Detection of Enterococcal High-Level Aminoglycoside Resistance with MicroScan Freeze-Dried Panels Containing Newly Modified Medium and Vitek Gram-Positive Susceptibility Cards

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Both conventional and modified MicroScan Type 5 panels and Vitek Gram-Positive Susceptibility cards were compared with agar dilution screen plates for their abilities to detect high-level resistance to gentamicin and streptomycin in 235 enterococcal isolates, including 167 *Enterococcus faecalis* and 63 *E. faecium* isolates. The modified Type 5 panels contained dextrose-phosphate broth instead of Mueller-Hinton broth in their high-level-resistance screen wells. The sensitivities for detection of gentamicin and streptomycin high-level resistance were 100 and 100% (*E. faecalis*) and 100 and 94% (*E. faecium*) for the modified MicroScan panels, 100 and 89% (*E. faecalis*) and 100 and 98% (*E. faecium*) for the conventional MicroScan panels, and 81 and 86% (*E. faecalis*) and 85 and 94% (*E. faecium*) for the Vitek cards. All specificities were 100% except for the Vitek cards with streptomycin, where it was 96%. Isolates that showed resistance on the streptomycin agar screen plates were rescreened on plates containing 32,000 μg/ml to detect ribosomally mediated resistance. For all three systems, every failure to detect streptomycin high-level resistance occurred in isolates with enzymatic, not ribosomal, resistance. The modified MicroScan Type 5 panels are a suitable method for detecting enterococcal high-level resistance to gentamicin and streptomycin. The Vitek cards are too insensitive for this purpose.

Enterococcal isolates from the blood and cerebrospinal fluid may indicate serious underlying infections such as endocarditis and meningitis (1, 7, 12, 17, 26). These infections require synergistic therapy with a combination of penicillin or ampicillin and an aminoglycoside, most frequently gentamicin (8, 14, 17, 26). For decades, many isolates have shown resistance to penicillin-streptomycin synergism, and, over the last 5 years, rising levels of resistance to penicillin-gentamicin synergism have been observed (9, 14, 18, 25, 27, 28, 30). These effects are the result of high-level aminoglycoside resistance, which may be mediated by plasmid-encoded, drug-altering enzymes or, in the case of streptomycin, by ribosomal mutation (3–5, 10, 11, 13, 17). The reported percentages of strains with resistance to synergism vary from 31 to 60% for streptomycin and 0 to 55% for gentamicin (14, 15, 17, 20, 23). It is therefore important to test blood and cerebrospinal fluid isolates for such resistance (9, 10, 14, 17). The gold standard for this determination is the time-kill study, but a more efficient screening procedure has been described: synergy failure is predicted by high-level resistance (HLR), which is defined as the ability of an enterococcal isolate to grow on agar containing 2,000 μg of the antibiotic in question per ml (16). In addition to agar dilution, current methods of assaying for aminoglycoside MICs greater than 2,000 μg/ml include macrodilution, in-house and commercial microdilution, and high-content disk susceptibility, with various media and inocula (6, 19, 21, 22, 24, 29).

MicroScan (Baxter Health Care Corp., MicroScan Division, West Sacramento, Calif.) has produced a microdilution susceptibility test panel for gram-positive organisms, con-

taining two wells with 2,000 μg of gentamicin or streptomycin per ml in Mueller-Hinton broth (MHB) to screen for enterococcal HLR. Two reports have suggested that the current Type 5 freeze-dried panel (6) as well as a frozen panel (Type 2) (6, 24) that is no longer produced both show low sensitivity (i.e., false susceptibility) compared with other methods. The MicroScan package insert instructions currently include a warning to disregard the results of the two HLR wells. Subsequent to these reports, it was suggested to MicroScan that the problem might be corrected by changing the medium in the two synergy-screen wells from MHB to dextrose-phosphate broth (DPB). DPB, the medium used in the original study defining the HLR screening procedure, is a more enriched medium than MHB. Both contain animal peptones and casein hydrolysates, but DPB also has yeast extracts and additional salts. Thus, if strains that were HLR positive by other methods failed to grow as expected in the MicroScan HLR wells, these false-negatives might have been due to a nutritional deprivation rather than solely to drug susceptibility.

