Antimicrobial Susceptibility Testing: Special Needs for Fastidious Organisms and Difficult-to-Detect Resistance Mechanisms

James H. Jorgensen¹ and Mary Jane Ferraro²

Clinical microbiology laboratories are faced with the challenge of accurately detecting emerging antibiotic resistance among a number of bacterial pathogens. In recent years, vancomycin resistance among enterococci has become prevalent, as has penicillin resistance and multidrug resistance in pneumococci. More recently, strains of methicillin-resistant *Staphylococcus aureus* with reduced susceptibility to vancomycin have been encountered. In addition, molecular techniques have demonstrated that there are still problems detecting methicillin resistance in staphylococci, especially in coagulase-negative species. Among members of the family Enterobacteriaceae, mutated β-lactamase enzymes may confer difficult-to-detect resistance to later-generation penicillins and cephalosporins. Anaerobic bacteria are no longer entirely predictable in their susceptibility to agents that might be selected for empiric therapy. Therefore, clinical microbiology laboratories may not be able to rely on a single susceptibility testing method or system to detect all those emerging resistant or fastidious organisms. For reliable detection, laboratories may need to employ conventional, quantitative susceptibility testing methods or use specially developed, single concentration agar screening tests for some resistant species. Certain of these screening tests are highly specific, while others may require additional confirmatory testing for definitive results. Therefore, laboratories must retain the versatility to apply several different approaches to detect resistance in both common and infrequently encountered bacterial pathogens.

Clinical microbiology laboratories are faced with the challenge of accurately detecting emerging antibiotic resistance among several important bacterial pathogens. Certain of these are fastidious organisms that require enriched media and modified growth conditions for reliable susceptibility testing (e.g., *Streptococcus pneumoniae*), whereas some other organisms have subtle or inducible resistance mechanisms that may require special screening tests for reliable recognition (e.g., vancomycin-resistant enterococci, staphylococci with reduced susceptibility to vancomycin, methicillin-resistant staphylococci, and gram-negative bacilli that produce extended-spectrum β-lactamases [ESBLs]). Clinical laboratories may not be able to rely on a single susceptibility testing method or commercial system to detect all of these emerging resistant organisms. For reliable detection, it may be necessary to employ conventional broth or agar dilution MIC procedures, special fixed–drug-concentration screening tests, and modified antibiotic interpretive breakpoints that increase the opportunity for recognizing resistant strains. However, with several of the screening methods it is important to confirm presumptive resistance by performing more definitive phenotypic tests (e.g., tests designed to detect vancomycin-resistance or ESBL-mediated resistance). Despite the need for special or alternative testing methods for several bacterial pathogens, practical test methods are available for use in both large and small laboratories. In the near future, it may become practical to use sensitive and specific molecular diagnostic methods capable of detecting the genes most often associated with resistance.

**When Standard Methods Must be Modified or Alternative Methods Applied**

In a previous article [1], methods for susceptibility testing of common, nonfastidious bacteria were reviewed. These included broth and agar dilution, antimicrobial gradient, disk diffusion, and automated instrument systems for performance of susceptibility tests in clinical laboratories. Those methods have been developed and standardized for testing bacteria that grow rapidly (in <24 h) in unsupplemented Mueller-Hinton broth or agar during incubation in ambient air. At least 90% of clinical isolates can be tested by these methods, including staphylococci, enterococci, Enterobacteriaceae, and *Pseudomonas* and *Acinetobacter* species. However, clinically important resistance has emerged in *Streptococcus pneumoniae*, certain other *Streptococcus* species, *Haemophilus influenzae*, *Neisseria gonorrhoeae*, and some anaerobic bacteria [2]. These organisms require more complex growth media, usually containing blood or blood...
products, and other growth additives, and depending on the method used, they may require a CO₂-enriched or strictly anaerobic atmosphere of incubation. However, despite the need for enriched media and different incubation atmospheres, other parameters of standardized susceptibility tests for nonfastidious bacteria are maintained. Necessary modifications of standard methods will be described in this review.

Some important mechanisms of antimicrobial resistance among nonfastidious bacteria can be difficult to detect reliably by standard dilution, diffusion, or instrument-based testing methods. Molecular diagnostic methods that demonstrate well-characterized genes encoding for resistance mechanisms are emerging as the new benchmarks defining resistance in individual bacterial isolates [3]. However, special resistance screens that use a critical concentration of an antimicrobial agent are a practical method to accurately recognize mexitilin resistance in staphylococci, vancomycin and aminoglycoside resistance in enterococci, or the presence of an ESBL in certain common gram-negative bacteria [4]. The phenotypic approaches to defining subtle or difficult-to-detect resistance properties in common bacterial pathogens are also described below.

