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Ability of Commercial and Reference Antimicrobial Susceptibility Testing Methods To Detect Vancomycin Resistance in Enterococci

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We evaluated the abilities of 10 commercially available antimicrobial susceptibility testing methods and four reference methods (agar dilution, broth microdilution, disk diffusion, and the agar screen plate) to classify enterococci correctly as vancomycin susceptible or resistant using 50 well-characterized strains of enterococci. There was a high level of agreement of category classification data obtained with broth-based systems (Sceptor, MicroMedia, Pasco, and Sensititre), agar dilution, and an antibiotic gradient method (E test) with data obtained by reference broth microdilution; no very major or major errors were seen, and minor errors were ≤6%. Increased minor error rates were observed with disk diffusion (12%), Alamar (16%), Uniscept (16%), and conventional (overnight) MicroScan panels (16%). The errors were primarily with *Enterococcus casseliflavus* strains and organisms containing the *vanB* vancomycin resistance gene. Very major error rates of 10.3 and 20.7% were observed with Vitek and MicroScan Rapid (MS/Rapid) systems, respectively; however, only the MS/Rapid system produced major errors (13.3%). On repeat testing of discrepant isolates, the very major error rate with the Vitek system dropped to 3.4%, while the very major error rate with the MS/Rapid system increased to 27.6%; major errors with the MS/Rapid system were not resolved. Many of the commercial systems had only 4 dilutions of vancomycin, which resulted in up to 84% of values being off scale (e.g., Uniscept). Of the methods tested, most conventional broth- and agar-based methods proved to be highly accurate when incubation was done for a full 24 h, although several of the tests had high minor error rates. Automated systems continued to demonstrate problems in detecting low-level resistance.

Enterococci are the second most common cause of nosocomial infections reported to the National Nosocomial Infections Surveillance System (11). In 1993, almost 14% of the enterococci isolated from patients in intensive care units in the United States were resistant to vancomycin (1), and many vancomycin-resistant enterococci (VRE) have been reported to be resistant to β-lactams, aminoglycosides, fluoroquinolones, and other antimicrobial agents as well (4, 6). Automated methods of susceptibility testing have difficulty detecting some strains of VRE, particularly those containing the *vanB* gene (10, 13, 14); however, the accuracies of nonautomated susceptibility testing systems for detecting vancomycin resistance are unknown.

Therefore, in the study described here we assessed the accuracies of commercially available antimicrobial susceptibility testing methods for detecting VRE and evaluated the newly described brain heart infusion agar screen method (12). The results of 10 commercially available test methods were compared with the results of agar dilution, disk diffusion, the agar screen method, and the broth microdilution reference method described by the National Committee for Clinical Laboratory Standards (NCCLS) (7).

MATERIALS AND METHODS

Bacterial strains. Fifty strains of enterococci were obtained from the reference collection of the Centers for Disease Control and Prevention. The *van* genotypes and MIC ranges for the strains are given in Table 1. The organisms were picked to represent the major phenotypes and genotypes of strains with vancomycin resistance seen in the United States. Organisms with a variety of *vanB* phenotypes were specifically chosen to challenge the susceptibility testing methods. Organisms were identified by the method of Facklam and Collins (3).

