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MINIREVIEW

Antimicrobial Susceptibility Testing for Enterococci

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ABSTRACT Enterococci are major, recalcitrant nosocomial pathogens with a wide repertoire of intrinsic and acquired resistance determinants and the potential of developing resistance to all clinically available antimicrobials. As such, multidrug-resistant enterococci are considered a serious public health threat. Due to limited treatment options and rapid emergence of resistance to all novel agents, the clinical microbiology laboratory plays a critical role in deploying accurate, reproducible, and feasible antimicrobial susceptibility testing methods to guide appropriate treatment of patients with deep-seated enterococcal infections. In this review, we provide an overview of the advantages and disadvantages of existing manual and automated methods that test susceptibility of *Enterococcus faecium* and *Enterococcus faecalis* to β-lactams, aminoglycosides, vancomycin, lipoglycopeptides, oxazolidinones, novel tetracycline-derivatives, and daptomycin. We also identify unique problems and gaps with the performance and clinical utility of antimicrobial susceptibility testing for enterococci, provide recommendations for clinical laboratories to circumvent select problems, and address potential future innovations that can bridge major gaps in susceptibility testing.

KEYWORDS *Enterococcus*, antibiotic resistance, antimicrobial activity, antimicrobial agents, clinical methods, diagnostics, gram-positive bacteria, hospital infections, multidrug resistance, susceptibility testing

Enterococci are Gram-positive facultative anaerobic commensals that reside in the gastrointestinal (GI) tracts of humans and animals. Human infection with *Enterococcus faecalis* was first reported in 1899, and even with the introduction of antibiotics in the early 20th century, enterococcal infections remained challenging to treat (1–5). Nowadays, due to an expansion of medical innovation and a parallel increase in the immunocompromised patient population, these recalcitrant organisms have persisted as major nosocomial pathogens (1). Enterococci are the third most common pathogen in health care associated infections and the second most common cause of bacteremia acquired in intensive care units (6–9).

These organisms have a wide repertoire of intrinsic mechanisms of antimicrobial resistance and the genomic plasticity to facilitate the acquisition of additional resistance determinants (1). Further, the ability of members of this genus to survive adverse conditions, such as high temperatures, osmolarity, and disinfectants, permits enterococci to persist on hospital surfaces and contributes to their rise as prominent hospital-associated pathogens in the late 1970s (10). This increased frequency of isolation roughly corresponded with the introduction of third-generation cephalosporins (to which enterococci are intrinsically resistant), with *E. faecalis* initially isolated in 90 to 95% of cases (11). Subsequently, the widespread use of vancomycin and broad-spectrum antimicrobials led to the emergence of vancomycin-resistant enterococci (VRE), particularly in *Enterococcus faecium*. Beginning in the mid-1980s, rates of vancomycin resistance in *E. faecium* increased rapidly from approximately 0% to more than 83% by 2016, while over this same time period only around 9% of *E. faecalis* exhibited the VRE phenotype (12). Importantly, clinical isolates of VR-*E. faecium*

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often exhibit resistance to other commonly used antienterococcal antibiotics such as ampicillin and aminoglycosides, raising a major therapeutic challenge (3, 11). Linezolid is the only currently U.S. Food and Drug Administration (FDA)-approved antibiotic for VRE infections. However, therapy with linezolid can be limited due to its bacteriostatic effect and associated toxicity. Thus, several other newer antibiotics have seen increased use against VRE, particularly in immunocompromised patients and deep-seated enterococcal infections such as infective endocarditis, where bactericidal activity is usually favored. Indeed, daptomycin has become a front-line antibiotic against VRE, either as monotherapy or in combination, but challenges with susceptibility testing and increasing rates of resistance continue to pose a problem for its widespread use (13–15). Other antibiotics such as oritavancin, tigecycline, omadacycline, and eravacycline have demonstrated *in vitro* activity against VRE. However, aside from sporadic case reports, there is a lack of both clinical data and an assessment of performance of manual or commercial susceptibility testing platforms with these newer agents. In our literature review, we included key studies that highlighted the advantages and disadvantages of manual and automated antimicrobial susceptibility testing (AST) for antimicrobials commonly used in clinical practice for the treatment of infections caused by *E. faecium* and *E. faecalis*, namely, β-lactams, aminoglycosides, vancomycin, lipoglycopeptides, oxazolidinones, novel tetracycline-derivatives and daptomycin. Of note, our knowledge on the performance of many AST platforms is limited to studies conducted in the early 2000s which highlights the severe paucity of contemporary data in this field.

Choosing the appropriate therapy for enterococcal infections is of paramount importance to improve clinical outcomes. Indeed, a recent prospective study showed that the lack of microbiological eradication was a major factor that correlated with poor outcomes in patients with *E. faecium* bacteremia (16). Thus, accurate, precise, and reproducible antimicrobial susceptibility testing of enterococci becomes critical to guiding therapy. Laboratories generally perform susceptibility testing on all enterococcal isolates from sterile body sites. However, a major issue with standard susceptibility testing is that enterococci do not grow optimally in Mueller-Hinton (17–19), the standard growth medium for antimicrobial susceptibility testing recommended by both the Clinical and Laboratory Standards Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST). Further, the majority of clinical laboratories in the United States rely on commercial automated systems (cASTs) for susceptibility testing, while low-resource settings primarily deploy manual disks and gradient strip-based methods which often have been shown to have poor reproducibility for enterococci (19, 20). In this review, we summarize the performance and limitations of manual and commercial susceptibility testing methods for clinically relevant antimicrobials against *E. faecium* and *E. faecalis* and future areas of innovation to overcome existing AST hurdles.

β-LACTAMS

Unlike streptococci, enterococci exhibit much higher MICs to β-lactams (with resistance to most cephalosporins) and aminoglycosides. Among the β-lactams, the aminopenicillins (ampicillin and amoxicillin), penicillin, piperacillin, and imipenem have the best activity against these organisms. A recent SENTRY study highlighted the rising rates of ampicillin resistance among contemporary *E. faecium* isolates (~89.8% worldwide susceptibility) likely associated with the acquisition (and possibly increased expression) of a "resistant" *pbp-5* allele (encoding penicillin-binding protein 5) (2, 3, 21). However, *E. faecalis* isolates worldwide largely remain susceptible to ampicillin (21). Of note, a gene encoding a β-lactamase enzyme that conferred resistance to ampicillin has been identified in several *E. faecalis* from the 1980s and in *E. faecium* in the 1990s but has been rarely reported in contemporary clinical isolates (22, 23).

Disk diffusion and gradient diffusion methods are feasible manual methods for the assessment of β-lactam susceptibility for enterococci, most commonly penicillin or ampicillin, that are still commonly used in low-resource settings. Early evaluations of these methods in the 2000s reported overall good performance for susceptibility to

ampicillin (24). Nonetheless, the disk diffusion method has been reported to "overcall" resistance to ampicillin (a common limitation of this method with many organisms). A study that evaluated the impact of the discordance in ampicillin disk content between EUCAST (2 μg) and CLSI (10 μg), reported that among 87 *E. faecium* isolates, 4 and 3 ampicillin-susceptible *E. faecium* (by reference broth microdilution method) were categorized resistant or intermediate by the disk using EUCAST and CLSI guidelines, respectively (25). The same study also concluded that the gradient strip method falsely categorized two ampicillin-resistant isolates as susceptible. Larger studies are needed to confirm the problematic impact of the discrepant ampicillin disk concentrations. Of note, rare instances of penicillin or ampicillin resistance due to β-lactamase production are not reliably detectable by disk or dilution-based methods and CLSI guidelines recommend the use of a direct, nitrocefin-based test in select cases (26).

