based on published literature and the experience of the authors of the document. This is in sharp contrast to the extensive body of clinical microbiologic, pharmacokinetic, and pharmacodynamic information typically used for the establishment of breakpoints as published in other CLSI standards. Because of the limited testing and nature of the potential agents of bioterrorism and *Helicobacter pylori*, the interpretive recommendations and testing approaches for these organisms were recently transferred from the standard CLSI M100 documents to the M45 guideline.[13]

As mentioned, in addition to standard disk and MIC methods, many species of fastidious bacteria

TABLE. Clinical and Standards Laboratory Institute Published Guidelines for Antimicrobial Susceptibility Testing of Fastidious and/or Infrequently Recovered Bacteria

Abiotrophia and *Granulicatella* spp (once referred to as thiol-dependent, pyridoxal-dependent, or nutritionally variant streptococci)
Aeromonas spp
Bacillus spp other than *Bacillus anthracis*
Campylobacter coli and *Campylobacter jejuni*
Corynebacterium spp
Erysipelothrix rhusiopathiae
The group of bacteria previously referred to as the HACEK organisms (*Aggregatibacter actinomycetemcomitans, Aggregatibacter aphrophilus*, *Cardiobacterium hominis*, *Eikenella corrodens*, and *Kingella kingae*)
Facultatively anaerobic *Lactobacillus* spp
Leuconostoc spp
Listeria monocytogenes
Moraxella catarrhalis
Pasteurella spp
Pediococcus spp
Plesiomonas shigelloides

may be tested by a gradient agar technique. The Etest method permits placement of strips on media optimal for the growth of the organism being tested and allows the use of various incubation conditions. A major limitation, however, to such an approach is lack of approval for such testing by the FDA. When FDA clearance has not been awarded, the results of such testing should be interpreted with caution, and an applicable qualifying comment should be an integral component of any resultant patient report.

SUSCEPTIBILITY TESTING OF ANAEROBIC BACTERIA

The importance of anaerobic bacteria as participants in and causes of significant infections and the need for specific antibiotic therapy for bacteremia and surgical prophylaxis against anaerobes are well documented.[14-19] As a rule, AST is considered a necessity for effective guidance of antibiotic therapy, but how and when susceptibility testing of anaerobic bacteria should be performed have been topics of debate, in part owing to a number of misconceptions and confounding factors.[20-24] Specimens collected from infections in which anaerobes are involved are typically polymicrobic, rendering isolation and identification of individual organisms slow and the results of AST too delayed to have a consistent positive effect on individual patient outcomes. For clinicians, the combination of surgical intervention and broad-spectrum antibiotics has limited the correlation of potential antibacterial resistance with outcome, directing many clinical microbiology laboratories away from the routine performance of anaerobic susceptibility testing. There is considerable evidence, though, that antibiotic resistance is common among many anaerobic species and that patient treatment with inactive agents results in poor clinical responses and increased rates of mortality.[14,16,19,25,26] Results of AST have also indicated that substantial differences exist in resistance patterns among hospitals on a local, regional, and national basis, suggesting that one medical center's patterns may not be applicable to those of other facilities.[27-30] Therefore, the need for anaerobic AST is of far more importance today than in the past.

If practical, individual hospitals should establish antibiograms for the more frequently recovered anaerobes on a periodic basis and test individual patient isolates as needed to assist in patient care. For purposes of presenting cumulative antimicrobial susceptibility data, an attempt should be made to include the results from 80 to 100 anaerobic isolates with recognized important resistance mechanisms (eg, clindamycin resistance among members of the *Bacteroides fragilis* group). Ideally, following CLSI guidelines for preparation of antibiograms, 30 isolates for each genus or species should be included.[31] When this is not possible, an effort should be made to present data for 30 isolates from the *B fragilis* group and at least 10 strains for other genera. Antibiotics in the report should reflect the hospital formulary. A recent CLSI document included an antibiogram for members of the *B fragilis* group generated from the results of testing of isolates collected at many health care facilities across the United States by 3 reference laboratories.[32] Clinicians

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may refer to this document when prescribing empirical therapy for suspected or proven *B fragilis* group infections in settings in which anaerobic AST is not available.

To assist in management, anaerobic susceptibility testing should be performed when (1) the selection of an antibiotic to which the isolate is susceptible is critical for treatment of the patient, (2) long-term therapy is under consideration, (3) anaerobes are recovered from specific, usually sterile, body sites (eg, bone, blood, joint, or brain), or (4) treatment with an antimicrobial agent typically active against the organism has failed.

The agar dilution susceptibility testing approach, which uses *Brucella* blood agar as the medium, has been designated the reference method by the CLSI anaerobe working group.[33] Because of the time-consuming, labor-intensive nature of this method, it is not generally considered practical for routine use in most clinical microbiology laboratories but serves as the reference method to which other more practical testing approaches can be compared. Alternative testing methods currently used include BMD (only standardized for members of the *B fragilis* group), limited agar dilution, and gradient strip diffusion assays, such as Etest. Disk diffusion and broth disk elution testing should not be used because the results generated from such methods do not correlate with the CLSI reference agar dilution method.[33] Although of limited use, β-lactamase testing may be of value for some organisms if therapy with ampicillin or penicillin is being considered.

METHODS FOR SUSCEPTIBILITY TESTING OF *NOCARDIA* SPP AND OTHER AEROBIC ACTINOMYCETES

Susceptibility testing of *Nocardia* spp and other aerobic actinomycetes (*Rhodococcus* spp, *Streptomyces* spp, *Gordonia* spp, and *Tsukamurella* spp) should be performed on clinically significant isolates. Susceptibility testing results serve to guide initial therapeutic choices and may document emergence of drug resistance. No commercially available broth systems have yet been cleared by the FDA for *Nocardia* spp or other aerobic actinomycetes. The CLSI BMD is the reference method for testing.[34] Recommended drugs for primary testing are amikacin, amoxicillin-clavulanate, ceftriaxone, ciprofloxacin, clarithromycin, imipenem, linezolid, minocycline, moxifloxacin, trimethoprim-sulfamethoxazole, and tobramycin. Second-line drugs for testing include cefepime, cefotaxime, and doxycycline. Vancomycin and rifampin results should also be reported on isolates of *Rhodococcus equi* because these drugs are particularly useful therapeutically. Microbiological breakpoints were established in 2003 and are available for *Nocardia* spp only.[34] When aerobic actinomycetes other than *Nocardia* spp are tested, the susceptibility categories should be listed as tentative. The breakpoints for *S aureus* should be adapted as provided in the current CLSI M100 document (M100-S21) for *R equi*, but they should also be reported as tentative. Susceptibility testing results for most aerobic actinomycetes are available within 3 to 5 days, whereas results for *R equi* and some isolates of *Tsukamurella* spp may be read at 24 or 48 hours.

