JOURNAL OF CLINICAL MICROBIOLOGY, Feb. 1988, p. 250–256
0095-1137/88/020250-07$02.00/0

Vol. 26, No. 2

Effects of Medium and Inoculum Variations on Screening for High-Level Aminoglycoside Resistance in *Enterococcus faecalis*

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Received 27 July 1987/Accepted 28 October 1987

Enterococcus faecalis **isolates that are refractory to aminoglycoside-penicillin synergy can be detected by their ability to grow in the presence of high concentrations of aminoglycoside (2,000 μg/ml). In past studies investigators have used a variety of media and inoculum sizes to perform high-level aminoglycoside resistance screens, but little is known about how these variations affect test accuracy. We screened 63 *E. faecalis* strains on different media by using various inoculum sizes and correlated the results with synergy test results obtained by time-kill studies. Screens were done with dextrose-phosphate agar, brain heart infusion agar, Trypticase soy agar with 5% sheep blood, Mueller-Hinton agar with 5% sheep blood, dextrose-phosphate broth, and Mueller-Hinton broth. Agar screens were inoculated with 10^2, 10^4, and 10^6 CFU; and broth screens contained a final inoculum of 10^5 CFU/ml. The *E. faecalis* isolates were tested for high-level resistance to streptomycin, kanamycin, amikacin, gentamicin, and tobramycin. Of the 63 isolates tested, 21 did not show high-level resistance to any of the aminoglycosides tested, and 42 demonstrated high-level resistance to one or more drugs. The sensitivity of most screens was ≥90%. Regardless of the inoculum size or medium used, false-resistance results were seldom encountered. Screen specificity, which was used as the indicator of false susceptibility, was markedly influenced by both the inoculum size and the drug being tested. Specificity was low whenever a 10^2-CFU inoculum was used, when amikacin was tested with any inoculum, and when tobramycin was tested in broth media. Data for kanamycin could be used to predict amikacin-penicillin synergy, and the highly accurate gentamicin screen obviated the need for the testing of tobramycin. We recommend a 10^6-CFU inoculum for agar screens and a 10^5-CFU/ml inoculum for broth screens. The type of medium used did not substantially influence screen accuracy. Among the aminoglycosides, only streptomycin, gentamicin, and, occasionally, kanamycin need to be used to screen *E. faecalis* isolates for aminoglycoside-penicillin synergy.**

The synergistic antienterococcal activity that aminoglycoside and penicillin combinations exhibit has been known for some time (7, 11, 14). Use of these antibiotics is the most effective treatment for serious entercoccal infections, and they are recommended as the treatment of choice (8, 12, 17–19). Enterococci can acquire high-level resistance to aminoglycosides (i.e., resistant to >2,000 μg/ml), however, and become refractory to penicillin-aminoglycoside synergy (1, 2, 6, 9, 15, 16, 20, 22). High-level resistance has been reported for every aminoglycoside that might be considered for combination therapy; these include streptomycin, kanamycin, amikacin, tobramycin, gentamicin, and netilmicin (1, 2, 6, 9, 12, 15, 16, 20, 22). The emergence of these resistant strains can significantly limit the therapeutic choices for serious enterococcal infections.

Because high-level aminoglycoside resistance is most frequently mediated by constitutively produced aminoglycoside-modifying enzymes that are encoded on transferable plasmids, the incidence of enterococci that are refractory to combination therapy is expected to increase (3, 10, 13, 15, 25). Indeed, reports by Zervos et al. (24) have indicated that as much as 55% of their enterococcal isolates demonstrated high-level gentamicin resistance. The probability that synergy-resistant enterococci will emerge in many health care facilities, coupled with the serious consequences that this resistance might have for the therapeutic management of patients, indicates that perhaps the most relevant susceptibility testing methods for enterococci are those that predict the susceptibility of the organism to synergy.

