JOURNAL OF CLINICAL MICROBIOLOGY, Nov. 1988, p. 2270–2274
0095-1137/88/112270-05$02.00/0

Vol. 26, No. 11

Laboratory Detection of High-Level Aminoglycoside-Aminocyclitol Resistance in *Enterococcus* spp.

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Received 16 May 1988/Accepted 9 August 1988

Methods for detection of high-level resistance to aminoglycoside-aminocyclitol antibiotics were evaluated using 104 blood isolates of enterococci (97 *Enterococcus faecalis* and 7 *Enterococcus faecium*). Kanamycin was used to predict resistance to amikacin. Discrepancies between methods were resolved by time-kill studies. Four methods (MicroScan, macrotube, microtiter, and disk diffusion) for detecting resistance to gentamicin and streptomycin were compared, using 51 consecutive strains. There were 13 gentamicin-resistant strains, all of which were detected by macrotube, microtiter, and disk diffusion. MicroScan detected 2 (15%) of the 13. Of the 18 streptomycin-resistant strains, 17 (93%) were detected by disk diffusion, 16 (89%) by microtiter, 9 (50%) by macrotube, and 6 (33%) by MicroScan. An additional 53 consecutive strains were examined only by disk diffusion and microtiter for resistance to gentamicin, streptomycin, and kanamycin. The entire population of 104 strains contained 35 gentamicin-, 22 streptomycin-, and 54 kanamycin-resistant enterococcal isolates. All 35 gentamicin-resistant strains were detected by both methods. Of the 22 streptomycin-resistant strains, 1 was detected only by microtiter, 2 only by disk diffusion, and 19 by both methods. Of the 54 kanamycin-resistant strains, 1 was detected only by microtiter, 2 only by disk diffusion, and 51 by both methods. One additional strain which was resistant only by disk diffusion was susceptible to amikacin plus penicillin by time-kill studies. Disk diffusion is a suitable method for detection of high-level aminoglycoside-aminocyclitol resistance in *E. faecalis* and is well suited for sporadic testing. Additional data are necessary to determine the suitability of these tests for *E. faecium*.

Most enterococci are inhibited but not killed by cell wall-active agents such as penicillin, ampicillin, and vancomycin and are resistant to achievable serum levels of aminoglycoside-aminocyclitol antibiotics such as gentamicin and streptomycin. In urinary tract infections, single therapy with ampicillin, for example, is adequate because ampicillin is concentrated by the kidneys. In serious infections such as septicemia and endocarditis, however, bactericidal therapy is recommended (2, 3, 13). Bactericidal activity can be achieved by combining a cell wall-active agent with an aminoglycoside or aminocyclitol when the combination shows synergistic killing in time-kill studies. Resistance to 2,000 μg of the aminoglycoside or aminocyclitol per ml predicts that there will be no synergy by time-kill (6). Because resistance has been increasingly reported, screening of enterococci for high-level resistance to streptomycin, gentamicin, and kanamycin is recommended (2, 5, 12, 15).

At least five screening methods are available for detection of high-level aminoglycoside-aminocyclitol resistance in enterococci. They include a commercially available microtiter method (MicroScan; Baxter Healthcare Corp., W. Sacramento, Calif.), a microtiter method prepared in house (14), macrotube dilution, disk diffusion (10, 12), and an agar screen (11). The first four methods have been compared on blood culture isolates of *Enterococcus* spp.

MATERIALS AND METHODS

Source of strains. Blood culture isolates of *Enterococcus* spp. from patients at the University of Wisconsin Hospital and Clinics from 1 January 1987 through 26 February 1988 were examined. Multiple strains from the same patient were tested and stored only if the blood cultures had been collected more than 2 days apart. After the strains were identified to species and routine susceptibility tests were performed, the strains were stored at −70°C in brain heart infusion broth with 10% glycerol. Strains were revived from frozen stock by subculture onto Trypticase soy agar with 5% sheep blood (BBL Microbiology Systems, Cockeysville, Md.) and incubated at 35°C in ambient air overnight. A commercially available kit was used to identify the strains (API 20S; Analytab Products, Inc., Plainview, N.Y.).

