

Resistance to Penicillin-Streptomycin Synergy Among Clinical Isolates of Viridans Streptococci

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Viridans streptococci are thought to be highly susceptible to penicillin and streptomycin. We recently encountered a unique group of 15 isolates from South Africa epidemiologically related to the isolation of penicillin-resistant pneumococci. These organisms were highly resistant to penicillin (PCN) (minimal inhibitory concentration, 1 to 32 µg/ml) and streptomycin (SM) (minimal inhibitory concentration, ≥2,000 µg/ml). Two additional organisms with high-level streptomycin resistance were identified when 168 clinical isolates from Boston were screened. Time-kill studies with four organisms resistant to high levels of SM demonstrated lack of synergy between PCN and SM but marked synergy between PCN and gentamicin. Adenylylating, acetylating, and phosphorylating activity could not be detected in three organisms studied, and novobiocin failed to cure the SM resistance. Protein synthesis by ribosomes isolated from these organisms was dramatically reduced in the presence of gentamicin but was relatively resistant to inhibition by SM.

Viridans streptococci continue to be the most frequent cause of native valve endocarditis and are a common cause of prosthetic valve endocarditis (3, 8). These organisms, like other non-enterococcal streptococci, have long been considered universally susceptible to penicillin; therefore, this agent has been the drug of choice for viridans streptococcal endocarditis for 40 years. Often streptomycin is used in combination with penicillin to produce more rapid (synergistic) killing of viridans streptococci (19). As a result, some authors have advocated shorter courses of therapy when penicillin-streptomycin combinations are utilized in the treatment of viridans streptococcal endocarditis (2).

During a survey of the prevalence of penicillin-resistant *Streptococcus pneumoniae* among the pharyngeal flora of South African children, a number of strains of viridans streptococci which were streptomycin resistant were also found. The simultaneous occurrence of high-level resistance to streptomycin among these organisms has potentially important clinical implications. In the current report we describe the characteristics of these organisms. We also document the fact that high-level streptomycin resistance among viridans streptococci predicts

resistance to penicillin-streptomycin synergy. Finally, we present data demonstrating that the ribosome may be the site of streptomycin resistance in clinical streptococcal isolates.

MATERIALS AND METHODS

Strains and antibiotics. Six isolates from a collection of South African strains of viridans streptococci were used in these studies. The organisms were collected from July 1977 through April 1978 in Durban and Johannesburg, South Africa. Each strain came from a different patient and was obtained by culture of the nasopharynx of hospitalized children. Although none was definitely recovered from children who also carried resistant pneumococci, the latter were very prevalent in this population. All strains were alpha-hemolytic on sheep blood agar and were identified by standard methods by R. Facklam (Centers for Disease Control, Atlanta, Ga.). None of the organisms was known to be the cause of invasive disease.

An additional 168 clinical isolates from the Massachusetts General Hospital Bacteriology Laboratory were screened for penicillin and streptomycin resistance. These organisms were also alpha-hemolytic streptococci and were identified by standard methods (15).

Streptococcus faecium 6400 was used as a control organism; it has known aminoglycoside adenylyltransferase activity (10). *Streptococcus faecalis* 8436 has known aminoglycoside phosphotransferase activity against amikacin (9). A plasmid-containing derivative of *Pseudomonas aeruginosa* PAO38 with known aminoglycoside acetyltransferase activity against gentamicin (7) was kindly provided by G. Jacoby.

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Antibiotics included penicillin G (Parke, Davis & Co., Morris Plains, N.J.), streptomycin (Eli Lilly & Co., Indianapolis, Ind.), amikacin (Bristol Laboratories, Syracuse, N.Y.), and gentamicin (Schering Corp., Bloomfield, N.J.).

Susceptibility tests. Susceptibility tests were performed by the agar dilution method using Mueller-Hinton agar (Difco Laboratories, Detroit, Mich.) supplemented with 5% horse or sheep blood (18). Overnight cultures of test strains in Todd-Hewitt broth were diluted in fresh broth and applied to plates by means of a Steers replicator, yielding a final concentration of approximately 10^4 CFU.

Synergism studies. Antibiotic combinations were tested in Todd-Hewitt broth supplemented with 5% sheep blood incubated in 5% CO_2 at 37°C, as described previously (11). Synergism was defined as a decrease of 10^2 CFU/ml produced by the combination after incubation for 24 h compared with the effect of penicillin alone. In addition, we required that a decrease of at least 10^2 CFU/ml be produced in the presence of the combination as compared with the initial colony count. In all cases, the concentration of the aminoglycoside was less than the minimal inhibitory concentration (MIC) and thus resulted in no inhibition of growth by itself.