Testing of Nutritionally Fastidious Organisms

Facultative bacteria. The standard broth microdilution, agar dilution, and disk diffusion tests can be adapted to test several nutritionally fastidious bacterial species by appropriate modification of the test medium and the incubation conditions. The NCCLS has developed and standardized testing methods for Haemophilus species, N. gonorrhoeae, Streptococcus species (including S. pneumoniae), and, more recently, Helicobacter pylori [5–7]. H. influenzae can be tested with use of a supplemented Mueller-Hinton medium referred to as haemophilus test medium (HTM; table 1). HTM includes hematin, nicotinamide adenine dinucleotide, and yeast extract as supplements for growth of Haemophilus species, and it requires incubation in a 5% CO₂ atmosphere when used in the agar formulation [6]. Despite these additives, the broth and agar versions of HTM are transparent and only slightly darker than Mueller-Hinton medium.

Quality control and interpretive criteria have been published for a large number of parenteral and oral agents useful in treating infections caused by these organisms [7], although routine testing of agents other than ampicillin and trimethoprim/sulfamethoxazole may not be needed to manage most respiratory infections due to Haemophilus species [7]. Indeed, H. influenzae isolates are predictably susceptible to a number of combinations that include the β-lactam/β-lactamase inhibitors, later-generation cephalosporins, and fluoroquinolones [8].

N. gonorrhoeae can be tested by the agar dilution or disk diffusion methods with use of a supplemented GC agar base medium and incubation in 5% CO₂ for 20–24 h [5, 6] (table 1). Growth of N. gonorrhoeae relies on the “XV-like” supplement, which contains numerous vitamins and coenzymes required for growth of this species. A cysteine-free version of the growth supplement is required for agar dilution testing of carbapenem antibiotics (e.g., imipenem and meropenem) or β-lactam combinations that include the β-lactamase-inhibitor clavulanic acid [5]. However, cysteine does not significantly interfere with disk diffusion testing of these antibiotics or with agar dilution testing of any other drugs [5, 6].

Many clinical microbiology laboratories now use nonculture methods (e.g., direct probe or nucleic acid amplification tests) to detect N. gonorrhoeae directly in clinical samples and therefore do not recover viable gonococci for susceptibility testing. For this reason and because gonococci continue to respond effectively to the empirical therapeutic regimens currently recommended for treatment of gonorrhea [9], susceptibility testing of N. gonorrhoeae isolates is performed primarily for surveillance purposes by public health reference laboratories.

S. pneumoniae and certain species of the viridans Streptococcus group have emerged with multiple antimicrobial resistance mechanisms within the past decade [10–12]. It is now important for laboratories to test routinely pneumococcal isolates from most infections and, as a minimum, viridans streptococci from patients with bacteremia or endocarditis for susceptibility to penicillin [7]. Pneumococci should also be tested against relevant extended-spectrum cephalosporins, macrolides, and possibly other drug classes, depending on the type of infection [7].

The National Committee for Clinical Laboratory Standards (NCCLS) has developed quality control and interpretive breakpoint criteria specific for pneumococci and other streptococci.

Table 1. Media and methods recommended for testing fastidious bacteria.

<table>
<thead>
<tr>
<th>Organism, test method</th>
<th>Recommended medium</th>
<th>Incubation atmosphere</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Haemophilus species</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Broth microdilution</td>
<td>Haemophilus test medium</td>
<td>Ambient air</td>
</tr>
<tr>
<td>E test</td>
<td>Haemophilus test medium</td>
<td>5% CO₂</td>
</tr>
<tr>
<td>Disk diffusion</td>
<td>Haemophilus test medium</td>
<td>5% CO₂</td>
</tr>
<tr>
<td><strong>Neisseria gonorrhoeae</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Agar dilution</td>
<td>GC agar with XV-like supplement</td>
<td>5% CO₂</td>
</tr>
<tr>
<td>Disk diffusion</td>
<td>GC agar with XV-like supplement</td>
<td>5% CO₂</td>
</tr>
<tr>
<td><strong>Streptococcus pneumonia</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Broth microdilution</td>
<td>MHB + 3% lysed horse blood</td>
<td>Ambient air</td>
</tr>
<tr>
<td>E test</td>
<td>MHA + 5% sheep blood</td>
<td>5% CO₂</td>
</tr>
<tr>
<td>Disk diffusion</td>
<td>MHA + 5% sheep blood</td>
<td>5% CO₂</td>
</tr>
<tr>
<td><strong>Other Streptococcus species</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Broth microdilution</td>
<td>MHB + 3% lysed horse blood</td>
<td>Ambient air</td>
</tr>
<tr>
<td>E test</td>
<td>MHA + 5% sheep blood</td>
<td>5% CO₂</td>
</tr>
<tr>
<td>Disk diffusion</td>
<td>MHA + 5% sheep blood</td>
<td>5% CO₂</td>
</tr>
<tr>
<td><strong>Anaerobic bacteria</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Agar dilution</td>
<td>Brucella agar with 5% sheep blood</td>
<td>Anaerobic</td>
</tr>
<tr>
<td>E test</td>
<td>Brucella agar with 5% sheep blood</td>
<td>Anaerobic</td>
</tr>
<tr>
<td>Broth microdilution</td>
<td>Wilkins-Chalgren broth</td>
<td>Anaerobic</td>
</tr>
</tbody>
</table>

NOTE: GC, [author will define]; MHA, Mueller-Hinton agar; MHB, Mueller-Hinton broth.