Susceptibility testing. Broth microdilution, agar dilution, and disk diffusion were performed as described in NCCLS documents M7-A3 (7) and M2-A5 (8) by using cation-adjusted Mueller-Hinton (MH) broth (Difco, Detroit, Mich.) or MH agar (Becton Dickinson Microbiology Systems [BDMS], Cockeysville, Md.). Disks were obtained from BDMS. The agar screen test was performed as described previously (12) by using 6 μg of vancomycin per ml in brain heart infusion agar (Acumedia, Baltimore, Md.) and an inoculum of either 1 μl (10^5 CFU) or 10 μl (10^6 CFU) of a suspension equivalent to a 0.5 McFarland standard. The commercial systems investigated included Alamar (Alamar Biosciences, West Sacramento, Calif.), E test (AB Biodisk, Piscataway, N.J.), MicroMedia Fox panel (AccuMed, Westlake, Ohio), MicroScan conventional and Rapid (MS/Rapid) panels (Dade International, West Sacramento, Calif.), Pasco (Difco, Wheatridge, Colo.), Sceptor (BDMS), Sensititre (AccuMed), Uniscept (bioMérieux, Hazelwood, Mo.), and Vitek (bioMérieux). Tests were performed as described by the manufacturers in the package inserts, except that each test other than the automated systems was read at both 18 and 24 h. The results reported herein were those recorded after 24 h of incubation. In the present study, E-test results were not converted to the next highest doubling dilution before conversion to interpretive categories of susceptible, intermediate, and resistant. All 14 test systems were inoculated with overnight growth from blood agar plates inoculated from the same colonies to ensure uniformity. *Enterococcus faecalis* ATCC 29212 was used for quality control in all dilution methods. The strain was tested by each method on each testing day. In addition, *E. faecalis* ATCC 51299 was used for quality control in the agar screen test. *Staphylococcus aureus* ATCC 25923 was used for quality control in the disk diffusion test on 1 testing day.

Repeat testing. Tests yielding results that differed from the results of the broth microdilution method either by ≥2 doubling dilutions (MIC methods) or by categorical interpretation (i.e., susceptible, intermediate, or resistant, even if they were within ±1 dilution) were repeated in duplicate. Because the MICs for *Enterococcus casseliflavus* and *Enterococcus gallinarum* tend to be clustered around the intermediate breakpoint, these 10 strains were retested with four different lots of MH broth on 5 consecutive days to determine the stabilities of the MICs and the influence of different lots of media on the results. For this part of the study, MH broth was obtained from Difco, BDMS, Acumedia, and Oxoid (Unipath, Ogdensberg, N.Y.).

Genetic testing. All strains were tested for the presence of the *vanA*, *vanB*, and *vanC1* genes by PCR as described by Clark et al. (2). The presence of the *vanC2* gene, known to be intrinsic in *E. casseliflavus* isolates (9), was not confirmed.

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TABLE 1. Bacterial strains used in the study

Organism	*van* genotype	No. of strains	Vancomycin MIC range (μg/ml)
E. faecalis	*vanA*	2	512
E. faecium	*vanA*	8	256–1024
E. faecalis	*vanB*	11	32–1024
E. faecium	*vanB*	9	16–256
E. gallinarum	*vanC1*	5	4–8
E. casseliflavus	*vanC2*	5	4
E. faecalis		6	1–2
E. faecium		4	≤0.5–1

RESULTS

The susceptibilities of 50 strains of enterococci to vancomycin were determined by broth microdilution, agar dilution, disk diffusion, the agar screen method with two different inocula, and 10 commercial susceptibility test systems. The percentage of very major, major, and minor errors for the various tests when compared with those for the broth microdilution method and the number of strains that did not grow in the automated systems are given in Table 2. The differences in the MICs obtained with the various systems, on-scale ranges for the tests, and the percentage of values that were on scale are given in Table 3. For the *E. gallinarum* strains, both susceptible and intermediate results were considered correct, since repeat testing consistently produced results of 8 μg/ml (intermediate) on four lots of media on 5 consecutive days, even though the original results indicated MICs of 4 μg/ml (susceptible) for these isolates (data not shown).

Results with nonautomated commercial systems. The results obtained by the Sceptor and MicroMedia broth microdilution tests showed a perfect correlation with those obtained by the NCCLS broth reference method. However, only 36% of the Sceptor MICs were within the range of 2 to 32 μg/ml used on the panels. In contrast, 46% of the values were on scale for the MicroMedia panel, which has a range of 1 to 32 μg/ml. Pasco showed a single minor error with a *vanB* strain that represented a 1-dilution change. Only 28% of the test results were within the range of 2 to 16 μg/ml provided on the Pasco panel. Sensititre showed two minor errors, both with *vanB* strains; one was a 1-dilution error and the other was a 2-dilution error. Sixty-two percent of the Sensititre results were within the range of 1 to 64 μg/ml on the panel. The E test showed three minor errors, two with *vanB* strains and one with an *E. casseliflavus* strain. One error was 1 dilution lower, one was 2 dilutions higher, and the third was 3 dilutions higher. The E-test range is from 1 to 256 μg/ml; however, only 54% of the values were on scale because test results for 10 strains were recorded as >256 μg/ml, which was 2 or 3 dilutions higher than the test result for the reference strains. These high values were reproduced on retesting. On 2 of the 5 testing days, the quality control results for *E. faecalis* ATCC 29212 were 6 μg/ml, which was 0.5 dilution higher than the established quality control range. On the remaining days, the results were 4 μg/ml. Retesting of the quality control strain along with the isolates that produced minor errors again yielded MICs of 6 μg/ml, and the three minor errors were reproducible, including the one result that was lower than that by the broth reference method. Because the results for the quality control strains and the minor errors for the test strains were reproducible and because they were only one-half dilution outside of the designated range, the test values were kept in the data set.

