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Detection of Vancomycin Resistance in *Enterococcus* Species

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***Enterococcus faecalis* and *Enterococcus faecium* isolates that are resistant to vancomycin have recently been identified in North America and Europe. Of 155 clinical isolates of enterococci (113 *E. faecium* and 42 *E. faecalis*), we found that 98 were resistant, 52 were moderately susceptible, and 5 had intermediate susceptibilities to vancomycin by using broth microdilution susceptibility testing according to the National Committee for Clinical Laboratory Standards (NCCLS) (Approved Standard M7-A2). Using NCCLS disk diffusion methodology (Approved Standard M2-A4), we evaluated the NCCLS supplemental M100-S3 revisions for zone diameter interpretive standards and incubation conditions and found 5.8% minor errors. A total of 234 isolates, which included an additional 79 *E. faecium* isolates that were moderately susceptible to vancomycin, were used to evaluate the Vitek GPS-TA card (bioMerieux, Inc., Hazelwood, Mo.) and the Pos MIC type 6 panel (MicroScan; Baxter Health Care Corp., West Sacramento, Calif.) for the detection of vancomycin resistance. The Vitek card was 100% specific and 72% sensitive, whereas the MicroScan panel with the Walk/Away system was 98% specific, with a sensitivity of 93% which increased to 99% when readings were performed manually. An agar screen plate method was evaluated with vancomycin concentrations of 6, 8, 10, or 12 μg/ml; plates were inoculated so as to obtain a final concentration of 10^5 CFU per spot. This method was found to be 100% sensitive and specific at all concentrations.**

Enterococcus faecium and *Enterococcus faecalis* are part of the normal gastrointestinal flora of humans, but they can cause serious infections such as bacterial endocarditis and bacteremia and are often difficult to treat because of inherent resistance to antimicrobial agents (10, 23). A combination of penicillin or ampicillin and an aminoglycoside is normally the treatment of choice. However, in cases in which a β-lactam antibiotic cannot be used because of intolerance or resistance, vancomycin in combination with an aminoglycoside is the recommended regimen (7, 8, 27, 28). It is, therefore of concern that, in addition to resistance to β-lactams (4, 15) and the aminoglycosides (2, 9, 23), there have been a number of reports of vancomycin resistance. Such strains of *E. faecium* and *E. faecalis* were originally isolated in Europe in 1986, and a clinically significant isolate of vancomycin-resistant *Enterococcus gallinarum* was reported in the United States in 1987 (10). Since then, vancomycin-resistant isolates of enterococci have been recognized in Europe (1, 5, 22) and the United States (5, 22, 26). In this study, we evaluated the revised National Committee of Clinical Laboratory Standards (NCCLS) disk diffusion recommendations (M2-A4 [17], M100-S3 [19]) for their accuracy in detecting vancomycin resistance. We also evaluated the Vitek Gram-Positive Susceptibility card (GPS-TA; bioMerieux, Inc., Hazelwood, Mo.) and the Pos MIC type 6 panel (MicroScan; Baxter Health Care Corp., West Sacramento, Calif.). In addition, we tested an agar screen plate method, in which vancomycin is incorporated into the medium, by using methodology analogous to that of the oxacillin screen plate method used in identifying methicillin-resistant *Staphylococcus aureus* (18).

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MATERIALS AND METHODS

Organisms. There were 155 enterococcal isolates obtained from the Bureau of Laboratories, New York City Department of Health, New York, N.Y., and Mount Sinai Hospital, Toronto, Ontario, Canada (104 *E. faecium* and 9 *E. faecalis* isolates from New York; 9 *E. faecium* and 33 *E. faecalis* isolates from Toronto). The New York City isolates were referred from 12 hospitals for confirmation of vancomycin resistance. These organisms were equally isolated from blood, urine, and wound specimens (11). The identification of isolates was confirmed by conventional methodology (3).

An additional 79 *E. faecium* isolates from nine tertiary-care hospitals across Canada were selected because of their known susceptibilities to vancomycin. They were tested so that we could better evaluate the accuracies of the bioMerieux-Vitek GPS-TA card and the MicroScan Pos MIC type 6 panel in detecting vancomycin, ampicillin, and high-level aminoglycoside resistance.

The control organisms used in this study were *E. faecalis* ATCC 29212, *S. aureus* ATCC 29213, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *E. faecalis* HH22 (high-level streptomycin and gentamicin resistant) (14), *E. faecalis* UWHC 1921 (high-level gentamicin resistant, intrinsic low-level streptomycin resistant) (24), *E. faecalis* UWHC 1936 (low-level gentamicin and streptomycin resistant) (24), *E. faecium* 228 (vancomycin resistant, low-level gentamicin resistant and high-level streptomycin resistant) (6), *E. faecium* 228-3 (moderately vancomycin susceptible, low-level gentamicin resistant, high-level streptomycin resistant) (6), and *E. faecium* NYC 2491 (vancomycin resistant, high-level gentamicin and streptomycin resistant).