Screening for antimicrobial resistance. One hundred sixty-eight clinical isolates of viridans streptococci were grown at 37°C in Todd-Hewitt broth overnight, and a Steers replicator was used to inoculate approximately 10^4 organisms onto Mueller-Hinton agar supplemented with 5% sheep blood containing 1 μg of penicillin per ml, 500 μg of streptomycin per ml, or 2,000 μg of streptomycin per ml. Plates were examined for growth after 24 h of incubation at 37°C in CO_2 .

Assays of aminoglycoside-modifying enzyme activity. One milliliter of an overnight culture of the test organisms was added to 100 ml of Todd-Hewitt broth and incubated for 4 to 6 h in 5% CO_2 at 37°C. The cells were harvested at $4,500 \times g$ for 15 min at 4°C and washed twice in TMN buffer (17). The cells were then frozen at -70°C until they were used. After thawing at room temperature, cells were sonically disrupted (Cell Disruptor-350; Branson Instruments Co., Danbury, Conn.), using three 30-s bursts. Unbroken cells and large cell wall fragments were removed by centrifugation at $4,500 \times g$ for 15 min at 4°C, and the supernatant was used as a crude enzyme preparation.

Assays were performed in borosilicate tubes by the method of Benveniste and Davies (1) with several modifications. Antibiotics were prepared at a concentration of 0.4 mg/ml. A reaction mixture consisted of 10 μl of antibiotic, 10 μl of radioactive substance, 10 μl of buffer, and 20 μl of sonicated enzyme preparation. Adenylylating assays were incubated at 30°C for 30 and 45 min, phosphorylating assays were performed at 35°C for 25 min, and acetylylating assays were incubated at 30°C for 20 min at pH 5.8 and 7.8.

Radiolabeled chemicals were obtained from New England Nuclear Corp., Boston, Mass. These included [^{32}P]ATP (1,200 mCi/mmol), [^{14}C]ATP (588 mCi/mmol), and [^{14}C]acetyl coenzyme A (56 mCi/mmol). For use in enzyme assays, these were diluted with distilled water and unlabeled ATP or acetyl coenzyme A to specific activities of 1.7 $\mu\text{Ci}/\mu\text{mol}$ for [^{14}C]acetyl coenzyme A, 19.3 $\mu\text{Ci}/\mu\text{mol}$ for [^{14}C]ATP, and 28 $\mu\text{Ci}/\mu\text{mol}$ for [^{32}P]ATP. ATP and acetyl coen-

zyme A were obtained from Sigma Chemical Co., St. Louis, Mo.

Curing experiments. Cells were grown in Todd-Hewitt broth at 37°C in 5% CO_2 overnight. The following morning approximately 10^6 CFU/ml were inoculated into broth containing 1 to 2 μg of novobiocin (Sigma) per ml. This was the highest concentration of novobiocin which permitted growth. The tubes were incubated overnight at 37°C, and serial dilutions were made onto sheep blood agar. The following day individual colonies were replicated onto Todd-Hewitt agar plates and Todd-Hewitt agar plates containing streptomycin (2,000 $\mu\text{g}/\text{ml}$). A total of 373 colonies were picked for replication: 234 of strain 531, 64 of strain 859, and 75 of strain 167.

Studies of ribosomal protein synthesis. Cells were grown in broth, harvested at logarithmic growth phase, and washed twice in 5 ml of 0.01 M Tris (pH 7.8) with 0.017 M magnesium acetate and 0.06 M potassium chloride at 3°C. They were stored at -70°C. After thawing, cells were disrupted by grinding for 15 min with twice their wet weight of alumina. The method of Nirenberg was used with several modifications (12). An S30 crude cell extract was prepared in 0.017 M magnesium acetate buffer. Protein synthesis was assessed by using [^{14}C]phenylalanine (New England Nuclear Corp.; 54 mCi/mmol) incorporation into trichloroacetic acid-precipitable material, with polyuridylic acid (Sigma) as the messenger. Each reaction mixture consisted of 0.25 ml and contained 4×10^{-9} mol of [^{14}C]phenylalanine and 5×10^{-9} mol of unlabeled phenylalanine. A mixture consisting of other essential amino acids at a concentration of 5×10^{-9} mol per tube was added to prime the reaction. Aminoglycoside inhibition of protein synthesis was measured by the reduction in counts per minute in trichloroacetic acid-precipitable material after the addition of streptomycin or gentamicin. Background counts were determined by using a control reaction which was handled in a similar manner without the addition of ribosomes. The entire trichloroacetic acid precipitate was filtered through a filter (0.45 μm ; Millipore Corp., Boston, Mass.) and washed with 25 ml of 5% trichloroacetic acid before drying. The filters were then placed in omnifluor-toluene and counted in a Beckman scintillation counter. All studies were run in duplicate, and background counts were subtracted.

