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Enterococci from Bangkok, Thailand, with High-Level Resistance to Currently Available Aminoglycosides

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Enterococcal endocarditis is usually treated with a combination of a penicillin and an aminoglycoside. Recent reports have documented the emergence of enterococci in France with high-level resistance to gentamicin, tobramycin, and kanamycin and the emergence of strains in Houston, Tex. with high-level resistance to all of these drugs and streptomycin. In this study, we examined strains from a geographic area where newer aminoglycosides have been less commonly used. Of 125 distinct patient isolates, 18 (14%) were resistant to >2,000 μg of gentamicin and most other aminoglycosides per ml. Four of these strains transferred gentamicin resistance to a laboratory recipient. One strain, chosen for further study, was resistant to synergism between penicillin and gentamicin, tobramycin, kanamycin, streptomycin, and amikacin and demonstrated the following enzymatic activities: 3′- and 2″-aminoglycoside phosphotransferases, 6′-aminoglycoside acetyltransferase, and adenylylation of streptomycin. Optimal therapy for endocarditis caused by such highly resistant strains is currently unknown.

Optimal therapy of enterococcal endocarditis includes the use of a cell wall synthesis inhibitor, such as penicillin or vancomycin, in combination with an aminoglycoside to which the organism is not highly resistant. The need for combination therapy, in contrast to the therapy of endocarditis caused by other streptococci, is due to the fact that enterococci are resistant, or relatively resistant, to most antimicrobial agents; for example, 10 to 100 or more times as much penicillin is required to inhibit enterococci than other streptococci, and even more penicillin is required for killing (12, 15, 21).

Traditionally, the combination of penicillin plus streptomycin has been recommended for enterococcal endocarditis (9, 13). The observation that many enterococci are highly resistant (minimal inhibitory concentration [MIC], >2,000 μg/ml) to streptomycin and fail to show enhanced killing with the combination of penicillin plus streptomycin relative to penicillin alone has led to the recommendation that penicillin plus gentamicin (GM) be used for such strains (14, 17). Surveys from this country in the 1970s failed to reveal any enterococci highly resistant to GM (3, 14, 15, 18), although one reported strain failed to show enhanced killing even without high-level resistance (MIC 8 μg of GM per ml) (16).

We have recently reported that 5% of enterococci isolated in a large teaching hospital in Houston, Tex. were highly resistant to GM as well as to kanamycin (KM), amikacin, tobramycin (TM), and streptomycin (SM) (B. D. Mederski and B. E. Murray, J. Infect. Dis., in press); an earlier report from France also documented enterococcal strains highly resistant to GM, KM, and TM, but not to SM (8). This study was designed to investigate the existence of high-level resistance to newer aminoglycosides in another geographic area; 14% of enterococci were highly resistant even though some of these aminoglycosides had been infrequently used, thus suggesting that such strains are indeed quite widespread.

(This work was presented in part at the First Annual Streptococcal Genetics Conference, Sarasota, Fla., 1980.)

MATERIALS AND METHODS

Bacterial strains. Enterococci were isolated from clinical specimens submitted to the Bacteriology Laboratory of Ramathibodi Hospital, Bangkok, Thailand, over a 4-month period in early 1980. Only a single isolate per patient was used. All isolates were identified as enterococci by standard bacteriological methods, and isolates with high-level GM resistance (MIC, >2,000 μg/ml) were serologically grouped and identified to species level by further biochemical testing (6, 7).

Susceptibility testing and synergy. Screening for high-level resistance was done by streaking onto brain heart infusion (BHI) agar (Difco Laboratories, Detroit, Mich.) containing 2,000 μg of antibiotic per ml. Susceptibility testing was performed with BHI agar and a

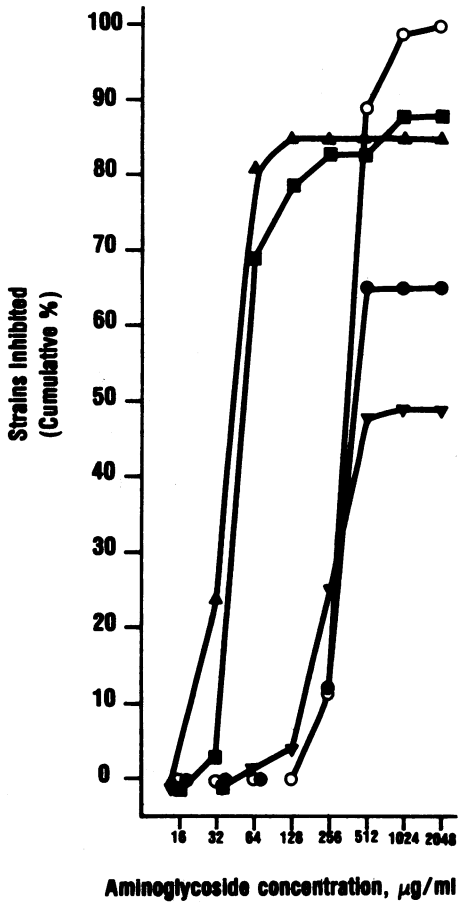

FIG. 1. Susceptibility of 125 clinical isolates of enterococci to amikacin (○), TM (■), GM (▲), KM (●), and SM (▼) as determined by agar dilution.