To evaluate this hypothesis, MicroScan supplied us with modified Type 5 panels containing 2,000 μg of gentamicin and streptomycin per ml in the HLR wells and differing from the commercially available panels only in the substitution of DPB for MHB in those wells. We tested the abilities of both the conventional and modified versions to detect enterococcal HLR to gentamicin and streptomycin as compared with the ability of an agar dilution standard. In addition, we further separated the enterococcal isolates that showed HLR to streptomycin at 2,000 μg/ml by rescreening at 32,000 μg/ml to detect extreme HLR to streptomycin associated with altered ribosomes (4).

We also evaluated another commercially available method for detecting enterococcal HLR, i.e., the Vitek Gram-Posi-

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TABLE 1. Classification of isolates for HLR to gentamicin and streptomycin by agar screen reference method

Resistance or susceptibility	No. of isolates			
	Total	E. faecalis	E. faecium	Other
Gentamicin resistance only	25	24	0	1
Streptomycin resistance only	50	36	14	0
Gentamicin and streptomycin resistance	87	44	39	4
Gentamicin and streptomycin susceptible	73	63	10	0

tive Susceptibility card (GPS-TA; Vitek Systems, Hazelwood, Mo.) used with the AutoMicrobic System.

MATERIALS AND METHODS

Isolates. Testing was performed on 143 unique clinical enterococcal strains isolated from blood, cerebrospinal fluid, and other sites at the Massachusetts General Hospital. In addition, 92 well-defined isolates were obtained from the Infectious Disease Laboratory at the New England Deaconess Hospital. The majority of these isolates had previously measured streptomycin and gentamicin MICs as well as synergy status determined by time-kill studies. The latter strains were from diverse geographical origins. Vitek Gram-Positive Identification (GPI) cards were used to identify species presumptively (Vitek Systems). All isolates were frozen in horse blood and DPB (50:50 [vol/vol]) and stored at −70°C.

Susceptibility testing. Before testing, each isolate was subcultured twice on brucella agar with 5% horse blood, and several colonies were then used to prepare a 0.5 McFarland suspension by using a spectrophotometer (Abbott A-just Turbidity Meter; Abbott Laboratories, North Chicago, Ill.). MicroScan POS Combo Type 5 and modified BP Combo Type 5 panels were inoculated by a standard technique with an inoculum of 5×10^5 CFU/ml. The same 0.5 McFarland suspension was used to prepare the inoculum for the Vitek GPS card according to the package insert. Simultaneously, dextrose-phosphate agar screening plates containing either streptomycin (Eli Lilly and Co., Indianapolis, Ind.) or gentamicin (Elkins-Sinn Inc., Cherry Hill, N.J.) at 2,000 μg/ml were spotted with 10 μl of the same 0.5 McFarland suspension for a final inoculum of approximately 10^6 CFU. MicroScan panels and agar plates were incubated for 20 h at 35°C in ambient air. Growth of 1 or more colonies (or confluent growth) on agar with streptomycin or gentamicin was considered evidence of HLR. For a very few strains, the agar plates were hard to interpret after 20 h. We found that incubating the plates for an additional 18 h greatly clarified the results. MicroScan panels were read by visual inspection by two observers, with any growth considered evidence of

HLR. Strains exhibiting streptomycin HLR at 2,000 μg/ml were retested on dextrose-phosphate agar containing 32,000 μg of streptomycin per ml. The Vitek GPS cards were incubated in and read by the AutoMicrobic System.

Quality control. A well-characterized clinical isolate with known HLR to both antibiotics was inoculated as a quality control strain on each agar screening plate. *Enterococcus faecalis* ATCC 29212 was inoculated as a quality control strain on MicroScan Pos Combo Type 5 panels and Vitek GPS cards each time panels were tested with unknown isolates.

Statistical definitions. Sensitivity was defined as the ability of a method to detect HLR when HLR was present by our reference method, agar dilution (sensitivity = true HLR positives by particular method/total HLR positives by agar dilution). Specificity was defined as the ability of a method to report absence of HLR when it was absent by agar dilution (specificity = true HLR negatives by particular method/total HLR negatives by agar dilution).