The two most common methods for determining synergy, the checkerboard technique and time-kill tests, are often too cumbersome, time-consuming, and labor intensive for routine use in many clinical laboratories. Because enterococcal resistance to synergy is most frequently mediated by resistance to high levels of aminoglycosides, however, a simple screening test originally described by Moellering et al. (16) can be used to predict the susceptibility of *Enterococcus faecalis* isolates to synergy. By this method, an agar medium is supplemented with a particular aminoglycoside to a final concentration of 2,000 μg/ml, and *E. faecalis* isolates are inoculated onto the agar surface. Strains that grow are exhibiting high-level aminoglycoside resistance and, therefore, would likely be refractory to drug synergy. On the other hand, strains that fail to grow are probably susceptible to aminoglycoside-penicillin synergy.

Although several investigators have used tests based on the principle of this high-level aminoglycoside resistance screen, the type of media and size of the inoculum employed have varied considerably. The different media used have included Trypticase soy yeast broth (BBL Microbiology Systems, Cockeysville, Md.) (22), cation-supplemented Mueller-Hinton broth (MBH) (9), Trypticase soy broth (8), dextrose-phosphate agar (DPA) (2, 16), 5% defibrinated horse blood agar (1), brain heart infusion agar (BHI) (15, 20), and nutrient agar (18). Similarly, the inoculum sizes have varied from 10^4 to 10^6 CFU/ml (8, 9, 15, 20, 22), and some investigators (2, 16, 18) have inoculated test agars simply by

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streaking a single enterococcal colony over the surface without quantitating the number of bacteria that were present in the inoculum.

The effect that these test variations have on the accuracy of the screen for predicting enterococcal resistance to synergy has not been thoroughly examined. The present study was undertaken to determine the effect that variation in the type of medium and inoculum size would have on the ability of the screen to detect high-level aminoglycoside resistance accurately and, therefore, to predict susceptibility to aminoglycoside-penicillin synergy. The information gained from this investigation could be useful for establishing the most reliable conditions for performing the screen.

MATERIALS AND METHODS

Organisms. For this study we used 63 strains of *E. faecalis*. Fifty-six of the isolates were obtained from patient specimens submitted to the University of Chicago Clinical Microbiology Laboratories, and the other seven strains were graciously provided by Steven R. Crider (Naval Hospital, Camp Pendleton, Calif.). We confirmed the identify of each *E. faecalis* isolate on the basis of colony morphology, Gram stain, and recommended biochemical characteristics (5).

Antibiotics and media. The antibiotics used for this investigation included gentamicin (Schering Corp., Bloomfield, N.J.), tobramycin (Eli Lilly & Co., Indianapolis, Ind.), streptomycin and penicillin (Sigma Chemical Co., St. Louis, Mo.), and kanamycin and amikacin (Bristol Laboratories, Syracuse, N.Y.). Mueller-Hinton agar (MHA) and MHB were obtained from BBL Microbiology Systems. All other media, which included Trypticase soy agar (TSA), DPA, BHI, and dextrose-phosphate broth (DPB), were provided by GIBCO Laboratories, Madison, Wis. MHA and TSA were the only media that were supplemented with 5% sheep blood.

High-level aminoglycoside resistance screen. For the high-level aminoglycoside resistance screen, each aminoglycoside was incorporated into each agar (MHA, TSA, DPA, BHI) and broth (DPB, MHB) medium to a final concentration of 2,000 μg of drug per ml. Inocula for the agar screens were prepared from overnight (18 to 24 h) *E. faecalis* cultures that had been grown in DPB at 35°C. By using a Steers replicator, portions of each overnight culture, which contained approximately 10^9 CFU/ml, were inoculated onto the various agars to give a final inoculum of 10^6 CFU. Subsequently, the remainder of each culture was diluted with sterile 0.85% saline to match the turbidity of a 0.5 McFarland standard (ca. 10^8 CFU/ml). From this suspension appropriate dilutions were made, and a Steers replicator was used to inoculate the agar media with the 10^2 and 10^4 CFU inoculum of each *E. faecalis* strain. Media that lacked an aminoglycoside supplement were used as positive growth controls. All media were incubated for 18 to 24 h in an ambient atmosphere at 35°C. Following incubation the agar screens were examined for the presence of bacterial growth; if bacterial growth was evident, the relative amount (i.e., light to heavy) was noted.