* A combination of vitamins and coenzymes needed for the growth of N. gonorrhoeae.
[5–7]. The NCCLS reference broth microdilution procedure for these organisms is to use Mueller-Hinton broth supplemented with 2%–5% lysed horse blood but with otherwise standard inoculum and incubation conditions for 20–24 h [5] (table 1). Commercial microdilution panels that closely resemble the NCCLS reference method have received clearance from the United States Food and Drug Administration (FDA) for use in clinical laboratories [13]. In addition, the E test (AB BIODISK, Solna, Sweden) has become very popular for testing pneumococci because of its simplicity and convenience [14].

Clinical laboratories may also employ the NCCLS disk diffusion test for most but not all drugs when testing pneumococci and other streptococci [6]. The disk diffusion test uses sheep blood supplemented with Mueller-Hinton agar and incubation for 20–24 h in 5% CO2 [6] (table 1). For streptococcal isolates, a CO2 atmosphere for incubation is important to prevent growth failures. This requirement has been incorporated into the standardized test and taken into account in the development of quality control diameter ranges for zones of inhibition.

The pneumococcal strain ATCC 49619 has been designated by the NCCLS for routine quality control of both MIC and disk diffusion tests for all streptococci [5–7]. Despite its simplicity, the disk diffusion test for pneumococci has 2 shortcomings. The oxacillin disk can be used to “screen” for penicillin susceptibility, which is indicated by a zone of inhibition ≥20 mm. Such strains are uniformly susceptible to other relevant β-lactam antibiotics, including amoxicillin and most cephalosporins [7]. However, if the oxacillin zone of inhibition is <20 mm, it is necessary to determine a penicillin MIC to clarify whether an isolate is resistant, intermediate resistant, or borderline susceptible to penicillin [7, 15]. Second, the disk test does not provide acceptable accuracy with some of the most important drugs (e.g., the extended-spectrum cephalosporins) when tested against pneumococci [7, 15].

A convenient approach used by some laboratories is to apply selected E test strips (e.g., penicillin and cefotaxime or ceftriaxone) along with disks for non-β-lactam drugs (e.g., erythromycin, trimethoprim/sulfamethoxazole, or a quinolone) on the same large Mueller-Hinton sheep blood agar plate. Although this is a very convenient test method, the laboratory personnel should verify that the particular drug and specific organism that they wish to test are a combination that has received FDA clearance for clinical use.

The NCCLS reference methods that have been recently developed for H. pylori [7] are relevant at this time primarily for research purposes, including for comparison of the in vitro activities of new compounds under development for treatment of H. pylori gastritis. Clinical laboratory testing of this species is not recommended at present. In cases in which there appears to be a clinical need for testing a particular isolate (such as with apparent failure of empirical therapy), a laboratory might consider using E test strips for ampicillin, clarithromycin, or metronidazole with Mueller-Hinton 5% sheep blood agar plates incubated in a microaerophilic environment at 35°C for 3–5 days [16]. However, it should be noted that the E test has not yet received FDA clearance for use with H. pylori.

Last, because of the predictable susceptibility of Moraxella catarrhalis to agents other than penicillin or amoxicillin [8], the NCCLS recommends direct β-lactamase testing of significant isolates but not the use of growth-based susceptibility tests with other antimicrobial agents [5–7].

Anaerobic bacteria. Susceptibility testing of obligately anaerobic bacteria is not a standard procedure in many clinical microbiology laboratories at this time. This is due in part to the success of many empirical antibiotic regimens recommended for treating anaerobic infections and also to the technical difficulty of performing anaerobic testing. Many times, cultures of samples taken from anaerobic infections reveal the presence of multiple facultative and anaerobic species that must be systematically isolated and tested separately, which can take several days [17]. In addition, the NCCLS reference MIC method for anaerobes is an agar dilution procedure that requires preparation of antibiotic dilutions and special media at the site of testing [18]. Indeed, there is a serious lack of practical, commercially available reagents for performing anaerobic susceptibility testing.

Some laboratories deal with these challenges by testing anaerobic bacterial isolates only selectively. Laboratories may test isolates from only selected sites (e.g., blood or material from brain abscesses), the anaerobic species most likely to harbor resistance mechanisms (e.g., members of the Bacteroides fragilis group or certain Clostridium species), or by specific requests of physicians on a case-by-case basis. Some other laboratories may choose to perform periodic surveillance testing of stored clinical isolates in order to generate local data to guide clinicians with empirical therapy. However, the need for some form of routine testing may become more important, since resistant anaerobes have been found at some centers [17, 19].

The NCCLS recommends an agar dilution method as the reference procedure for anaerobes. The method employs brucella agar supplemented with sheep blood and other growth additives [18]. It uses an inoculum 1 log10 higher (i.e., 105 cfu/spot) than is used with aerobic organisms and requires incubation in an anaerobic gas atmosphere for 48 h before determination of MICs. Because interpretation of the MIC endpoints can be more difficult than with aerobic bacteria, the NCCLS publication on anaerobic testing includes color photos that depict various endpoints and guides to their appropriate interpretation.