Challenges in the identification and AST of *Nocardia* spp have become apparent within the past 10 years. Before the year 2000, conventional phenotypic methods were largely used to identify *Nocardia* spp; however, molecular identification is now the preferred means of reliable identification to the species level.[35] Both 16s ribosomal RNA sequencing and matrix-assisted laser desorption/ionization-time of flight are among the techniques currently used, but limitations exist.[36] Because routine molecular testing is difficult to implement in many clinical microbiology laboratories, isolates typically must be sent to a reference laboratory for identification. To address this issue, Wallace et al[37] proposed a series of various susceptibility patterns that could predict placement of *Nocardia* spp within particular groups, but, as new strains are identified, these groupings have not proved to be valid, particularly for the *Nocardia nova* complex.

The method for susceptibility testing of *Nocardia* spp also presents a challenge to many clinical microbiology laboratories because BMD is somewhat impractical owing to cost, availability of supplies, and expertise needed to perform and interpret the results. Moreover, false resistance with BMD has been noted for ceftriaxone when testing *Nocardia brasiliensis* and imipenem when testing *Nocardia farcinica*.[34] In addition, sulfonamide results are inconsistent when using BMD; for this compound, disk diffusion testing with sulfisoxazole may be performed concurrently. Investigators have evaluated use of the Etest method compared with BMD, with varying results.[38] However, both lack of agreement with BMD and the absence of standardized methods have restricted routine use of the Etest. The CLSI has proposed that the agar proportion method be used to confirm questionable results from commercial broth systems and to test additional antibiotics or concentrations of drugs.

The major taxonomic revisions within the last 10 years have further complicated testing because clinical and epidemiological differences exist among species. Furthermore, resistance to trimethoprim-sulfamethoxazole and other antimicrobials is increasing among various species; thus,

identification of isolates to the species level remains imperative.[39]

METHODS FOR SUSCEPTIBILITY TESTING OF MYCOBACTERIA

According to the most recent Centers for Disease Control and Prevention mycobacterial susceptibility testing guidelines, initial isolates from patients with tuberculosis should be tested for susceptibility to isoniazid, rifampin, ethambutol, and pyrazinamide.[40] This guidance overrides the prior practice of performing susceptibility testing for only 3 drugs (isoniazid, rifampin, and ethambutol) and then only when a pulmonary or infectious disease clinician requested it. Current guidelines also state that susceptibility testing should be repeated after 3 months if the patient remains culture-positive despite appropriate therapy. However, susceptibility testing may be performed earlier if the patient appears to be failing to respond to therapy or if intolerance to the drug regimen is evident. First-line susceptibility test results should be available for isolates of the *Mycobacterium tuberculosis* complex within 15 to 30 days of original receipt of the specimen in the laboratory.[41] However, ideally, susceptibility results should be available within 7 to 14 days of specimen receipt.[34] If resistance to any of the 4 initially tested agents is discovered, testing of secondary drugs should be performed as soon as possible. If the isolate is resistant only to pyrazinamide, *Mycobacterium bovis* should be ruled out because most *M tuberculosis* isolates are susceptible to pyrazinamide. Further specific guidelines regarding secondary drug testing and follow-up are outlined in CLSI M24-A2.[34]

The agar proportion approach has traditionally been considered the standard method for antimycobacterial susceptibility testing, but as an agar-based system, the time to result reporting is lengthy (approximately 3 weeks).[42] Both the agar proportion method and the radiometric method define resistance as growth of more than 1% of the inoculum of bacterial cells in the presence of an antitubercular drug. The antitubercular drugs are inoculated at specific in vitro concentrations, the values of which correlate to clinical responsiveness. If more than 1% of the bacterial population grows in the presence of a drug, that particular drug will not be of therapeutic utility.[42] The agar proportion method is used primarily to confirm results from commercial liquid broth systems and to test additional drugs that may not be available for testing using other systems. The FDA-cleared broth systems for *M tuberculosis* testing have shorter incubation times than the agar proportion method; however, these commercial testing systems are only cleared for certain drugs.[43]

There are several molecular assays that have been developed for the detection of mutations associated with drug resistance in *M tuberculosis*, including both real-time polymerase chain reaction (PCR) methods and line probe assays.[44] Molecular-based methods for detection of *M tuberculosis* drug resistance are more rapid than traditional methods of susceptibility testing. Mutations associated with resistance to isoniazid or rifampin are typically detected by such methods. These tests may be run using growth from positive cultures but may also be performed directly on acid-fast smear-positive specimens if the patient is highly suspected of having drug-resistant tuberculosis. It is currently suggested that molecular testing of drug susceptibility be backed up by culture, with performance of phenotypic culture-based drug susceptibility testing when the isolate is retrieved from culture.[34]

Susceptibility testing of nontuberculous mycobacteria (NTM) should be performed on isolates considered clinically significant. The American Thoracic Society criteria for clinical significance of NTM are positive cultures from at least 2 sputum specimens or 1 bronchial wash or bronchial lavage specimen. Alternatively, a transbronchial or lung biopsy with histopathologic findings consistent with mycobacteria and positive on culture for NTM is sufficient to be interpreted as clinically significant. In addition, NTM isolates from usually sterile body sites, such as cerebrospinal fluid, are considered clinically significant. However, routine susceptibility testing need not be performed on *Mycobacterium marinum* because acquired resistance is uncommon with this organism.[34] Initial susceptibility testing of *Mycobacterium kansasii* to isoniazid, rifampin, and ethambutol need not be performed but can be offered if treatment has failed.

