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Chromosomally Mediated High-Level Gentamicin Resistance in *Streptococcus mitis*

ACHIM KAUFHOLD[1]* AND ELSA POTGIETER[2]

Institute of Medical Microbiology, Technical University (RWTH) Aachen, Pauwelsstrasse 30, D-52057 Aachen, Germany,[1] and The South African Institute for Medical Research, University of the Witwatersrand, Parktown, Johannesburg 2193, South Africa[2]

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Four blood culture isolates of *Streptococcus mitis* were found to be resistant to penicillin (MIC, 16 to 32 μg/ml) and gentamicin (MIC, 128 or 1,000 μg/ml), and the two antibiotics demonstrated a lack of in vitro synergy. As shown by polymerase chain reaction assays, the structural gene known to encode high-level gentamicin resistance in *Enterococcus faecalis*, *Enterococcus faecium*, and *Streptococcus agalactiae* was also present in all four *S. mitis* strains. Attempts to isolate plasmids were unsuccessful, but an oligonucleotide probe derived from the gentamicin resistance gene hybridized to distinct restriction fragments of genomic DNA, suggesting that the resistance genes in these strains are integrated into the bacterial chromosome.

Although penicillin resistance was found in nasopharyngeal isolates of viridans group streptococci as early as 1978 (5, 6, 18), in most parts of the world the vast majority of viridans group streptococci remain highly susceptible to penicillin (MICs, ≤0.12 μg/ml).

The emergence of numerous incidences of penicillin resistance in viridans group streptococci isolated from blood cultures in South Africa has been described recently (18). It was of particular concern that four *Streptococcus mitis* strains resistant to penicillin simultaneously exhibited increased (MICs, 64 to 128 μg/ml) or high-level (MIC, 1,000 μg/ml) resistance to gentamicin. The latter resistance trait was observed for the first time among viridans group streptococci, and these MICs exceed those commonly encountered for *S. mitis*. For example, in a recent study the MIC of gentamicin for 90% of the *S. mitis* blood culture isolates tested was 4 μg/ml (21).

The present study was undertaken to examine the phenotypic and genetic characteristics of the four South African *S. mitis* strains with respect to their gentamicin resistance.

The *S. mitis* strains 56, 160, 256, and 265 investigated in the present study were clinical blood culture isolates recovered at Johannesburg hospital laboratories between 1988 and 1991, and their antibiotic susceptibilities were previously described in detail (18). All strains were resistant to penicillin; penicillin MICs for the strains were 16 to 32 μg/ml. The MIC of gentamicin was 1,000 μg/ml for strains 56 and 160, while strains 256 and 265 were inhibited by gentamicin at a concentration of 128 μg/ml. Strain 3735 was a high-level gentamicin-resistant *Enterococcus faecium* isolate that was used as a control organism; it was recently shown to harbor the structural gene encoding the bifunctional aminoglycoside-modifying 6′-acetyltransferase-2″-phosphotransferase (6′AAC-2″APH) enzyme (13). Eight different *S. mitis* strains (MICs of gentamicin, 1 to 4 μg/ml) isolated from patients' blood cultures at Aachen, Germany, and Johannesburg, South Africa, hospitals served as negative controls in polymerase chain reaction (PCR) and hybridization assays.

The susceptibilities of all *S. mitis* strains to gentamicin were retested by the E test (AB Biodisk, Solna, Sweden) according to the instructions of the manufacturer's package insert.

As described previously (18), tests of antibiotic synergy were performed in cation-supplemented Mueller-Hinton broth by determining time-kill curves (16) for organisms exposed to penicillin and gentamicin alone and in combination. In all cases, the concentration of the aminoglycoside was less than the MICs for the organisms, and thus resulted in no inhibition of growth by itself. Synergy was defined as a decrease of 10^2 CFU/ml produced by the combination of antibiotics after incubation for 6 h compared with decrease in CFU per milliliter obtained with the most effective agent alone (18).

