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Factors Influencing Determination of High-Level Aminoglycoside Resistance in *Enterococcus faecalis*

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The ability of seven methods to detect high-level gentamicin (58 strains) and streptomycin resistance (56 strains) among 107 *Enterococcus faecalis* isolates was investigated at the University of Chicago Medical Center and the University of Nebraska Medical Center. Methods included a standard agar screen plate, high-content disk diffusion, Remel (Lenexa, Kans.) EF Synergy Quad plates, standard microdilution panels prepared in house, Pasco MIC Gram-Positive panels (Difco Laboratories, Detroit, Mich.), MicroScan MIC Type 5 dry panels (Baxter Healthcare Corp., MicroScan Div., West Sacramento, Calif.), and Vitek GPS-TA cards (Vitek Systems Inc., Hazelwood, Mo.). Results indicating false resistance were not obtained by any method, and there was 100% agreement between the results of the disk diffusion and standard agar screen methods. Prolonging incubation from 24 to 48 h increased resistance detection for both agar and microdilution screens. EF Synergy Quad plates inoculated with micropipettes detected 100% of the streptomycin- and gentamicin-resistant isolates. Resistance detection for streptomycin and gentamicin, respectively, was 93 and 96% by standard microdilution, 93 and 98% by Pasco panels, 88 and 89% by MicroScan panels, and 88 and 91% by Vitek GPS-TA cards. False susceptibility occurred more frequently with streptomycin-resistant isolates than it did with gentamicin-resistant strains and appeared to be strain related in some instances. The use of an increased inoculum size enhanced resistance detection with these strains, but it complicated interpretation of results and led to the selection of streptomycin-resistant mutants. Until results of further studies delineate optimum test conditions, a delay in the final interpretation of agar and microdilution screen results until 48 h for isolates showing no or light growth at 24 h may help to minimize the occurrence of false susceptibility reporting.

An increasing number of enterococci are exhibiting the high-level resistance to aminoglycosides (i.e., MIC, >2,000 μg/ml) that is responsible for resistance to the synergy usually achieved between a cell wall-active agent and the corresponding aminoglycoside (3, 6, 22). The consequences of such resistance for effective treatment of serious enterococcal infections makes screens designed to detect this resistance among the most relevant of the susceptibility testing procedures performed with clinically significant isolates. Evaluating and establishing test accuracy are therefore of clinical importance.

Test methods that have been evaluated include agar screen (12, 15), broth microdilution (2, 15, 17, 18, 23), broth macrodilution (12, 15, 21), and disk diffusion (9, 10, 13, 15). Comparisons of results reported from these different studies indicate that the accuracy of the screen may vary with the method being used. However, the causes for these variations in accuracy have not been thoroughly investigated. The purpose of this study was to evaluate the accuracy of several available screening methods, determine whether their accuracies varied when the tests were performed in different laboratories, establish potential causes for the suboptimal performance of certain tests, and investigate what methodological changes might enhance accuracy.

(This study was presented, in part, at the 30th Interscience Conference on Antimicrobial Agents and Chemotherapy [11].)

MATERIALS AND METHODS

Organisms. In all, 107 single isolates of *Enterococcus faecalis* obtained from cultures of patient blood specimens submitted to the Clinical Microbiology Laboratories of either the University of Chicago Medical Center (UC) or the University of Nebraska Medical Center (UN) were used. *E. faecalis* ATCC 29212 and UC 73 (a strain previously shown to be highly resistant to both gentamicin and streptomycin) served as susceptible and resistant controls, respectively.

Screen methods. For inoculum preparation, four to five colonies of each isolate were selected from overnight growth on Trypticase soy agar supplemented with 5% sheep blood (Becton Dickinson Microbiology Systems, Cockeysville, Md.) and suspended in 0.85% sterile saline to a turbidity matching that of a 0.5 McFarland standard. Aliquots of this suspension (ca. 10^8 CFU/ml) were diluted appropriately to achieve the final desired inoculum sizes.