The standard susceptibility testing method for NTM is BMD. The American Thoracic Society and CLSI guidelines exist for susceptibility testing of members of the *Mycobacterium avium* complex, *M kansasii*, *M marinum*, and the rapidly growing mycobacteria.[34] However, accurate susceptibility predictions for other slowly growing mycobacteria cannot be made. The macrolides are the only antimicrobial agents that should be tested against *M avium* complex because they are the only agents for which correlations have been demonstrated between in vitro susceptibility tests and clinical response.[45] Because the mutation leading to resistance is the same for clarithromycin and azithromycin, only 1 drug need be tested. Generally, clarithromycin is tested because azithromycin demonstrates poor solubility. If the isolate is macrolide resistant, testing for susceptibility to the secondary agents moxifloxacin and linezolid may be considered. Susceptibility testing of *M avium* complex may also be performed if the patient

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relapsed while undergoing macrolide therapy. In addition, susceptibility testing should be repeated after 3 months of therapy for patients with disseminated *M avium* complex disease and after 6 months of therapy for patients with chronic pulmonary disease caused by *M avium* complex. Commercially available broth systems have not yet been cleared by the FDA for slowly growing NTM.

PHENOTYPIC AND GENOTYPIC METHODS FOR DETECTION OF ANTIMICROBIAL RESISTANCE

A number of phenotypic tests are available to the clinical microbiology laboratory to characterize a pathogen's susceptibility to an antibiotic by screening for a specific resistance mechanism or phenotype. Although such screening tests do not result in determination of an MIC, some have sufficient specificity and sensitivity that confirmatory testing is not required and the screening test result can be reported without further testing. Other assays require additional or confirmatory testing. For example, tests for inducible clindamycin resistance among staphylococci and screening tests for high-level gentamicin and streptomycin resistance in enterococci are generally considered to be comparable to standard methods for the detection of clinically significant resistance, and they do not require confirmatory testing. By comparison, laboratories that use ertapenem resistance as a surrogate marker for carbapenemase production among certain species of Enterobacteriaceae must confirm resistance to meropenem, imipenem, or doripenem by another more standardized approach because ertapenem-resistant strains are not always resistant to these other agents.

In addition, methods for the direct detection of antibiotic-resistant bacteria in clinical samples have progressed rapidly in recent years, largely because of the continued evolution and spread of multidrug-resistant pathogens, such as methicillin-resistant *S aureus* (MRSA). The development of commercial assays that facilitate rapid detection of such pathogens directly from clinical specimens, often generating results in a few hours or less, has positively enhanced surveillance efforts and patient management. Other genotypic assays for detection of specific antimicrobial resistance genes in gram-negative bacteria (eg, bla_{KPC}-containing *Klebsiella pneumoniae*) have the potential to improve therapeutic patient decisions and assist in epidemiological investigations of resistance gene dissemination in the hospital and community setting.

β-LACTAMASE TESTS

A positive β-lactamase test result indicates that the organism is resistant to applicable β-lactam agents, but a negative reaction is inconclusive because other mechanisms of resistance to the β-lactams may exist. For example, a positive β-lactamase test result for a strain of *N gonorrhoeae* means that the isolate is resistant to penicillin, ampicillin, and amoxicillin and that these drugs would not be appropriate therapeutic choices. However, a β-lactamase test only detects one form of penicillin resistance in *N gonorrhoeae*. Strains with chromosomally mediated resistance with penicillin-binding protein modifications can only be detected by the disk diffusion or the agar dilution MIC method.[46,47]

Three direct β-lactamase tests—the acidometric, iodometric, and chromogenic methods—have been widely used. All 3 methods involve the testing of isolates grown on nonselective media, and results are typically available within 1 to 60 minutes. Although some bacteria (eg, *N gonorrhoeae* and *H influenzae*) produce β-lactamase constitutively, others (eg, staphylococci) may produce detectable levels of enzyme only after exposure to an inducing agent, typically a β-lactam.[48] Even after induction, though, direct β-lactamase tests may not be sufficiently sensitive to detect β-lactamase production in all staphylococci.[49] Therefore, for serious infections that require penicillin therapy, clinical microbiology laboratories should perform both MIC and induced β-lactamase tests on all subsequent isolates from the same patient. In addition, PCR testing of the isolate for the presence of the *blaZ* β-lactamase gene may also be an option.

DETECTION OF METHICILLIN RESISTANCE IN *STAPHYLOCOCCUS* SPP

The most common currently used method for the detection of MRSA is culture.[50] Traditional MRSA detection methods consist of culture from a selective liquid or solid medium. Recently, chromogenic agars have shown improved sensitivity and specificity over nonchromogenic media for detection of MRSA.[51] Chromogenic agars contain selective antibiotic(s) and various chromogenic substrates, which provide easy visual identification of colonies. An additional advantage of chromogenic agar is the faster time to detection of the organism. MRSA is often detected within 20 to 48 hours on chromogenic media, with a high percentage of cases identified within 24 hours.[52] Several chromogenic media are available from different manufacturers.

The use of an overnight preenrichment step with selective broth medium before inoculation of the agar has been shown to increase sensitivity of the testing by 15% to 30%.[53] However, the delay in detection of an additional 18 to 24 hours and the cost of the selective broth medium represent disadvantages.