TABLE 2. Error rates of 13 susceptibility test methods when compared with broth microdilution results[a]

Method	No. of strains with no growth	Percent error		
		Very major	Major	Minor
Sceptor	0	0	0	0
MicroMedia	0	0	0	0
Pasco	0	0	0	2
Sensititre	0	0	0	4
Agar dilution	0	0	0	4
E test	0	0	0	6
Disk diffusion	0	0	0	12
Alamar	0	0	0	16
Uniscept	0	0	0	16
MicroScan, conventional	0	0	0	16
Vitek	2	10.3	0	18
MS/Rapid	1	20.7	13.3	18
Agar screen				
1-μl inoculum	0	0	33.3 (0)[b]	
10-μl inoculum	0	0	73.3 (0)	

[a] The denominator for calculating very major errors was 29 (the number of resistant strains); that for major errors was 15 (the number of susceptible strains), and that for minor errors was 50 (the total number of isolates tested).

[b] Numbers in parentheses reflect percent errors observed when tests were reread after training of the microbiologist reading the results.

The Alamar panel produced eight minor errors; six were with *vanB* strains and two were with *E. casseliflavus* strains. Four of the errors were within 1 dilution of the reference result, three were 2 dilutions lower, and one was 4 dilutions lower. On repeat testing, for all four strains with errors that were >1 dilution different, higher MICs were shown and the errors were resolved. Fifty-eight percent of the Alamar test results were within the dilution scheme of 1 to 32 μg/ml on the panel.

The Uniscept system also demonstrated eight minor errors, but only one was with a *vanB*-containing isolate; the remainder were three *E. gallinarum* strains for which MICs were >16 μg/ml and four *E. casseliflavus* strains for which MICs were 8 μg/ml. Only 16% of the Uniscept results were within the scale of 2 to 16 μg/ml on the panel. Why the system produces higher MICs for strains with intrinsic resistance is unclear; however, these errors were reproducible.

The remaining nonautomated broth system consisted of the MicroScan conventional panels. Seven of the eight minor errors were with *vanB* strains and one was with *E. casseliflavus*. Three of the errors were within 1 dilution, four were 2 dilutions lower, and one was 4 dilutions lower. Thirty-two percent of the results were within the range of 2 to 16 μg/ml on the panel. On repeat testing, only two of the five errors of >1 dilution were resolved.

Results with automated commercial systems. The Vitek system showed a very major error rate of 10.3%, but no major errors were noted. Two *vanB* strains failed to grow in the Vitek cards on initial testing, although one grew on retesting. Tests with two additional strains resulted in very major errors; the MIC for one strain was 5 dilutions lower and that for the other strain was 6 dilutions lower than those obtained by the broth reference method. Tests with five other *vanB* strains produced minor errors that were ±1 dilution different from those obtained by the broth reference method. Four *E. casseliflavus* strains also produced minor errors; three were 1 dilution higher and one was 2 dilutions higher. On repeat testing, one of the two very major errors resolved to a minor error, one of the strains that was a growth failure grew and produced the correct result, and one minor error was resolved.