Interpretive breakpoints specific for anaerobic bacteria and anaerobic quality control strains have been recommended by the NCCLS [18]. Because agar dilution is not a practical method for most clinical laboratories, the NCCLS has also described a broth microdilution procedure that may represent a more practical choice for routine use [18]. It includes use of Wilkins-Chalgren broth, an inoculum of 1 × 105 cfu/mL (rather than
Difficult-to-Detect Resistance Mechanisms in Nonfastidious Organisms

Methicillin/oxacillin resistance in staphylococci. Staphylococci continue to be among the most common of all nosocomial and community-acquired bacterial pathogens. Both *Staphylococcus aureus* and the coagulase-negative species of *Staphylococcus* may be methicillin/oxacillin-resistant because of the product of a special, low-affinity penicillin-binding protein, PBP 2a [21]. Production of PBP 2a results in broad resistance to semisynthetic penicillins, cephalosporins, and carbapenem. Methicillin-resistant strains are often (but not always) multiply resistant to several other drug classes, including macrolides, clindamycin, aminoglycosides, chloramphenicol, fluoroquinolones, and trimethoprim/sulfamethoxazole [21].

Accurate methods for detection of methicillin resistance have been a particular concern of many laboratories. Because of lingering difficulties with standard phenotypic susceptibility testing methods and concern over the possibility of missing some highly heterogeneous strains, the most definitive method is now recognized to be the detection of the gene (*mecA*) that encodes production of PBP 2a [3]. This gene can be detected by a PCR method that uses published primers or by a direct DNA probe method [3]. However, such methods are beyond the scope of many clinical laboratories, and FDA-cleared commercial kits for performing them are not yet available.

The most practical and reliable phenotypic test for detection of methicillin-resistant *S. aureus* (MRSA) appears to be the NCCLS oxacillin-salt agar screening procedure [5]. The test is performed by spot-inoculating the surface of a Mueller-Hinton agar plate containing oxacillin (6 µg/mL) and 4% NaCl with a 0.5-McFarland-density direct inoculum suspension [5]. After a full 24-h incubation at a temperature no higher than 35°C, any growth on the plate, when examined with transmitted light, indicates MRSA. FDA-cleared oxacillin screening plates are available commercially from several sources. It is important to note that although this procedure is referred to as a screening test, it is sufficiently accurate that no further testing is required to confirm that an isolate is indeed resistant to oxacillin [4, 5].

The second most reliable phenotypic method for detection of MRSA appears to be the NCCLS broth microdilution test procedure with incorporation of 2% NaCl in cation-adjusted Mueller-Hinton broth for testing of oxacillin [5]. The NCCLS disk diffusion procedure that employs a 1-µg oxacillin disk is also generally reliable for detection of MRSA but cannot practically take advantage of salt-supplemented medium because of adverse affects on drugs of other classes that might also be tested on the same plate [6]. Last, the E test has been shown to be reliable for detection of MRSA if 2% NaCl is included in the Mueller-Hinton agar [22]. With any of the above methods, the direct inoculum-suspension procedure should be used, and tests should be incubated for a full 24 h at 35°C (not 37°C) [4–6].

Detection of methicillin-resistant coagulase-negative staphylococci (MR-CNS) by phenotypic methods is even more difficult than recognition of MRSA. Several studies have shown that oxacillin MICs with MR-CNS may be lower and disk diffusion zone-of-inhibition diameters larger than those with MRSA [23, 24]. Therefore, the MIC and zone of inhibition diameter breakpoints developed for detection of MRSA (i.e., oxacillin MIC of ≥ 0.5 µg/mL) may not detect a number of MR-CNS isolates. Moreover, oxacillin-salt agar screening also fails to detect a number of CNS strains that have the *mecA* gene [24].

The NCCLS has recently proposed that the most sensitive phenotypic method for detection of MR-CNS is either a broth microdilution determination of the oxacillin MIC or the oxacillin disk diffusion test with specially derived breakpoints specific for CNS [7]. Oxacillin susceptibility of CNS is now defined by an MIC ≤ 0.25 µg/mL or zone of inhibition ≥ 18 mm, whereas resistance is indicated by an oxacillin MIC > 0.5 µg/mL or zone of inhibition ≤ 17 mm [7]. It is important to note that these breakpoints differ substantially from those still recommended for *S. aureus* and that the breakpoints were derived on the basis of the presence or absence of *mecA* rather than the pharmacokinetic properties of oxacillin [7]. Clinical outcome data, however, are sparse for strains of CNS with MICs of 0.5–1 µg/mL.

In the past, the NCCLS provided a cautionary statement indicating that in vitro testing of various cephalosporins, penicillin-β-lactamase–inhibitor combinations, and carbapenems could lead to false-susceptible results with methicillin-resistant strains. In such cases, laboratories were urged to not report the apparent susceptibility of these agents or to report them as resistant on the basis of the recognized poor clinical response of methicillin-resistant staphylococci to any of the β-lactam antibiotics. Because this practice was sometimes confusing to laboratory personnel and physicians and because it involved unnecessary testing, the NCCLS now states that penicillin and oxacillin are the only β-lactams that need be tested routinely against staphylococci [5–7].