Many clinical laboratories in the United States utilize automated or semiautomated commercial systems, which in general, produce accurate and reproducible results for susceptibility testing of β-lactams and aminoglycosides. The BD Phoenix system has been reported to perform accurately and reliably for enterococci with 100% categorical agreement (CA) for ampicillin and penicillin relative to the agar dilution reference method (27). The Vitek 2 system was also reported to have high concordance for ampicillin (93 to 100% EA) against multidrug-resistant enterococcal isolates (28, 29). The Vitek 2 has a tendency to "undercall" ampicillin nonsusceptibility for *E. faecium* isolates with low-level MICs and isolates with β-lactamase production (rare), where detection is inoculum dependent (25). On the other hand, it can "overcall" ampicillin resistance in *E. faecalis*, as another study reported (9 MEs out of 60 isolates) (28), which can be clinically problematic. However, there is a paucity of contemporary data evaluating the performance of any modified or updated commercial AST systems.

Penicillin-resistant, ampicillin-susceptible *E. faecalis* phenotype. *E. faecalis* is generally susceptible to penicillin, amino-penicillins, and imipenem. Thus, CLSI guidelines recommend using ampicillin susceptibility results as a surrogate marker to predict susceptibility to amoxicillin, imipenem, and piperacillin-tazobactam for non-β-lactamase-producing *E. faecalis* (30). Importantly, β-lactamase production in *E. faecalis* is rare and not tested by most clinical microbiology laboratories, supporting the practical usage of ampicillin susceptibility as a surrogate marker of susceptibility to the other β-lactam antibiotics (see above) in *E. faecalis*. Of note, the EUCAST clinical breakpoints for ampicillin are lower than CLSI and there are no EUCAST breakpoints for penicillin (Table 1). Indeed, a 2001 study reported that all 201 ampicillin-susceptible *E. faecalis* isolates from a single U.S. institution were reported to have imipenem MICs $\leq$ 4 mg/L by broth microdilution and presumed to be imipenem-susceptible (31). Of note, there are no distinct enterococcal breakpoints for imipenem. Another multicenter follow-up study in the United States. with 633 diverse, contemporary enterococcal strains showed $\geq$ 94% CA by broth microdilution and $\geq$ 98% CA by disk diffusion for penicillin or ampicillin results, predicting imipenem susceptibility in both *E. faecalis* (n = 442) and *E. faecium* (n = 151) (32). However, starting in 2006, some clinical reports emerged from Greece, Denmark, and Brazil of *E. faecalis* isolates that exhibited penicillin resistance but were susceptible to ampicillin. The mechanistic basis of this phenomenon seems to be due to alterations and overproduction of PBP4 (33, 34). Further, a 2012 study using 317 *E. faecalis* clinical isolates from Brazil showed that 11% of isolates exhibited the phenotype of penicillin resistance (MICs 16 to 32 μg/mL) and ampicillin susceptibility (MICs 1 to 8 μg/mL). According to CLSI breakpoints (35), MIC values $\geq$ 16 mg/L were considered resistant. Interestingly, broth microdilution and Etest categorized 100% of these strains as susceptible to amoxicillin (91.2% were susceptible by standard disk diffusion), indicating a good correlation between ampicillin and amoxicillin susceptibility results. However, only 73.5, 61.8, and 38.2% of the isolates were susceptible to imipenem when using broth microdilution, gradient diffusion, and disk diffusion, respectively. None of the isolates were susceptible to piperacillin by broth and gradient diffusion, while 27% were determined to be piperacillin-susceptible by disk diffusion. Another study with 59

TABLE 1 Clinical breakpoints recommended by CLSI or EUCAST for relevant antimicrobials against enterococci (30, 39)[a]

Antimicrobial	Disk content	Interpretive categories and zone diam breakpoints in mm			Interpretive categories and MIC breakpoints in μg/mL			
		S	I	R	S	SDD	I	R
Penicillins								
Penicillin CLSI	10 U	≥15	–	≤14	≤8	–	–	≥16
Ampicillin CLSI	10 μg	≥17	–	≤16	≤8	–	–	≥16
Ampicillin EUCAST	2 μg	≥8	–	<8	≤4	–	–	>8
High-level aminoglycoside resistance[b]								
Gentamicin CLSI	120 μg	≥10	–	≤6	≤500	–	–	>500
Gentamicin EUCAST	30 μg	≥8	–	<8	≤128	–	–	>128
Streptomycin CLSI	300 μg	≥10	–	≤6	≤1,000 (broth) ≤2,000 (agar)	–	–	>1,000 (broth) >2,000 (agar)
Streptomycin EUCAST	300 μg	≥14	–	<14	≤512	–	–	>512
Glycopeptides and lipoglycopeptides								
Vancomycin CLSI	30 μg	≥17	15–16	≤14	≤4	–	8–16	≥32
Vancomycin EUCAST	5 μg	≥12	–	<12	≤4	–	–	>4
Dalbavancin, vancomycin-susceptible *E. faecalis* only CLSI	–	–	–	–	≤0.25	–	–	–
Oritavancin CLSI	–	–	–	–	≤0.12	–	–	–
Televancin CLSI	–	–	–	–	≤0.25	–	–	–
Teicoplanin CLSI	30 μg	≥14	11–13	≤10	≤8	–	16	≥32
Teicoplanin EUCAST	30 μg	≥12	–	<12	≤4	–	–	>4
Lipopeptides								
Daptomycin, *E. faecium* only CLSI	–	–	–	–	–	≤4	–	≥8
Daptomycin, *Enterococcus* spp., except *E. faecium* CLSI	–	–	–	–	≤2	–	4	≥8
Streptogramins and oxazolidinones								
Linezolid CLSI	30 μg	≥23	21–22	≤20	≤2	–	4	≥8
Linezolid EUCAST	10 μg	≥20		<20	≤4			>4
Tedizolid CLSI					≤0.5			
Tetracyclines								
Tigecycline, *E. faecalis* EUCAST	15 μg	≥20	–	<20	≤0.25	–	–	>0.25
Tigecycline, *E. faecium* EUCAST	15 μg	≥22	–	<22	≤0.25	–	–	>0.25
Eravacycline, *E. faecalis* EUCAST	20 μg	≥22	–	<22	≤0.125	–	–	>0.125
Eravacycline, *E. faecium* EUCAST	20 μg	≥24	–	<24	≤0.125	–	–	>0.125
Fosfomycin								
E. faecalis urinary tract only, CLSI	200 μg	≥16	13–15	≤12	≤64	–	128	≥256
Nitrofurantoins								
CLSI	300 μg	≥17	15–16	≤14	≤32	–	64	≥128
E. faecalis uncomplicated UTI only, EUCAST	100 μg	≥15	–	<15	≤64			>64

[a]If an agent has breakpoints listed by CLSI or EUCAST but not both, that implies that the agency does not have established breakpoints for the agent. CLSI guidelines recommend reading results for disk diffusion at 16 to 18 h and for dilution based methods at 16 to 20 h. EUCAST recommends reading all results at 16 to 20 h. Both agencies recommend reading vancomycin test results at 24 h. Please see CLSI M100 document and EUCAST guidelines for further detailed recommendations on testing for each agent. A dash indicates that no value is given for that interpretive category. For instance a dash under "I" means there is no intermediate range, only a susceptible or resistant one. For disks, it means these particular antibiotics do not have a corresponding disk manufactured, thus no concentration can be given.

[b]High-level aminoglycoside resistance is interpreted as a positive or negative result based on the indicated cutoff values. The CLSI recommends reading gentamicin and streptomycin disk diffusion results at 16 to 18 h, gentamicin broth or agar dilution methods results at 24 h, and streptomycin broth or agar dilution methods results at 24 to 48 h (reincubation required if susceptible at 24 h).

penicillin-resistant *E. faecalis* isolates reported that 27 and 98% were resistant to imipenem and piperacillin (MICs ≥ 16 mg/L), respectively, while none were resistant to ampicillin or amoxicillin by BMD (36). Thus, the current evidence suggests that in penicillin-resistant isolates, ampicillin susceptibility is an unreliable predictor of *in vitro* susceptibility to imipenem or piperacillin.