Early diagnosis of MRSA in the laboratory is crucial in guiding appropriate antimicrobial therapy. Rapid molecular methods of MRSA detection are increasingly used and are available for testing from a variety of sources.[54] Traditional detection of MRSA from automated blood culture instruments is time-consuming because 24 to 72 hours are required for subculture, biochemical identification, and AST of the isolate once the result turns positive. Several techniques are available for identification of MRSA directly from blood culture bottles that have flagged as positive for growth when gram-positive cocci in clusters are seen on Gram stain.[55] Several commercial molecular methods are available, including nucleic acid amplification and hybridization assays.[51] A commercial penicillin-binding protein 2a latex agglutination kit, which facilitates the detection of the protein product expressed by the *mec*A gene, is available for detection of MRSA directly from blood culture.[56] Other systems are currently under investigation.[50]

HIGH-LEVEL AMINOGLYCOSIDE RESISTANCE IN ENTEROCOCCI

Successful treatment of enterococcal endocarditis and other serious enterococcal infections requires the use of an aminoglycoside with a cell wall–active agent, such as ampicillin, penicillin, or vancomycin. All enterococci demonstrate innate low-level resistance to aminoglycosides because of their facultative anaerobic metabolism, which reduces transmembrane potential, thereby limiting drug uptake. However, the bactericidal combination of an aminoglycoside and a cell wall–active antimicrobial, which allows for markedly enhanced uptake of the aminoglycoside, leads to enhanced killing of the organism in the absence of high-level aminoglycoside resistance (HLAR).

The CLSI recommends HLAR screening of enterococci with both gentamicin and streptomycin from blood cultures or other specimens submitted for the evaluation of endocarditis, such as heart valve tissue. The BMD may be performed by assessing growth of the organism in the presence of 1000 μg/mL of streptomycin or 500 μg/mL of gentamicin in brain heart infusion broth.[5] The recommended screening concentrations for streptomycin and gentamicin using other methods are also covered.[57] Performance data of commercially available media and systems for HLAR screening have been reviewed.[58]

In enterococci, HLAR is mediated by aminoglycoside-modifying enzymes (AMEs), which modify the aminoglycoside by acetylation, adenylation, or phosphorylation. The most prevalent AME gene among enterococci with HLAR to gentamicin is *aac(6')-Ie-aph(2")-Ia*, which has both acetyltransferase and phosphotransferase activity.[59] This common AME confers resistance to all available aminoglycosides, except streptomycin. Commonly, HLAR to streptomycin is mediated by either *ant(6')-Ia* or *ant(3")-Ia*; in such cases, streptomycin should not be used in combination with a β-lactam agent. Detection of HLAR to both gentamicin and streptomycin precludes the use of aminoglycosides for synergism in any clinical situation.

Enterococcus faecium possesses a naturally occurring AME, resulting in moderate resistance to tobramycin (MICs, 64-1000 μg/mL). The presence of the *aac(6')-Ii* gene precludes synergistic treatment with tobramycin, kanamycin, netilmicin, or sisomicin. In addition, many enterococci possess the *aph(3')-IIIa* gene, which confers high-level resistance to kanamycin and abolishes any synergistic effect with amikacin. Thus, gentamicin and streptomycin are the only 2 aminoglycosides to test and consider for synergistic therapy. Novel genes carrying AMEs mediating resistance to gentamicin, such as *aph(2")-Ib*, *aph(2")-Ic*, *aph(2")-Id*, and *aph(2")-Ie*, have been discovered and may further complicate HLAR testing because the susceptibilities to various aminoglycosides differ.[60] Because of the large numbers of AME-encoding genes, molecular HLAR screening in enterococci remains investigational and has not yet been widely available in clinical microbiology laboratories.[61]

Issues that continue to challenge HLAR screening in enterococci include the isolation of multiple AMEs within single enterococcal isolates, isolates that harbor infrequent AMEs but do not demonstrate the HLAR phenotype, and the increasing prevalence of various HLAR enzymes in enterococci.

DETECTION OF VANCOMYCIN-RESISTANT ENTEROCOCCI

The vancomycin-resistant enterococci (VRE) are important opportunistic pathogens in many health care facilities and common colonizers of the gastrointestinal tract. The VRE are among the most common causes of hospital-acquired infections in the United States, and patients colonized with VRE in the gastrointestinal tract may serve as reservoirs for nosocomial transmission.[62,63]

Two common patterns of enterococcal resistance exist, both of which result in elevated vancomycin MICs. The first, and most clinically important, is vancomycin resistance due to acquisition of genetic information, usually on a plasmid or other transmissible genetic element.[64] This acquired trait is most commonly observed in strains of *E faecium* and *Enterococcus faecalis* harboring the *vanA* or *vanB* genes that encode for high-level vancomycin resistance. Expression of the *vanA* gene results in elevated vancomycin MICs (>128 μg/mL) and is the

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dominant resistance factor in enterococci. By comparison, expression of the *vanB* gene results in lower vancomycin MICs, typically in the range of 16 to 64 μg/mL. The second pattern of resistance, intrinsic (inherent) in nature, is characteristically seen in *Enterococcus gallinarum* and *Enterococcus casseliflavus*. Most frequently encoded by *vanC*, this pattern of resistance may also be due to the expression of genes other than *vanC* (eg, *vanE*, *vanG*, and *vanL*) and results in either low-level resistant or intermediate MICs, typically in the 2- to 16-μg/mL range.[65] Contact precautions are generally only required for patients harboring enterococci with acquired resistance, such as *E faecium* or *E faecalis*. Guidelines for susceptibility testing of enterococcal pathogens when grown in culture from blood or other sites have remained fairly standard over time. However, there have been several recent advances in both culture-based and molecular-based screening of stool for VRE, facilitating identification of patients who are potential reservoirs for infection and transmission.

The CLSI guidelines for vancomycin susceptibility testing of enterococci isolated from various sites suggest the use of standard BMD or disk diffusion testing.[5] If disk diffusion or Etest is performed, the susceptibility plates must be held for a total of 24 hours to obtain accurate readings. Organisms for which vancomycin MICs are in the range of 8 to 16 μg/mL should be further identified by biochemical testing because infection control precautions will differ based on identification of the organism as an *Enterococcus* sp other than *E faecalis* or *E faecium*. Accordingly, isolates with intermediate zones on disk testing should be tested by an MIC method and/or further identified by biochemical and other identification tests to guide appropriate infection control practices.