Bacterial whole-cell DNA was extracted by the protocol of Huang et al. (11), while the preparation of plasmid DNA (starting from 50-ml bacterial cultures) was attempted by using a modification of the method originally described by Birnboim and Doly (1). This method was previously shown to reliably extract plasmid DNA from enterococci (12), and the same procedure is routinely used in the laboratory of A.K. to extract recombinant plasmids from *Streptococcus sanguis*. Genomic DNA (10 μg) was digested with restriction enzymes according to the specifications of the manufacturer (Boehringer, Mannheim, Germany). PCR assays, including the use of amplification primers that flank a 1,472-bp fragment comprising the entire gentamicin resistance gene in *Enterococcus faecalis* (8), were carried out exactly as described previously (13). For DNA-DNA hybridization experiments, an oligonucleotide (internal to the PCR primers) which corresponds to the gene portion encoding the 6′-acetylating activity of 6′AAC-2″APH served as the probe (designated the AAC probe; see reference 13). Agarose gel electrophoresis, synthesis of oligonucleotides, nonradioactive labeling of the probe with digoxigenin-dUTP, and hybridization assays were performed as described previously (13, 14).

When genomic template DNAs of the four gentamicin-resistant *S. mitis* strains and the positive control strain *E. faecium* 3735 were subjected to PCR assays, a single 1.47-kb DNA fragment was amplified from all investigated strains. The specificities of the PCR products were further proven by subsequent Southern blot hybridization experiments, in which the nonradioactively labeled AAC probe hybridized

* Corresponding author.

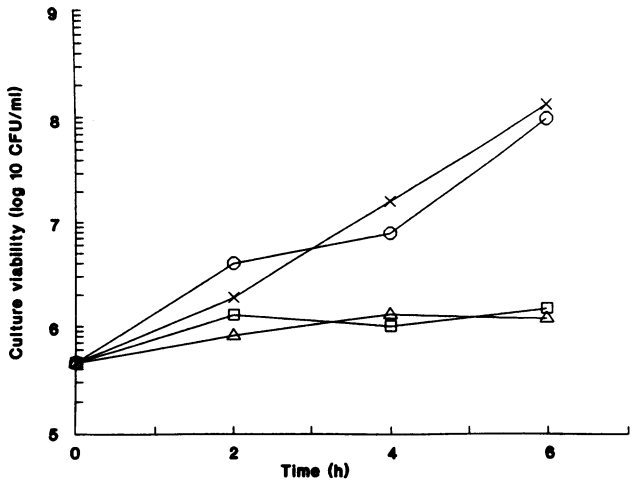

FIG. 1. Time-kill study of strain *S. mitis* 256 with 16 μg of penicillin per ml (□), 16 μg of gentamicin per ml (○), penicillin plus gentamicin (16 μg/ml each) (△), or control (×).

strongly with the amplified DNA fragments. In contrast, no PCR amplicon was detected in any of the control *S. mitis* strains for which the gentamicin MIC was 1 to 4 μg/ml.

Whole-cell DNAs of all *S. mitis* strains were digested with restriction enzyme *Hin*dIII, *Hae*III, *Eco*RI, or *Xba*I. Regardless of the restriction enzyme used, both strains for which gentamicin MICs were 128 μg/ml (strains 256 and 265) showed identical restriction enzyme digestion profiles, indicating the genetic relatedness between these two isolates, whereas *S. mitis* strains 56 and 160 differed in their molecular fingerprints. Subsequently, Southern blots of digested whole-cell DNA were hybridized with the AAC probe. The probe hybridized to distinct digestion fragments of genomic DNA from the four gentamicin-resistant strains, whereas no hybridization signal was detected in any of the gentamicin-susceptible *S. mitis* strains that served as controls. Several attempts to detect plasmids in the four gentamicin-resistant *S. mitis* isolates were unsuccessful.