All isolates were stored at −70°C in buffered glycerol and were subcultured onto Columbia-based agar containing 5%

sheep blood (Woodlyn Laboratories Ltd, Guelph, Ontario, Canada) at least twice prior to being tested.

Broth microdilution. Broth microdilution susceptibility testing was performed in accordance with NCCLS guideline M7-A2 (18) by using cation-adjusted Mueller-Hinton broth (Difco Laboratories, Detroit, Mich.). Microdilution panels were prepared by using the Quick Spense IIe System (Sandy Springs Instrument Co., Belco Inc., Vineland, N.J.). The antimicrobial agents tested were vancomycin (Sigma Chemical Co., St. Louis, Mo.), teicoplanin (Marion Merrel Dow Inc., Cincinnati, Ohio), and ampicillin (Sigma).

Disk diffusion. Standard disk diffusion testing was performed on Mueller-Hinton II agar (Becton Dickinson Microbiology Systems, Cockeysville, Md.) according to NCCLS guideline M2-A4 (17) by using commercially prepared 30-μg vancomycin disks (Oxoid, Unipath Ltd., Basingstoke, United Kingdom). After 18 and 24 h of aerobic incubation at 35°C (according to NCCLS supplement M100-S3 [19]), the zone sizes were measured with electronic calipers (Fowler Ultra-Cal II; Sylvac). Any haze or growth within the zone of inhibition was considered resistant. Scattergrams were created by plotting the zone sizes against the MICs obtained by broth microdilution for each organism (17). The NCCLS breakpoints for vancomycin are as follows: resistance, ≤14 mm and ≥32 μg/ml; intermediate susceptibility, 15 to 16 mm and 8.0 to 16 μg/ml; moderate susceptibility, ≥17 mm and ≤4.0 μg/ml (19). A very major error was defined as an isolate that was resistant by broth microdilution but that was moderately susceptible by disk diffusion. A major error was defined as an isolate that was moderately susceptible by broth microdilution but resistant by disk diffusion. A minor error was defined as a discrepancy between results obtained by the two methods that differed only by one interpretation category (i.e., a microdilution result of resistance and a disk diffusion result of intermediate susceptibility or a microdilution result of intermediate susceptibility and a disk diffusion result of moderate susceptibility) (16).

Vancomycin agar screen plates. Plates containing vancomycin concentrations of 6, 8, 10, and 12 μg/ml in Mueller-Hinton agar were prepared. Bacterial suspensions were adjusted to a 0.5 McFarland turbidity standard with a turbidometer (A-Just; Abbott Diagnostics, North Chicago, Ill.). A 1.0-μl aliquot of each suspension was spotted onto the agar surface to achieve a final inoculum of approximately 10^5 CFU per spot. Equivalent results were obtained when a Steers replicator, a 1.0-μl sterile loop, or a sterile swab was used to deliver the inoculum. The inoculum was allowed to absorb into the agar prior to aerobic incubation at 35°C for 18 h. Plates were read against a dark, nonreflecting background, and any growth or haze within the zone was considered to indicate resistance.

Vitek system. Susceptibility testing was performed according to the manufacturer's instructions for all enterococcal isolates by using the Vitek GPS-TA card. The cards were read by the Vitek system after an average of 6 h of incubation at 35°C.

MicroScan panels. Susceptibility testing by use of the freeze-dried MicroScan POS MIC type 6 panels was performed on all isolates in accordance with the manufacturer's instructions. These panels incorporate glucose phosphate broth for the detection of high-level aminoglycoside resistance. The panels were incubated for 18 h and were then read by the automated Walk/Away system and by visual inspection. Any visible growth was considered to indicate resistance.

Aminoglycoside agar screen plates. Single-concentration agar plates for the detection of high-level resistance to gentamicin (HLGR) and streptomycin (HLSR) were prepared as described previously (25). Each plate contained 2,000 μg of gentamicin or streptomycin (Sigma) per ml in Mueller-Hinton agar. By using a Steers replicator, 1.0 μl of a 10^9-CFU/ml suspension of each organism was inoculated onto the agar surface to produce a final inoculum of approximately 10^6 CFU per spot. Colony counts were performed by using appropriate dilutions of the original inoculum to confirm the inoculum size. The plates were allowed to dry and were then incubated aerobically for 24 h at 35°C. If there was growth of two or more colonies on the plate, the organism was considered to be resistant.