RESULTS

A total of 235 isolates were tested: 167 *E. faecalis*, 63 probable *E. faecium*, 4 probable *E. avium*, and 1 *E. gallinarum*. Previous studies in our laboratory have shown that the Vitek GPI card used for identification identifies *E. faecalis* with high sensitivity and specificity; it is somewhat less accurate in subclassifying the remaining species into *E. faecium* and others (20). All 235 strains were tested for gentamicin and streptomycin HLR with conventional Micro-Scan Type 5 panels containing MHB in the HLR wells, modified Type 5 panels with DPB in those wells, Vitek GPS cards, and agar dilution. One strain would not grow in the Vitek GPS card despite our two attempts.

Table 1 gives the number of isolates, by species, showing HLR to gentamicin, streptomycin, or both drugs, as determined by our agar screen reference method. The sensitivities and specificities of all three methods for gentamicin and streptomycin HLR detection are given in Table 2. In Table 3 are given the sensitivities of all three methods for detecting streptomycin HLR categorized by the following two different resistance mechanisms: enzymatic, with MICs between 2,000 and 32,000 μg/ml, and ribosomal, with MICs above 32,000 μg/ml. For all three methods, all failures to detect streptomycin HLR (lack of sensitivity) occurred in isolates with enzymatic resistance. Table 4 shows the sensitivities of the three methods for streptomycin HLR by species.

DISCUSSION

MicroScan microdilution panels and Vitek AutoMicrobic System cards make up the majority of commercially available systems used for susceptibility testing (2). Both have methods for detection of enterococcal HLR to gentamicin

TABLE 2. Detection of HLR to gentamicin and streptomycin by three methods

Method	Gentamicin		Streptomycin	
	Sensitivity (%)	Specificity (%)	Sensitivity (%)	Specificity (%)
Modified MicroScan Type 5 panels with DPB	100 (112/112)	100 (123/123)	98 (134/137)	100 (98/98)
Conventional MicroScan Type 5 panels with MHB	100 (112/112)	100 (123/123)	93 (127/137)	100 (98/98)
Vitek GPS cards[a]	82 (92/112)	100 (122/122)	90 (122/136)	96 (94/98)

[a] One isolate did not grow in Vitek GPS cards.

TABLE 3. Sensitivity of three methods to detect different mechanisms of HLR to streptomycin

Method	Sensitivity (%)	
	Enzymatic[a]	Ribosomal[b]
Modified MicroScan Type 5 panels with DPB	96 (82/85)	100 (52/52)
Conventional MicroScan Type 5 panels with MHB	88 (75/85)	100 (52/52)
Vitek GPS cards[c]	83 (70/84)	100 (52/52)

[a] MIC = 2,000 to 32,000 μg/ml.
[b] MIC > 32,000 μg/ml.
[c] One isolate did not grow in Vitek GPS cards.

and streptomycin. No evaluations of this aspect of the Vitek GPS card have appeared in the literature to date. One report has evaluated the performance of an earlier, frozen version of the MicroScan (Type 2) panel and found it deficient (24). The only study evaluating the present, freeze-dried conventional MicroScan Type 5 panel reported poor sensitivity in determining enterococcal HLR and recommended that the product not be used for this purpose (6).

The modified MicroScan Type 5 panels that we tested contained DPB instead of the conventional MHB in the aminoglycoside HLR wells. DPB is the more enriched medium, so any failure of an enterococcal isolate to grow, presumptive evidence of susceptibility, is likely to be due to true drug susceptibility and not inadequate medium. In our study, the modified Type 5 panel with DPB was 100% sensitive for gentamicin HLR detection and 98% sensitive for streptomycin. The conventional Type 5 panels with MHB showed HLR detection sensitivities of 100% for gentamicin but only 93% for streptomycin. Even when HLR was detected, for many strains growth in the conventional HLR well was sparse compared with growth in the corresponding modified DPB well. Thus the modified Type 5 panels containing DPB were more sensitive and easier to read for streptomycin HLR. This is at variance with an earlier study concluding that aminoglycoside HLR detection with either an agar or broth dilution method does not depend on the medium (21). That study, however, used a macrotube dilution system (1 ml) with a final inoculum of 10^5 CFU/ml and only tested E. faecalis species. Although the authors concluded that all media performed well, their data suggest that systems using MHB or Mueller-Hinton agar are somewhat less sensitive (especially at lower inocula) than those using DPB or dextrose phosphate agar. Also, results from strains producing 1 to 10 colonies on agar or a fine film of growth were considered ambiguous and not included in the analysis. This eliminated from the study strains with border-