Susceptibility to all other currently available β-lactams can be accurately deduced from the results of testing only penicillin and oxacillin, as follows. Penicillin-susceptible strains can be
safely assumed to be susceptible to all β-lactams with recognized activity against staphylococci. Penicillin-resistant, oxacillin-resistant strains are susceptible to various β-lactams, with the exception of β-lactamase-labile penicillins such as ampicillin, mezlocillin, piperacillin, and ticarcillin. Staphylococci resistant to oxacillin are resistant to all currently available β-lactam antibiotics, including the penicillin–β-lactamase-inhibitor combinations. Therefore, while susceptibility breakpoints for various cephalosporins or other β-lactam drugs have been retained for research or epidemiological purposes, it is not necessary or useful for clinical laboratories to test those agents against staphylococci on a routine basis [7].

**Diminished vancomycin susceptibility in staphylococci.** There has been great concern that the genes encoding vancomycin resistance in enterococci could be transferred to staphylococci, especially to MRSA, thereby creating vancomycin-resistant *S. aureus*. The vanA gene cluster has been transferred in vitro on a plasmid to a recipient *S. aureus*, thereby creating a perfectly functional vancomycin-resistant strain [25]. While this has not yet occurred in nature, strains with reduced vancomycin susceptibility have been reported from Japan and several sites within the United States [26–28]. These strains are now referred to as vancomycin-intermediate *S. aureus* (VISA) or glycopeptide-intermediate *S. aureus* (GISA). These strains have demonstrated a vancomycin MIC of 8 µg/mL and are associated with a thickened cell wall and increased levels of some cell wall precursors [28, 29]. They have not been shown to contain the vanA, vanB, or vanC sequences from *Enterococcus* species [27, 28]. In addition, vancomycin-intermediate isolates of coagulase-negative staphylococci, particularly *Staphylococcus epidermidis* and *Staphylococcus haemolyticus*, have been reported in the past [30, 31].

The NCCLS changed the vancomycin disk diffusion breakpoints in 1998 to facilitate the detection of vancomycin resistance in staphylococci, should it emerge. However, even the modified disk breakpoints have not proven to be sufficient for detection of the VISA strains reported to date [29]. Tenover et al. [29] have examined several conventional and commercial susceptibility testing methods with the limited collection of VISA isolates. Their findings can be summarized by saying that most MIC methods that incorporate at least an overnight incubation period (≥16 h) appear to offer reliable detection of VISA. In addition, the vancomycin screening agar developed for detection of vancomycin-resistant enterococci (VRE) seems to offer a very simple and inexpensive way to screen for VISA.

Those instruments currently marketed in the United States that provide rapid susceptibility results should be used with caution [29]. With any of the procedures, the Centers for Disease Control and Prevention (CDC) recommends that *S. aureus* isolates with vancomycin MICs ≥4 µg/mL should be regarded as “possibly resistant” and forwarded to a public health reference laboratory for further characterization [32].

**Vancomycin resistance in enterococci.** The prevalence of VRE has increased sharply in the United States in the past decade [33]. A committee of the CDC has published recommendations for preventing the spread of VRE within health care facilities, and these include prompt and accurate detection of such strains by clinical microbiology laboratories [33]. However, there have been problems with accurate detection of VRE with use of certain conventional and commercial antimicrobial susceptibility testing methods [34].

For this reason, the NCCLS has recommended that the simplest and most sensitive test for recognition of VRE is the test that uses vancomycin screening agar, originally described by Willey et al. [5, 35]. This method incorporates use of 6 µg/mL of vancomycin in brain-heart infusion (BHI) agar, spot inoculation with 10⁵ or 10⁶ cfu, and incubation for a full 24 h at 35°C (table 2). Studies comparing use of this method with the presence of the vanA, vanB, or vanC genes have confirmed the sensitivity of this approach for detection of VRE [36]. However, it is important to note that vancomycin screening agar is not entirely specific for vanA- and vanB-type resistance.

*Enterococcus casseliflavus* and *Enterococcus gallinarum* contain the vanC₁ and vanC₂ genes that result in an intrinsic intermediate level of vancomycin susceptibility (e.g., usually a vancomycin MIC of 8 µg/mL) that does not represent “true” vancomycin resistance (usually indicated by a vancomycin MIC ≥32 µg/mL) [37]. Therefore, it is important to perform several confirmatory tests on isolates of presumed VRE found on vancomycin screening agar. These tests should include a gram stain (to detect *Lactobacillus* species, also vancomycin-resistant), biochemical tests for species-level identification (most VRE in the United States are *Enterococcus faecium*), and determination of the vancomycin MIC by either broth microdilution or the E test with 24-h incubation [5, 38, 39]. It may also be helpful to perform a test for motility and determine the ability to acidify methyl-α-D-glucopyranoside (MGP), which are properties of *E. casseliflavus* and *E. gallinarum* [40].