For the broth (i.e., DPB and MHB) screening method, the inoculum was prepared in saline from cultures grown overnight in DPB, as was done for the agar screen inocula. However, the final inoculum was 10^5 CFU in 1.0 ml of broth. Media that were not supplemented with an aminoglycoside were used as positive growth controls. Broths were incubated in an ambient atmosphere at 35°C and, following 18 to 24 h of incubation, were examined visually for the presence of bacterial growth.

Time-kill studies. To perform time-kill studies with each *E. faecalis* isolate that was screened for high-level aminoglycoside resistance, we used a method based on a procedure described previously by Moellering et al. (17). After the organisms were grown in DPB overnight at 35°C, the turbidity of the broth cultures was adjusted to equal a 0.5 McFarland standard. A 0.1-ml portion of the overnight culture was added to 0.9 ml of fresh DPB to give a final organism concentration of 10^7 CFU/ml. Prior to inoculation, each tube of fresh DPB was supplemented with the appropriate aminoglycoside, either alone or in combination with penicillin. A DPB tube that did not contain antibiotic was used as a positive growth control. The concentration of antibiotic that was used closely approximated the maximum achievable serum level of each drug in serum and was less than the MIC for the *E. faecalis* strains tested. The drug concentrations used were as follows: streptomycin, 25 μg/ml; amikacin, 20 μg/ml; kanamycin, 20 μg/ml; gentamicin, 5 μg/ml; tobramycin, 5 μg/ml; penicillin G, 10 U/ml.

Inoculated broths were incubated in an ambient atmosphere at 35°C. At 0-, 4-, and 24-h intervals after inoculation, a 0.01-ml portion was removed from each of the DPB tubes and diluted and 0.1 ml of diluent was plated on TSA. By using the viable counts determined at each time interval, a 24-h time-kill curve was established for each *E. faecalis* strain. Susceptibility to aminoglycoside-penicillin synergy was defined as a ≥100-fold increase in killing by the drug combination over the killing accomplished by the most active of the two drugs when tested separately. Resistance to synergy was a <100-fold increase in killing (18).

Data analysis. Results of the high-level resistance screens were correlated with results of the time-kill synergy studies. Based on the correlations, screen results were classified as true positive, true negative, false positive, or false negative. True positive results were those in which both the screen test and the time-kill test showed an isolate to be synergy susceptible. True negative results were those in which an isolate was synergy resistant by both tests. Results in which an isolate was synergy susceptible by the screen test but resistant by the time-kill test were considered to be false positive, and those that were synergy resistant by the screen test but susceptible by the time-kill method were considered to be false negative.

The specificities and sensitivities of each of the various high-level aminoglycoside resistance screens were calculated by using the following formulas: sensitivity (%) = [number of true positives/(number of true positives + number of false negatives)] × 100; specificity (%) = [number of true negatives/(number of true negatives + number of false positives)] × 100. By these formulas, sensitivity indicated the incidence of false resistance observed with the various screens, and specificity indicated the incidence of false susceptibility.

RESULTS

Examples of time-kill curves generated for two *E. faecalis* isolates are given in Fig. 1. Results obtained with a strain that was susceptible to the synergistic activity of every aminoglycoside-penicillin combination tested are shown in Fig. 1A, and results obtained with a strain that was resistant to every antibiotic combination tested are shown in Fig. 1B. Such time-kill plots were generated for each *E. faecalis* isolate and were used as the standard by which isolates were characterized as being susceptible or resistant to the synergy of various aminoglycoside-penicillin combinations.