The result of synergy studies performed on *S. mitis* 256 is shown in Fig. 1. The combination of penicillin plus gentamicin was not synergistic, with no increase in killing by the antibiotic combination compared with the killing produced by penicillin (at a concentration of 16 μg/ml) alone. Additional time-kill studies with the other resistant *S. mitis* strains confirmed this lack of antimicrobial synergy against any of the organisms tested. When time-kill studies were done with two *S. mitis* strains for which the gentamicin MIC was 1 or 4 μg/ml, an additive effect (i.e., a 10-fold increase in killing) of the antibiotic combination was observed.

The gentamicin MICs for all *S. mitis* strains investigated were accurately predicted by the E test. Thus, the E test clearly distinguished the *S. mitis* strains displaying increased and high-level gentamicin resistance from the eight gentamicin-susceptible *S. mitis* strains.

There are three accepted antibiotic regimens for the treatment of endocarditis caused by penicillin-susceptible viridans group streptococci (2). These include penicillin alone for 4 weeks, penicillin for 4 weeks plus streptomycin or gentamicin for 2 weeks, or a short-course (2-week) combination therapy with penicillin and an aminoglycoside.

The first report of high-level resistance to streptomycin (MIC, >2,000 μg/ml) among several species of streptococci, including viridans group streptococci, was published in 1982 (10). Subsequently, additional strains of viridans group streptococci (mostly *S. mitis*), primarily isolated in South Africa, were encountered (5, 6); these strains were highly resistant to both penicillin and streptomycin. In time-kill studies with selected organisms, a lack of synergy between this antibiotic combination was demonstrated, whereas marked synergistic killing by penicillin plus gentamicin was achieved (5, 7). The occurrence of high-level streptomycin resistance led to the use of gentamicin as the drug of choice in combination therapy for viridans group streptococcal endocarditis. Therefore, the emergence of high-level gentamicin and penicillin resistance in viridans group streptococci has potentially important clinical implications.

Resistance to gentamicin in enterococci and staphylococci is due to a bifunctional enzyme with 6′-acetyltransferase and 2″-phosphotransferase activities (4, 8, 20). The gene encoding high-level gentamicin resistance has been shown to reside on a transposon in *E. faecalis* similar to the staphylococcal transposons Tn*4001* and Tn*4031* (9). In *E. faecalis*, the gene may be integrated into the bacterial chromosome (19), although in the majority of strains, it is found on conjugative and nonconjugative plasmids (17). The gene was shown to be located on the chromosome in the single *Streptococcus agalactiae* strain with high-level gentamicin resistance identified thus far (3).

Recently, we have demonstrated by PCR and hybridization experiments that the high-level gentamicin resistance genes of *E. faecalis*, *E. faecium*, and *S. agalactiae* are highly homologous (13). Applying the same experimental methodology, the results of the present study proved for the first time that the homologous gene appears in viridans group streptococci as well. Despite several attempts, we were not able to demonstrate plasmids in the four *S. mitis* blood culture isolates investigated by the methods described above, suggesting that increased or high-level gentamicin resistance was chromosomally mediated. Although there is currently no definite proof, it seems likely that the gene resides on a transposon in *S. mitis* as well, so that it can be anticipated that this resistance trait has the potential of further spread among viridans group streptococci. Whereas in enterococci the presence of the gentamicin resistance gene virtually always results in the expression of resistance to gentamicin at concentrations of >2,000 μg/ml, the gene expression in *S. mitis* was apparently diminished; i.e., the gentamicin MICs were only 1,000 or 128 μg/ml. Nevertheless, it was of note that the increased or high-level gentamicin resistance predicted resistance to the bactericidal synergistic combination in all instances. Analogous to the situation in enterococci (15, 17), this finding may preclude the use of combination therapy in the clinical setting.

In view of the data presented here, detailed antibiotic susceptibility testing, including the testing for high-level aminoglycoside resistance, of all clinically significant blood culture isolates of viridans group streptococci seems to be warranted. Although further studies are needed to fully explore the potential of the E test for detecting increased or high-level gentamicin resistance in viridans group streptococci, our preliminary data indicate that this method may offer the laboratory a convenient and reliable screening test.

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