Susceptibility testing. Strains were tested for high-level resistance to gentamicin, streptomycin, and kanamycin. Kanamycin was used to detect high-level resistance to amikacin (12). All susceptibility tests were incubated overnight at 35°C in ambient air. All macrotube, microtiter, disk diffusion, and time-kill studies were performed in the Microbiology Research Laboratory. Antimicrobial powders were obtained from Sigma Chemical Co. (St. Louis, Mo.). The MicroScan tests were performed in the Clinical Microbiology Laboratory.

Screening methods. (i) Micro Scan. Susceptibility of some strains to 500 μg of gentamicin per ml and 2,000 μg of streptomycin per ml was determined using MicroScan POS MIC2 panels (Baxter Healthcare Corp., MicroScan Div., W. Sacramento, Calif.). The inoculum was prepared in saline using the Turbidity Standard Technique described in the package insert. This gives a final concentration of approximately 10^5 CFU/ml.

(ii) Macrotube dilution. Standard methods (8) were used to determine the susceptibility of strains to 500 μg of gentamicin and 2,000 μg of streptomycin per ml. This method produces a final inoculum of 7.5×10^5 CFU/ml.

(iii) Microtiter. Fifty microliters of inoculum prepared as described above was added to 50 μl of antimicrobial agents dispensed in microtiter trays (Dynatech Laboratories, Inc.,

Alexandria, Va.) to give a final concentration of 500 μg of gentamicin, 2,000 μg of streptomycin, or 2,000 μg of kanamycin per ml. A drug-free growth control well was also included. Plates were incubated overnight and examined as described above. Each organism was tested at least once. If the gentamicin or streptomycin results disagreed with those of the MicroScan or macrotube dilution screens, the microtiter and macrotube tests were repeated. When discrepancies continued to occur, time-kill studies were performed.

(iv) Disk diffusion. Susceptibility to aminoglycoside-aminocyclitol antibiotics was determined on Mueller-Hinton agar using standard disk diffusion methods (7) with disks containing 120 μg of gentamicin, 120 μg of kanamycin, and 300 μg of streptomycin per disk. Strains were defined as having high-level resistance if the diameter of the zone of inhibition was <10 mm (10, 12). Discrepancies between microtiter and disk diffusion test results were resolved by the use of time-kill studies.

Confirmatory testing: time-kill studies. Time-kill studies were done in Mueller-Hinton broth inoculated with log-phase growth adjusted to give an inoculum of about 7.5×10^5 CFU/ml. Antimicrobial agents were tested at clinically achievable concentrations (gentamicin, 10 μg/ml; amikacin, 20 μg/ml; streptomycin, 25 μg/ml; penicillin, 100 U/ml), singly and in pairs. At 0, 4, 7, and 24 h after inoculation and incubation at 35°C, colony counts were determined on brain heart infusion agar (Difco Laboratories, Detroit, Mich.) supplemented with 10^4 IU of penicillinase (Difco; BBL) per ml and 5% sodium chloride, the latter to inactivate aminoglycoside-aminocyclitol antibiotics (4). A blood agar plate was inoculated to verify purity. Plating was done with a Spiral Plater (Spiral Systems, Inc., Cincinnati, Ohio). Synergy was defined as a 100-fold reduction in CFU at 24 h by the combination of agents as compared with the most active single drug (5).

Quality control. Two well-characterized clinical isolates were used for quality control of the macrotube, microtiter, disk diffusion, and time-kill studies. *Enterococcus faecalis* UWHC 1921 had been shown to be resistant to penicillin plus gentamicin and penicillin plus amikacin and susceptible to penicillin plus streptomycin by time-kill. *E. faecalis* UWHC 1936 had been shown to be susceptible to penicillin plus gentamicin and resistant to penicillin plus streptomycin by time-kill. Both of these organisms were included each time tests were performed. *E. faecalis* ATCC 25213 was used as the penicillin-plus-amikacin-susceptible strain in the time-kill studies. It was also the quality control organism used for the MicroScan panels, as per the manufacturer's instructions. Each lot number was tested upon receipt in the laboratory. When retesting was done, quality control was repeated.

RESULTS

A total of 104 strains from 93 patients were studied; 97 (93.3%) were *E. faecalis* and 7 (6.7%) were *Enterococcus faecium*.