(i) Standard agar screen. Mueller-Hinton quadrant agar (Difco Laboratories, Detroit, Mich.) plates were prepared at the UN Medical Center. Three quadrants were supplemented with an aminoglycoside, 500 μg of gentamicin per ml, 2,000 μg of gentamicin per ml, or 2,000 μg of streptomycin per ml, and the fourth quadrant served as a growth control. Each quadrant was inoculated with a 10-μl aliquot of the suspension of 10^8 CFU/ml, for a final inoculum of 10^6 CFU. At UC this was done by using a micropipette; at UN a 10-μl calibrated loop (Difco) was used.

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TABLE 1. Detection of high-level aminoglycoside resistance after 24 h of incubation[a]

Aminoglycoside (concn [μg/ml]) tested	No. of resistant strains	% Resistant strains detected:						
		With EF plates inoculated by[b]:			By microdilution			By Vitek
		Loop	Pipette	Swab[c]	Standard[c]	Pasco[c]	MicroScan[c]	
Gentamicin (500)	58	83	98	98	92	NT[d]	NT	91
Gentamicin (2,000)	58	79	98	97	88	96	59	NT
Streptomycin (2,000)	56	71	98	84	79	79	65	88

[a] As established by standard agar screens performed at UC and UN; Vitek data are based on a 6-h incubation.
[b] Inoculation methods are as described in the text.
[c] Data are averages of results obtained at UC and UN.
[d] NT, not tested; concentration not included in the panel.

(ii) High-content disk diffusion. Disks containing 120 μg of gentamicin and 300 μg of streptomycin were prepared at the UC Medical Center as described previously (13). The disks were used with Mueller-Hinton agar plates (Becton Dickinson Microbiology Systems) to perform disk diffusion testing by recommended guidelines (7). For both disks, zones of inhibition of <10 mm were interpreted as high-level resistance.

(iii) Standard broth microdilution. An MIC 2000 Dynatech microdilution system (Dynatech Laboratories, Alexandria, Va.) was used to prepare broth microdilution panels containing gentamicin at 500 and 2,000 μg/ml and streptomycin at 2,000 μg/ml in 0.1 ml of cation-adjusted Mueller-Hinton broth. The final inoculum for each microdilution well was 5×10^5 CFU/ml (5×10^4 CFU per well).

(iv) Commercial systems. Remel (Lenexa, Kans.) EF Synergy Quad (EF) plates contained Mueller-Hinton agar supplemented with the same aminoglycoside concentrations as were used in the standard agar plates. Each plate was inoculated by two methods. At UC, a micropipette was used to apply 10 μl of the suspension of 10^8 CFU/ml to the agar surface of each quadrant (pipette method). By the second method, a sterile swab was immersed and saturated in the organism suspension, excess liquid was expressed against the side of the tube, and the tip of the swab was applied to the agar surface. This procedure (swab method) was repeated for the inoculation of each quadrant. At UN, the swab method was performed as described above for UC, but a 10-μl calibrated loop (loop method) instead of the pipette method was used. Unless otherwise indicated, Pasco MIC Gram-Positive panels (Difco), MicroScan MIC Type 5 dry panels (Baxter Healthcare Corp., MicroScan Div., West Sacramento, Calif.), and Vitek GPS-TA cards (Vitek Systems Inc., Hazelwood, Mo.) were inoculated, incubated, and read according to the recommendations of each manufacturer.

With each test isolate, screening methods were performed at both UC and UN, except that testing by Vitek GPS-TA was performed only at UC. Vitek GPS-TA results were obtained after a 6-h incubation, and disk diffusion readings were made at 24 and 48 h. Agar and microdilution screens were incubated at 35°C in an ambient atmosphere and were examined for growth after 18 to 24 h of incubation. Those showing no growth were reincubated for 18 to 24 h longer and were reexamined for growth. In all instances, isolates showing light growth on agar screens (i.e., individual colonies or a light but definite haze on the agar surface) or in microdilution panels (i.e., a small but definite button, or haze, on the well bottom) were considered resistant.

