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Detection of Vancomycin Resistance in Enterococci by the Alamar MIC System

RONALD J. ZABRANSKY,* ANTHONY R. DINUZZO, AND GAIL L. WOODS

Department of Pathology, University of Texas Medical Branch, Galveston, Texas 77555-0743

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The ability of the Alamar microdilution MIC system to detect vancomycin resistance in enterococci was evaluated by comparing the results with an agar dilution screen method. Of 100 strains tested, 41 were resistant and 47 were susceptible by both tests. Five strains were intermediate and one was resistant by the Alamar MIC system but susceptible by the agar screen. Three strains each were susceptible or intermediate by the Alamar MIC system but resistant by the agar screen. The predictive values for the Alamar MIC system were 94% (susceptible) and 88% (combined intermediate and resistant). The Alamar MIC system does not appear to have sufficient accuracy for the detection or confirmation of vancomycin resistance in enterococci.

Enterococci are generally considered part of the normal gastrointestinal flora of humans and animals. They are the third most common clinical isolate associated with nosocomial infections (1). Their significance as pathogens associated with serious infections such as bacteremia or endocarditis has been well recognized, but when they are isolated in conjunction with other organisms their clinical importance has been debated (4). However, the increased incidence of enterococci that are resistant to therapy with the usually prescribed agents (beta-lactams, aminoglycosides, vancomycin) has further magnified the significance of these organisms as pathogens (12, 13). It is therefore critical that clinical laboratories accurately report antimicrobial susceptibilities for enterococci as well as periodically publish data on changes in susceptibility patterns (11).

The increasing prevalence of oxacillin (methicillin)-resistant *Staphylococcus aureus*, serious infections caused by coagulase-negative staphylococci, and *Clostridium difficile* disease requiring therapy has prompted the increased use of vancomycin. The most disturbing result of this practice is the recent reports of vancomycin-resistant enterococci (VRE) isolated from patients in Europe and the United States (2, 8). While the incidence is low, it does not appear to be limited to certain areas because as interest was piqued by the earlier reports, recent evidence shows that vancomycin resistance in these organisms is universal (3, 5).

Several methods for detecting VRE have been studied: an agar dilution screen with a single concentration of vancomycin, instrumented susceptibility testing systems, broth microdilution, and disk diffusion (9–11). The newest revisions of the standards of the National Committee for Clinical Laboratory Standards (NCCLS) for antimicrobial susceptibility testing describe in detail the recommended approaches for detecting vancomycin resistance by disk diffusion (6) or broth and agar dilution (7). Moreover, those documents describe a new zone size and MIC breakpoints for defining vancomycin resistance. Since the adoption of these new standards further evaluations of new susceptibility testing methods are needed to determine their efficacies for detecting VRE.

One such new method is the Alamar microdilution MIC system (Alamar Biosciences, Sacramento, Calif.). The system consists of microdilution panels with wells containing disks impregnated with various concentrations of antimicrobial agents and an oxidation-reduction color indicator. The indicator measures the metabolic reduction of the growth medium, changing from blue to red as growth occurs. This color change can be read with the unaided eye or by a computer-controlled colorimeter. This study compares the Alamar MIC system with an agar dilution screen for the detection of VRE.

MATERIALS AND METHODS

Organisms. One hundred recent clinical isolates of enterococci were included in the study; 40 previously called vancomycin resistant by microdilution (MicroScan, Sacramento, Calif.) were provided by the Medical College of Pennsylvania, and 10 resistant strains were provided by Ronald N. Jones (University of Iowa). The other 50 isolates were susceptible, as determined by disk diffusion at the University of Texas Medical Branch. The organisms were identified by using the Vitek AMS system (bioMerieux, Hazelwood, Mo.) and were maintained frozen (−70°C) in skim milk. The strains tested included 49 *Enterococcus faecalis*, 37 *Enterococcus faecium*, 6 *Enterococcus avium*, 3 *Enterococcus durans*, 3 *Enterococcus casseliflavus-gallinarum*, and 2 *Enterococcus gallinarum*. Two control organisms were used: *E. faecalis* ATCC 29212 (vancomycin susceptible) and *E. faecalis* ATCC 51299 (borderline vancomycin resistant; MIC, 16 to 32 μg/ml) (10, 11). The control organisms were included in each test run.