A variety of culture-based and molecular methods have been studied to support active surveillance efforts to identify VRE from the gastrointestinal tract.[66,67] Although culture-based methods are not as rapid as molecular-based screening methods, isolates obtained from culture can be stored for further study or identification. Molecular-based VRE screening methods decrease the time to identification but are costly. Culture remains the screening method of choice for VRE stool screening, but molecular methods are becoming increasingly recognized and used. Some investigators advocate the use of a broth enrichment step before inoculation of a culture plate or a molecular assay to increase sensitivity.[66]

Traditional screening agars for VRE from stool specimens include *Campylobacter* medium with 5 antibiotics, including 10 μg/mL of vancomycin, and various types of bile esculin azide agar with vancomycin.[68,69] The traditional VRE screening agars require 24 to 48 hours to identify colonies preliminarily, with additional time required for confirmatory identification and susceptibility testing and up to 5 days to final identification. Various chromogenic VRE media demonstrate adequate sensitivity and specificity, with reduced turnaround time to results through early visual colony identification. There are many selective and differential chromogenic VRE agars that appear promising for use in VRE stool screening. However, performance data vary according to whether prior overnight broth enrichment of the specimen in liquid media was performed.[67]

Real-time PCR is a sensitive and rapid approach to the identification of VRE from gastrointestinal tract specimens.[66] Recently, the BD GeneOhm VanR (BD Diagnostics, Spark, MD) and Xpert *vanA/vanB* (Cepheid, Sunnydale, CA) assays, which detect isolates carrying *vanA* and *vanB* genes within 2 to 4 hours, have been introduced.[70] Most FDA-cleared assays have been marketed for direct detection of VRE from rectal or perianal swabs, although detection from stool specimens has shown comparable results. Some studies have demonstrated improved performance of molecular assays after overnight aerobic or anaerobic preenrichment of stool in broth media; however, the preenrichment step increases the turnaround time to results.[70] Occasionally, lower specificity has been shown with the use of perianal sampling due to the presence of anaerobes that carry the *vanB* gene.[69]

In summary, there are a variety of VRE screening methods available, the most promising of which appear to be chromogenic media and molecular assays, due to rapid result reporting. However, some assays require an overnight preenrichment step to maximize sensitivity.

CLINDAMYCIN RESISTANCE IN STREPTOCOCCI AND STAPHYLOCOCCI

Clindamycin and erythromycin resistance in streptococci may either be due to *erm* genes, which lead to the production of macrolide ribosomal methylases, or to expression of the *mef* gene, which encodes an efflux pump targeting only the macrolides. By comparison, erm enzymes methylate the 23S ribosomal RNA component of the 50S bacterial ribosomal subunit, which causes decreased binding of macrolides, lincosamides (clindamycin), and streptogramin B antibiotics (designated the MLS_B phenotype). In staphylococci, inducible clindamycin resistance is also due to the MLS_B phenotype, but its efflux pump is encoded by the *msrA* gene.

The MLS resistance phenotype can be either induced or constitutively expressed. Inducible clindamycin resistance cannot be detected by routine MIC or disk testing of clindamycin.[71] Therefore, the 14-

and 15-member macrolides, which are better inducers of clindamycin resistance than is clindamycin itself, must be used for induction of clindamycin in the clinical laboratory.[72] Testing of streptococci and staphylococci for inducible clindamycin resistance is important because clindamycin is frequently used to treat staphylococcal, group B streptococcal, and group A streptococcal infections. In addition, resistance to clindamycin is increasing in prevalence, with a recent estimate of 12.8% inducible clindamycin resistance among *S aureus* in the United States.[73] Both inducible and constitutive clindamycin resistance has likewise become increasingly common among β-hemolytic streptococci, with less resistance reported for group A streptococcal than for group B streptococcal infections.[74]

The CLSI has recommended 2 different methods for detection of inducible clindamycin resistance in staphylococci and β-hemolytic streptococci.[5] One method is the disk approximation test, also known as the D-zone test. With this approach, separate erythromycin and clindamycin disks are placed specific distances apart on an agar plate, depending on whether staphylococci or streptococci are being tested. If there is flattening of the zone of inhibition between the 2 disks and the zone resembles the letter "D," the test result is interpreted as positive for induction of clindamycin resistance. The second method suggested by the CLSI is the use of a single-well microdilution test containing both erythromycin and clindamycin. Whenever inducible MLS_B resistance is detected, clindamycin treatment should be avoided, if possible. However, the CLSI states in their guidelines that clindamycin may still be clinically effective in some patients, despite a positive induction test result.

Automated methods for identification of inducible clindamycin resistance have also recently been introduced.[75] Molecular assays using primers for various *erm* genes have been used in various research settings for the purpose of following resistance trends or validating BMD assays.[71] However, PCR assays for inducible clindamycin resistance are not currently considered the standard of care in the clinical microbiology laboratory.

Constitutive or inducible resistance to clindamycin may also be seen in *S pneumoniae* due to expression of a ribosomal methylase encoded by the *ermB* gene. The methylase alters the binding site on the ribosomes for the macrolides and clindamycin, similar to that seen for β-hemolytic streptococci and staphylococci.[76] Clindamycin is recommended as a second- or third-line antibiotic choice for pediatric patients receiving long-term antibiotic therapy for some conditions, such as pneumococcal osteomyelitis and/or joint infections.[77] Because pneumococci are capable of expressing inducible clindamycin resistance, some authors suggest that isolates of *S pneumoniae* be tested for *erm*-mediated resistance in certain clinical situations involving pediatric patients. Jorgensen et al[77] recently assessed performance of the CLSI-suggested disk and BMD methods for detection of inducible clindamycin resistance in pneumococci.