RESULTS

There was complete agreement between standard agar screen results obtained at UN and UC. In all, 49 isolates were susceptible to gentamicin and 51 isolates were susceptible to streptomycin. Fifty-eight strains showed gentamicin resistance after 24 h of incubation, and this number did not change when the incubation was prolonged to 48 h, regardless of whether 500 or 2,000 μg of gentamicin per ml was used. At 24 h, 41 and 53 isolates showed resistance to streptomycin when they were tested at UN and UC, respectively. After 48 h of incubation, 56 resistant strains were detected at both test sites. On the basis of this complete agreement in standard agar screen results, the gentamicin and streptomycin resistance profiles of the 107 isolates were taken as those obtained by this method. These profiles, which included 20 isolates susceptible to both drugs, 27 isolates resistant to both drugs, 31 isolates resistant to gentamicin only, and 29 isolates resistant to streptomycin only, were used to compare and evaluate results obtained by the other screening methods.

Results of high-content disk agar diffusion testing at UC and UN were combined for analysis. Inhibition zone diameters obtained with the 49 gentamicin-susceptible and the 51 streptomycin-susceptible strains ranged from 10 to 30 mm for both aminoglycosides. A zone of 10 mm, which was used as the breakpoint zone diameter between resistance and susceptibility, was noted with one gentamicin-susceptible strain and three streptomycin-susceptible strains. The gentamicin-susceptible strain gave a zone of 10 mm at UN and a zone of 13 mm at UC. Of the three streptomycin-susceptible isolates, one gave a 10-mm zone at both UC and UN; the other two gave 13- and 14-mm zones at UN. The inhibition zone diameters exhibited by 58 gentamicin-resistant strains were all <10 mm and ranged from 6 to 9 mm. Fifty-six of these isolates gave no inhibition zone (diameter, 6 mm). Similarly, the zone diameters of the 56 streptomycin-resistant isolates ranged from 6 to 9 mm, with 49 and 55 strains showing no inhibition zone when they were tested at UN and UC, respectively. Extending incubation from 24 to 48 h did not notably alter the zone sizes obtained with either aminoglycoside.

The percentages of resistant strains detected by the EF plates and the microdilution panels after 24 h of incubation and with the Vitek GPS-TA card after 6 h of incubation are shown in Table 1. On EF plates, the highest proportion of gentamicin and streptomycin-resistant strains (98%) was detected by pipette inoculation. By swab inoculation, detection of gentamicin resistance was comparable to that obtained by pipette inoculation, but streptomycin resistance

TABLE 2. Detection of high-level aminoglycoside resistance after 48 h of incubation[a]

Aminoglycoside (concn [μg/ml]) tested	No. of resistant strains	% Resistant strains detected:					
		With EF plates inoculated by[b]:			By microdilution		
		Loop	Pipette	Swab[c]	Standard[c]	Pasco[c]	MicroScan[c]
Gentamicin (500)	58	98	100	100	97	NT[d]	NT
Gentamicin (2,000)	58	93	100	98	96	98	89
Streptomycin (2,000)	56	98	100	99	93	93	88

[a] As established by standard agar screens performed at UC and UN.
[b] Inoculation methods are as described in the text.
[c] Data are average of results obtained at UC and UN.
[d] NT, not tested; concentration not included in the panel.

detection was lower, only 84%. The lowest percent detection obtained with EF plates occurred by loop inoculation. By microdilution, only the standard panel with gentamicin at 500 μg/ml and the Pasco panel with gentamicin at 2,000 μg/ml detected greater than 90% of the aminoglycoside-resistant strains after 24 h of incubation. Vitek GPS-TA cards detected 88 and 91% of the streptomycin- and gentamicin-resistant isolates, respectively. For all methods except MicroScan, fewer streptomycin-resistant strains than gentamicin-resistant strains were detected, and no false resistance occurred with any system.