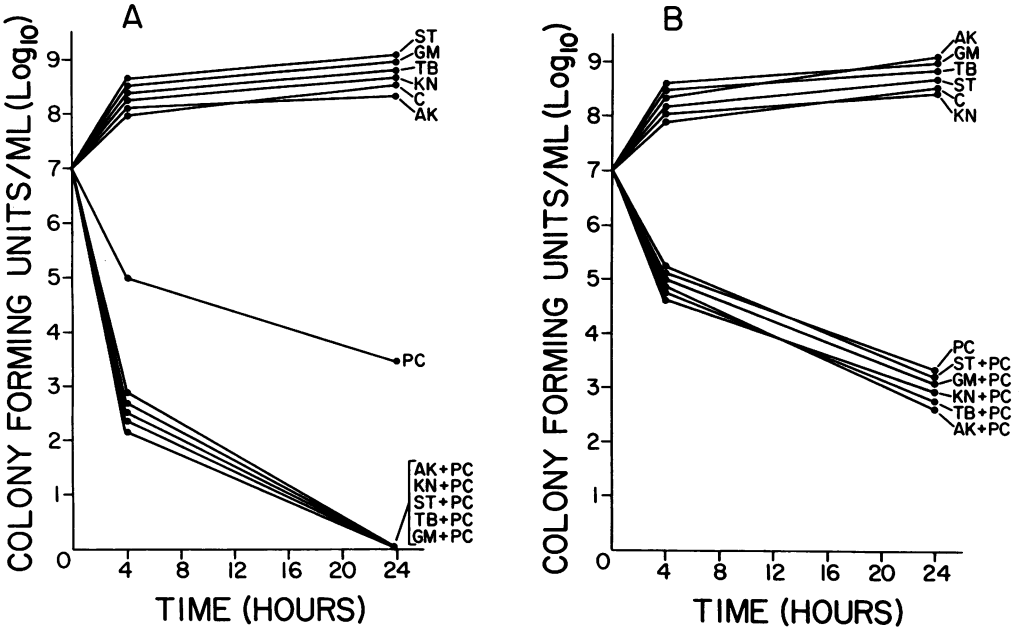

FIG. 1. Effect of penicillin and each aminoglycoside, tested alone and in combination, on the growth of a *E. faecalis* strain that was susceptible to synergy between penicillin and each aminoglycoside tested (A) and a *E. faecalis* strain that was resistant to synergy between penicillin and each aminoglycoside tested (B). The following antibiotics were tested: penicillin (PC), 10 U/ml; streptomycin (ST), 25 μg/ml; amikacin (AK), 20 μg/ml; kanamycin (KN), 20 μg/ml; gentamicin (GM), 5 μg/ml; tobramycin (TB), 5 μg/ml; control (C), no antibiotics. All time-kill studies were conducted by using DPB.

Of the 63 *E. faecalis* isolates selected for this study, 21 were susceptible to every drug combination tested, and 42 strains exhibited resistance to one or more aminoglycoside-penicillin combinations (Table 1). Among these 42 isolates, two were resistant only to streptomycin-penicillin synergy, whereas the other 40 were resistant to more than one aminoglycoside-penicillin combination. All 40 of these multiply-resistant isolates showed kanamycin-penicillin and amikacin-penicillin resistance, 33 showed streptomycin-penicillin resistance, and 22 showed both gentamicin-penicillin and tobramycin-penicillin resistance.

The effects that various agar media and inoculum sizes had on the specificities of the high-level resistance screens are given in Table 2. Regardless of the aminoglycoside tested or the agar medium employed, use of the 10^2 inoculum frequently resulted in specificities that were well below 90% and several that were less than 80%. Extremely low specificities were also observed with all of the amikacin screens. No matter which agar medium or inoculum size was used for these screens, the specificity never exceeded 23%. When kanamycin was substituted for amikacin to predict amikacin-penicillin synergy, however, the specificity increased sub-

TABLE 1. Aminoglycoside resistance patterns[a] of *E. faecalis* isolates

Aminoglycoside resistance pattern of:	No. of strains
Streptomycin	2
Streptomycin, kanamycin, amikacin	18
Streptomycin, kanamycin, amikacin, gentamicin, tobramycin	15
Kanamycin, amikacin, gentamicin, tobramycin	7
Susceptibility to all	21

[a] As determined by time-kill studies (Fig. 1).