All 46 strains of *E. faecalis* stocked from blood cultures collected between July 1987 through 26 February 1988 were examined for high-level resistance to gentamicin and streptomycin, using all four screening methods. Thirteen (28.3%) and 15 (32.6%) of the strains were resistant to gentamicin and streptomycin, respectively. No strain was resistant to both agents. The ability of each method to detect high-level aminoglycoside-aminocyclitol resistance is shown in Table 1. All 13 gentamicin-resistant strains were detected by the macrotube, microtiter, and disk diffusion methods. There were 11 instances in which strains were susceptible to gentamicin by MicroScan and resistant by macrotube. On retesting there were no changes in the results of the macrotube dilution test results, but three strains became gentamicin resistant by MicroScan. Time-kill studies confirmed that the combination of penicillin plus gentamicin did not show synergistic killing against any of these 11 strains. Three strains, however, were killed by penicillin alone.

TABLE 1. Detection of high-level gentamicin and streptomycin resistance in *Enterococcus* spp. by four methods[a]

Test method	No. of resistant strains detected (%)		
	Streptomycin		Gentamicin: *E. faecalis*[b]
	E. faecalis	*E. faecium*	
MicroScan	4 (27)	2 (67)	2 (15)
Macrotube	7 (47)	2 (67)	13 (100)
Microtiter	13 (87)	3 (100)	13 (100)
Disk diffusion	14 (93)	3 (100)	13 (100)
Any method	15	3	13

[a] Data on 51 strains.
[b] There were no strains of gentamicin-resistant *E. faecium*.

No method was able to detect all 15 streptomycin-resistant strains. Two were detected only by disk diffusion and one only by microtiter. The single resistant strain not detected by disk diffusion had a 10-mm (susceptible) zone diameter. On retesting the zone was 9 mm (resistant). Time-kill studies showed no synergy between penicillin and streptomycin against this strain. There were two instances in which strains were susceptible to streptomycin by MicroScan and resistant by macrotube dilution. On retesting there were no changes in the results of either microtube or MicroScan results. Time-kill studies were performed on these strains. The combination of penicillin plus streptomycin did not show synergistic killing against either of these two strains. One strain, however, was killed by penicillin alone.

The 51 strains of *E. faecalis* isolated from blood cultures collected between January and June 1987 were tested for resistance to high-level gentamicin and streptomycin only by the microtiter and disk diffusion methods. All of the 22 gentamicin-resistant strains were detected by both methods. Of the four streptomycin-resistant strains, all four were detected by disk diffusion and three were detected by microtiter.

All 97 strains of *E. faecalis* from the entire study period were tested for high-level kanamycin resistance only by the microtiter and disk diffusion methods. Of the 50 kanamycin-resistant strains, 49 were detected by disk diffusion and 49 by microtiter. Neither the strain detected only by disk diffusion nor the one detected only by microtiter was synergistically killed by penicillin plus amikacin.

Three of the seven strains of *E. faecium* isolated during the entire study period were streptomycin resistant by both the microtiter and disk diffusion methods. One of these was not detected by MicroScan. The combination of penicillin plus streptomycin did not show synergistic killing against any of these three strains. Three strains were resistant to high-level kanamycin by both microtiter and disk diffusion. Two strains were resistant by disk diffusion but not microtiter. In one case the strain was synergistically killed by penicillin plus amikacin. The other strain was killed by penicillin as a single agent. None of the seven strains was resistant to gentamicin. One strain grew on Mueller-Hinton agar with but not without blood.

TABLE 2. Disk diffusion zone sizes for aminoglycoside-aminocyclitol-susceptible and -resistant enterococci

Agent and response[a]	*E. faecalis*				*E. faecium*			
	No. tested	Zone of inhibition[b] (mm)			No. tested	Zone of inhibition[b] (mm)		
		Range	Mean ± SD	Mode		Range	Mean ± SD	Mode
Gentamicin								
Resistant	35	6	6	6				
Susceptible	62	17–22	19.7 ± 1.0	20	7	19–23	20.7 ± 1.4	20
Streptomycin								
Resistant	19	6–10	7.3 ± 1.2	8	3	6–9	7 ± 1.7	6
Susceptible	78	16–23	19.7 ± 1.2	20	4	18–20	19.5 ± 1.0	20
Kanamycin								
Resistant	50	6–19[c]	6.3 ± 1.8	6	4	6–8	6.5 ± 1.0	6
Susceptible	47	12–20	18.3 ± 1.8	20	3	6–19[d]	13.7 ± 5.8	

[a] As determined by disk diffusion, microtiter, and, where necessary, time-kill studies. See text for methods.
[b] 6 indicates no zone of inhibition.
[c] Forty-nine strains had 6-mm zone sizes, and one which was resistant to kanamycin by microtiter and time-kill studies had a 19-mm zone of inhibition.
[d] One strain which was susceptible my microtiter and synergistically killed by penicillin plus amikacin had a 6-mm zone of inhibition.

