Comparison of Microscan Broth Microdilution, Synergy Quad Plate Agar Dilution, and Disk Diffusion Screening Methods for Detection of High-Level Aminoglycoside Resistance in *Enterococcus* Species

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We compared the dried MicroScan microdilution panel, Synergy Quad plate agar dilution, and high-potency disk diffusion screening methods for the detection of high-level aminoglycoside resistance in 815 enterococcal bloodstream isolates. Agreement between the three methods was 99% when testing for high-level gentamicin resistance and 96% when testing for high-level streptomycin resistance.

There are currently three screening methods approved by the National Committee for Clinical Laboratory Standards for detection of high-level aminoglycoside resistance in enterococci, viz., broth microdilution, agar dilution and disk diffusion (15). Although each method has been evaluated in numerous settings, few studies have compared all three methods on the same group of enterococcal isolates. Using each of the three methods, therefore, we tested 815 enterococcal bloodstream isolates for high-level resistance to gentamicin and streptomycin.

The enterococcal isolates had been evaluated as part of another study (14) and were tested for high-level gentamicin and streptomycin resistance by three different screening methods based on National Committee for Clinical Laboratory Standards recommended guidelines (15). For each method, bacterial suspensions were made in the following manner. Frozen isolates of *Enterococcus* were thawed, inoculated onto 5% sheep blood agar, and incubated for 18 to 24 h at 35°C in an atmosphere containing 5% CO₂. Each isolate was subcultured onto fresh 5% sheep blood agar and incubated for an additional 18 to 24 h. A total of 5 to 10 colonies were then emulsified into 3 ml of deionized water to a level of turbidity equivalent to that of a 0.5 McFarland standard.

The broth microdilution screening method used was the dried MicroScan Pos Combo Panel Type 13 panel (Dade Behring, Inc., West Sacramento, Calif.) that incorporates wells containing gentamicin and streptomycin diluted in dextrose phosphate broth to final concentrations of 500 and 1,000 µg/ml, respectively. Microdilution panels were inoculated with 0.5 McFarland standard suspensions diluted to a final well concentration of approximately 5×10^5 CFU/ml, incubated in a WalkAway system at 35°C, and read at 18 h. Panels that indicated susceptibility to streptomycin were placed in an ambient air incubator at 35°C, and the streptomycin well was manually read at 48 h.

The second method for detecting high-level aminoglycoside resistance was the agar dilution screening method using Synergy Quad plates (Remel, Lenexa, Kans.). These plates use brain heart infusion as the basal medium and incorporate quadrants containing gentamicin and streptomycin at concentrations of 500 and 2,000 µg/ml, respectively. A 0.5 McFarland standard suspension (10 µl) was spotted onto the agar surface and incubated in ambient air at 35°C for 24 h. Isolates that showed no growth in the streptomycin quadrant after 24 h were reincubated for an additional 24 h. The presence of more than one bacterial colony on the aminoglycoside quadrants indicated high-level resistance.

The third method for detecting high-level aminoglycoside resistance was the disk diffusion screening method. A 0.5 McFarland standard suspension was streaked onto Mueller-Hinton agar, and disks containing 120 µg of gentamicin and 300 µg of streptomycin (BBL Sensi-Disk, Becton Dickinson, Cockeysville, Md.) were applied. Plates were incubated at 35°C in ambient air for 18 h. A zone size of 6 mm in diameter indicated high-level resistance, and ≥ 10 mm indicated susceptibility. Zone sizes of 7 to 10 mm in diameter were considered intermediate.

Enterococcus faecalis ATCC 51299 and *E. faecalis* ATCC 29212 were used as controls for all three methods on each day of testing.

Isolates with one or more discordant results among the three phenotypic methods were further tested for the presence of high-level aminoglycoside resistance genes. The aminoglycoside resistance genes tested for included all those known to cause mid-level and high-level gentamicin and streptomycin resistance (2). Using a multiplex PCR (S. M. Donabedian, M. J. Zervos, R. Kariyama, J. W. Chow, Abstr. 41st Intersci. Conf. Antimicrob. Agents Chemother., abstr. 197, 2001), the *aac(6')-Ie-aph(2'')-Ia*, *aph(2'')-Ib*, *aph(2'')-Ic*, and *aph(2'')-Id* genes were tested for. PCRs were performed at a volume of 50 µl with the following mixture: 1.25 U of *Taq* DNA polymerase, 0.2 mM each deoxynucleoside triphosphate, 1.5 mM MgCl₂, 1× PCR buffer, 5 pmol of each primer, and 2 colonies of each isolate. PCR was performed with an initial lysing-denaturing step of 10 min at 94°C and 35 cycles of 1 min at 94°C, 1 min at

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TABLE 1. PCR primers used to amplify aminoglycoside resistance genes