DETECTION OF EXTENDED-SPECTRUM β-LACTAMASE PRODUCTION AMONG ENTEROBACTERIACEAE

Members of Enterobacteriaceae (and other organisms, including *P aeruginosa*) can produce β-lactamases, referred to as extended-spectrum β-lactamases (ESBLs), capable of hydrolyzing penicillins, the monobactam aztreonam, and cephalosporins (including expanded-spectrum cephalosporins, such as cefotaxime, ceftriaxone, ceftizoxime, and ceftazidime).[78] The CLSI guidelines specify screening criteria and confirmatory testing approaches for detection of ESBL production by *Escherichia coli*, *K pneumoniae*, and *Proteus mirabilis*.[5] For organisms such as *Enterobacter* spp and *Serratia* spp, which produce AmpC-type enzymes, ESBL screening should not be performed because false-negative results can occur. These screening and confirmatory tests were necessary because standard disk diffusion and MIC tests did not uniformly identify isolates producing ESBLs. Because ESBLs are usually inhibited by clavulanic acid, the CLSI made use of this property in developing the tests recommended to clinical laboratories for their detection and recommending that isolates producing ESBLs be reported as resistant to all penicillins, cephalosporins, and aztreonam. On establishment of new, lower interpretive criteria for many of these compounds, largely based on pharmacokinetic and pharmacodynamic principles and limited clinical data, the CLSI revised their recommendations for reporting.[5] When the new breakpoints are adopted by clinical laboratories, the CLSI recommends that results for specific cephalosporins and aztreonam be reported and interpreted as they are tested and that the ESBL screening and confirmatory tests need only be performed for epidemiological and infection control purposes. By comparison, although the European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints for the cephalosporins are similar to those now recommended by the CLSI, EUCAST recommends that laboratories continue to screen and confirm ESBL production due to the limited supporting clinical data and that cephalosporin reports be changed from susceptible to intermediate or intermediate to resistant if an isolate tests positive for ESBL production.[79]

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DETECTION OF CARBAPENEMASE ACTIVITY AMONG ENTEROBACTERIACEAE

Carbapenemases, enzymes that hydrolyze carbapenem class antibiotics (ertapenem, imipenem, meropenem, and doripenem), usually hydrolyze all other currently available β-lactams, with the exception of aztreonam for some metallo-β-lactamases. The genome encoding for the production of these enzymes may be located on plasmids (eg, *K pneumoniae* carbapenemases), a feature that makes them of particular concern from an infection control perspective. There are 3 classes of carbapenemases: serine class A (including KPC, SME, IMI, GES, and NMC), class B enzymes known as the metallo-β-lactamases (such as VIM, IMP, and NDM), and the class D OXA enzymes.[80] Carbapenemases have been identified in a wide range of gram-negative genera. The KPC enzyme, the most frequently identified class A carbapenemase in the United States, is most often found in the Enterobacteriaceae but has also been detected in *P aeruginosa*.[81] Metallo-β-lactamases are most frequently seen in *Acinetobacter* spp and *P aeruginosa*, but recently NDM has become widespread in some regions of the world among species of Enterobacteriaceae, particularly *K pneumoniae*. OXA carbapenemases are most frequently found in *Acinetobacter* spp but have also been reported among isolates of Enterobacteriaceae.

In 2009 the CLSI Subcommittee on Antimicrobial Susceptibility Testing (SAST) recommended the modified Hodge test for the detection of carbapenemase activity in Enterobactericeae.[82] Advantages of this assay include its ease of performance, the ability to test several isolates on a single plate, and the detection of different classes of carbapenemases with one test.[83,84] The primary disadvantages are subjectivity in reading the results, its inability to differentiate the various carbapenemases (potentially useful from an epidemiological perspective), and the false-positive results that can occur with some organisms producing AmpC or ESBL enzymes.

The modified Hodge test was originally recommended for the detection of carbapenemases in bacteria for which carbapenem MICs were elevated but still fell within the susceptible range. When isolates tested positive, the SAST recommended that they be designated carbapenemase-producing strains in the patient report with a warning indicating that the therapeutic outcomes of patients infected with such organisms and treated with the relevant carbapenem were unknown, particularly when alternative dosing regimens were used (eg, continuous or prolonged infusion). In 2010, though, the SAST lowered the carbapenem breakpoints to capture most carbapenemase-producing strains, which would now test either as resistant or intermediate to these compounds.[5] Implementation of the revised breakpoints eliminates the need for laboratories to routinely perform the modified Hodge test, although such testing may in some cases still be of value from an epidemiological or infection control perspective. A number of phenotypic tests allow detection and differentiation of class A (eg, inhibition by boronic acid) and class C (eg, inhibition by chelating agents such as ethylenediaminetetraacetic acid) carbapenemases, but these tests fail to detect class D (OXA) and are primarily used for strain characterization rather than for clinical purposes.

DETECTION OF PLASMID-MEDIATED AMPC-TYPE β-LACTAMASES

Chromosomally mediated, inducible, AmpC-type β-lactamases are produced by several gram-negative species. Often referred to as the ASPACE or ASPICE organisms, these include, but are not restricted to, *Aeromonas* spp, *Serratia* spp, *P aeruginosa*, *Acinetobacter* spp indole-positive Proteae (*Providencia* spp, *Morganella morganii*, and *Proteus vulgaris*), *Citrobacter* spp, and *Enterobacter* spp.[85] Resistant to the currently available β-lactamase inhibitors, such as tazobactam, sulbactam, and clavulanic acid, these enzymes result in resistance to a wide range of β-lactam antibiotics. The combination of a porin deletion with an AmpC-type enzyme can result in resistance to carbapenems. The genome encoding AmpC-type β-lactamases may also be harbored on transmissible plasmids and has been reported among a number of species that do not naturally produce inducible chromosomally mediated AmpC-type enzymes, including *K pneumoniae*, *E coli*, *P mirabilis*, and *Salmonella* spp.[86] Similar to the plasmids carrying the genes encoding ESBLs, those with AmpC-type genes often harbor resistance determinants for multiple classes of drugs.

Because detection of plasmid-mediated AmpC-producing pathogens may have epidemiological and infection control importance, several assays have been developed in an attempt to accurately detect this resistance type.[87-94] None, however, has been sufficiently standardized or tested against an adequate number of organisms producing plasmid-mediated AmpC-type enzymes to be considered superior in performance to the other assays.