The ability of the microtiter and disk diffusion methods to detect aminoglycoside-aminocyclitol resistance was compared for all 104 strains. All 35 gentamicin-resistant strains were detected by both methods. Of the 22 streptomycin-resistant strains, 1 was detected only by microtiter, 2 only by disk diffusion, and 19 by both methods. The resistant strain detected by microtiter only was susceptible by both methods on initial testing, kanamycin resistant on repeat testing, and resistant to synergistic killing by penicillin plus amikacin and penicillin plus streptomycin by time-kill studies. Of the 54 kanamycin-resistant strains, 1 was detected only by microtiter, 2 only by disk diffusion, and 51 by both methods. One additional strain, an *E. faecium* isolate discussed above, which was resistant only by disk diffusion was susceptible to amikacin plus penicillin by time-kill studies.

Details of the disk diffusion screening test results for the 104 strains of enterococcus are presented in Table 2. Gentamicin- and kanamycin-resistant strains showed no zone of inhibition around the antibiotic-containing disks. Eight of the 19 streptomycin-resistant strains of *E. faecalis* gave no zone, and 9 had 8-mm zones. Aminoglycoside-aminocyclitol-susceptible strains usually had zones that were clearly larger than those for the resistant strains. There were two instances of overlapping zone sizes between susceptible and resistant strains (Table 2, kanamycin). One streptomycin-resistant strain of *E. faecalis* had a zone size of 10 mm, which is susceptible by the criteria of Sahm and Torres (12). On repeat testing, a 9-mm zone of inhibition, indicating resistance, was obtained. In one other instance, a strain with an 8-mm zone of inhibition around the streptomycin disk had a 10-mm zone on repeat testing. Ten other strains tested two times had no changes in interpretive categories.

DISCUSSION

Penicillin, ampicillin, gentamicin, streptomycin, and amikacin are rarely bactericidal against enterococci when used alone. The susceptibility of *E. faecalis* to high levels of aminoglycoside-aminocyclitol antibiotics can be used to predict synergistic killing when a cell wall-active agent is used in combination with that aminoglycoside-aminocyclitol. We began reporting the results of synergy screens using MicroScan panels after reports of increasing resistance appeared in the literature (15).

As part of a research effort to examine the prevalence of aminoglycoside-aminocyclitol-resistant enterococci in our institution, stocked blood isolates were tested using a macrotube method. When the prevalence of aminoglycoside-aminocyclitol-resistant strains was noted to be lower during the time when MicroScan results had been reported, further investigation was performed and a more suitable method was sought.

Discrepancies were scattered over the 7-month period during which nine lot numbers of panels were used. All lot numbers of panels were in control using the recommended quality control organism. The reason for the discrepancies is unclear. There was approximately a half-log lower inoculum in the MicroScan method than in the macro- and microtube methods. The data of Sahm and Torres (11) indicate that this should not significantly alter the results. Methods for inoculum preparation were comparable and consistent with the recommendations in the literature (4, 8). Standard incubation time and temperature were used (8). Of the four screening methods used, only MicroScan was unable to detect some gentamicin-resistant strains. False susceptibility to gentamicin could have been the result of an excess amount of antimicrobial agent in the well. This would be difficult to detect with a quality control organism such as *E. faecalis* ATCC 25213, which has a gentamicin MIC of 4 μg/ml and, in fact, is killed by 10 μg of gentamicin per ml (data not shown). Gentamicin assays were not performed on contents of the wells to confirm this hypothesis.

All of the methods had some degree of difficulty in detecting streptomycin resistance. Differences in inoculum size cannot explain all the discrepancies among the broth methods because the macro- and microtube methods used the same size of inoculum. The other factors affecting the accuracy of these tests are unknown.

On the basis of our data, the currently available frozen MicroScan panels should not be used to screen for high-level gentamicin and streptomycin resistance in *E. faecalis*.

Because of the increase in gentamicin resistance in *E. faecalis*, it is clear that laboratory testing of clinical isolates should be made available. Well-standardized and well-controlled methods are necessary. On-panel endpoints are needed for adequate quality control of MIC panels. The more active and broad-spectrum the antibiotic and the narrower the range of concentrations tested, the harder this becomes. Adequate control of single dilution tests would require the use of two strains, one resistant only to high-level

gentamicin and one resistant only to high-level streptomycin.

