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Ability of Clinical Laboratories To Detect Antimicrobial Agent-Resistant Enterococci

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To test the ability of clinical laboratories to detect antimicrobial resistance among enterococci, we sent four vancomycin-resistant enterococcal strains and one β-lactamase-producing enterococcus to all 93 nongovernment, hospital-based clinical laboratories in New Jersey; 76 (82%) participated in the study. Each organism was tested by the laboratory's routine antimicrobial susceptibility testing method. The proportion of laboratories that correctly reported that an isolate was resistant to vancomycin varied according to the resistance level of the isolate: high-level resistance (MIC for *Enterococcus faecium* = 512 μg/ml), 96% of laboratories correct; moderate-level resistance (MIC for *E. faecium* = 64 μg/ml), 27% correct; low-level resistance (MIC for *Enterococcus faecalis* = 32 μg/ml), 16% correct; and intrinsic low-level resistance (MIC for *Enterococcus gallinarum* = 8 μg/ml), 74% correct. The β-lactamase-producing *E. faecalis* isolate was identified as resistant to penicillin and ampicillin by 66 and 8% of laboratories, respectively, but only three laboratories recognized that it was a β-lactamase producer. This survey suggests that many laboratories may fail to detect antimicrobial agent-resistant enterococci.

Enterococci are now the second most common cause of surgical wound infections and nosocomial urinary tract infections and the third most common cause of nosocomial bacteremias reported by hospitals participating in the National Nosocomial Infections Surveillance System (19). Recently, enterococci resistant to multiple antimicrobial agents have been recognized, including strains resistant to vancomycin, beta-lactams, and aminoglycosides (5, 7, 11, 13, 16, 17, 22). Such strains pose therapeutic dilemmas for clinicians, particularly when the strains cause outbreaks in intensive care units (1–3, 6, 8, 10, 12, 16). It is crucial for laboratories to provide accurate antimicrobial resistance patterns for enterococci so that effective therapy and infection control measures can be initiated. However, detecting β-lactamase-producing and vancomycin-resistant enterococci can be difficult (7, 14). Recent reports have documented the failures of several automated susceptibility testing systems to detect some enterococci with low-level vancomycin resistance (18, 23). Disk diffusion testing also has been shown to fail to detect some vancomycin-resistant enterococci (18, 21). In 1992, new breakpoints for testing enterococci against vancomycin were established (20) and were adopted by the National Committee for Clinical Laboratory Standards (NCCLS) (15). A recent report suggests that these new breakpoints are both sensitive and specific in detecting vancomycin-resistant enterococci (23). Since outbreaks of infection due to resistant enterococci are likely to increase in the United States (2, 6, 14), the ability of various laboratory systems to detect resistance is of paramount importance.

To determine the abilities of various antimicrobial susceptibility testing methods to detect ampicillin, penicillin, and vancomycin resistance, we asked a large sample of laboratories in New Jersey to test five isolates of enterococci with different antimicrobial susceptibility patterns. The results of the survey are reported here.

MATERIALS AND METHODS

Study protocol. Five enterococci (two *Enterococcus faecalis* isolates, two *Enterococcus faecium* isolates, and one *Enterococcus gallinarum* isolate) representing the four most common vancomycin resistance phenotypes and a β-lactamase-producing isolate from the Centers for Disease Control and Prevention (CDC) strain collection were coded as organisms 1 through 5, inoculated onto Trypticase soy agar slants (Becton Dickinson Microbiology Systems, Cockeysville, Md.), and sent to all 93 hospital laboratories in New Jersey along with a susceptibility test result form. New Jersey laboratories were chosen because of the strong laboratory surveillance and reporting system already in place in that state. The characteristics of the isolates and relevant MIC patterns are shown in Table 1. Each laboratory supervisor was instructed to test the five organisms for resistance to ampicillin, penicillin, and vancomycin by using the antimicrobial susceptibility testing method routinely used in her or his laboratory and to indicate the zone size and interpretation (moderately susceptible, intermediate, or resistant) or the MIC and interpretation on the form provided, using NCCLS interpretive guidelines (15). After completion of the testing, the forms were sent to CDC for analysis.