Steers replicator to inoculate an undiluted overnight culture (20). Serial twofold dilutions of aminoglycosides were used at concentrations of 4 to 2,048 μg/ml. Synergism studies were performed by the protocol of Moellering et al. (18) with glucose phosphate broth. Synergism was defined as a 2-$\log_{10}$ (100-fold) increase in killing at 24 h with the combination of penicillin and an aminoglycoside as compared with the killing achieved with penicillin alone; the concentration of GM used did not inhibit growth when used alone. Concentrations of penicillin used were 10 U/ml for clinical isolates and 4 U/ml for strain JH2-7.

Transfer studies. For conjugation studies, a single colony of GM-resistant donor strains and of enterococcal strain JH2-7 (*thy* Rif, fusidic acid resistant) (10) were inoculated into BHI broth and grown overnight at 37°C. An 0.05-ml sample of donor strains and 0.5 ml of JH2-7 were added to 4.5 ml of BHI broth and then collected on a membrane filter (HA, 0.45-μm; Millipore Corp., Bedford, Mass.). The filter was placed on BHI agar and incubated overnight at 37°C. The bacteria on the filter were then dispersed in 2 ml of 0.85% NaCl, and 0.2 ml was plated onto BHI agar plates containing 100 μg of rifampin and 1,000 μg of GM per ml.

RESULTS AND DISCUSSION

A total of 125 strains of enterococci were studied. Susceptibility of these enterocci to aminoglycosides is shown in Fig. 1, and the correlation of highly resistant strains and their clinical sources are shown in Table 1. Of the 125 strains tested, 63 (50%) were highly resistant (MIC >2,000 μg/ml) to SM, 44 (35%) to KM, 18 (14%) to GM, and 15 (12%) to TM. All of the KM-resistant strains were also highly resistant to SM. Of even greater importance, all 15 of the TM-resistant strains were also highly resistant to GM, KM, and SM; 3 strains resistant to >2,048 μg of GM, SM, and KM per ml and an MIC of TM of 1,000 μg/ml. The MICs of amikacin for the 18 GM-resistant strains ranged from 512 to 2,048 μg/ml and were >2,048 μg/ml for sisomicin and netilmicin. The 18 strains with high-level resistance to GM, KM, and SM all reacted with group D antisera and were found to be *Streptococcus faecalis*; 8 of these strains produced beta-hemolysis of horse and human (but not sheep) blood (*S. faecalis* var. *zymogenes*), whereas 10 did not produce beta-hemolysis with any of the blood used.

Four of the 18 strains with high-level GM resistance transferred this resistance to strain JH2-7; resistance to high levels of TM, KM, and SM were all cotransferred. Transfer frequencies ranged from 10^{-1} to 10^{-4} transconjugants per recipient. MICs of GM, TM, KM, and SM were >2,048 μg/ml for the transconjugants. Beta-hemolysis was not transferred, although three of the four successful donors produced beta-hemolysis.

One of the clinical isolates with transferable high-level GM resistance, strain BE133, isolated from urine, was shown to be resistant to the combination of penicillin and GM, KM, and SM, even when 1,000 μg of aminoglycoside per ml was used (Fig. 2); this strain was also resistant to synergism when amikacin, TM, and netilmicin were used. The laboratory strain JH2-7 was synergistically killed by the combination of penicillin plus GM (5 μg/ml) (Mederski and Murray, in press), but the GM-resistant transconjugants were resistant to penicillin-GM synergism.

Strain BE133 was also examined for the production of aminoglycoside-modifying enzymes as previously described (19; Mederski and Murray, in press) and was found to have four enzymatic activities. The presence of a 3′-aminoglycoside phosphotransferase [APH(3′)] was inferred because of a marked reduction in phosphorylation of compounds without a 3′-hydroxyl, in comparison with their analogs containing a 3′-hydroxyl (e.g., 71,000 cpm with kanamycin B versus 1,650 cpm with TM). Because lividomycin A, butirosin, and ribostamycin are excellent substrates and amikacin is also a substrate, the

TABLE 1. Prevalence of high-level resistance to four aminoglycosides among enterococci

Origin of isolate	No. of strains tested	No. (%) of strains with high-level resistance to[a]:			
		SM	KM	GM	TM
Urine	49	28 (57)	18 (37)	11 (22)	9 (18)
Wound (pus, trachea, ascites, placenta)	22	12 (55)	8 (36)	3 (14)	2 (9)
Blood, cerebrospinal fluid	7	3 (43)	2 (29)	2 (29)	2 (29)
Miscellaneous (rectal, cervix, unknown)	47	20 (43)	16 (34)	2 (4)	2 (4)

[a] High-level resistance was defined as an MIC of >2,000 μg/ml. The total percentages of strains highly resistant to SM, KM, GM, and TM were 50, 35, 14, and 12%, respectively.

enzyme is likely an APH(3')-III (1, 5; Mederski and Murray, in press). This inference is somewhat complicated by the fact that an additional phosphotransferase was present; this enzyme appeared to be acting at the 2" position because there was persistent phosphorylation of compounds lacking the 3'OH but absence of phosphorylation in derivatives also lacking the 2"OH (2,961 cpm with sisomicin versus 140 cpm with 2" deoxysisomicin; background, 130 cpm) (1, 5).