TABLE 1. Comparative in vitro activities of antimicrobial agents against *E. faecium* and *E. faecalis*

Microorganism (no.)	Antimicrobial agent	MIC (μg/ml)[a]		
		Range	50%	90%
E. faecium (102), vancomycin resistant[b]	Vancomycin	16–1,024	512	1,024
	Ampicillin	8.0–512	64	512
	Teicoplanin	≤0.5–>64	32	>64
E. faecium (11), vancomycin moderately susceptible	Vancomycin	1.0–4.0	2.0	4.0
	Ampicillin	1.0–256	64	64
	Teicoplanin	≤0.5–1.0	≤0.5	≤0.5
E. faecalis (42)[c]	Vancomycin	1.0–512	2.0	4.0
	Ampicillin	1.0–32	1.0	2.0
	Teicoplanin	≤0.5–32	≤0.5	≤0.5

[a] 50% and 90%, MICs for 50 and 90% of the isolates tested, respectively.
[b] The five strains for which the MICs were in the intermediate susceptibility category were included in the resistant category.
[c] Only one isolate of *E. faecalis* was resistant to vancomycin (MIC, 512 μg/ml) and teicoplanin (MIC, 32 μg/ml).

RESULTS

The results of susceptibility testing by broth microdilution testing are summarized in Table 1. Of the 103 enterococcal isolates that were either resistant or that had intermediate susceptibility to vancomycin, only one isolate was *E. faecalis*. For five *E. faecium* isolates, the vancomycin MIC was considered to be intermediate. All the vancomycin-resistant isolates were from New York City.

Of the 103 strains that had intermediate susceptibility or that were resistant to vancomycin, 31 (30%) isolates were found to be moderately susceptible to teicoplanin (MICs, ≤1.0 μg/ml). This included all five isolates in the category of intermediate susceptibility to vancomycin. Of the 31 moderately susceptible isolates, vancomycin MICs were 512 μg/ml for 4 isolates and ranged from 16 to 128 μg/ml for the remaining 27 isolates. The remaining 70% (72 of 103) of the vancomycin-resistant isolates were found to be resistant to teicoplanin. Teicoplanin MICs for these isolates were ≥32 μg/ml, whereas the vancomycin MICs were all ≥256 μg/ml. All strains found to be resistant to vancomycin were also resistant to ampicillin (MICs, ≥16 μg/ml).

The zone sizes obtained by disk diffusion testing are presented in Fig. 1. There were nine (5.8%) minor errors, three of which occurred with organisms categorized as intermediately susceptible and six of which were caused by organisms which were moderately susceptible. The majority (61%) of the remaining moderately susceptible isolates produced zone diameters equal to 17 mm. No major or very major errors occurred. These error rates would be consid-

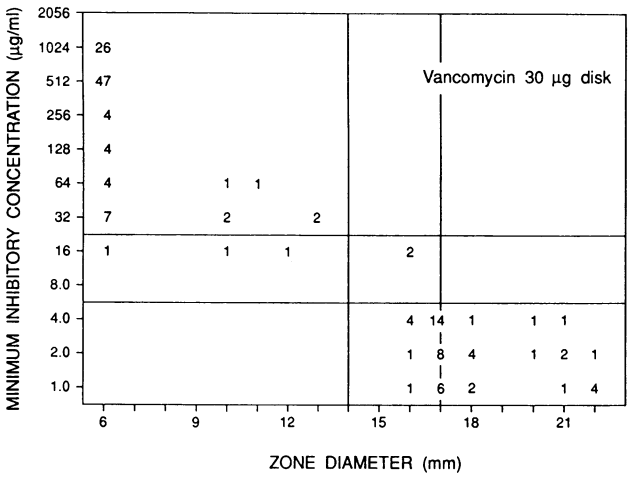

FIG. 1. Scattergrams showing correlation among MICs and zone diameters by using a 30-μg vancomycin disk. Numbers are the number of datum points at each location (155 isolates were tested).

ered acceptable by NCCLS guidelines for the evaluation of susceptibility testing criteria (16–18).

Table 2 summarizes the sensitivities and specificities of the Vitek system and the MicroScan panel for the detection of vancomycin resistance as well as ampicillin and high-level aminoglycoside resistance in the 234 strains of enterococci tested.

The vancomycin screen plate was able to differentiate moderately susceptible from resistant and intermediately susceptible isolates with 100% sensitivity and specificity at each concentration of vancomycin tested. However, lighter growth was obtained on plates containing the higher vancomycin concentrations (10 and 12 μg/ml) when organisms for which MICs were within 2 dilutions of the intermediate category were tested. Plates containing 6 and 8 μg of vancomycin per ml produced heavy confluent growth for all isolates for which MICs were in the resistant and intermediate susceptibility categories and were, therefore, easier to read.