Reference methods. MICs were determined by broth microdilution using cation-adjusted Mueller-Hinton broth (Becton Dickinson Microbiology Systems) performed according to NCCLS guidelines (15). Disk diffusion testing was also performed by using Mueller-Hinton agar according to NCCLS guidelines (15). No susceptible category was used for enterococcal susceptibility testing during this period; isolates giving these results were classified as moderately

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TABLE 1. Characteristics of enterococcal study isolates

Organism no. and species	MIC (μg/ml) of:			β-Lactamase production[a]	Vancomycin phenotype
	Vancomycin	Penicillin	Ampicillin		
1. *E. faecium*	512	32	4	−	VanA
2. *E. faecium*	64	>256	64	−	VanB-like
3. *E. faecalis*	16–32	4	1	−	VanB
4. *E. gallinarum*	8	2	1	−	VanC
5. *E. faecalis*	2	4[b]	1[b]	+	N/A[c]

[a] −, negative reaction; +, positive reaction.
[b] MICs of penicillin and ampicillin were determined by using a standard inoculum (5×10^5 CFU/ml). An inoculum of 10^7 CFU/ml resulted in MICs of both penicillin and ampicillin of ≥64 μg/ml for this organism.
[c] N/A, not applicable.

susceptible, intermediate, or resistant. Categorical errors, such as very major, major, and minor errors, were not calculated for the data because of the small sample size for most methods.

A polymerase chain reaction assay was used to confirm the presence of the *vanA* resistance determinant in organism 1 (4).

The vancomycin screen test was performed by using the swab method as described by Willey et al. (23) on Mueller-Hinton and brain heart infusion (BHI) agar plates containing 6 or 8 μg of vancomycin per ml. β-Lactamase tests were performed by using the colorimetric cephalosporin nitrocefin (Cefinase; Becton Dickinson Microbiology Systems).

RESULTS

Detection of vancomycin resistance. Seventy-six hospitals participated in the study; the methods they used for antimicrobial susceptibility testing are shown in Table 2.

The overall results of the study are shown in Table 3, and the results obtained with the most commonly used systems are shown in Table 4. The *E. faecium* isolate with high-level vancomycin resistance and teicoplanin resistance that contains the *vanA* gene was identified as vancomycin resistant by 96% of laboratories (Table 3). One WalkAway user classified this organism as moderately susceptible to vancomycin, and two Vitek users classified it as intermediate to vancomycin (Table 4).

All systems had difficulty in detecting vancomycin resistance in organisms 2 and 3 (Table 3). Organism 2 is an *E. faecium* isolate with a novel phenotype similar to VanB in that the organism is susceptible to teicoplanin; however, the MIC of vancomycin for this isolate is higher than those for typical strains of the VanB phenotype (24). Only 29% of laboratories correctly identified this isolate as vancomycin resistant (Table 3), although 43% (including 24 Vitek users, 1 WalkAway user, and 8 users of other MicroScan products) classified it as intermediate (Table 4). Two of four disk diffusion users classified this isolate as moderately susceptible with a zone size of 19 mm, one classified it as intermediate with a zone size of 16 mm, and one classified it as resistant, noting the presence of a haze within the zone of inhibition, as described in the most recent NCCLS guidelines (15). Two of three Unisept users classified the organism as resistant; the other classified it as intermediate.

Organism 3 is an *E. faecalis* isolate manifesting a more typical VanB phenotype with low-level vancomycin resistance (vancomycin MIC = 32 μg/ml) and teicoplanin susceptibility (MIC, ≤2 μg/ml). Overall, only 17% of study participants classified it as vancomycin resistant (Table 3). All Vitek users classified this organism as moderately susceptible. Of participants using the other systems, only 1 WalkAway user and 8 users of other MicroScan products classified it as vancomycin resistant, while 4 WalkAway users and 11 users of other MicroScan products classified it as intermediate (Table 4). Three of four disk diffusion users classified the organism as moderately susceptible with zone sizes of 19 to 20 mm, and one disk diffusion user reported it as intermediate (zone size, 16 mm), as did the CDC laboratory, which reported a zone size of 15 mm for this organism. Two of three Unisept users classified this organism as resistant; the other classified it as moderately susceptible.

Organism 4 was an *E. gallinarum* isolate with *vanC*-mediated vancomycin resistance (MIC = 8 μg/ml). None of

TABLE 2. Methods used for susceptibility testing

Method	No. of laboratories (%)
Vitek (AutoMicrobic system)	38 (50.0)
MicroScan	
AutoSCAN (Touchscan)	21 (27.6)
WalkAway	7 (9.2)
Dry panel (no reader)	1 (1.3)
Disk diffusion	4 (5.3)
Unisept	3 (4.0)
Autobac	1 (1.3)
MicroMedia	1 (1.3)

TABLE 3. Results of enterococcal susceptibility testing reported by category

Organism no.	% of laboratories reporting susceptibility to[a]:						
	Vancomycin			Penicillin[b]		Ampicillin[b]	
	MS	I	R	MS	R	MS	R
1	1	3	**96**	43	**57**	**95**	5
2	28	43	**29**	2	**98**	0	**100**
3	62	**21**	**17**	**98**	2	**99**	1
4	29	**71**	0	**98**	2	**99**	1
5	**99**	1	0	37	**63**	92	**8**

[a] MS, moderately susceptible; I, intermediate; R, resistant. Boldface type indicates correct answers.
[b] No intermediate category recognized by the NCCLS for penicillin and ampicillin.