As suggested above, vancomycin MIC determinations based on a conventional overnight incubation period are generally reliable for detection of vancomycin resistance [34]. In addition, the rapid Vitek instrument (bioMerieux Vitek, Hazelwood, MO) has received FDA clearance for its updated approach to detection of VRE. The reliability of the disk diffusion method can be improved by careful inspection of apparent zones of inhibition around a standard vancomycin disk after a full 24-h incubation at 35°C [6]. Some strains of enterococci with inducible vanB-type resistance may give rise to tiny, pinpoint colonies within an otherwise apparent zone of inhibition. Therefore, all vancomycin disk diffusion tests should be read with use of a strong transmitted light source to allow visualization of subtle in-growth colonies.

**High-level aminoglycoside resistance in enterococci.** Enterococci may be resistant to high concentrations of aminoglycosides and thus be refractory to the synergistic interaction of aminoglycosides and cell wall–active antibiotics. Resistance to
zymes, particularly ANT(3’/9) or ANT(9) [21]. Resistance to gentamicin is most often mediated by production of the bifunctional enzyme AAC(6’)/APH(2’α) that inactivates all aminoglycosides except streptomycin [21].

Laboratories can test clinically important isolates of Enterococcus species for high-level gentamicin resistance with use of a single 500-μg/mL concentration in either a BHI broth microdilution format or a BHI agar plate containing that concentration of gentamicin [5, 7]. Growth after 24 h in the presence of that concentration of the drug confirms high-level gentamicin resistance, without the need for further testing. Gentamicin results predict those for tobramycin against gentamicin resistance, without the need for further testing. Genetic resistance to one or both of the aminoglycosides, without necessarily referencing the test concentrations of the drugs. Laboratories should also create concise comments that briefly explain the implications of high-level resistance for inclusion on susceptibility reports. Testing for high-level aminoglycoside resistance is normally restricted to isolates from the blood or CSF or isolates from bone or joint infections [5, 6]. There is no evidence that other infections (e.g., urinary tract or wound infections) require bactericidal therapy with an aminoglycoside in combination with a cell wall–active agent.

Bush group I β-lactamases in some gram-negative bacteria. Resistance to broad-spectrum penicillins and cephalosporins may occur in some gram-negative bacteria that produce large amounts of chromosomal Bush group I β-lactamase. This has been described most often with regard to Enterobacter species, Citrobacter freundii, Serratia marcescens, and Pseudomonas aeruginosa [41]. The gene for production of Bush group I β-lactamase is usually under the control of a second, repressor gene that allows production of only small amounts of enzyme [42]. However, large amounts of the enzyme can be produced by induction or derepression of the gene by certain highly enzyme-stable compounds, most notably cefoxitin and imipenem. This can be demonstrated by performance of disk induction tests that simply confirm that these organisms have the capa-

<table>
<thead>
<tr>
<th>Organism</th>
<th>Resistance trait</th>
<th>Screening method</th>
<th>Medium</th>
<th>Confirmatory testing</th>
</tr>
</thead>
<tbody>
<tr>
<td>Staphylococcus aureus</td>
<td>Methicillin/or oxacillin resistance</td>
<td>Oxacillin agar screen</td>
<td>Mueller-Hinton broth + 2% NaCl + 6 μg/mL oxacillin</td>
<td>None</td>
</tr>
<tr>
<td>CNS</td>
<td>Methicillin/or oxacillin resistance</td>
<td>Oxacillin MIC ≥0.5 μg/mL</td>
<td>Mueller-Hinton broth</td>
<td>None</td>
</tr>
<tr>
<td>Enterococcus species</td>
<td>Vancomycin intermediate resistance</td>
<td>Vancomycin agar screen</td>
<td>BHI agar + 6 μg/mL vancomycin</td>
<td>Vancomycin MIC; species-level identification</td>
</tr>
<tr>
<td>Enterococcus species</td>
<td>High-level aminoglycoside resistance</td>
<td>Vancomycin agar screen</td>
<td>BHI agar + 500 μg/mL gentamicin</td>
<td>None</td>
</tr>
<tr>
<td>Enterococcus species</td>
<td>High-level aminoglycoside resistance</td>
<td>Broth screen (microdilution)</td>
<td>BHI broth + 500 μg/mL gentamicin</td>
<td>None</td>
</tr>
<tr>
<td>Escherichia coli and Klebsiella species</td>
<td>Extended-spectrum β-lactamase production</td>
<td>MIC ≥2 μg/mL (cefepoxide, ceftazidime, aztreonam, cefotaxime, ceftaxime)</td>
<td>Mueller-Hinton broth</td>
<td>≥3-fold decrease in MIC with 4 μg/mL clavulanate</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Zone of inhibition (cefepoxide, ≤22 mm; ceftazidime, ≤22 mm; aztreonam, ≤27 mm; cefotaxime, ≤27 mm; ceftaxime, ≤25 mm)</td>
<td>Mueller-Hinton agar</td>
<td>≥5-mm increase in ceftazidime or cefotaxime zone of inhibition with 10 μg clavulanate</td>
</tr>
</tbody>
</table>

NOTE. BHI, brain-heart infusion; CNS, coagulase-negative staphylococci.
bility of producing the enzyme in large amounts after induction [42].