TABLE 2. Specificity of high-level aminoglycoside resistance screens tested with various agar media and inoculum sizes[a]

Aminoglycoside[b]	Inoculum (CFU)	Specificity (%) on the following agar media:			
		DPA	BHI	TSA	MHA
Streptomycin	10^2	100	93	79	82
	10^4	100	100	100	97
	10^6	100	100	100	97
Gentamicin	10^2	90	81	76	76
	10^4	95	95	90	95
	10^6	95	95	95	95
Tobramycin	10^2	82	72	81	50
	10^4	91	91	91	88
	10^6	95	95	91	91
Kanamycin	10^2	90	69	78	79
	10^4	100	97	97	100
	10^6	100	100	100	100
Amikacin	10^2	17	10	21	17
	10^4	17	15	10	17
	10^6	22	23	20	21
Kanamycin-amikacin[c]	10^2	91	68	83	81
	10^4	100	96	97	100
	10^6	100	100	100	100

[a] The formula used to calculate specificity (incidence of false susceptibility) is given in the text.
[b] Each aminoglycoside screen was tested at 2,000 μg/ml.
[c] For kanamycin-amikacin, kanamycin was used in place of amikacin to predict amikacin-penicillin synergy.

stantially. This increase was especially evident with the use of 10^4- and 10^6-CFU inocula, which resulted in specificities of ≥96%. Similarly, the specificities of the other aminoglycoside screens were frequently ≥95% when either 10^4- or 10^6-CFU inocula were used. In general, when all of the aminoglycoside screens are considered, the specificities obtained with the 10^6-CFU inoculum tended to be slightly higher than the specificities obtained with 10^4-CFU inoculum.

The sensitivities obtained with various aminoglycoside screens are given in Table 3. Regardless of the medium and inoculum size used, streptomycin, gentamicin, tobramycin, and amikacin screens all had high sensitivities, with most being 100%. In general, the sensitivity of the kanamycin screens was also high. Only when a 10^6-CFU inoculum was tested on BHI did kanamycin sensitivity fall to a relatively low 80%. For the screens in which kanamycin was used in place of amikacin to predict amikacin-penicillin synergy, the sensitivities were ≥95% with 10^2- and 10^4-CFU inocula, but were lower with the 10^6-CFU inoculum. Most notable was the 77% sensitivity obtained with the use of BHI.

When the *E. faecalis* isolates were screened for high-level resistance, some of the tests resulted in very light growth at the site of inoculation. These instances of light growth, which were characterized by the presence of ≤10 individual colonies or a fine film of growth, were categorized as questionable screen results. Instead of including these results in the data for calculating the specificities and sensitivities given in Tables 2 and 3, respectively, these questionable results were tabulated according to both the agar medium used and the aminoglycoside tested and were correlated with results of the time-kill synergy studies (Table 4). Because of the low specificities associated with all of the amikacin screens and with the screens inoculated with 10^2 CFU (Table 2), the questionable results obtained with these tests were not included in Table 4. Of the 2,520 remaining screen results, only 43 (1.7%) were questionable. Of these 43 questionable results, 26 occurred with strains that were characterized as being synergy resistant to particular aminoglycoside-penicillin combinations, and the remaining 17 occurred with synergy-susceptible strains. The kanamycin screens most frequently resulted in light growth, and among the agar media, questionable results occurred most often with TSA and MHA.

The sensitivities and specificities of the broth screens for high-level aminoglycoside resistance are given in Table 5. Because agar screen results showed that inocula of 10^4 to 10^6 CFU generally resulted in high specificities and sensitivities (Tables 2 and 3, respectively), and because 10^5 CFU/ml is the final inoculum recommended for broth dilution susceptibility testing (21), the broth screens were tested with a single inoculum size of 10^5 CFU/ml. With the exception of the streptomycin screen results obtained in DPB, the sensitivities of these tests were 100%. However, the specificities of the screens varied considerably with the aminoglycoside being tested. Whereas streptomycin, gentamicin, kanamycin, and kanamycin substituted for amikacin synergy screens demonstrated specificities above 90%, the specificities of the amikacin and tobramycin screens were considerably lower, regardless of the broth that was used. The low specificities of the amikacin screens performed in broth were consistent

TABLE 3. Sensitivity of high-level aminoglycoside resistance screens tested with various agar media and inoculum sizes[a]