TABLE 4. Agreement of test MIC results with reference MIC results

Organism and antimicrobial agent	MIC (μg/ml)	% Agreement with reference MIC results (n)[a]		
		Vitek (38)	AutoSCAN (21)	WalkAway (6)
Organism 1				
Vancomycin	512	94.7	100	83.3
Penicillin	32	72.4	17.0	25.0
Ampicillin	4	100	100	83.3
Organism 2				
Vancomycin	64	0	57.9	66.7
Penicillin	>256	100	100	100
Ampicillin	>256	100	100	100
Organism 3				
Vancomycin	16–32	0	36.8	16.7
Penicillin	4	100	100	100
Ampicillin	1	100	100	83.3
Organism 4				
Vancomycin	8	97.4	52.6	66.7
Penicillin	2	100	100	100
Ampicillin	1	100	100	100
Organism 5				
Vancomycin	2	100	100	83.3
Penicillin	4[b]	96.6	27.8	0
Ampicillin	1[b]	10.8	5.6	0

[a] *n*, number of laboratories using this method in the study.
[b] MIC when tested with a standard inoculum; organism is β-lactamase positive.

the participants classified the organism as resistant (Table 3). However, 97% of Vitek users classified the isolate as intermediate to vancomycin (Table 4), as did the CDC laboratory. Of the MicroScan users, 50% also identified the organism as intermediate to vancomycin, while the remainder reported the organism as moderately susceptible. Three of four disk diffusion users classified the organism as moderately susceptible with a zone size of 19 mm. One laboratory reported the organism as intermediate with a zone size of 16 mm. The CDC laboratory reported a zone size of 17 mm and classified the organism as moderately susceptible.

The fifth organism, an *E. faecalis* isolate moderately susceptible to vancomycin, was reported by one WalkAway user as intermediate to vancomycin, while all other laboratories classified it correctly as moderately susceptible.

Detection of beta-lactam resistance. Although the CDC laboratory classified organism 1 as resistant to penicillin but susceptible to ampicillin, 43% of the laboratories, including 11 Vitek users, 4 WalkAway users, and 15 users of other MicroScan products, classified it as susceptible to both penicillin and ampicillin. Only one WalkAway user classified the organism as resistant to both penicillin and ampicillin. Most of the penicillin or ampicillin results for organisms 2, 3, and 4 were correct (Tables 3 and 4).

Of the eight laboratories that indicated that they routinely tested *Enterococcus* isolates for β-lactamase production, only three reported organism 5 as a β-lactamase-producing *E. faecalis* organism. Two of the three laboratories reported using a nitrocefin test; the third laboratory did not indicate which test was used. The MICs of penicillin and ampicillin for organism 5 placed it in the moderately susceptible range when a standard inoculum of 5×10^5 CFU/ml was used, but penicillin and ampicillin MICs for the organism were ≥64 μg/ml (indicating resistance) when the inoculum was increased to 10^7 CFU/ml. Approximately 97% of Vitek users classified the organism as resistant to penicillin, but only 10.8% classified it as ampicillin resistant (Table 4). All WalkAway users classified organism 5 as moderately susceptible to both drugs; the other MicroScan products were only slightly more effective in detecting beta-lactam resistance. All four disk diffusion users classified the organisms as moderately susceptible to ampicillin with zone sizes of 18 to 19 mm, as did the CDC laboratory. Only two disk diffusion users reported testing this organism against penicillin by disk. One classified it as resistant (zone size, 14 mm) and the other classified it as moderately susceptible (zone size, 15 mm).