The precise global epidemiological prevalence of penicillin-resistant, ampicillin-susceptible *E. faecalis* strains remains unclear. A prospective observational study in South Korea with 295 patients with *E. faecalis* bloodstream infections showed that 22.7% of recovered isolates were penicillin-resistant and ampicillin-susceptible using CLSI breakpoints, and that infections involving these isolates were associated with a >2-fold higher 30-day mortality rate (adjusted odds ratio 2.27, 95% confidence interval,1.01 to 5.02) relative to penicillin-susceptible strains (37). Of note, a significant decrease in survival was also observed in patients infected with penicillin-resistant isolates that were treated with ampicillin and/or piperacillin-based regimens likely due to therapeutic failure ($P = 0.011$). However, these data should be taken with caution since, as mentioned above, AST methods used by clinical laboratories have variable performance for the detection of penicillin resistance. A 2019 study showed that the gradient strip method consistently miscategorized isolates as penicillin resistant, while all 24 of them were determined to be susceptible by reference broth microdilution (38). The study also showed that all isolates identified as penicillin resistant by the gradient strip method had piperacillin-tazobactam MICs $\leq$ 16/4 μg/mL by broth microdilution which, despite the lack of CLSI breakpoints for enterococci, is considered a wild-type MIC range below the epidemiological cutoff value (39). The Vitek 2 seems to have similar performance issues as was reported in a study of 49 isolates where 94% were falsely categorized by the Vitek 2 as penicillin resistant with CLSI breakpoints relative to the reference method (40). Thus, in clinical practice common AST methods are inaccurate and unreliable for detection of penicillin resistance and should not be used to predict susceptibility to other agents nor to preclude therapeutic use of penicillin or piperacillin-tazobactam without verification with alternative reference methods. These issues highlight a general limitation of using surrogate AST methods to guide treatment decisions where performance issues can be amplified and have major clinical repercussions.

AMINOGLYCOSIDES

High-level resistance to aminoglycosides can vary geographically with rates between 40 and 68% but the frequency seems to be increasing in both *E. faecium* and *E. faecalis* (16, 21, 41, 42). Indeed, due to the intrinsically high MICs to aminoglycosides in most clinically relevant enterococci, clinical laboratories only tests for high-level resistance to gentamicin (>500 μg/mL) and streptomycin (>1,000 μg/mL by broth, >2,000 μg/mL by agar) to determine the possibility of synergism with a cell wall acting agent (Table 1). The most common gene associated with high-level resistance to gentamicin is *aac(6′)-Ie–aph(2″)-Ia* which encodes a bifunctional aminoglycoside-modifying enzyme (often located on conjugative transposons) conferring resistance to all commercially available aminoglycosides, including amikacin and tobramycin with the exception of streptomycin (Table 2) (43). High-level resistance to streptomycin alone is possible due to ribosomal mutations or the presence of a streptomycin-nucleotidyltransferase enzyme (1). Of note, high-level resistance to gentamicin or streptomycin is sufficient to abolish the bactericidal activity observed between a cell wall active agent and the respective aminoglycoside.

When testing for high-level aminoglycoside resistance, CLSI recommends using MH agar for disk diffusion and agar dilution while brain heart infusion broth is recommended for microdilution since it yields better growth and concordance compared to MH broth (44). Early evaluations of disk diffusion based methods in the 2000s reported overall good performance for identification of high-level resistance to aminoglycosides (45). Indeed, gentamicin testing via gradient strip has consistently been reported as an accurate marker of high-level aminoglycoside resistance while the streptomycin strip is less reliable (46). Further, a 2001 study that assessed six national committee recommended disk diffusion procedures from the United States and Europe (54 *E. faecalis* and 7 *E. faecium* clinical isolates) showed poor performance for gentamicin, relative to broth microdilution (47). This discordance is expected because global disk diffusion recommendations differ in terms of media, inoculum density, antimicrobial disk concentration and interpretative clinical breakpoints.

TABLE 2 Summary of antimicrobial agents covered in the review, common mechanisms of resistance and associated challenges to antimicrobial susceptibility testing[a]

Antibiotic class	Resistance in enterococci: Common mechanism	Resistance in enterococci: Associated gene(s)	Challenges to antimicrobial susceptibility testing
β-Lactams			
Ampicillin	PBP modification or overproduction	*pbp-5* (*E. faecium*)	• Disk diffusion method "overcalls" resistance to ampicillin • Gradient strips can falsely characterize resistant isolates as susceptible • Vitek 2 "undercalls" ampicillin nonsusceptibility in *E. faecium*
Penicillin w/AMP-S	PBP modification or overproduction	*pbp-4* (*E. faecalis*)	• Vitek 2 "overcalls" ampicillin resistance in *E. faecalis* • Gradient strip method and Vitek 2 both consistently miscategorize PCN-S isolates as PCN-R (compared to broth microdilution) • BMD, gradient strips, and disk diffusion methods for ampicillin may not reliably predict susceptibility to imipenem or piperacillin in in PCN-R AMP-S phenotype enterococci
Aminoglycosides			
Gentamicin	Aminoglycoside-modifying enzyme (high-level resistance)	*aac(6′)-Ie–aph(2″)-Ia*	• Gradient strips less useful for the detection of high-level resistance due to max tested concn of 256 μg/mL
Streptomycin		Ribosomal mutations and streptomycin adenyltransferase	
Glycopeptides (vancomycin and teicoplanin)	Remodeling of peptidoglycan precursors by production of D-Ala-D-Lac (high level) or D-Ala-D-Ser-ending precursors. Destruction of normal D-Ala-D-Ala ending precursors	*vanABCDEGMNO*, (*vanA*, *vanB*, and *vanC* being the most commonly identified)	• Vitek 2 does not identify all *vanB*-carrying enterococcal isolates, missing up to 25% of isolates • Vitek 2 tends to misidentify enterococci with low-level vancomycin resistance (i.e., *E. gallinarum* or *casseliflavus*) • Gradient strips identify <50% of *vanB*-positive isolates • BMD identifies ~60% of *vanB*-positive isolates
Lipoglycopeptides	Remodeling of peptidoglycan precursors similar to glycopeptide resistance. For oritavancin increased expression of *vanA* operon.	*vanAB*	• Gradient strips are not FDA-approved for oritavancin • Automated commercial AST systems do not yet include oritavancin and dalbavancin on their panels • BMD has increased complexity due to the lipophilic tail unique to the class, which necessitates low-binding plastic microtiter plates, and addition of p-80 to block drug binding to plastic. DMSO is also required as a drug stabilizer • Many clinical labs send out AST due to complexity
Oxazolidinones	Mutations in 23S rRNA genes Changes in ribosomal proteins Modification of rRNA Ribosomal protection	23S rRNA, *rplC*, *rplD*, *cfr*, *optrA*, *poxtA*	• Agar-based diffusion methods increase in accuracy and precision after prolonged incubation (>42 h) • Broth-based methods with CAs of 75–85% compared to BMD (EUCAST breakpoints) • Phoenix and Vitek 2 have reduced CA to BMD (64.2%, 79%) due to lack of 2-day incubation (EUCAST) • No agar-based methods have acceptable CA to BMD when using CLSI breakpoints

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TABLE 2 (Continued)