Aminoglycoside[b]	Inoculum (CFU)	Sensitivity (%) on the following agar media:			
		DPA	BHI	TSA	MHA
Streptomycin	10^2	100	96	100	100
	10^4	100	96	100	100
	10^6	96	96	100	96
Gentamicin	10^2	100	100	100	100
	10^4	100	100	100	100
	10^6	100	100	100	100
Tobramycin	10^2	100	100	100	100
	10^4	100	100	100	100
	10^6	100	100	100	100
Kanamycin	10^2	100	100	100	100
	10^4	100	100	100	100
	10^6	94	80	95	95
Amikacin	10^2	100	100	100	100
	10^4	100	100	100	100
	10^6	100	100	100	100
Kanamycin-amikacin[c]	10^2	100	100	100	100
	10^4	100	96	100	95
	10^6	94	77	90	90

[a] The formula used to calculate sensitivity (incidence of false resistance) is given in the text.
[b] Each aminoglycoside screen was tested at 2,000 μg/ml.
[c] For kanamycin-amikacin, kanamycin was used in place of amikacin to predict amikacin-penicillin synergy.

TABLE 4. Number of questionable screen results obtained with the use of various aminoglycosides and agar media

Aminoglycoside	Questionable results on the following agar media[a]:				Totals by aminoglycoside[a]
	DPA	BHI	TSA	MHA	
Streptomycin	0/0	0/0	3/4	0/0	3/4
Gentamicin	0/1	0/1	2/3	2/2	4/7
Tobramycin	1/1	1/1	1/1	6/6	9/9
Kanamycin	1/6	1/3	5/8	3/6	10/23
Totals by medium	2/8	2/5	11/16	11/14	26/43[b]

[a] Questionable screen results were defined as ≤10 colonies per spot inoculum or very light haze of growth. Results obtained with amikacin and with the 10^2-CFU inoculum were excluded. Results are expressed as the number of questionable results obtained with synergy-resistant strains/total number of questionable results.
[b] The total number of questionable results was 1.7% of all results.

TABLE 5. Sensitivities and specificities[a] of high-level aminoglycoside resistance screens tested in broth media[b]

Aminoglycoside	Results on the following broth media:			
	DPB		MHB	
	Sensitivity (%)	Specificity (%)	Sensitivity (%)	Specificity (%)
Streptomycin	96	94	100	91
Gentamicin	100	95	100	95
Kanamycin	100	95	100	97
Tobramycin	100	82	100	43
Amikacin	100	10	100	7
Kanamycin-amikacin[c]	100	97	100	100

[a] Formulas used to calculate sensitivity (incidence of false resistance) and specificity (incidence of false susceptibility) are given in the text.
[b] Final inoculum for all tests, 10^5 CFU/ml.
[c] For kanamycin-amikacin, kanamycin was used in place of amikacin to predict amikacin-penicillin synergy.

with the low specificities noted with various agar media (Table 2). In contrast, the specificities of the broth screens for tobramycin were much lower than the specificities obtained with agar media (Table 2). The 43% specificity of the tobramycin screen in MHB resulted from the fact that 13 truly resistant *E. faecalis* isolates failed to grow in the broth screen. These isolates were retested by using higher inocula (10^6 and 10^7 CFU/ml). With the 10^6-CFU/ml inoculum, none of the 13 isolates grew, but all strains grew when the inoculum was 10^7 CFU/ml. However, with the 10^7-CFU/ml inoculum, several strains that were susceptible to tobramycin-penicillin synergy also grew. A similar attempt to increase the specificity of the tobramycin screen in DPB by increasing the inoculum size also resulted in a number of false-resistant results.

DISCUSSION

As indicated by the high sensitivities given in Table 3, very few instances of false resistance occurred when various agar media and inoculum sizes were used to test *E. faecalis* isolates for synergy resistance. Exceptions were noted when a 10^6-CFU inoculum was used in kanamycin screens for determining both kanamycin-penicillin synergy and for amikacin-penicillin synergy (kanamycin substituted for amikacin), particularly when BHI was employed. Because the same inoculum size did not result in low sensitivities for the other aminoglycoside screens, regardless of the agar medium that was used, the explanation for the false resistance observed with kanamycin screens on BHI is unclear. In any case, our results indicate that if BHI is used, the sensitivities of the kanamycin screens may be significantly influenced by the inoculum size. In contrast, sensitivities of the other aminoglycoside synergy screens are not notably affected by the agar medium or inoculum size.