TABLE 4. Sensitivity by species of 3 methods to HLR to streptomycin

Method	Sensitivity (%)	
	E. faecalis	E. faecium
Modified MicroScan Type 5 panels with DPB	100 (80/80)	94 (50/53)
Conventional MicroScan Type 5 panels with MHB	89 (71/80)	98 (52/53)
Vitek GPS cards[a]	86 (68/79)	94 (50/53)

[a] One isolate did not grow in Vitek GPS cards.

line HLR that might have caused discrepant results on different media.

Although in our study the conventional Type 5 panels performed worse than the modified version, their sensitivities for detecting HLR were higher than those found in a previous study: 100% compared with 90% for gentamicin and 93% compared with 41% for streptomycin (6). Several explanations are possible. (i) The MicroScan production facilities were moved between the time of the previous study and the time that we performed ours, and thus different production methods may have altered the panels. (ii) We considered any growth in the HLR wells as indicating resistance. Other users may have higher thresholds and interpret more strains as susceptible. The package insert instructions require distinguishing between a "white haze" or "fine granular growth" throughout the wells (indicating growth) from either "slight whiteness" or "clear broth" (indicating no growth). Although most isolates gave rise to easily interpretable wells, ambiguous cases did exist, especially in the conventional panels. To challenge the panels in a way consistent with normal laboratory work-flow, our data are based only on readings made after 20 h of incubation, but for some difficult strains we found that an additional 24 h of incubation clarified or changed the initial reading. (iii) The sensitivity of detection of HLR to streptomycin is dependent on the mechanism of resistance. The two versions of Micro-Scan panels and Vitek cards always detected strains with ribosomal HLR and streptomycin MICs greater than 32,000 μg/ml. All three systems, however, occasionally failed to detect strains with enzymatic HLR and MICs between 2,000 and 32,000. The results of the evaluations of these systems may vary, therefore, depending in part on the proportion of isolates with these two mechanisms of streptomycin HLR.

All three strains for which the modified Type 5 panels failed to detect streptomycin HLR were probable E. faecium with streptomycin MICs of 4,000 μg/ml. For two of these strains, incubating the panel for an additional 24 h (total, 44 h) resulted in positive growth in the streptomycin HLR well. For the same two strains, the conventional Type 5 panels detected streptomycin HLR where the modified panels failed, although the growth even in the conventional HLR wells was weak. It is thus possible that some strains of E. faecium, unlike the majority of enterococci, grow better at 20 h in MHB than DPB. In support of this is the fact that both the conventional Type 5 panels and the Vitek cards showed marginally better sensitivity with E. faecium. Nonetheless, the streptomycin HLR sensitivity of the modified Type 5 panels with E. faecium is quite good, i.e., 94%. Moreover, the percentage of E. faecium strains in our study, 26%, is much higher than the reported clinical prevalence (20). Thus, even when the prevalence of streptomycin HLR in a given hospital is 50%, the modified Type 5 panels will show positive and negative predictive values greater than 99% when applied to clinical isolates with smaller numbers (i.e., 10%) of E. faecium.

The third of the three systems we tested, the Vitek GPS cards, performed least well, with sensitivities for detecting gentamicin and streptomycin HLR of 82 and 90%, respectively, and specificity for streptomycin HLR of 96%. It was the only system that failed to identify all instances of gentamicin HLR and the only system that lacked 100% specificity for both drugs.

In sum, we found that the MicroScan Type 5 panels modified with DPB were a sensitive and specific method for detecting enterococcal HLR to gentamicin and streptomycin. The conventional Type 5 panel with MHB performed

less well, although considerably better than reported in a previous study (6). The Vitek GPS cards performed poorly in detecting enterococcal HLR and cannot be recommended for this purpose. Failure to detect streptomycin HLR with any of the three methods occurred only with enzymatically mediated HLR and never with ribosomal HLR. The sensitivity of all three methods in detecting streptomycin HLR will depend somewhat on the proportions of strains with the two resistance mechanisms in a particular institution.

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