MS/Rapid panels showed a very major error rate of 20.7% and a major error rate of 13.3%; one *vanA* strain failed to

TABLE 3. On-scale ranges, off-scale values, and MIC differences for 12 test susceptibility testing methods

Method	On-scale range (μg/ml)[a]	Off-scale values (no. low/no. high)[b]	% On scale	No. of strains with the following differences in MIC dilutions compared with MIC by the broth reference method:									
				−6	−5	−4	−3	−2	−1	0	+1	+2	+3
Broth dilution	1–1,024	3/0	94										
Agar dilution	1–1,024	4/0	92						2	31	9	4	
Alamar	1–32	1/20	58			1		4	3	9	10		
E test	1–256	0/23	54						1	5	7	8	2
MicroMedia	1–32	3/24	46				1		1	18	3		
MicroScan conventional	2–16	12/22	32				1	4	2	4	5		
MS/Rapid	2–16	19/19	24			3	2		1	1		4	
Pasco	2–16	7/29	28							7	7		
Sceptor	2–32	7/25	36						1	13	4		
Sensititre	1–64	1/18	62					5	4	18	2		
Uniscept	2–16	9/33	16								5	3	
Vitek	1–16	3/22	50	1	1	1		1	2	5	11	1	

[a] Dilution range on the panels.
[b] Low, number of strains for which MICs were less than the lowest dilution on the panel (e.g., ≤1 μg/ml); high, number of strains for which MICs were greater than the highest dilution on the panel (e.g., ≥32 μg/ml).

produce adequate growth. There were six very major errors with *vanB* strains, for all of which the MICs obtained with the MS/Rapid system were ≤2 μg/ml. If the MICs for the strains were assumed to be 2 μg/ml, then the errors are as large as 9 dilutions. None of the very major errors were resolved on retesting, and for two strains with minor errors the result changed to very major errors. The two major errors, both of which were with *E. casseliflavus* strains, also were not resolved. Only 24% of the MS/Rapid results were within the range of 2 to 16 μg/ml represented on the panel.

Reference methods. The results of agar dilution demonstrated only two minor errors, one with a *vanB* strain for which the MIC was 2 dilutions higher than that by the broth reference method and one with a susceptible *E. faecalis* strain that was called intermediate by agar dilution. The latter error was resolved on retesting. Disk diffusion testing showed six minor errors, four with *vanB* strains, one with an *E. casseliflavus* strain, and one with a susceptible *E. faecalis* strain (the same strain for which an error was noted by agar dilution).

The vancomycin agar screen test was performed with inocula of 10^5 CFU (1 μl) and 10^6 CFU (10 μl). The presence of nonenterococcal contaminants and the haze produced by the larger 10-μl inoculum led to reading errors on initial testing by the microbiologist reading the test. The tests were read as negative by other microbiologists. On repeat testing after reviewing enterococcal morphology more closely, the first microbiologist was able to differentiate positive and negative results more clearly and read the tests, which were performed in a blinded fashion, with both inocula without errors.

DISCUSSION

The present study was a stringent challenge of the ability of commercially available susceptibility testing methods to differentiate vancomycin-susceptible enterococci from VRE. Test strains were chosen carefully to represent a wide variety of genotypes and phenotypes and included a relatively high percentage of *vanB*-containing isolates and *E. gallinarum* and *E. casseliflavus* strains. Although the last two organisms are only rarely isolated from clinical samples, the rate of isolation of *vanB*-containing strains is now higher than that of *vanA*-containing strains in several U.S. cities (1a). In addition, *E. gallinarum* and *E. casseliflavus* strains are being detected with increasing frequency from surveillance cultures of stool samples in the United States (1a). Thus, we felt that it was important to ascertain how commercial methods would classify these strains with respect to their susceptibility or resistance to vancomycin.

NCCLS recommends a full 24 h of incubation before reading vancomycin MIC results for enterococci (7). In the present study, 12 results produced by five different systems increased by 1 dilution between the 18- and 24-h readings, which changed the interpretive category. Thus, we believe that delaying readings until incubation has reached a full 24 h is critical for accurate interpretation of the results.