TESTING TO DETECT BACTERICIDAL ACTIVITY

The AST methods used in the clinical microbiology laboratory typically assess only the inhibitory activities of antimicrobial agents. This is generally sufficient for patient management for most bacterial infections encountered by clinicians. In some, albeit rare, situations it may also be of value to determine

the bactericidal activity of an antibiotic against a specific patient bacterial pathogen. Serious infections in which a bactericidal effect is generally considered necessary for optimal treatment include bacteremia in neutropenic patients, patients with chronic osteomyelitis, and patients with bacterial endocarditis.[95-97]

Minimum Bactericidal Concentration Testing

After determination of MICs in a broth system under standard conditions, measured aliquots of growth media may be subcultured quantitatively to solid media to assess bactericidal activity. To calculate the extent of killing at each antibiotic concentration, plates are incubated under appropriate conditions, colony counts are performed, and results are compared to that of the growth control tube or well. The accepted definition of the minimum bactericidal concentration (MBC) is the lowest concentration of antibiotic at which a 99.9% (3 log) or greater reduction in growth compared with the initial inoculum is observed.[98] As with the MIC, the antibiotic MBC is reported in micrograms per milliliter or in some regions of the world in milligrams per liter. Determination of the MBC allows one to detect potential tolerance by the patient's specific isolate to an antibiotic usually considered bactericidal, a phenomenon reportedly leading to clinical failure among some patients.[99-101] Tolerance occurs when the antibiotic MIC for an organism is low (within the susceptible range) but the MBC is elevated, frequently at a concentration beyond that generally considered clinically achievable from a pharmacokinetic perspective. Tolerance is specifically defined as an MBC 32-fold or more higher than the MIC for the antibiotic under consideration.[98,100] Failure to demonstrate at least a 3-$\log_{10}$ decrease in colony-forming units per milliliter in the time-kill assay is also considered to represent tolerance.[98]

Time-Kill Kinetic Assays

Time-kill assays allow one to assess the rate of bactericidal activity at varying antibiotic concentrations over time. Although these assays are time-consuming and laborious to perform because they require subculture of media at specific times during a 24-hour period, results of combinations of antimicrobial agents can also be assessed. In 1999, standard methods for performance of the assay were published by the CLSI (previously the National Committee for Clinical Laboratory Standards).[98] Results of time-kill assays are typically presented graphically, plotting colony counts for each antimicrobial agent and concentration tested at each time point at which subcultures were performed (usually at 0, 4, 8, 12, and 24 hours). As with the MBC, bactericidal activity is defined as a 99.9% or greater killing at a specified time. When antibiotics are tested in combination using the time-kill approach, synergy is typically defined as a 2-log decrease or more in the number of colony-forming units achieved with the combination of antibiotics when compared with that achieved by the most active agent tested alone.

TESTS FOR ASSESSMENT OF INTERACTIONS AMONG ANTIMICROBIAL AGENTS

The value of in vitro testing to assess antibiotic interactions (ie, synergy testing) remains highly controversial. With a few bacterial species and antibiotic combinations, synergistic bactericidal activity is actually predictable and need not be routinely determined (eg, ampicillin, penicillin, or a glycopeptide plus gentamicin or streptomycin against susceptible strains of enterococci). When synergy testing is performed, the results should be interpreted with caution because they do not take into account drug interactions from a pharmacokinetic or a patient safety (adverse effect) perspective.

The checkerboard BMD test, wherein 2 antimicrobial agents are serially diluted in a 2-dimensional fashion to include all combinations during a specified clinically relevant range, is a somewhat less labor-intensive approach to assessing antibiotic interactions in vitro than the time-kill assay. This same approach to drug interaction testing can be taken using an agar dilution approach, although the method is even more laborious. Using these methods, one is able to recognize synergistic, additive, indifferent, or antagonistic interactions occurring with the agents being tested. By this method, a fractional inhibitory concentration (ΣFIC) is calculated by comparing the MIC of each drug alone to the MIC of that drug in combination with the second agent. Synergy is usually defined as a 4-fold decrease in the MIC of the agents in combination when compared with the antibiotics tested alone.[102] The FIC is calculated and interpreted as follows:

$$\Sigma\text{FIC} = \text{FIC of agent A} + \text{FIC of agent B}$$

$$\text{FIC of agent A} = \frac{\text{MIC of agent A in combination}}{\text{MIC of agent A alone}}$$

$$\text{FIC of agent B} = \frac{\text{MIC of agent B in combination}}{\text{MIC of agent B alone}}$$

Synergy is defined as $\Sigma\text{FIC} \leq 0.5$

Indifference is defined as $0.5 < \Sigma\text{FIC} \leq 4$

Antagonism is defined as $\Sigma\text{FIC} > 4$

Some investigators consider compounds additive when >0.5 ΣFIC ≤ 1.

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The time-kill approach appears to correlate more closely with in vivo studies of combined antibiotic effects than does the checkerboard method.[103,104] A simpler approach, albeit less standardized, is the use of Etest strips. Using this method, synergy is again defined by the FIC as described earlier.[105]

SERUM BACTERICIDAL TESTING (SCHLICHTER ASSAYS)

Another approach to assessing bactericidal activity and determining the effects of antibiotic combinations is serum bactericidal testing. Determinations of serum inhibitory titers and serum bactericidal titers (SBTs) are performed in a manner analogous to that for MIC and MBC testing, except that the patient's serum, rather than serially diluted concentrations of antibiotic, is used. A guideline outlining the specifics for performance of SBT testing has been published by the CLSI.[106] To perform the testing, 1 or more blood samples are collected from the patient (usually attempting to draw the specimens when antibiotic peak and trough levels are achieved). Serial 2-fold dilutions of the patient serum in pooled, pretested human serum are then prepared in tubes or wells of microtiter plates. Each tube or well is then inoculated with a standardized suspension of the patient's infecting pathogen in an applicable growth medium. The serum inhibitory titer is defined as the highest dilution of the patient's serum preventing visible growth after an appropriate incubation period. Similar to MBC testing, quantitative subcultures are then performed from each dilution of the patient's serum that prevented visible growth. The SBT is defined as that dilution resulting in a 99.9% or greater decrease in the original inoculum based on standardized rejection value tables. Results are then reported as titers (dilutions) of the patient's serum. The purpose of this assay is to determine whether the dosage regimen chosen for treatment of the infection results in sufficient bactericidal activity in the patient's blood.[96,107] Higher serum titers suggest that adequate patient dosing has been achieved, unexpected antibiotic elimination has not occurred, and the bacterial pathogen is not tolerant. A peak SBT of 1:64 or higher and a trough SBT of 1:32 or higher have been shown to correlate with rapid bacterial eradication from the blood and optimal time to cardiac vegetation sterilization in cases of endocarditis.[96,108] Lower bactericidal titers, however, do not necessarily predict a poor clinical response. Limited data have indicated that in cases of acute and chronic osteomyelitis a peak SBT of 1:16 or higher and a trough SBT of 1:4 or higher are predictive of therapeutic efficacy.[96,109] Major disadvantages of this assay include its labor-intensive nature and the requirement to obtain, prepare, and pretest pooled human serum for use in the procedure.