In every instance, when incubation was prolonged from 24 h (Table 1) to 48 h (Table 2), the percentage of resistant strains detected by EF plates and by the three microdilution systems increased. The most substantial increases occurred by loop inoculation of EF plates and with the MicroScan panels. Although MicroScan was the only test procedure by which resistance detection remained below 93% with 48 h of incubation, this method showed the greatest detection increase at 48 h (23% for streptomycin and 30% for gentamicin). Except for MicroScan, prolonging of incubation to 48 h enhanced streptomycin resistance detection to a greater extent than it did gentamicin resistance detection. Use of either 500 or 2,000 μg of gentamicin per ml in the EF plates and standard microdilution panels resulted in comparable resistance detection percentages. No false resistance was detected when incubation was increased to 48 h for any of the methods.

The percentage of resistant strains detected by those screen methods performed at both UC and UN are presented in Table 3 by method and test site. In most instances, the results obtained at UC and UN were comparable. The greatest differences occurred with MicroScan testing of gentamicin (100% detection of resistance at UC; 78% detection of resistance at UN) and streptomycin (93% detection of resistance at UC; 84% detection of resistance at UN) and testing of streptomycin by the standard microdilution panel (96% detection of resistance at UC; 89% of resistance at UN). On average, only for the MicroScan testing done at UN was the proportion of resistant strains detected (81%) below 90%.

We investigated the possibility that the false susceptibility encountered in this study was strain related. Resistant strains (as defined by their standard agar dilution results) that showed light or no growth at 24 or 48 h by two or more test methods at both sites were selected for further study. Twenty strains met these criteria (14 resistant to streptomycin only, 5 resistant to gentamicin only, 1 resistant to both gentamicin and streptomycin) and were retested at UC by all methods. Of the 15 streptomycin-resistant strains, 9 still gave light or no growth by two or more methods, as did 3 of 6 gentamicin-resistant isolates. The methods that yielded light or no growth were not always the same methods that demonstrated these growth patterns during original testing. Additionally, 10 isolates (6 resistant to streptomycin, 3 resistant to gentamicin, 1 resistant to both) that showed false susceptibility by Vitek GPS-TA were retested by the Vitek System. Of these 10 strains, 7 were among the 20 strains that had met the other criteria for repeat testing. Three of the seven streptomycin-resistant isolates again were falsely sus-

TABLE 3. Comparison of high-level resistance detection by test site[a]

Aminoglycoside (concn [μg/ml]) tested	Test site	% Resistant strains detected by:				Avg % detected by aminoglycoside
		EF swab[b]	Microdilution			
			Standard	Pasco	MicroScan	
Gentamicin (500)	UC	100	98	NT[c]	NT	97
	UN	100	97	NT	NT	98
Gentamicin (2,000)	UC	98	98	98	100	99
	UN	98	93	98	78	92
Streptomycin (2,000)	UC	100	96	91	93	95
	UN	98	89	95	84	93
Avg % detected by method	UC	99	97	94	96	
	UN	99	93	96	81	

[a] Includes results obtained by methods performed only at both UC and UN; resistance was interpreted after 48 h of incubation.
[b] The Remel EF plate was inoculated with a swab as described in the text.
[c] NT, not tested; concentration not included in the panel.

ceptible, as were two of the four gentamicin-resistant isolates.

To investigate the effect of an increased inoculum size, isolates for which false susceptibility persisted were tested on EF plates and in microdilution systems by using 100-fold greater inocula than is routinely recommended. Vitek GPS-TA cards were inoculated with a 10-fold greater inoculum size, and all inoculum sizes were confirmed by colony counts. In nearly every instance, use of a greater inoculum size resulted in detection of the resistant strains. However, with susceptible controls the larger inoculum size resulted in small buttons on the bottom of the microdilution panels that could be mistaken for growth. Use of a higher inoculum (10^8 CFU) with six susceptible control strains tested on EF plates resulted in the appearance of 2 to 10 individual colonies on the streptomycin quadrant. Subsequent testing of subcultures of these colonies indicated that they were spontaneous streptomycin-resistant mutants (data not shown).