In contrast to the sensitivities of the screens, the specificities were substantially influenced by the inoculum size (Table 2). Use of 10^2 CFU frequently resulted in false-susceptible test results (i.e., low specificity). Such results indicate that ineffective aminoglycoside-penicillin combinations could be used for the treatment of serious *E. faecalis* infections. Therefore, the 10^2-CFU inoculum size must be considered inadequate for synergy screening. Although good specificities were achieved with both 10^4- and 10^6-CFU inocula, slightly fewer false-susceptible results were noted with the larger inoculum.

Regardless of the inoculum size or the agar medium used, the amikacin screens showed extremely low specificities (Table 2). The high incidence of false susceptibility that we observed agrees with results reported by Basker et al. (1) and Calderwood et al. (2). Results of their studies showed that some *E. faecalis* strains failed to grow in the presence of 2,000 μg of amikacin per ml, but when tested in time-kill studies, the strains were refractory to amikacin-penicillin synergy. Why these strains are inhibited by 2,000 μg of amikacin per ml but are not synergistically killed by the combination of penicillin and serum-achievable concentrations of amikacin is not known. In any case, as demonstrated previously (1, 2) and in our study, kanamycin can be used in place of amikacin to accurately predict amikacin-penicillin synergy (Tables 2, 3, and 5). Two major factors are responsible. First, *E. faecalis* resistance to kanamycin and amikacin is mediated by either one of two aminoglycoside-modifying enzymes, 3′-phosphotransferase-III or the bifunctional enzyme 2″-phosphotransferase–6′-acetyltransferase (3, 10, 13). Therefore, because any kanamycin-resistant strain is also amikacin-resistant, the sensitivity of the kanamycin screen for amikacin-penicillin synergy should be comparable to the sensitivity observed for kanamycin-penicillin synergy, as shown by the results given in Table 3. Second, in contrast to the 2,000-μg amikacin screen, most strains that are susceptible to 2,000 μg of kanamycin per ml are synergistically killed by the kanamycin-penicillin combination, as well as by the amikacin-penicillin combination. Thus, with the kanamycin screen for amikacin-penicillin synergy, few instances of false susceptibility would be expected and the specificities should be high (Table 2).

Except for the amikacin screens and those performed with the 10^2-CFU inoculum, the specificities and sensitivities of most agar screens were high (Tables 2 and 3). Of 2,520 tests that were performed, in only 43 (1.7%) was questionable growth shown (Table 4). Thus, results of most high-level screens performed on agar media demonstrate either synergy resistance or susceptibility. However, when questionable growth does occur, neither the resistance nor the susceptibility of the isolate can be presumed. Organisms that give such results should be tested by the time-kill method.

Most questionable results occurred with TSA and MHA (Table 4), but they represented only 3% of the screen results obtained with these two media. Similarly, most questionable results occurred when kanamycin was tested, but the 23 questionable results consisted of only 5% of the results obtained with this particular aminoglycoside. These data indicate that TSA and MHA and kanamycin are acceptable for reliable synergy screening.

Except for amikacin and tobramycin, the sensitivities and specificities of the aminoglycoside resistance screens performed in broth media were high (Table 5). The same mechanism that was responsible for the low specificities in the agar screen was probably responsible for the low specificities observed for the broth amikacin tests. As was true for the agar screens, kanamycin can be used in the broth method to predict amikacin-penicillin synergy. The reason for the high number of false-susceptible results observed with the tobramycin screens is unknown. An increase in the inoculum to 10^7 CFU/ml resulted in growth of the truly resistant strains that failed to grow when a 10^5-CFU/ml inoculum was used, but use of the higher inoculum increased the incidence of false resistance seen among synergy-susceptible strains. The problems encountered with the tobramycin broth screens could be eliminated by using gentamicin to screen for tobramycin-penicillin synergy. Because the bifunctional aminoglycoside-modifying enzyme (2″-phosphotransferase–6′-acetyltransferase) that mediates high-level resistance to gentamicin is the enzyme that also mediates tobramycin resistance (3), isolates that are resistant to gentamicin-penicillin synergy are also resistant to tobramycin-penicillin synergy. Therefore, gentamicin screens, which showed high specificities and sensitivities in both agar and broth media (Tables 2, 3, and 5), could be used to predict not only gentamicin-penicillin synergy but also tobramycin-penicillin synergy. Of importance is the fact that because the broth screens were performed in 1-ml volumes, the results obtained might be different from those that may occur with the use of a broth microdilution system. Studies to investigate the accuracy of microdilution procedures for detecting synergy resistance are needed.