Gene	Primer	Product size (bp)	Positive control organism	Reference
<i>aac(6')-Ie-aph(2'')-Ia</i>	5'-CATTATACAGAGCCTTGGAAGATG-3' 5'-GTCTTAAAAAACTGGCAATATCTCGTT-3'	778	<i>E. faecalis</i> SF350	7
<i>aph(2'')-Ib</i>	5'-CTTGACGCTGAGATATATGAGCAC-3' 5'-GTTTGTAGCAATTCAGAAACACCCTT-3'	867	<i>E. faecium</i> SF11770	11
<i>aph(2'')-Ic</i>	5'-CCACAATGATAATGACTCAGTTCCC-3' 5'-CCACAGCTCCGATAGCAAGAG-3'	444	<i>E. gallinarum</i> SF9117	3
<i>aph(2'')-Id</i>	5'-GTGGTTTTTACAGGAATGCCATC-3' 5'-CCCTCTTCATACCAATCCATATAACC-3'	641	<i>E. casseliflavus</i> SF11300	21
<i>aadE</i>	5'-ACTGGCTTAATCAATTTGGG-3' 5'-GCCTTCCGCCACCTCACCG-3'	597	<i>E. faecalis</i> ATCC 49533	6
<i>aadA</i>	5'-TGATTGCTGGTTACGGTGAC-3' 5'-CGCTATGTTCTCTTGCTTTTG-3'	284	<i>E. coli</i> C600(pHP45 Ω)	10

55°C, and 3 min at 72°C. PCR products were run on a 2% agarose gel at 60 V for 2 h. PCR assays were used as previously described (4) to test for the *aadE* and *aadA* genes. Table 1 shows the primer sequences used in the PCR assays.

Enterococcal isolates were considered to be susceptible or resistant to high-level aminoglycosides when concordant results were obtained with all three phenotypic methods. The remaining isolates were classified as resistant when an appropriate resistance gene was detected. A very major error (false susceptibility) was defined as a susceptible result from any phenotypic method when the isolate possessed an appropriate resistance gene. The opposite pattern (false resistance) was considered a major error.

The 815 isolates comprised 639 *E. faecalis* strains, 123 *Enterococcus faecium* strains, 18 *Enterococcus casseliflavus* strains, 12 *Enterococcus avium* strains, 11 *Enterococcus gallinarum* strains, 7 *Enterococcus durans* strains, 3 *Enterococcus raffinosus* strains, and 2 strains whose species were not determined. Overall, 124 isolates were gentamicin resistant and 255 were streptomycin resistant (105 were resistant to both gentamicin and streptomycin). Of the 51 enterococcal isolates other than *E. faecalis* and *E. faecium*, none were gentamicin resistant and 6 were streptomycin resistant (3 of *E. avium*, 2 of *E. gallinarum*, and 1 of *E. casseliflavus*). Six isolates (five of *E. faecalis* and one of *E. faecium*) had discordant gentamicin resistance results, and five of these possessed the *aac(6')-Ie-aph(2'')-Ia* gene. Of the 32 isolates (25 of *E. faecalis*, 6 of *E. faecium*, and 1 of *E. avium*) with discordant streptomycin resistance results, 22 possessed the *aadE* gene. No other resistance genes were detected in any of the isolates tested. All discordant results were confirmed by repeat testing. The results for the testing of the isolates for high-level aminoglycoside resistance with the three phenotypic methods are shown in Table 2.

Our findings show that agreement between the three phenotypic tests was 99% when testing for high-level gentamicin resistance and 96% when testing for high-level streptomycin resistance. These findings are better than those documented in a recent comparison of MicroScan microdilution panels and a reference agar dilution method (5) but are similar to those of another recent evaluation of MicroScan microdilution panels and the Synergy Quad plate agar dilution screen (1). Collec-

tively, these results indicate that the current MicroScan panels have improved sensitivity for detecting high-level aminoglycoside resistance compared with earlier panels (9, 12, 16, 20). Our findings also indicate that the disk diffusion method using high-potency disks performs at least as well as the other methods, and this is consistent with the findings of other investigators (13, 16–19).

Consistent with the findings from other studies (1, 5, 8, 9, 12, 13, 16, 17, 20, 22, 23), testing for high-level resistance to streptomycin had higher error rates than testing for gentamicin. In our hands, the agar dilution screening method was the least accurate of the three methods for detecting high-level streptomycin resistance and did not perform as well as reported by others (1, 8).

Our findings indicate that the broth microdilution, agar dilution, and high-potency disk diffusion screening methods perform well for detecting high-level aminoglycoside resistance in enterococci. In particular, all three methods had a low error rate for detecting high-level resistance to gentamicin.

TABLE 2. Categorical error rates, sensitivity, and specificity of the three testing methods for detecting high-level aminoglycoside resistance

Aminoglycoside and screening method	No. of errors (% error rate) ^a that were:		Sensitivity ^c	Specificity ^d
	Very major	Major		
Gentamicin				
Broth microdilution	5 (0.6)	1 (0.1)	119/124 (96.0)	690/691 (99.9)
Agar dilution	0	1 (0.1)	124/124 (100)	690/691 (99.9)
Disk diffusion	1 (0.1)	0	123/124 (99.2)	691/691 (100)
Streptomycin				
Broth microdilution	5 (0.6)	6 (0.7)	250/255 (98.0)	554/560 (98.9)
Agar dilution	17 (2.1)	3 (0.4)	238/255 (93.3)	557/560 (99.5)
Disk diffusion	2 (0.2) ^b	7 (0.9) ^b	253/255 (99.6)	553/560 (98.8)

^a Error types are defined in the text.

^b Results include three intermediate zone sizes (one resistant and two susceptible isolates).

^c Values are numbers of isolates in which high-level resistance was detected/total number of resistant isolates. Values in parentheses are percentages.

^d Values are numbers of isolates giving specific results/total number of isolates. Values in parentheses are percentages.

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