Antibiotic class	Resistance in enterococci		Challenges to antimicrobial susceptibility testing
	Common mechanism	Associated gene(s)	
			• Gradient strips are effective, but have brand variability, and interpretation is difficult due to trailing enterococcal growth within the halo of growth inhibition • Tedizolid susceptibility testing platforms have lagged, and linezolid has been used as a surrogate marker
Tetracycline derivatives Glycylcyclines (tigecycline)	Displacement of antibiotic from site of action: • Efflux pumps • Ribosomal protection proteins	*tet*(L), *rpsJ*	• In the limited multipathogen studies available, gradient strips appear to have lower performance than agar dilution • Further studies are needed overall • There is a paucity of published data evaluating susceptibility testing platforms • Existing gradient strips tend to report MICs 1-fold lower than the reference BMD
Aminomethylcyclines Lipopeptides (daptomycin)	Alteration of cell wall synthesis and cell membrane homeostasis	*liaFSR*, *cls*	• The requirement of calcium for daptomycin activity leads to variation of MIC determination between lots of media due to calcium concn • MIC determinations by BMD and gradient strip have been demonstrated to vary significantly across institutions/labs • Genotype risk factors for daptomycin resistance are not consistently reflected in MICs by any method

[a]ABC, adenosine-triphosphate binding cassette; Ala, alanine; AMP, ampicillin; AST, antimicrobial susceptibility testing; BMD, broth microdilution; CA, categorical agreement; DMSO, dimethyl sulfoxide; Lac, lactate; p-80, polysorbate-80; PCN, penicillin; Ser, serine; -R, resistant; -S, susceptible. Further studies are needed regarding mechanisms of resistance in enterococci. Most common clinical mechanisms/gene associations listed. For a comprehensive review and discussion, please see references 1 and 140.

Among automated AST systems, the BD Phoenix system has been reported to perform accurately and reliably for enterococci with 100% categorical agreement (CA) for gentamicin relative to the agar dilution reference method (48). The Vitek2 system was reported to have acceptable performance against multidrug-resistant enterococcal isolates with high concordance for high-level gentamicin resistance (essential agreement [EA] of 97 to 100%) and high-level streptomycin resistance (96 to 100% EA) (28, 29). The Vitek2 yields low rates of very major (VME) or major errors (ME) for high-level gentamicin and streptomycin resistance (only 1 isolate in a collection of 84) (29).

VANCOMYCIN

Vancomycin was the front-line agent for the treatment of ampicillin-resistant enterococcal infections or in patients with severe β-lactam allergies until the emergence and dissemination of VRE (1, 49). Vancomycin resistance in the clinical setting is due, in part, to the synthesis of modified, low vancomycin binding-affinity cell wall precursors (terminating in D-alanine-D-lactate or D-alanine-D-serine) encoded by several gene clusters, of which the *vanA* and *vanB* operons are the most commonly encountered (often carried on mobile transposons) (50). A chromosomally encoded *vanC* operon confers low-level resistance to vancomycin (MICs 2 to 32 μg/mL) and is found in *E. gallinarum* and *E. casseliflavus*, which are less commonly reported as causes of clinical infections. The presence of additional *van* gene clusters (i.e., *vanD*, *vanE*, *vanG*, *vanL*, *vanM*, and *vanN*) in enterococci is possible but rare (51). The *vanA* genotype is the most prevalent, but *vanB* harboring isolates are increasingly reported as the cause of sporadic outbreaks in Europe and Australia (52–55). The main difference is that isolates exhibiting the VanB phenotype test susceptible to teicoplanin with variable MICs to vancomycin

(4 to 1,024 μg/mL). Molecular testing has helped in differentiating the genotypes. An early study with 150 diverse enterococcal isolates (60 *E. faecalis*, 55 *E. faecium*, 26 *E. gallinarum*, and 9 other *Enterococcus* spp.) from 35 states, including 99 vancomycin-resistant strains reported a CA for vancomycin of 95.3, 96.6, and 96% for the gradient strip, Vitek, and disk diffusion methods, respectively (using CLSI breakpoints, which is consistent with later studies) (28, 56). The gradient strip and Vitek errors were due to "overcalling" vancomycin nonsusceptibility relative to the reference method. The BD Phoenix commercial system is reported to have good performance for all antimicrobials for enterococci, including >98% CA for vancomycin and teicoplanin (57, 58). In a study using Vitek2, the performance of vancomycin susceptibility showed >94% CA for both *E. faecalis* and *E. faecium*. Of note, Vitek2 detected 96% of the *vanA*-carrying isolates but only 81% of *vanB* isolates (28). In another study, the Vitek2 correctly identified 78 of 80 isolates with the *vanC* phenotype but was unable to distinguish between *E. gallinarum* or *E. casseliflavus* (59), although the clinical relevance of distinguishing between these two *vanC* species is unclear. Of note, a study showed that misidentification of 13 vancomycin-resistant *E. faecium* isolates with low-level, inducible vancomycin resistance over a 5 month period from patients in the transplant unit led to spread of VRE through the unit and inappropriate management (60). After the issue was identified, the laboratory tested the isolates and found that the BD Phoenix accurately identified 12 out of 13 isolates as *E. faecium*, while the Vitek2 correctly identified only 2 isolates, with the remaining misidentified either as *Aerococcus viridans* or "unable to be identified." Further, the Rapid ID 32 Strep system accurately identified all 13 isolates. Larger contemporary studies are needed to determine whether the Vitek2 performance has improved performance in the last few years.

In another study from 2019 that included 68 *vanB*-carrying VRE isolates, the Vitek2 correctly identified 52 as being vancomycin resistant, while testing by broth microdilution identified only 41 isolates as VRE. The Etest (bioMérieux) and MIC test strip (Liofilchem) gradient strips correctly identified 26 and 24 *vanB* positive isolates, respectively (61). A multicenter study using 12 *E. faecalis* and 18 *E. faecium* isolates compared Vitek2, EUCAST disk diffusion, and the CLSI recommended screen (brain heart infusion agar [BHI] supplemented with 6 μg/mL vancomycin) for the detection of low-medium level *vanB*-mediated resistance. The results showed that the disk and agar method (which only yielded errors due to low-level MICs of 4 to 8 μg/mL), performed better than the Vitek2, which yielded more errors in the intermediate level resistance category (16 to 32 μg/mL) (62). Furthermore, the study showed that disk diffusion detected vancomycin resistance better on MH-II agar from Oxoid ($P < 0.0001$) or Merck ($P = 0.027$) compared to BD BBL. Similarly, the CLSI agar screen performed better on Difco BHI agar than the Oxoid or BD BBL brands ($P = 0.017$). These variations associated with the agar or disk manufacturer used by laboratories highlights a major limitation in the interpretation of phenotypic testing (63, 64).

It is worth noting that some *vanA*-carrying enterococci exhibit phenotypic susceptibility to vancomycin, and can revert to a vancomycin-resistant phenotype (designated vancomycin-variable enterococci [VVE]) (65). Furthermore, some enterococci exhibit loss of function mutations in the *ddl* genes (encoding the native D-Ala-D-Ala ligases) and can only survive in the presence of vancomycin capable of inducing the van clusters and synthesize cell wall precursors ending in D-lactate. These isolates have been termed vancomycin-dependent enterococci (VDE) (66).