DISCUSSION

Antimicrobial agent-resistant enterococci are being reported with increasing frequency throughout the United States (2, 6, 9, 10, 12, 14, 16, 17, 19, 22). Several recent outbreaks of vancomycin-resistant enterococci, some caused by enterococcal strains demonstrating high-level vancomycin resistance (*vanA*) and others caused by strains manifesting one of the low-level resistance phenotypes, emphasize the need for laboratories to be able to detect all levels of vancomycin resistance. As demonstrated here, both the automated and nonautomated commercial antimicrobial susceptibility testing systems and the disk diffusion method using the new vancomycin breakpoints for enterococci have difficulty detecting vancomycin resistance other than high-level resistance mediated by *vanA*. Previous reports (18, 23) noted failures of the MicroScan and Vitek systems to detect low-level vancomycin resistance. These deficiencies have been confirmed in our study. In addition, we encountered a problem in detecting intrinsic vancomycin resistance in *E. gallinarum* with automated methods. Our laboratory repeated the vancomycin susceptibility tests on all five organisms, using the newly released Vitek software (version R07.1). With the revised software, the Vitek system detected vancomycin resistance in organism 2, reporting an MIC of >32 μg/ml, but it did not detect resistance in organism 3.

With the exception of the penicillin resistance results for organism 1 and the vancomycin resistance results for organism 2, the Vitek results were highly consistent from laboratory to laboratory. Less consistent results were reported by WalkAway users and those using other MicroScan panels and readers, although the small number of WalkAway users in this study suggests that caution must be used when interpreting the data. MIC ranges of up to 4 doubling dilutions for penicillin and ampicillin and up to 3 dilutions for vancomycin were reported. This may be due to differences in inoculum size between laboratories or to differences in interpreting endpoints.

The NCCLS recommends that all isolates of enterococci from blood and cerebrospinal fluid be tested for β-lactamase production by using nitrocefin and an inoculum of 10^7 CFU/ml (15). Recently, Handwerger et al. described a β-lactamase-producing, vancomycin-resistant *E. faecalis* isolate that did not produce a positive nitrocefin test (7). Only a bioassay for penicillin hydrolysis and differing beta-lactam MICs when higher inocula were used in broth microdilution testing indicated the presence of the enzyme.

Although the nitrocefin test may not be 100% sensitive, it still remains the most effective method for screening for β-lactamase-producing enterococci in the clinical laboratory and the only method recommended by the NCCLS. Most susceptibility testing systems do not recognize β-lactamase-producing enterococci as resistant to ampicillin.

It is interesting that approximately 97% of Vitek users called organism 5 resistant to penicillin but moderately susceptible to ampicillin, since the β-lactamase is effective in hydrolyzing both drugs. While some β-lactamase-negative enterococci show differential resistance to penicillin and ampicillin (such as organism 1, for which the reference MIC testing indicated penicillin resistance [penicillin MIC = 32 μg/ml] but moderate susceptibility to ampicillin [ampicillin MIC = 4 μg/ml]), the clinical significance of this is not clear. Similar differential susceptibility patterns have been noted for other enterococci (1, 6–8). Grayson et al. (6) have noted that penicillin MICs for *E. faecium* are often 1 to 2 dilutions higher than those of ampicillin. However, when the organism is β-lactamase positive, it should be considered resistant to beta-lactam drugs.

Microbiologists need to assess the abilities of their routine susceptibility testing methods to detect resistant enterococci. Since the NCCLS had not yet completed their studies on methods for determining high-level aminoglycoside resistance, the issue of detecting streptomycin and gentamicin resistance was not addressed in this study.

In our hands, the agar screen test described by Willey et al. (23) using 6 μg of vancomycin per ml detected all vancomycin-resistant organisms, including the *E. gallinarum* isolate, when BHI agar was used but not when Mueller-Hinton agar was used. When 8 μg of vancomycin per ml was tested, organisms 3 and 4 showed poor growth, suggesting that 6 μg/ml may be the optimum concentration for this screening test. This test is currently being evaluated by an NCCLS working group.

In conclusion, we strongly support the NCCLS recommendation to test enterococcal isolates from normally sterile body sites for β-lactamase production by using nitrocefin (15). While this may not be a perfect test, it is more sensitive than current automated methods. On the basis of our preliminary studies, we also support the use of a BHI screen plate containing 6 μg of vancomycin per ml as an alternative to automated susceptibility testing systems for the detection of all classes of vancomycin resistance, but we recommend that the BHI agar be used in place of Mueller-Hinton agar.

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ADDENDUM IN PROOF

The sequence of the *vanB* gene was published after this manuscript had been accepted (S. Evers, D. F. Sahm, and P. Courvalin, Gene **124**:143–144, 1993). By using primers that we developed that are specific for the *vanB* gene, we have shown by polymerase chain reaction assay that organisms 2 and 3 both carry the *vanB* gene.

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