Antibiotic. Stock and working solutions of vancomycin susceptibility powder (Sigma Aldrich, St. Louis, Mo.) were prepared as described in NCCLS document M7-A3 (7). All solutions were stored at −70°C.

Agar screen. The agar screen was performed by the procedure described by Swenson et al. (10) and recommended by NCCLS (7) with brain heart infusion agar (Difco, Detroit, Mich.) containing 6 μg of vancomycin per ml. Plates were stored at 4°C for up to 1 week until they were needed. Plates were spot inoculated with 10 μl of a Mueller-Hinton broth suspension of the test and control organisms equivalent to a 0.5 McFarland standard by using a calibrated pipettor. Inoculated plates were incubated in an ambient atmosphere at 35°C for up to 48 h.

Alamar MIC system. The testing of the isolates with the Alamar MIC system was performed according to the manufacturer's specifications. The Alamar trays were specially prepared for this evaluation and contained only vancomycin disks with concentrations ranging from 0.25 to 32 μg/ml. Briefly, a suspension of the test or control organisms equivalent to a 0.5 McFarland standard was prepared in 0.85% saline and was then diluted 100-fold in Alamar inoculum broth for a final inoculum concentration of approximately 5×10^6 CFU/ml. Each of the panel wells was inoculated with 100 μl of the final inoculum broth, covered, and incubated at 35°C in an ambient atmosphere. All strains were tested in duplicate; approximately 20 strains were included in each run.

Reading, interpretation, and recording of results. The agar screen plates were read at 24 and 48 h according to the specifications in the NCCLS standard (7); any haze or minute colonies within the inoculum spot were considered to be growth. The Alamar test panels were read according to the manufacturer's directions without instrumentation after 24 and 48 h of incubation; any change in color from blue to red was considered evidence of growth. For the agar screen method, organisms that grow in the presence of 6 μg of vancomycin per ml are considered resistant; in MIC systems such as the Alamar system, the breakpoint for resistance is 32 μg/ml; MICs of 8 and 16 μg/ml are considered intermediate

* Corresponding author. Present address: Pathology and Laboratory Medicine Service (113), VA Medical Center, 10701 East Blvd., Cleveland, OH 44106. Phone: (216) 791-3800, ext. 4920. Fax: (216) 231-3489.

TABLE 1. Enterococci susceptible, intermediate, or resistant to vancomycin by the agar screen and Alamar MIC methods

Agar screen result	No. of strains			
	Alamar MIC system			Total
	Susceptible (<8 μg/ml)	Intermediate (8 to 16 μg/ml)	Resistant (>16 μg/ml)	
Susceptible (MIC, ≤6 μg/ml)	47	5	1	53
Resistant (MIC, >6 μg/ml)	3	3	41	47

(7). Intermediate and resistant results by the Alamar system were combined for some of the analyses.

Adjudication of discrepant results. Strains with results that did not agree (agar screen susceptible, Alamar system intermediate or resistant; agar screen resistant, Alamar system susceptible) were retested by a broth microdilution procedure with cation-supplemented Mueller Hinton broth and a full 24 h of incubation (7).

RESULTS AND DISCUSSION

No differences were observed in the results obtained at the 24- and 48-h readings for the agar screen and the Alamar MIC system methods. The control strains gave identical results by the respective methods in each run. The susceptible control strain (ATCC 29212) produced the expected results by both methods; however, the borderline-resistant control strain (ATCC 51299), for which the vancomycin MIC is purported to be 16 to 32 μg/ml (10, 11), was consistently inhibited by 8 μg/ml on the Alamar MIC plates. This strain was inhibited by 16 μg/ml by standard broth microdilution testing.

Of the 100 strains of enterococci tested, 41 were resistant and 47 were susceptible by both test systems. For 12 isolates, the results of the agar screen and those of the Alamar system did not agree; of 6 isolates resistant by the agar screen, 3 were susceptible and 3 were intermediate by the Alamar system. The other six strains were susceptible by the agar screen; one of these was resistant and five were intermediate by the Alamar system (Table 1). This gave a specificity of 88.7% and a sensitivity of 93.6% for the Alamar system when intermediate and resistant results are combined. The ability of the Alamar system to predict the correct susceptible and resistant (combined with intermediate) results was 94 and 88%, respectively.