VRE screening in hospital settings and molecular assays. The clinical laboratory plays a critical role in preventing VRE nosocomial infections by screening patients and hospital environments for VRE colonization and relaying that information to the infection control team. Colonization of the GI tract with VRE is associated with many factors that include prior use of broad-spectrum agents such as cephalosporins, antibiotics with antianaerobic activity (e.g., metronidazole), and vancomycin (67). While the majority of VRE-colonized patients may not develop symptomatic infections, colonization and domination (where >30% of GI microbiome taxa are composed of enterococci) of

the GI tract increases the risk for VRE bacteremia in immunocompromised patients, particularly in neutropenic and bone marrow transplant patients (68, 69). Active surveillance for VRE colonization and isolation are the primary methods to prevent widespread VRE transmission. Chromogenic media for direct culturing of stool samples like ChromID (bioMérieux) allows for colorimetric differentiation between *E. faecalis* and *E. faecium* with 90 and 85.4% sensitivity, respectively, and 100% specificity for both. These media perform better than bile esculin azide agar containing vancomycin which has higher false-positive rates (42). False positives are possible with *Candida* spp. and Gram-negative rods but contaminants tend to be colorless and likely to be interpreted incorrectly as VRE. CHROMagar (BD) detects VanA and VanB phenotypes while inhibiting the growth of VanC. Overall, culture-based methods have inherent limitations of time to reporting with delays of up to 5 days and PCR-based molecular assays offer faster turn-around times. Select chromagars which can identify VRE in 1 day can be useful for clinical laboratories that batch PCR assays and perform them once a day.

Rapid identification of pathogenic organisms and differentiation from contaminants is especially critical in bloodstream infections to decrease time to appropriate therapy. Further, rapid molecular tests applied on samples taken to evaluate colonization status decrease the time to implement targeted infection control procedures. The performance of molecular assays varies with sensitivities ranging from 61.5 to 100% and specificity ranging from 14.7 to 99.5% (42). The FDA-approved Xpert *vanA* assay (Cepheid) was reported to have 100% sensitivity and 96.9% specificity in a study with 101 VRE-positive rectal swabs from patients at high-risk for VRE colonization (70). The Roche LightCycler VRE detection platform for rectal swabs has a high negative predictive value (NPV) for *vanA* (99.9%) and *vanB* (99.9%) and low positive predictive value (PPV) for *vanA* (68.5%) and *vanB* (1.4%) compared to conventional culture (71). Interestingly, the Cepheid *vanA* and *vanB* assay had a high PPV (92%) when the reference culture method involved a broth enrichment step prior to plating but not otherwise (32%), reflecting the variable performance of the "gold standard" culture-based test (70, 72). Thus, these molecular assays are most useful to "rule out" VRE colonization in patients. A study with 1027 swab specimens from 3 distinct sites reported a BD GeneOhm VanR assay sensitivity, specificity, PPV, and NPV of 93.2, 81.9, 54.4, and 98.1%, respectively (73). The specificity was limited by false positives due to *vanB*, suggesting that secondary confirmation with other methods is necessary. Of note, in this study, perianal swabs had higher specificity than rectal swabs for *vanA* and *vanB* detection (87.1% versus 74.7%). A 2019 European multicenter external quality control assessment conducted over 6 years reported that accurate detection of *vanA* isolates has remained high over the years (sensitivity 94 to 97%), the sensitivity of detecting *vanB* increased from 82% in 2013% to 93% in 2018 and commercial or in-house assays performed equally well (74).

The Verigene Gram-positive blood culture assay is a multiplex microarray-based molecular test with 12 Gram-positive organism targets including *E. faecium* and *E. faecalis* and 3 resistance markers including *vanA* and *vanB*. In a multicenter study with 1,252 blood cultures containing Gram-positive bacteria the Verigene blood culture assay had a sensitivity of 94.8% (55/58) and 92.6% (26/28) for the organism identification of *E. faecalis* and *E. faecium*, respectively, with a 100% specificity for both in addition to a 100% sensitivity and specificity (55/55) for accurate detection of *vanA* or *vanB* (75). These findings were confirmed in a study with 203 positive blood cultures from pediatric patients (76). However, a major hurdle of all PCR-based methods is the presence of *van* clusters in organisms other than enterococci like *Streptococcus mitis/oralis*, *Eubacterium lenta*, *Ruminococcus spp*, *Lactococcus spp*, and some *Clostridium* spp. which are all ubiquitous commensal colonizers of the GI tract (71, 77). Indeed, the lower values of specificity and PPV in the *vanB* assays described above may be due to higher carriage of *vanB* by gut anaerobes and other commensals (78). Selective broth enrichment of VRE rectal swabs (consisting of inoculating swabs into broth containing amoxicillin, amphotericin B, aztreonam, and colistin and incubating for 24 h) increases the Cepheid Xpert's PPV to 100% from 41% compared to using nonenriched samples

(direct swab medium application), with no significant change in NPV (99.5% and 100% for enriched and nonenriched samples, respectively) (79). Of note, the study authors suggested a $C_T \leq 25$ for a true positive for *vanB*, and recommended C_T values between 25 and 30 to be confirmed by culture results (75). However, this approach requires independent validation of the enrichment process, the modified specimen and increases turnaround time by 24 h. Furthermore, PCR-based assays cannot recover organisms for pulsed-field gel electrophoresis, multilocus sequence typing, or whole-genome sequencing to track outbreak epidemiology (80). Alternative immunologic methods like lateral flow devices may bridge the gap between genotypic and phenotypic testing for vancomycin resistance. The NG Biotech lateral *vanA* and *vanB* assay showed 100% sensitivity and specificity in a study with 135 isolates, including 24 *vanA* and 33 *vanB*, when broth enrichment or vancomycin-selective agar were used prior to the assay (81).

LIPOGLYCOPEPTIDES

Telavancin and dalbavancin only retain activity against *vanB* harboring enterococci but not *vanA* genotypes, while oritavancin is active *in vitro* against all VRE, including *vanC* phenotypes, with ≥16-fold more potency (82). A study with 1,086 enterococci reported that seven vancomycin-intermediate isolates had low oritavancin MICs of 0.004 to 0.015 μg/mL and 200 VRE isolates had oritavancin MICs between 0.002 and 1 μg/mL (83). A recent SENTRY study reported 92.3 to 98.3% susceptibility to oritavancin in global collection of VRE strains (21). Broth microdilution testing is more complex for these agents as revised CLSI guidelines include adding DMSO as a solvent and diluent for drug solubilization, in addition to supplementing MHII media with 0.002% polysorbate-80 (p-80) to minimize adherence of the drug to plastic surfaces of the 96-well plates (84–87). The revised CLSI guidelines state lower dalbavancin and oritavancin MICs with the addition of p-80. Thus, in addition to problems with accuracy and reproducibility due to the medium manufacturer, lot-to-lot variation, inoculum density and antibiotic stock preparation, the type of 96-well microtiter plate is an important source of variation for these agents. For example, for dalbavancin, tissue-culture treated and polystyrene plates have worse performance than Corning's nonbinding surface plates that have a nonionic hydrophilic surface to minimize nonspecific molecular interactions (88). Manual methods like the dalbavancin gradient diffusion strip (Etest) was reported to show high agreement with broth microdilution (92.5% MICs within one doubling dilution, 100% within two doubling dilutions) in a study with 200 Gram-positive bacteria (including 20 enterococci), while agar dilution on average yielded 2-fold higher MICs (89). There are no FDA-approved oritavancin gradient strips for clinical use. Long-acting lipoglycopeptides (dalbavancin and oritavancin) are not yet included in panels for testing on commercial systems like the Vitek2, Phoenix, or MicroScan. Thus, due to the complexities mentioned above and due to the low testing volume for these agents, many clinical laboratories outsource lipoglycopeptide testing to larger reference or commercial laboratories.