The findings of this study indicate that if laboratories are going to routinely screen clinically significant *E. faecalis* isolates for resistance to aminoglycoside-penicillin synergy, they should follow certain guidelines and take specific precautions. With respect to inoculum size, use of an insufficient inoculum (i.e., 10^2 CFU) can have an adverse effect on

the specificity of the agar screens. Therefore, we do not recommend the use of unquantifiable inoculation procedures such as the streaking of a single colony over the agar surface of an antibiotic-containing plate. Because the fewest instances of false-susceptible results were obtained with 10^6 CFU, this inoculum size is recommended for agar screens. With the 10^6 CFU inoculum, the instances of false-resistant screen results may be increased when kanamycin is tested on BHI. However, use of an agar medium other than BHI (DPA, TSA, MHA) would alleviate this problem. For synergy screens performed in broth media, we recommend a final inoculum size of 10^5 CFU/ml, the same as that which is used for other broth dilution susceptibility testing procedures (21).

Because the specificities and sensitivities of the synergy screens were not notably affected by the type of agar or broth medium used, except when a 10^6-CFU inoculum was used to test kanamycin on BHI, in general, the medium that is available may be used. In many clinical microbiology laboratories, this would be TSA, MHA, or MHB.

The aminoglycosides selected for screening depend on the drugs that are being considered for therapy and the antibiotics that give the most reliable screen results. The aminoglycosides that might be considered for combination therapy with a cell wall-active agent are streptomycin, kanamycin, amikacin, gentamicin, tobramycin, and netilmicin. Among these drugs, only streptomycin, gentamicin, and occasionally, kanamycin should be considered for synergy screening. Regardless of whether *E. faecalis* resistance to streptomycin is mediated by ribosomal mutation (4, 25) or enzymatic inactivation (13), only streptomycin can be used to screen for streptomycin-penicillin synergy. Because the same bifunctional enzyme (i.e., 2″-phosphotransferase–6′-acetyltransferase) that mediates high-level gentamicin resistance also mediates tobramycin and netilmicin resistance (3), a gentamicin screen alone currently is sufficient to reliably predict synergy between penicillin and gentamicin, tobramycin, or netilmicin. In addition, this enzyme also mediates resistance to kanamycin and amikacin so that gentamicin-resistant *E. faecalis* isolates are also resistant to these two aminoglycosides (3). Some *E. faecalis* isolates are susceptible to gentamicin (i.e., lack 2″-phosphotransferase–6′-acetyltransferase), however, but are resistant to kanamycin and amikacin (Table 1). These isolates produce 3′-phosphotransferase-III, an enzyme that inactivates kanamycin and amikacin but not gentamicin (13). Therefore, although all gentamicin-resistant isolates are also resistant to kanamycin and amikacin, not all gentamicin-susceptible isolates are susceptible to kanamycin and amikacin. The susceptibilities of these gentamicin-susceptible strains to kanamycin and amikacin can be determined only by a screen for high-level kanamycin resistance.

Finally, we must emphasize that the results of this study pertain only to *E. faecalis* isolates and not to the less commonly encountered *Enterococcus faecium*. *E. faecium* is resistant to most aminoglycoside-penicillin combinations, and is often susceptible only to gentamicin-penicillin synergy. High-level resistance screens performed with these organisms frequently do not correlate with results of synergy studies (23). Further studies are needed to establish reliable screening procedures that may be used to test *E. faecium* susceptibility to aminoglycoside-penicillin synergy.

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