While we would usually consider minor error rates of 16 to 18% to be unacceptably high, it is important to note that many of the errors in the study occurred with *E. casseliflavus* strains that are known to contain an intrinsic vancomycin resistance gene (9) and with the *vanB* strains, the MICs for which cluster around the resistance breakpoint. Since the clinical significance of these organisms is unknown, we do not consider these minor errors, most of which classified the *vanB*-containing organisms as resistant, to detract from the accuracies of these systems. This is particularly true for the Alamar system, in which the majority of errors resolved on retesting. Conversely, the errors with the Uniscept system, which showed a large number of errors with *E. gallinarum* and *E. casseliflavus* strains, are more of a concern because these errors were not resolved on retesting. The interpretation of the *E. gallinarum* results was problematic in the present study. While the initial broth microdilution results for the *E. gallinarum* strains were 4 μg/ml, which is considered susceptible, repeat testing on four lots of media showed, almost exclusively, MICs of 8 μg/ml (intermediate). Since the other three reference methods (agar dilution, disk diffusion, and agar screen) classified all five isolates as intermediate (with the exception of one strain by agar dilution, for which the MIC was 4 μg/ml), the broth dilution results were considered minor errors. However, since broth dilution was the reference method for the study, both susceptible and intermediate were considered correct results for these strains.

As is emphasized by NCCLS, disk diffusion test results can be difficult to read with *vanB*-containing enterococcal strains because of light growth. It was interesting in the present study that two susceptible strains were classified as intermediate by the disk method, although both were within 1 mm of the breakpoint. Taking this into account, and given the overall performance of the test, we believe that disk diffusion testing is

acceptable for laboratories that do not routinely perform methods that determine MICs.

The agar screen plates were read initially by a microbiologist who usually does not perform susceptibility tests and who was not familiar with enterococcal growth patterns. This was done on purpose to determine the difficulty of reading the test result. In several cases, a nonenterococcal contaminant present on the plates was read as positive growth. In other cases, the inoculum spot was read as weakly positive for two susceptible strains with the 1-μl inoculum and for five susceptible strains with the 10-μl inoculum. On repeat testing, after training, both the first microbiologist and several other microbiologists with more experience with susceptibility testing read all of the weakly positive results as negative. Thus, it is important to train technologists how to read the screen plates. Technologists who are proficient in differentiating enterococci from staphylococci should not have difficulty in interpreting the results of the agar screen test, especially if the lower inoculum of 1 μl instead of the inoculum of 10 μl is used. Contamination was noted with only one set of agar screen plates and was not detected by any of the other susceptibility testing methods.

Most commercial nonautomated broth- and agar-based susceptibility testing systems accurately detected vancomycin resistance in these enterococci, although the number of systems with only 4 dilutions on the plate was surprising. It is difficult to determine the true accuracy of a method when 84% of the values are off scale. This also makes it impossible to differentiate high-level from low-level vancomycin resistance, which could be helpful for epidemiologic studies of the transmission of VRE in hospitals.

The present study confirmed previous reports showing that automated systems have difficulty in detecting low-level vancomycin resistance (10, 13, 14). MS/Rapid panels demonstrated both very major and major errors, while only very major errors were noted with the Vitek system. Until new algorithms can be established for these methods, it will be necessary for laboratories that use these systems to seek alternate methods of testing enterococci for their susceptibility to vancomycin.

Finally, results with quality control strains were in range by all methods on all test days, with the exception of the E-test results for *E. faecalis* ATCC 29212 on 2 of the 5 testing days. On these occasions, the results were repeatedly one-half dilution out of range. Since one of the minor errors was repeatedly 1 dilution lower than the broth microdilution reference result and the categorical classification (MICs) for the remaining 15 enterococcal strains from those testing days agreed with those obtained by the broth reference method, the test appeared to be performing in an acceptable manner, so the data were accepted. For routine testing, however, an MIC for a quality control strain of 6 μg/ml should be considered an indication that the test result is out of range and that the tests should be repeated. Although MICs for quality control strains that were out of range were not observed in a previous study of MICs for enterococci obtained by the E-test method (5), five of six of the daily quality control results for *E. faecalis* ATCC 29212 in that study were 4 μg/ml and the remaining value was 3 μg/ml. Thus, by the E test, the MICs for this quality control organism are consistently at the upper end of the acceptable range. Occasional out-of-range values are likely to be observed as random errors or may be related to the characteristics of the lot of MH agar used. Clinical laboratories that use the E test need to be aware of this potential problem.

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