OXAZOLIDINONES

As mentioned above, linezolid is the only FDA-approved antibiotic indicated for VRE infections with 98 to 99.6% susceptibility reported globally in a recent SENTRY surveillance study of 7,615 VRE isolates using CLSI breakpoints (21). Tedizolid also has *in vitro* activity against VRE with 99.5 to 100% global susceptibility. Of note, there are no EUCAST breakpoints for tedizolid and CLSI only has a susceptible breakpoint (≤0.5 mg/L). CLSI guidelines recommend that susceptibility to linezolid can be used as a surrogate marker for susceptibility to tedizolid for *E. faecalis*, though isolates that are intermediate or resistant to linezolid can retain susceptibility to tedizolid (26). Several mechanisms of resistance that affect oxazolidinones have been reported and include mutations in the 23S rRNA subunit (the G2576T change is the most common in clinical strains), changes in L3 or L4 ribosomal genes and horizontal acquisition of plasmid-borne determinants such as *cfr*, *optrA*, and *poxtA* genes (2, 90). The accumulation of mutated rRNA alleles in combination with other

resistance mechanisms leads to high-level resistance. Of note, *cfr* confers *in vitro* resistance only to linezolid, while the *optrA* and *poxtA* genes confer resistance to both linezolid and the newer antibiotic tedizolid (91, 92). A unique caveat reported for oxazolidinone susceptibility testing in enterococci is increased accuracy and precision when results are read after a prolonged incubation of 2 days (>42 h) due to hazy and trailing growth in agar diffusion-based methods (93).

A recent study assessed performance of commercial tests with 80 *E. faecium* and 20 *E. faecalis* isolates, including 20 carrying *optrA*, 17 with *poxtA* and 1 with both (93). Using EUCAST breakpoints for linezolid (susceptible $\leq$ 4 μg/mL, $\geq$19 mm), the Etest gradient strip had the highest CA (87%) with broth microdilution (on day 1), followed by disk diffusion. Two other broth-based methods (Sensititre [Thermo Fisher] and UMIC [Biocentric]) and MicroScan exhibited a CAs of 85, 82, 83, and 75%, respectively, relative to the reference broth microdilution method. Etest, disk, Sensititre, and UMIC had acceptable CAs of 92, 90, 92, and 92%, respectively, by day 2 of reading results while MicroScan exhibited a CA of 89%. Phoenix and Vitek2, for which results interpretation on day 2 was not possible, had CAs of 64.2 and 79%, respectively. Notably, when using the CLSI breakpoints (susceptible $\leq$ 2 μg/mL, intermediate 4 μg/mL, resistant $\geq$ 8 μg/mL), none of the methods yielded an acceptable CA. Sensititre had the highest CA at 76%, while the automated methods had the highest very major error (VME) rates. CLSI breakpoints yielded the lowest VME rates of 1.7% at day 2 with both Sensititre and Etest, while EUCAST breakpoints yielded a VME rate of 8.5% for Sensititre and 11.9% for Etest. Thus, the study confirmed that extended incubation times, up to 2 days, whenever possible, enhance performance of methods for linezolid susceptibility testing, though it is not currently included in recommendations by CLSI or EUCAST. Indeed, in the above study, the automated methods failed to identify several resistant isolates that harbored *optrA* and *poxtA*. Other studies, however, have shown that the MicroScan, agar dilution, and Vitek 2 show the best performance in the detection of resistant isolates with 23S rRNA mutations (94, 95). This discrepancy highlights the risk of solely using automated methods to detect linezolid resistance.

Gradient strips have been shown to be an effective method for linezolid MIC determination although with reported brand variability. Etest, however, can be harder to interpret due to the trailing of enterococcal growth within the halo of growth inhibition. Interestingly, after observing significantly higher linezolid nonsusceptibility rates relative to published literature, an institution transitioned their testing from the Etest to Vitek2 and evaluated the impact of such change in a clinical outcome study of 116 VRE patients. The change in susceptibility platforms increased linezolid susceptibility rates from 36 to 98% ($P < 0.001$) and decreased time to appropriate therapy from 1.1 to 0.8 days ($P = 0.007$), suggesting that laboratories should optimize AST algorithms if their local antibiogram diverges significantly from regional or global rates (96).

Tedizolid is a later generation oxazolidinone that has been shown to be noninferior to linezolid in the treatment of soft tissue infections (97, 98). Tedizolid also appears to retain activity against *cfr*-containing isolates of *S. aureus* (99). The approval of tedizolid susceptibility testing platforms has lagged and linezolid has been used as a surrogate marker. In a study encompassing 1,241 enterococcal isolates, including VRE, CA between tedizolid and linezolid susceptibility results by broth microdilution was 98.3% using CLSI breakpoints with 3 VME (susceptible to tedizolid but nonsusceptible to linezolid) and 18 minor errors (MiE). These results suggest that linezolid is a viable surrogate marker of tedizolid susceptibility and that the VME were likely due to *cfr* harboring isolates (91). In another study that included 220 enterococcal strains, (100 vancomycin-resistant *E. faecalis* and 120 vancomycin-resistant *E. faecium*, of which 25 were daptomycin nonsusceptible and 10 were linezolid resistant), the tedizolid MIC_{50}/MIC_{90} values were 0.25/0.25 μg/mL for *E. faecalis* and 0.5/1 μg/mL for *E. faecium* (100). Compared with linezolid, the tedizolid MIC_{90} values were 8- and 4-fold lower for *E. faecalis* and *E. faecium*, respectively. Of note, the 10 linezolid-resistant *E. faecium* (MICs between 8 and 32 μg/mL) exhibited tedizolid MICs between 4 and 8 μg/mL (all *cfr* negative), suggesting other mechanisms of resistance and confirming the higher potency of tedizolid *in vitro*.

TETRACYCLINE DERIVATIVES

Tigecycline is the first bacteriostatic glycylcycline approved for clinical use with a 20-fold higher ribosomal affinity than tetracycline (101). This antibiotic retains activity against ~99.5% of VRE with a MIC_{90} of 0.25 μg/mL. Resistance is associated with mutations in efflux pumps (similar to the multidrug and toxin extrusion pumps in *S. aureus*) and mutations in *rpsJ*, a gene encoding ribosomal protein S10, which often occur in response to tigecycline exposure. Mutations in *rpsJ* often in combination with other resistance mechanisms in both Gram-positive and Gram-negative pathogens (102, 103). A multicenter study with a large collection of a variety of Gram-positive and Gram-negative aerobes, anaerobes, nonpneumococcal streptococci, *Streptococcus pneumoniae*, and *Haemophilus influenzae* showed that the Etest gradient diffusion strip showed excellent inter and intralaboratory reproducibility with an EA of 99.3% across three study sites with a MiE rate of 1.9% (104). Another large study (2010) using EUCAST breakpoints that included 6,151 isolates (391 VRE) showed that disk diffusion overcalls resistance, with 5.1% of isolates falsely categorized as resistant relative to agar dilution that called 100% of the VRE as susceptible to tigecycline (105). Another study, which included enterococcal isolates, also showed a similar lower performance for Etest gradient strips compared to agar dilution with EUCAST breakpoints (106). The Vitek2 was reported to have 100% EA and CA with CLSI breakpoints in a study with 78 enterococcal isolates (29), with a paucity of contemporary data for other automated systems.