The most troublesome of our results were with the three strains that were susceptible by the Alamar system (MIC, ≤2 μg/ml) but that grew weakly in the presence of 6 μg of vancomycin per ml on the agar screen plates. When they were retested by an alternate broth microdilution method the MICs for these strains were 8, 16, and >64 μg/ml, respectively, confirming the agar screen results. Another strain was susceptible by the agar screen, but the MIC for this strain by the Alamar system was 32 μg/ml; the MIC for this strain by the standard microdilution method was 2 μg/ml, confirming the agar screen results. Together, these constituted a major error rate of 4%. The minor errors involved the five Alamar-intermediate strains that were susceptible by the agar screen (5%). For two additional strains that grew weakly on the agar screen an intermediate MIC (8 μg/ml) and a susceptible MIC (4 μg/ml) were obtained by the Alamar system. The MICs for four of these weakly growing strains were thus close to the 6-μg/ml breakpoint of the screen plate, suggesting that the results may be related to the growth characteristics of the organism or its inability to express resistance rather than the accuracy of the Alamar test system.

The Alamar system was very reproducible; for only six strains were MICs different on duplicate testing. For four strains MICs were ≤4 μg/ml in both testing events and were therefore susceptible; two strains were susceptible (MICs, 4 μg/ml) on one run but intermediate (MICs, 8 μg/ml) on the second run. Since these latter two strains were susceptible by the agar screen and the confirmatory microdilution test, the Alamar test data indicating susceptibility were used for the calculations in this report.

As has been described previously (4, 5), *E. faecium* is more likely than *E. faecalis* to be resistant to vancomycin. This was confirmed in our evaluation, in which 35 of 49 *E. faecalis* strains were susceptible by both the agar screen and the Alamar system and 22 of 37 *E. faecium* strains were intermediate or resistant by both methods (Table 2). Of the strains with discrepant results (the results of the Alamar system and the agar screen did not agree), eight were *E. faecalis*, one was *E. faecium*, and one was *E. avium.*

For the borderline-resistant control strain MICs were consistently lower in the Alamar system than what has been described previously (8 versus 16 to 32 μg/ml), and this fact may illustrate a problem with the vancomycin concentrations in the intermediate range in the Alamar trays. This may also have contributed to the false-susceptible or false-intermediate results obtained with the test strains.

Growth on the Alamar panels is easy to read, with clearly red or discernible red hues. This is especially true for staphylococci and gram-negative bacilli. In the present study, most isolates gave clear-cut endpoints, but some strains had endpoints that were difficult to read, with purple or pink hues in a blue background. These presentations may be analogous to the trailing endpoints seen with some organism-antibiotic combinations. When examining trailing growth endpoints the investigator has the prerogative to decide what represents growth. This option is not clear when one is reading the Alamar indicator, although the manufacturer has instrumentation that can be programmed to read such results consistently, which could resolve this issue. Alternatively, some of the difficult-to-read

TABLE 2. Enterococcal species susceptible or resistant to vancomycin as determined by the Alamar MIC system and the agar screen

Organism	No. of strains				Total
	Alamar susceptible		Alamar resistant[a]		
	Screen susceptible[b]	Screen resistant	Screen susceptible	Screen resistant	
Enterococcus faecalis	35	3	5	6	49
Enterococcus faecium	4	1	0	32	37
Enterococcus avium	5	0	1	0	6
Enterococcus durans	3	0	0	0	3
Enterococcus casseliflavus-gallinarum	0	0	0	3	3
Enterococcus gallinarum	0	0	0	2	2

[a] Includes Alamar-intermediate and -resistant results (MICs, ≥8 μg/ml).
[b] Screen susceptible, MIC < 6 μg/ml.

endpoints could be related to the slight pigmentation of vancomycin and the fact that there were no other drugs on the panel which would have provided a source for comparison.

Currently, the Alamar system does not appear to perform with sufficient accuracy for detecting or confirming vancomycin resistance in enterococci. Additional studies are required to determine if this relates to the ability of the test medium to support the growth of the organisms, the sensitivity of the redox indicator, the peculiarities of specific strains of enterococci, or the specific concentrations of the antibiotics in the disks.

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