DISCUSSION

Results of this investigation and those of previous studies have shown that false aminoglycoside susceptibility, not false resistance, is the problem most frequently encountered with available screening methods (2, 4, 12, 15, 17, 18, 21). In this investigation prolonging of the incubation time to 48 h substantially increased the percentage of resistant strains detected. This effect was observed at both UC and UN for the standard agar screen, the EF Synergy Quad plates, and the three microdilution methods and was most notable for detection of streptomycin resistance. In contrast, other investigators have not observed that prolonged incubation of MicroScan panels improves detection of resistant strains (2, 17). The reason for these findings is unknown, but it may be related to differences in the strains studied or how the results were interpreted when light growth was present in the microdilution wells.

Throughout this study, light growth on the agar surface or in the microdilution well was interpreted as resistance. This approach, which has also been used for an agar screen method in a recent study by Weissmann et al. (18), was used here to establish consistency and minimize the occurrence of false susceptibility. However, there is an increased risk of false resistance. There was complete agreement between UC and UN when these criteria were used for interpreting standard agar screen results, but difficulties in interpreting light growth may account for the differences between these test sites in determining streptomycin resistance by the standard microdilution method and gentamicin and streptomycin resistance by using MicroScan panels (Table 3). Such difficulties may also have contributed to the wide disparity in the percentage of gentamicin- and streptomycin-resistant strains detected by MicroScan in previous studies. With the frozen MicroScan Gram-Positive Type 2 panel, detection of gentamicin resistance has ranged from 15% (15) to 84% (2), and that of streptomycin resistance has ranged from 27% (15) to 46% (19). Comparable differences have been reported for the MicroScan Type 5 panel. Fuller et al. (2) reported detection of 41 and 90% of the streptomycin- and gentamicin-resistant strains, respectively, while in our current study the respective proportions were 93 and 100% at UC and 84 and 78% at UN. The decision as to how light growth should be interpreted and how this interpretation affects falsely susceptible and resistant readings in various screening methods must await establishment of a true standard for determining synergistic resistance. Even the current standard, time-kill studies, may be hampered by technical and interpretive artifacts. One approach would be to collect strains whose high-level aminoglycoside resistance profiles have been established through the use of a battery of nucleic acid probes for genes encoding aminoglycoside-modifying enzymes, such as those described by Ounissi et al. (8). These well-characterized isolates could then be used to investigate the accuracy of different screening methods. Until such studies are undertaken, the appropriate interpretation of light growth in clinical situations remains problematic.

With prolonged incubation, most methods investigated detected more than 93% of the resistant isolates. Micropipette inoculation of EF plates showed complete agreement with standard agar screen results and appears to provide a convenient and reliable method for resistance screening. The standard microdilution and Pasco panels were comparable in detecting resistance (93 to 98%), but the reasons why these two systems detected more resistant strains than the MicroScan panel (ca. 89%) did are unknown. Recent evaluations of MicroScan panels in which dextrose phosphate broth replaced Mueller-Hinton broth in the screening wells revealed enhanced resistance detection (17, 18), but in the report by Szeto et al. (17), streptomycin resistance detection was still only 85%. In our current study, all three microdilution systems used a Mueller-Hinton broth formulation, but MicroScan panels showed the lowest detection percentage. These findings and those of Szeto et al. (17) suggest that, in addition to medium, other factors may contribute to problems encountered with the MicroScan system in the detection of resistance.