Omadacycline is a novel aminomethylcycline tetracycline derivative approved in the United States for the treatment of community-acquired pneumonia and acute skin and soft tissue infections. It retains broad-spectrum activity against Gram-positive and Gram-negative aerobic and anaerobic pathogens including VRE with modal MICs of $\leq$0.25 μg/mL. Omadacycline has MIC_{50} values for enterococci between 0.03 and 0.12 μg/mL and MIC_{90} values between 0.12 and 0.5 μg/mL, as reported in large multicenter surveillance studies (21, 107–109). Eravacycline is a novel synthetic fluorocycline recently approved for the treatment of complicated intraabdominal infections that retains broad-spectrum antimicrobial activity in the presence of mechanisms of tetracycline resistance like efflux pumps and ribosomal protection mutations (110). It achieves higher serum concentration levels compared to tigecycline and displays more potent *in vitro* activity with an MIC_{90} value of 0.06 μg/mL against *E. faecalis* and *E. faecium*, while MIC_{90} values of tigecycline are on average 2-fold higher (111). A report with 986 enterococci, including 189 VRE, also reported MIC_{50}/MIC_{90} values for eravacycline of 0.06/0.12 μg/mL, which were 2-fold lower than tigecycline (112). Besides macroscale epidemiological surveillance studies, there is a paucity of published data evaluating susceptibility testing platforms for these novel tetracycline derivatives. A recently published study evaluated the omadacycline MTS gradient strip (Liofilchem) with *E. faecalis* FDA breakpoints ($\leq$0.25 mg/L susceptible, 0.5 mg/L intermediate, $\geq$1 mg/L resistant) reporting essential agreement (EA) of 100% for *E. faecium* and 98.3% for *E. faecalis* with MTS results within 1 dilution of the modal MIC for 95.2% of a total of 474 Gram-positive isolates (113). Thermo Fisher validated their Sensititre microdilution assay for omadacycline against 603 clinical Gram-positive isolates, including 132 enterococci, showing high reproducibility with an evaluable essential agreement of 98.3% by manual interpretation and 99.2% by automated reading (114). The eravacycline MTS gradient strip was also validated with a CA of 99.3%, evaluable EA of 92% and a 0.8% ME rate for 288 enterococcal isolates using FDA breakpoints (115). The MTS gradient strip had a tendency to report MICs 1-fold lower than the reference broth microdilution method. A study that evaluated performance of the Mast and Bio-Rad eravacycline disks against a diverse collection of 1,441 isolates (including 129 enterococci), reported a 97% agreement between the disk brands with no discordance reported between the disks and broth microdilution (116). Of note, CLSI does not have clinical breakpoints for any newer tetracycline derivatives and EUCAST has distinct *E. faecalis* and *E. faecium* breakpoints for tigecycline and eravacycline (Table 1).

DAPTOMYCIN

Daptomycin is a cyclic lipopeptide antibiotic FDA approved for *S. aureus* infections but used off-label as a front-line agent for the treatment of multidrug-resistant enterococcal infections. Wild-type *E. faecium* daptomycin MICs are often 8- to 16-fold higher than *S. aureus* with an MIC_{90} of 4 μg/mL (18). Daptomycin resistance seems to be on the rise in VRE, especially in institutions with higher rates of daptomycin use, with some major cancer centers reporting rates of daptomycin nonsusceptibility between 15 and 21% (15, 117).

The methodology and interpretation of routine susceptibility to daptomycin has been challenging. This issue is due, in part, by the requirement of calcium for daptomycin activity and the variability in calcium concentrations between different lots of standard media. Indeed, early studies also reported problems for daptomycin testing due to significant variability in the concentrations of Ca^{2+} in commercial MHA between manufacturers and within lots or batches (118).

Although the original daptomycin breakpoint established by CLSI was 4 μg/mL, clinical data suggested that patients infected with enterococcal isolates within the "susceptible" range (MICs between 2 and 4 μg/mL) failed therapy (119, 120). Mechanistic studies have indicated that most clinical strains develop resistance or tolerance (i.e., loss of bactericidal activity of daptomycin) via mutations in genes encoding a three-component regulatory system involved in orchestrating the cell envelop response to antimicrobial peptides (the LiaFSR system). Mutations seem to be "selected" by multiple factors, including exposure to cationic antimicrobial peptides produced by the innate immune system, suggesting that enterococci are primed to develop resistance to daptomycin and, hence, the higher MICs observed in clinical strains compared to other Gram-positive pathogens. This phenomenon also potentially explains the documentation of daptomycin resistance in isolates recovered from patients who were never exposed to daptomycin.

A primary challenge to establish more accurate daptomycin clinical breakpoints was that the *E. faecium* wild-type modal population MIC is 2 to 4 μg/mL (18). A multicenter study evaluated the correlation of *liaFSR* mutations with daptomycin susceptibility testing with 40 clinical *E. faecium* isolates across 3 sites. Daptomycin MICs spanned 3-$\log_2$ dilutions by broth microdilution for 60% of isolates, 4 dilutions, for 17.5%, 5 dilutions for 2.5% and 6 or more dilutions for 20% (19). The Oxoid Mueller-Hinton broth supplemented with Ca^{2+} (CA-MHB) yielded MICs that were 1-fold higher, on average, than the CA-MHB from BD BBL or Difco. The modal MIC was 4 μg/mL and 16.7% of the high-level resistant isolates (MICs $>$ 32 μg/mL) were interpreted as susceptible by broth microdilution. If all isolates with *liaFSR* mutations were categorized as "daptomycin tolerant" based on previous data, 86.8% of these isolates were miscategorized as susceptible which resulted in an overall 59.8% VME rate. Interestingly, the use of alternative breakpoints did not improve concordance. For example, a breakpoint of 1.5 μg/mL resulted in a lower VME rate (3.3%) at the cost of a high ME rate (74%); a breakpoint of 2 μg/mL resulted in a VME rate of 32.1% with an ME rate of 15.1% and a breakpoint of 1 μg/mL resulted in a VME rate of 15.8% with a ME rate of 73.8%. It is especially concerning that results are not reproducible even by the reference method, which suggests that the problems with variability and lack of reproducibility are multifaceted and independent of any single factor like technical interpretation, medium brand, or breakpoints.

The aforementioned study also showed that the Etest (bioMérieux) and MTS (Liofilchem) gradient strips both failed to identify isolates that were daptomycin tolerant or resistant by genotype (19). Furthermore, BD MHA yielded MICs that were on average 1-fold higher than the MHA from Remel or Hardy with both Etest and MTS. Of note, gradient strips also showed a high degree of variability (120). An additional finding was that enterococci have poor growth on MHA from Remel and Hardy, indicating that MHA might not be the ideal medium for enterococcal AST (as mentioned above).

E. faecium daptomycin MICs range from 1 to 4 μg/mL with an epidemiological cutoff of $\leq$4 μg/mL and there are significant difficulties in differentiating the isolates with

MICs of 1, 2, and 4 μg/mL with any method. On the other hand, the *E. faecalis* epidemiological cutoff is lower than *E. faecium* ($\leq$2 μg/mL). Despite these differences, CLSI was hesitant to set distinct clinical breakpoints for *E. faecium* and *E. faecalis* over concerns that some clinical laboratories may not identify enterococcal isolates to the species level. In addition, since daptomycin was not FDA-approved for *E. faecium* for any indication (as opposed to *E. faecalis*), commercial systems would be unable to obtain FDA clearance for susceptibility testing. Studies also reported that isolates with MICs $<$ 1 μg/mL harbor *liaFSR* mutations and may predispose to therapeutic failure with daptomycin at the FDA approved dosage of 6 mg/kg/day (121, 122). Thus, it was essential to provide physicians with breakpoint guidance to effectively deploy daptomycin against enterococcal infections.

A special working group at CLSI was tasked to provide recommendations. The work culminated with the release of distinct CLSI breakpoints in 2019 for *E. faecium* (susceptible dose dependent [SDD] $\leq$ 4 μg/mL and resistant $\geq$ 8 μg/mL based on daptomycin doses of 8 to 12 mg/kg/day) and other *Enterococcus* spp (susceptible $\leq$ 2 μg/mL; intermediate 4 μg/mL; resistant $\geq$ 8 μg/mL) (Table 1) with the hopes that clinicians will consider using higher doses of daptomycin against all vancomycin-resistant *E. faecium* infections. Overall, the difficulty in developing enterococcal daptomycin breakpoints highlights the problems with the stochastic nature of MIC distributions and relying on this single measurement as a precise number for subsequent clinical decision making. While useful, the S/I/R categorization scheme for AST has notable weaknesses and alternative, innovative systems to predict antimicrobial susceptibility are clearly needed.