ANTIFUNGAL SUSCEPTIBILITY TESTING

There have been major advances in the standardization and clinical interpretation of antifungal susceptibility testing in recent years. The number of antifungal agents is increasing as the incidence of systemic and other infections due to *Candida* spp, *Aspergillus* spp, *Zygomycetes*, and other filamentous fungi is increasing.[110] Susceptibility testing of clinically significant isolates, especially those from usually sterile sites, is important both epidemiologically and clinically for guiding treatment. Antifungal susceptibility testing standards for yeasts and molds have been developed by both the CLSI and European Committee on Antimicrobial Susceptibility Testing (EUCAST) for a variety of antifungal agents.[111-115]

The standard antifungal reference testing method against which many other susceptibility testing methods are measured is the BMD method.[112] The BMD methods proposed by different organizations vary by the media used, supplements and inoculum added, incubation conditions, and end point interpretations. The CLSI provides MIC breakpoint and interpretive data for *Candida* spp for several antifungal agents, including fluconazole, itraconazole, voriconazole, anidulafungin, caspofungin, micafungin, and flucytosine.[111] The CLSI breakpoints for fluconazole, voriconazole, and the echinocandins have recently been revised for *Candida* species.[116-118] The susceptible dose-dependent category for the azoles infers that susceptibility to these antifungals by *Candida* spp is dependent on achieving the maximal possible blood level. In addition, the fluconazole data for the CLSI breakpoints were gathered from studies involving patients with oropharyngeal candidiasis and invasive candidal infections in nonneutropenic patients. Therefore, the clinical relevance of fluconazole breakpoints in clinical situations other than those mentioned has not been established. Interpretive data for the echinocandin class of drugs are based primarily on experience with nonneutropenic patients with candidemia, and clinical relevance of these data in other patient populations is uncertain. Finally, itraconazole data are based on experience with mucosal infections only, and data supporting the CLSI breakpoints for invasive infections are not available. Although there are no CLSI breakpoints currently approved for amphotericin B when testing *Candida* spp, isolates for which the MICs are greater than 1.0 μg/mL are generally considered resistant.[111] Although the CLSI documents only provide guidelines for *Candida* spp, some investigators have applied the CLSI breakpoints to *Cryptococcus* spp, and correlations have

been demonstrated between higher MICs and treatment failures.[119]

Because the BMD testing method is difficult to perform in daily practice in clinical microbiology laboratories, other testing approaches have been investigated. Disk diffusion antifungal susceptibility testing is a simple and cost-effective method for both yeasts and molds. Fluconazole disk diffusion testing of *Candida* spp has been available for several years. The CLSI has also established guidelines for disk diffusion testing of filamentous fungi, which is a relatively simple, rapid, and cost-effective alternative to BMD testing.[115] Etests are likewise less labor intensive than BMD and are relatively simple and reproducible for the testing of antifungal agents, especially against molds.[120] Sensititre YeastOne (Trek Diagnostic Systems) is a colorimetric antifungal susceptibility testing MIC plate that exhibits high agreement with the CLSI BMD method.[121] A new disk agar diffusion method, the Neo-Sensitabs tablet diffusion assay (Rosco, Taastrump, Denmark) has also been developed and tested for antifungal testing of molds and yeasts.[122] Finally, commercial automated systems for MIC determination of yeasts are simple alternative methods and are comparable to other established antifungal susceptibility testing methods.[123]

Antifungal susceptibility testing of *Candida albicans* and other *Candida* spp is fairly simple to perform, with determination of fluconazole susceptibility as the most important first step. If the *C albicans* isolate is fluconazole resistant or if the isolate is non–*C albicans*, further susceptibility testing will often be required. In contrast, antifungal susceptibility testing of molds is not currently as useful clinically because of both the long turnaround time of such testing and the difficulties in developing accurate breakpoints for molds.[124]

CONCLUSION

The goal of this article was to provide a review of current concepts in laboratory methods and approaches that serve to assist clinicians in making optimal antibiotic decisions for treatment of infections in this era of ever-evolving antimicrobial resistance. By its nature, the review could not be all-inclusive. For example, a standard has been developed for the susceptibility testing of *Mycoplasma* spp and *Ureaplasma urealyticum*.[125] Until recently, *M pneumoniae*, an important cause of community-acquired pneumonia, was thought to be universally susceptible to the macrolide class of antibiotics. Not only has macrolide resistance now been reported, but it is in fact widespread in some countries, including China and Israel, rendering availability of standardized methods for testing, heretofore of limited value but now of clinical importance.[126,127] A topic that warrants consideration is the influence and effect of standards-setting organizations (eg, CLSI, EUCAST, and FDA) on AST and reporting. This is, however, beyond the scope of this article but has been addressed in another recent publication.[128] Many of the testing approaches reviewed in these discussions have been used, essentially unchanged, for decades. As molecular techniques increasingly become a part of the daily routine in clinical laboratories, the day may come when such highly sensitive and specific methods replace many of the assays currently being used to predict antimicrobial susceptibility and resistance. As bacterial pathogens continue to exhibit increasing antibiotic resistance and appropriate empirical antibiotic decisions become more and more difficult, AST will take on an even more important role in managing patient infections.

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The Symposium on Antimicrobial Therapy will continue in an upcoming issue.

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