The phenomenon of derepression, however, is a reversible induction associated with a return to production of low levels of enzyme when the inducing drugs are removed. More significant is the fact that spontaneous mutations may occur in the repressor gene, leading to irreversible constitutive production of Bush group I \(\beta\)-lactamase [42]. These spontaneous mutants may be selected during therapy for serious infections with later-generation penicillins or cephalosporins, leading to therapeutic failures in some patients [43]. In such cases, susceptibility testing will indicate that the patient’s isolate is susceptible to several later-generation \(\beta\)-lactams at the outset of therapy, but resistance can be readily demonstrated in isolates 4–10 days later, during therapy, if spontaneous mutants occur and are “selected” by the presence of the drug [43].

There is no real value in performing the disk induction tests on clinical isolates of Enterobacter, Citrobacter, or P. aeruginosa, since virtually all isolates have the gene for producing the Bush group I enzyme. However, laboratories may assist physicians by including a brief comment on routine laboratory reports that such resistance may occur during therapy with a penicillin or cephalosporin [7]. In addition, laboratories should repeat susceptibility tests on any isolates that appear to be the same strains of these genera recovered from patients undergoing prolonged therapy for serious infections. Such repeated tests should be performed every 3–4 days to detect the emergence of resistance as soon as possible [7].

Extended-spectrum \(\beta\)-lactamas es in some Enterobacteriaceae. Virtually every strain of Klebsiella pneumoniae produces a plasmid-mediated \(\beta\)-lactamase known as SHV-1, which normally confers resistance to only ampicillin and ticarcillin [42]. A substantial percentage of contemporary Escherichia coli isolates also produce a plasmid-mediated \(\beta\)-lactamase, known as TEM-1, that confers ampicillin resistance [42]. However, spontaneous mutations may occur in these very common enzymes that “extend” the spectrum to include hydrolysis of later-generation penicillins, cephalosporins, and aztreonam.

These enzymes are now referred to as ESBLs, and they have been subjected to the latest enzymology and genetic sequencing techniques to characterize the precise mutations and unique hydrolytic activities of each enzyme [44]. The unique ESBLs TEM-2 through TEM-69 and SHV-2 through SHV-23 have now been described [45]. These enzymes all appear capable of hydrolyzing penicillins, cephalosporins, and aztreonam at a higher-than-normal inoculum density in standard MIC tests and have been associated with adverse outcomes for patients treated with those agents and in nosocomial infection outbreaks [46, 47]. For these reasons, it is important that clinical laboratories be able to recognize ESBL-producing isolates and alert physicians to their presence.

Because of differences in drug substrate affinities of some ESBLs and the resulting inoculum effects with standard susceptibility tests, ESBL-producing isolates may give rise to inconsistent or illogical results when tested by standard MIC and disk diffusion tests. For example, an isolate might appear to be resistant or intermediate to ceftazidime but susceptible to cefotaxime and aztreonam. Such differences in activities with those drugs should not occur with Klebsiella species or E. coli. The NCCLS has dedicated considerable effort in the past few years to standardizing methods for reliable recognition of ESBL. The current recommendations are that the drugs that are most readily hydrolyzed by ESBL should be tested as indicators of possible ESBL [5–7]. These include (in order of preference for greatest sensitivity of detection of North American strains) cefpodoxime, ceftazidime, aztreonam, cefotaxime, and ceftriaxone.

It is further suggested that special “screening” breakpoints be applied to interpretation of the results of tests with the “indicator” drugs [5–7]. For example, MICs of 2–8 \(\mu\)g/mL for those compounds are very unlikely against Klebsiella or E. coli strains that have only native TEM-1 or SHV-1 enzymes, but those MICs do not reach the breakpoint that has been used until now to separate the susceptible from the intermediate or resistant categories of those drugs (except cefpodoxime). The NCCLS suggests that MICs \(\geq 2 \mu\)g/mL for those agents are presumptive evidence of the presence of an ESBL [7]. The same principle may be applied to disk testing by use of smaller, “screening” zone-of-inhibition diameters [6, 7] (table 2).

Most recently, the NCCLS has described phenotypic confirmatory tests for possible ESBL-producing strains detected by means of the screening procedures described above [5–7]. Either MIC or disk diffusion tests may be performed with a substrate cephalosporin tested alone and with the addition of clavulanic acid. Since ESBLs are plasmid-mediated enzymes, they are inhibited by the \(\beta\)-lactamase inhibitors, clavulanate, sulbactam, and tazobactam. The NCCLS recommends use of clavulanate added in fixed concentration to both ceftazidime and cefotaxime for optimal confirmation of most ESBLs [7] (table 2).

With the MIC procedure, the MIC of the cephalosporin plus clavulanate should be at least 3 log, dilutions (or 8-fold) lower than when the cephalosporin is tested alone [5, 7]. In the disk test, the diameter of the zone of inhibition should increase by at least 5 mm in the presence of the clavulanate [6, 7]. These confirmatory tests are probably most important with E. coli and Klebsiella oxytoca isolates, in which excess production of native \(\beta\)-lactamase (chromosomal AmpC and K1, respectively) may occasionally give rise to resistance to cefpodoxime. In contrast, cefpodoxime is both a sensitive and specific indicator of an ESBL in K. pneumoniae.