The disk diffusion test for high-level aminoglycoside-aminocyclitol resistance is an appealing alternative. As recommended by Sahm and Torres (12), we used disks containing 120 or 300 rather than 2,000 μg of antimicrobial agent for the disk diffusion test. However, using their recommended cutoff (≥10 mm indicates synergy), we had one strain which was falsely susceptible to high-level streptomycin. No other streptomycin-susceptible strain had a zone size of <16 mm. In our hands with our strains, a cutoff for synergy of ≥11 mm would have been the most accurate. Because we followed the published method in performance of this test, the difference in optimum zone size breakpoints may reflect differences in the susceptibilities of our strains to these agents.

The macrotube method, which would be appropriate for both *E. faecalis* and *E. faecium*, was able to detect all gentamicin-resistant strains but only 50% of the streptomycin-resistant strains. The microtiter method, which would also be appropriate for both species, detected all gentamicin-resistant and 89% of the streptomycin-resistant strains, but is not well suited to low-volume testing. The disk diffusion test was able to detect all of the gentamicin-resistant strains and 94% of the streptomycin-resistant ones, but has not been recommended for use with *E. faecium*, which made up 6.7% of our enterococcal strains. Laboratories that do not identify their enterococcal isolates to species should not use the disk diffusion method until its suitability for *E. faecium* has been documented.

In our hands microtiter was preferred because it was accurate and could be used for both *E. faecalis* and *E. faecium*. However, because it is not well suited to low-volume testing and because gentamicin resistance has not been reported in *E. faecium*, we are using disk diffusion with the following provisos: (i) a zone of ≥11 indicates synergy, (ii) results of the gentamicin screen are reported for both *E. faecalis* and *E. faecium*, (iii) results for the streptomycin screen are reported only for *E. faecalis*, and (iv) susceptibility to other aminoglycoside-aminocyclitol antimicrobial agents is determined by special arrangement using the microtiter method.

Sahm and Torres recommended testing gentamicin, streptomycin, and in some cases kanamycin, the latter to indicate high-level amikacin resistance (12). They suggest that the gentamicin results can be used to predict high-level resistance to tobramycin and netilmicin because 2″-phosphotransferase-6′-acetyltransferase mediates resistance to all three (1). We have strains that by the microtiter method are resistant to high-level gentamicin but not tobramycin, tobramycin but not gentamicin, and gentamicin but not netilmicin (data not shown). Time-kill studies, however, did not substantiate bactericidal activity of penicillin plus netilmicin. Netilmicin has been shown to be more active than gentamicin against enterococci by some authors (9). These strains may be gentamicin resistant by a different method than that described by Courvalin et al. Our data indicate that the agents should be tested individually but that other aminoglycoside-aminocyclitol agents should not be used for synergy screening unless results have been validated by time-kill studies.

The present data point out a little-discussed shortcoming of high-level aminoglycoside-aminocyclitol antibiotic screening. Penicillin was bactericidal as a single agent against 5 of 29 strains evaluated by time-kill studies. Therefore, although there was no growth in the presence of penicillin plus an aminoglycoside-aminocyclitol antibiotic, the definition of synergy was not met by these strains. While one would expect combination therapy with ampicillin plus an aminoglycoside-aminocyclitol antibiotic to be effective in these cases, addition of the latter agent may be unnecessary. Because of the toxicity associated with these agents, avoidance of unnecessary administration of them is desirable. Animal studies evaluating the effectiveness of penicillin alone against such strains are needed before tests for the bactericidal activity of penicillin or ampicillin are added.

Clinical laboratories need a method that can be used to detect high-level aminoglycoside-aminocyclitol resistance in both *E. faecalis* and *E. faecium*. Disk diffusion is easy to perform and is well suited to testing individual isolates. The suitability of this method for *E. faecium* needs to be documented, particularly for agents other than gentamicin, since resistance to some has been documented. In addition, it must be verified that there are breakpoints which will function appropriately in all institutions. A multicenter study performed under the guidance of the National Committee for Clinical Laboratory Standards may be appropriate.

ACKNOWLEDGMENT

I am indebted to Debby Taniguchi for excellent technical assistance.

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