Additional activity included acetylation of compounds with a 6'NH_2 group (but not their 6'NH_2-lacking derivatives) and adenylylation of SM. A 3'-phosphotransferase and an SM adenylyltransferase were previously found in the enterococci with high-level KM and SM resistance reported by Krogstad et al. (11); the APH(3') conferred resistance to penicillin-amikacin synergy even without high-level amikacin resistance. 6'-Aminoglycoside acetyltransferase [AAC(6')] and APH(2") activities were reported by Courvalin et al. (4) in one of the strains from France (8) with high-level GM, KM, and TM resistance. Our study in Houston revealed strains with all four of these modifying activities, similar to strain BE133 described here (Mederski and Murray, in press).

The finding of high-level resistance to newer aminoglycosides in this population is of particular concern since some of these agents had been infrequently used in Thailand at the time of this study. Since the enzymes active against GM, TM, netilmicin, and sisomycin in these strains are also active against KM, the relatively frequent use of this agent may have provided a

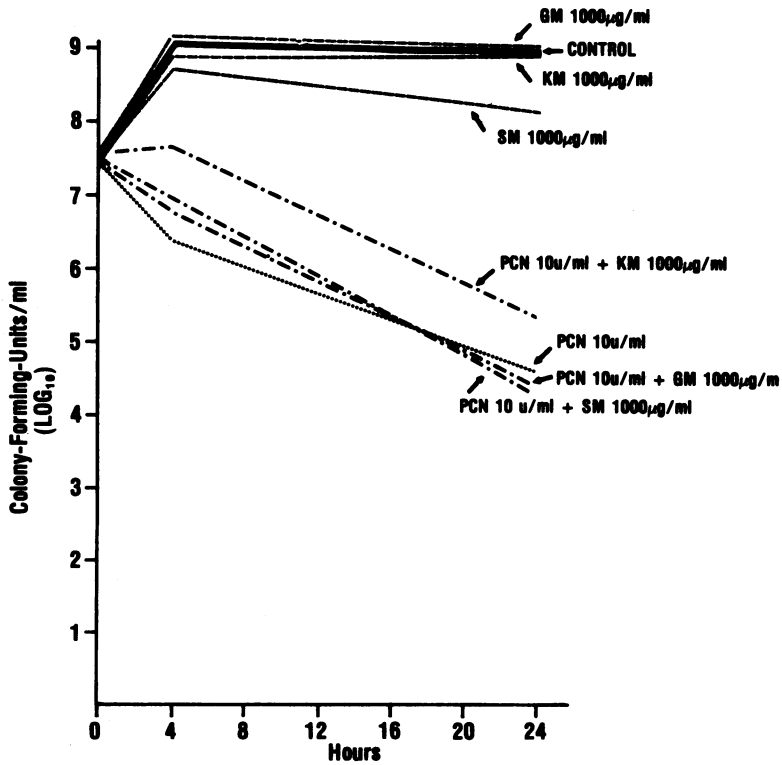

FIG. 2. Effect of GM, KM, TM, and SM alone and in combination with penicillin (PCN) against the clinical isolate BE133.

positive selective pressure for APH(2″) and AAC(6′) as well as for APH(3′). The major difference between these strains and our Houston isolates is that only 4 of 18 of these strains transferred GM resistance, whereas it was transferable from all 9 of the highly resistant Houston isolates that were tested (Mederski and Murray, in press).

Although no clinical information is available on any of these patients, two of the highly resistant strains were from blood or cerebrospinal fluid, sites likely to benefit from combination therapy. Since, however, synergism with commercially available aminoglycosides could not be demonstrated, and in fact, antagonism has been previously reported with highly resistant strains, the addition of an aminoglycoside would not appear to be justified (2). Optimal therapy of severe enterococcal infections caused by strains highly resistant to all available aminoglycosides is currently unknown. Although some patients are likely to be cured by penicillin alone, a high relapse rate and the accompanying morbidity would be expected. To identify such high-risk patients, screening for high-level resistance, particularly with isolates from cases of endocarditis, should be performed.

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ADDENDUM IN PROOF

We have recently examined 65 strains of enterococci obtained in Chile from the Instituto de Salud Publica. Twenty strains were highly resistant to SM and 14 to KM; 10 were highly resistant to each of GM, TM and amikacin. Of these strains, six were resistant to only SM, and none were resistant to only KM. Four were resistant to SM plus KM but not other aminoglycosides, and 10 were resistant to SM, KM, GM, and TM as well as amikacin, thus extending the documentation of such strains to another geographical area. In addition, a strain has recently been described in France with transferable high-level resistance to all of the above aminoglycosides and has been shown to produce modifying enzymes similar to the activities reported in this paper (3a).

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