Another potential clue to the presence of an ESBL is co-resistance to gentamicin and trimethoprim/sulfamethoxazole that may be associated with resistance genes carried on the same plasmid as those encoding the ESBL [48].

Laboratories should make reasonable efforts to detect ESBLs
in these 2 very common gram-negative genera. When they are detected and confirmed by the methods described above, the laboratory should change the result category to “resistance” for all of the penicillins, aztreonam, and the true cephalosporins (but not the cephamycins, i.e., cefotetan, cefmetazole, and cefoxitin) [5–7]. Alternatively, a laboratory could include a warning statement that an isolate produces an ESBL and should be considered as clinically resistant to the compounds noted above.

Direct detection of some enzymes that mediate resistance. Simple, rapid tests are available that allow direct detection of 2 bacterial resistance agents; specifically, some β-lactamases and chloramphenicol acetyl transferase [4]. β-lactamase tests may incorporate penicillin as a substrate along with an indicator system that detects its destruction by bacterial enzymes or may use a chromogenic cephalosporin (e.g., nitrocefin) that changes color when it is hydrolyzed by a β-lactamase [4]. With either of these reagents, direct testing of bacterial colonies taken from an initial growth plate can provide evidence within a few minutes that an enzyme is present that hydrolyzes penicillin as well as ampicillin and amoxicillin.

Unfortunately, there are only a few bacterial species for which this approach can be applied: H. influenzae, N. gonorrhoeae, M. catarrhalis, staphylococci, enterococci, and some anaerobic bacterial species [4, 5, 17]. These tests do not detect resistance to penicillins due to other mechanisms (e.g., alteration of PBPs) or resistance to extended-spectrum β-lactam antibiotics; for example, these tests cannot be used to detect ESBL.

Chloramphenicol acetyl transferase (CAT) can be detected by a rapid tube or filter paper test within 1–2 h and provide evidence of chloramphenicol resistance in H. influenzae, S. pneumoniae, and some Enterobacteriaceae [4]. While not all resistance to chloramphenicol is mediated by CAT, the majority of resistance among H. influenzae and S. pneumoniae isolates can be attributed to production of that enzyme. However, because the use of chloramphenicol has diminished dramatically in developed countries, the CAT test is rarely performed in clinical laboratories.

Other problem organisms. There are several other species of bacteria that may harbor important intrinsic or acquired resistance mechanisms that make therapy problematic. Unfortunately, little has been accomplished toward standardization of susceptibility testing of Corynebacterium species, Bacillus species, fastidious gram-negative bacilli of the Haemophilus-Acinobacillus-Cardiobacterium-Eikenella-Kingella (HACEK) group, or Pasteurella species. In addition, Stenotrophomonas maltophilia and Burkholderia cepacia present problems because the interpretative-category results of disk diffusion or automated-instrument tests may not agree with categories of susceptibility based on conventional MIC determinations. The NCCLS currently recommends that only MIC testing should be performed against S. maltophilia [5, 6]. Indeed, with organisms for which disk diffusion interpretive breakpoints have not been specifically derived, it may be most prudent to rely on empirical regimens supported by literature citations. If it is deemed necessary to do a susceptibility test on a particular isolate, the most reliable approach may be to determine MICs of relevant drugs by a conventional MIC procedure (e.g., broth microdilution). Interpretation of the resulting MICs must then lie with the infectious diseases specialist in light of the patient’s condition and the previous experiences of the clinician.

Future Approaches to Detection of Antimicrobial Resistance

The newest and perhaps most definitive approach to detection of antimicrobial resistance is the direct detection of genes known to encode certain resistance mechanisms. It is possible to detect mecA (the gene responsible for methicillin resistance in Staphylococcus species), vanA and vanB (the genes responsible for vancomycin resistance in enterococci), and various β-lactamases and aminoglycoside-inactivating enzymes [3]. Methods that employ the use of genetic probes or nucleic acid amplification techniques offer the promise of excellent sensitivity, specificity, and speed in the detection of resistance genes.

The detection of the genes for methicillin and vancomycin resistance have become the benchmarks for evaluating new or improved phenotypic tests for those resistance properties. Despite the accuracy of this approach with some resistance traits, no molecular genetic tests are yet commercially available or FDA-cleared for clinical laboratory use. Potential limitations of this approach include the large number of different resistance mechanisms that would need to be detected (e.g., many different β-lactamases produced separately or together) and the possibility that certain genes that could be detected may not be expressed or may not result in phenotypic resistance [3]. In some instances multiple mutations result in remodeling of the molecular targets affected by antibiotics (e.g., PBPs in pneumococci) [21]. Therefore it might be necessary to sequence the genes of interest in order to detect subtle mutations.

A recent technological advance that may facilitate both the ability to probe for a large number of genes conveniently and the ability to sequence other genes quickly and cheaply is the application of computer chip technology to genetic analyses [49]. Several companies are developing computer chip–based products that will allow detection of as many as several hundred resistance genes simultaneously or rapid sequencing of some genes involved with resistance mediators. Therefore, the performance of highly definitive tests for antimicrobial resistance may become a practical approach for routine but selective application in the future and could herald a new paradigm in antimicrobial susceptibility testing.

References


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