TABLE 2. Comparative sensitivities and specificities of the Vitek GPS-TA and MicroScan Pos MIC type 6 panel for the detection of vancomycin, ampicillin, and high-level aminoglycoside resistance in 234 *Enterococcus* spp.[a]

Antimicrobial agent (no. of isolates in each susceptibility category)[b]	Vitek system		MicroScan system			
			Walk/Away[c]		Visual[d]	
	Sensitivity (%)	Specificity (%)	Sensitivity (%)	Specificity (%)	Sensitivity (%)	Specificity (%)
Vancomycin (R, 98; MS, 131; I, 5)	72	100	93	98	99	96
Ampicillin (R, 132; MS, 102)	96	93	82	99	92	99
HLGR (106)	98	99	45	100	78	100
HLSR (126)	93	100	49	99	82	99

[a] There were 192 *E. faecium* isolates and 42 *E. faecalis* isolates. For the determination of sensitivities and specificities of the commercial systems, the moderately susceptible and intermediate susceptibility categories were combined.

[b] R, resistant; MS, moderately susceptible; I, intermediate; HLGR, high-level gentamicin resistance; HLSR, high-level streptomycin resistance.

[c] Interpretation by the Walk/Away system at 18 h.

[d] Interpretation by visual inspection.

As determined by the high-level aminoglycoside agar screen method, 106 (45%) of a total of 234 enterococci tested had HLGR, 126 (54%) had HLSR, and 80 (34%) had high-level resistance to both aminoglycosides. Of the 103 isolates that were vancomycin resistant or that had intermediate susceptibility, 76 (74%) had both HLGR and HLSR, 16 (16%) had HLGR only, 9 (9%) had HLSR only, and 2 (1%) displayed no high-level resistance to either of the aminoglycosides tested.

DISCUSSION

Vancomycin-resistant *Enterococcus* spp. have been reported at several centers in the United States (5, 22, 26), and it is therefore important that clinical laboratories are able to accurately identify such isolates. The 102 vancomycin-resistant and vancomycin-intermediate *E. faecium* isolates and the one vancomycin-resistant *E. faecalis* isolate used in this study were obtained from 12 different hospitals in New York City over 8 months in 1991. Analysis of restriction fragment length polymorphisms of chromosomal DNAs from 25 of these isolates demonstrated genetic diversity (11). It is of importance to note the high prevalence of high-level aminoglycoside resistance among the vancomycin-resistant and intermediately susceptible strains of enterococci as well as their uniform resistance to ampicillin. This means that there are no antimicrobial agents or combinations of antimicrobial agents that would have in vitro activity against the majority of these isolates. In addition, 70% of the vancomycin-resistant isolates were found to be resistant to teicoplanin, a new glycopeptide that has not yet been released.

The revised NCCLS zone diameter guidelines for the testing of vancomycin, M100-S3 (19), were found to be acceptable. However, future consideration may be made to reduce the moderately susceptible breakpoint from ≥17 to ≥16 mm. This would decrease the number of minor errors without increasing the number of major or very major errors. It would also decrease the number of strains that cluster at the breakpoint.

The agar screen plate containing 6 or 8 μg of vancomycin per ml is a reliable and inexpensive alternative to disk diffusion. The method is simple to perform and fits with ease into the normal work flow of the laboratory. However, this method needs further evaluation with larger numbers of enterococcal strains for which vancomycin MICs are within the intermediate susceptibility range.

By using the manufacturers' instructions, neither the Vitek nor the MicroScan Walk/Away system can be relied upon to adequately detect vancomycin resistance in enterococci. However, reading of the MicroScan panels by visual inspection increased the sensitivity to 99%. The Walk/Away system failed to detect resistance, despite the visual observation of obvious growth in many of the wells. This suggests that the problems are, in some cases, due to the Walk/Away software and/or the reader and not the medium or growth conditions. Improvement in the sensitivity of the Vitek system will probably be achieved only by changes to the software program to alter current standards for growth curve interpretation and/or revision of the length of incubation. Therefore, in those areas where vancomycin-resistant enterococci are endemic, laboratories that use the Vitek system should use an alternate method, such as disk diffusion or the agar screen plate, to detect vancomycin resistance. Personnel in laboratories that use the Walk/Away system should check the panels visually.

The results presented here also demonstrate the inadequa-

cies inherent in the MicroScan and Vitek systems for the detection of enterococcal resistance to other antimicrobial agents commonly used for therapy. The problem of detecting high-level aminoglycoside resistance has been addressed previously, with the recommendation that a single-concentration agar screen plate or a high-potency disk be used in conjunction with the commercial systems (12, 13, 20, 21, 25). Similarly, the lack of sensitivity of the MicroScan panels and the lack of specificity of the Vitek cards to detect ampicillin resistance have been noted previously (12).

In view of this and other reports of vancomycin-resistant isolates in North America, the universal susceptibility of enterococci to vancomycin can no longer be presumed. Routine susceptibility testing of clinically relevant isolates is therefore warranted. Disk diffusion and vancomycin agar screen plates are the most reliable methods available for the detection of vancomycin resistance.

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