SUSCEPTIBILITY TESTING FOR ANTIMICROBIALS USED TO TREAT URINARY TRACT INFECTIONS

Enterococcus spp. are a leading cause of hospital-acquired urinary tract infections (UTI) with *E. faecalis* being the most prevalent (123). However, the identification of an enterococcal isolate from a urine culture often represent colonization with unclear clinical significance (124). When treatment is indicated in cases of uncomplicated UTI, there are limited options including fosfomycin and nitrofurantoin whose activity is largely limited to *E. faecalis*. CLSI has clinical breakpoints for *E. faecalis* isolates from urine cultures with agar dilution recommended with supplementation of the media with 25 mg/L glucose-6-phosphate. Fosfomycin disks contain 50 μg of glucose-6-phosphate and broth microdilution is not recommended. The most common resistance mechanism of fosfomycin resistance include plasmid-mediated carriage of *fosB* (MICs to $>$1,024 mg/L), encoding a magnesium (Mg-II)-dependent inactivating enzyme that adds L-cysteine to the epoxide ring of fosfomycin. Acquired mutations in the active site of UDP-*N*-acetylgluosamine enolpyruyl transferase have also been described (125–127). Studies elucidating mechanisms of nitrofurantoin resistance are largely limited to *Enterobacterales*. Limited reports implicate chromosomally encoded nitroreductases in nitrofurantoin resistance in *E. faecalis* and *E. faecium* (128). There are limited contemporary studies assessing the efficacy of or performance of susceptibility testing methods with nitrofurantoin and fosfomycin against enterococcal urinary isolates. Studies from the 2000s report low rates of resistance to nitrofurantoin. A 2003 multicenter U.S. study with 697 VRE (616 *E. faecium*, 81 *E. faecalis*) reported 2.4% resistance to nitrofurantoin (129). A study in the U.K. conducted between 2005 and 2014, with, 5,528 enterococcal isolates from urine cultures at a tertiary hospital, of which 542 were VRE, reported a drastic decrease in susceptibility to nitrofurantoin over time (from 100% in 2005 to as low as 60%) and observed that it was more effective against *E. faecalis* than *E. faecium* (130). A recent study from India conducted over a 10-year period with 239 *E. faecalis* isolates reported MIC_{50}/MIC_{90} values with agar dilution of 8/64 and 8/16 mg/L for nitrofurantoin and fosfomycin, respectively (131).

BEYOND S/I/R: INNOVATIONS IN SUSCEPTIBILITY TESTING

The current gold standard for antimicrobial susceptibility testing in clinical laboratories is phenotypic tests that measure microbial growth in the presence of an antibiotic relying on culture-based isolation of the pathogen. The entire process may take 48 to 72 h (sometimes longer) depending on the physiological properties of the organism and the antimicrobial. The delays in reporting may cause serious setbacks in terms of guiding the appropriate antimicrobial therapy. Innovation in AST has focused on the development of syndromic rapid molecular tests that detect the presence of genetic markers of antimicrobial resistance directly from specimens and function as surrogates to predict susceptibility of organisms, reducing turnaround time to results by at least 1.5 days compared to conventional methods (132). However, rapid molecular assays are limited by our incomplete knowledge of the vast array of resistance mechanisms harbored across organisms to various antimicrobials and potential discrepancies with phenotypic AST results.

Our previous studies have also demonstrated the failure of gold standard MIC determination to predict daptomycin susceptibility in *E. faecium* due to lack of reproducible results. Isolates with daptomycin MICs < 1 μg/mL harbor mutations in the *liaFSR* cell envelope stress response system associated with treatment failure and genetic and biochemical studies have shown that alterations in the *liaFSR* system are early evolutionary indicators of the eventual development of high-level daptomycin resistance (19, 133, 134). Innovative tools like whole-genome sequencing (WGS) have been used to predict phenotypic antimicrobial resistance and to track the epidemiological patterns of multidrug-resistant pathogens (135). WGS tools either utilize a rules-based approach to predict susceptibility based on the presence of one or more AMR genetic determinants derived from a database or deploy machine-learning modeling algorithms to train programs to predict susceptibility by incorporating complex data on single nucleotide polymorphisms, indels, and other genetic features (136). A recent WGS study with a set of 177 derivation and 205 validation clinical *E. faecium* isolates yielded an average CA of $>$97% for a rules-based genomic prediction of susceptibility across six antimicrobials relative to phenotypic results generated by the Vitek2 and confirmed by broth microdilution (137). All discrepancies were resolved in the validation set except for tetracycline and doxycycline which had false-positive rates of 14 and 27%, respectively, largely due to extensive variability in the *tet*(M) sequence. A major limitation of the study was that many clinically relevant agents like daptomycin and tigecycline were not assessed. Another study on Resfinder 4.0, a resistance database that generates *in silico* antibiograms, reported an overall genotype-phenotype concordance of 92.8 to 96.2% for *E. faecium*, ranging from 64% for chloramphenicol to 100% for ampicillin, ciprofloxacin, erythromycin, gentamicin, and vancomycin with two data sets of 522 combined observations (138). The predicted and observed AMR phenotypes matched in 91 to 97% of cases in which AMR determinant were detected and in 95 to 97% of cases in which no determinant was detected. ResFinder 4.0 performed similarly for *E. faecalis* with an overall genotype-phenotype concordance of 97% from 235 observations across five agents, ranging from 96% for erythromycin to 98% for tetracycline and vancomycin. Agents like daptomycin, tigecycline, and lipoglycopeptides were also not assessed in this study. Thus, the current gap in research on WGS-based susceptibility prediction tools is their application for agents with complex and multifaceted resistance mechanisms. Studies have reported the utility of mRNA-based tools that combined machine learning analysis of early antibiotic-induced transcriptional alterations with simultaneous detection of key resistance determinants in a single assay to detect resistance with 94 to 99% accuracy for multidrug-resistant Gram-negative pathogens but there is a lack of data on enterococci (139). Integration of WGS tools into the clinical microbiology lab shows promise but will require further studies, increased method evaluation and validation, enhancement of next-generation sequencing platforms and reduced cost, particularly with special attention given to the needs of low-resource settings. With the emergence of novel AST diagnostics, clinical outcome studies are increasingly critical to assess their ability to advance patient care.

CONCLUDING REMARKS

Enterococci are commensals of the GI tract of humans and animals that have rapidly emerged as problematic nosocomial pathogens due to their genomic plasticity, facilitating enhanced adaptation to hostile ecological niches. The rise of vancomycin-resistant *E. faecium* and, to a lesser extent, the increased prevalence of antimicrobial resistance determinants in *E. faecalis* often leaves clinicians with few to no treatment options. This situation creates an urgent need for novel tools that improve early detection, targeted treatment and global surveillance of these pathogens. Clinical microbiology laboratories are a primary source of information in our fight against multidrug-resistant enterococcal infections. Indeed, accurate, precise and accessible AST is critical for optimal patient care. Personalized medicine-based approaches like the development of precise testing algorithms to guide clinical laboratories to test susceptibility of pathogens to clinically relevant antimicrobials based on the precise clinical scenario would be a major advance in the field. Improved standardization of manual methods, reliance on local and regional antibiograms to inform AST, use of automation, and increased deployment of rapid point-of-care molecular tests in appropriate settings has greatly advanced AST for enterococci. However, there is a dire need for contemporary studies to evaluate the performance of commercial AST systems which have undergone technical improvements and software updates over the years. With the emergence of novel AST diagnostics, larger validation and clinical outcome studies are increasingly critical to assess their ability to advance patient care. Future advances that show promising potential include shifts in the genotype-phenotype binary paradigm with the introduction of complex methods that combine both pieces of information, genomic tools that predict susceptibility, innovations in direct specimen testing to decrease time to results, and the adaptation of *in vitro* AST media to more accurately mimic physiological host environments encountered by drugs and pathogens.

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