There was complete agreement between disk diffusion and standard agar screen results, which is consistent with previously published studies (10, 13, 15). Although most resistant strains showed no inhibition zone, some resistant and susceptible isolates may give inhibition zones at or near the 10-mm breakpoint. For strains giving such zone sizes, repeat disk testing and screening by another method may be warranted. The 90% average detection of resistant strains by the Vitek GPS-TA card was less than the 97% average reported by Solliday et al. (14), but it was comparable or slightly higher than the detection average reported by others (4, 17, 18). Further studies to establish alterations needed to enhance resistance detection to levels comparable to those of other screening methods are in advanced stages of investigation (5). Until appropriate adjustments are made, isolates determined to be susceptible by Vitek GPS-TA cards should be tested by an alternative method.

Regarding the aminoglycoside being tested, for almost every screen investigated, false susceptibility occurred more frequently with streptomycin-resistant strains than it did with gentamicin-resistant strains after 24 h of incubation. In most instances, prolonging of the incubation time to 48 h increased detection of streptomycin resistance to a greater extent than detection of gentamicin resistance. These findings are consistent with those of several previous studies which showed that false susceptibility occurs more frequently with streptomycin testing (2, 4, 14, 15, 17–19). In an earlier study, false streptomycin susceptibility occurred most frequently with the use of a low inoculum (10^2 CFU/ml) on either Mueller-Hinton or Trypticase soy agar (12). However, it is not yet known whether discrepancies in the results for streptomycin are related to media, inoculum size, or strain characteristics. Given that streptomycin therapy has been used to treat infections by gentamicin-resistant, streptomycin-susceptible isolates (16, 20), resolution of this false streptomycin susceptibility problem is important. For gen-

tamicin, results were comparable with the use of 500 and 2,000 μg/ml. These findings and those of Fuller et al. (2), who reported no difference with 500, 1,000, or 2,000 μg of gentamicin per ml in the MicroScan panels, suggest that the use of any of these concentrations should reliably detect gentamicin resistance.

Results obtained with the 20 isolates selected for repeat testing indicated that, in some instances, false susceptibility may be strain related. Eliopoulos et al. (1) have shown that the level of streptomycin resistance expressed by an isolate varies with the resistance mechanism. If resistance is enzymatically mediated by a streptomycin adenylyltransferase, MICs are ≤16,000 μg/ml, and if it is mediated by ribosomal mutation, MICs are >64,000 μg/ml. Although it is not yet proven by definitive molecular studies, a recent report suggests that strains exhibiting ribosomally mediated resistance are readily detected by some screening methods (18). Conversely, the streptomycin MICs for those strains whose resistance is enzyme mediated are lower and may be at or near the currently used breakpoint of 2,000 μg/ml, and their resistance may not always be detected. However, when we tested five of the falsely susceptible isolates by full-range MIC (62.5 to 2,000 μg/ml), MICs for all strains were >2,000 μg/ml. The potential effect that the underlying resistance mechanisms may have on the level of resistance expressed, and thus on detection, underscores the need for investigations with strains whose resistance mechanisms have been well characterized.

An increase in the inoculum size 10-fold for the Vitek and 100-fold for the microdilution and EF plate methods enhanced resistance detection for nearly every isolate that had repeatedly shown false susceptibility by two or more screening methods. These findings agree with those of Fuller et al. (2), who found that an increase in the inoculum size increased resistance detection by both MicroScan Type 2 and Type 5 panels. However, interpretation difficulties, the tendency for spontaneous mutants resistant to streptomycin to be selected on agar screens, and the unknown effect that an increased inoculum might have on other nonaminoglycoside antibiotics included in gram-positive testing batteries contraindicate this approach for the enhancement of resistance detection.

Because prolonging incubation to 48 h and interpreting light growth as resistance increased detection of resistant strains, these factors should be considered when high-level resistance screens are studied. However, it must be emphasized that the difficulties in interpreting light growth as resistance preclude the use of this approach in clinically relevant testing situations. Although a 48-h turnaround time for susceptibility results is not optimal, this approach best allows increased detection with minimal errors in the interpretation of results. Until further studies better define optimal test conditions, delaying final interpretations of agar screen and microdilution results until 48 h on all isolates showing no or light growth at 24 h may minimize the occurrence of false susceptibility reporting.

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