RESULTS

Susceptibility and screening studies. The antimicrobial susceptibilities of six South African strains of *Streptococcus viridans* are given in Table 1. All organisms were resistant to penicillin (MIC, 2 to 16 $\mu\text{g}/\text{ml}$) and streptomycin (MIC, $\geq 2,000$ $\mu\text{g}/\text{ml}$), but none had an MIC of gentamicin of greater than 64 $\mu\text{g}/\text{ml}$. Screening of 168 clinical isolates from Boston identified one additional strain (MGH-167) with high-level streptomycin (MIC, $\geq 4,000$ $\mu\text{g}/\text{ml}$) and moderate penicillin (MIC, 1 $\mu\text{g}/\text{ml}$) resistance. In addition, another penicillin-susceptible (MIC, ≤ 0.5 $\mu\text{g}/\text{ml}$) but highly streptomycin-resistant (MIC, $> 4,000$ $\mu\text{g}/\text{ml}$) strain was identified (MGH-148), and one penicillin-resistant (MIC, 1 $\mu\text{g}/\text{ml}$)

TABLE 1. Antimicrobial susceptibility of South African strains of viridans streptococci

Strain	Species	MIC ($\mu\text{g/ml}$)		
		Penicillin	Streptomycin	Gentamicin
531	<i>S. mitis</i>	4	8,000	4
859	<i>S. mitis</i>	4	8,000	16
L1	<i>S. sanguis</i> II	16	8,000	2
L2	<i>S. sanguis</i> II	2	2,000	64
L3	<i>S. mitis</i>	16	8,000	2
20220	<i>S. mitis</i>	8	8,000	2

streptomycin-“sensitive” (MIC, 250 $\mu\text{g/ml}$) organism was isolated. Therefore, the incidence of high-level streptomycin resistance among the Boston strains was 1.2%.

Synergy studies. The results of synergy studies performed on strain 531 are shown in Fig. 1 and 2. The combination of penicillin plus streptomycin was not synergistic, but the combination of penicillin plus gentamicin demonstrated bactericidal synergism. Additional time-kill studies with strains 859, L1, and MGH-167 all confirmed that the combination of penicillin plus streptomycin was not synergistic against organisms with high-level resistance to streptomycin (MIC, $\geq 2,000 \mu\text{g/ml}$). Studies with strains L1, 859, L3, and MGH-167 also confirmed that the

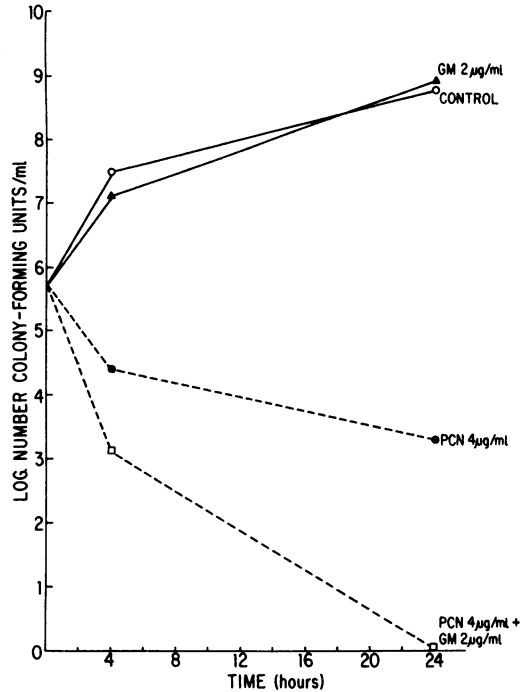


FIG. 2. Effect of penicillin (PCN) and gentamicin (GM) alone and in combination against *S. mitis* 531.

combination of penicillin plus gentamicin was synergistic.

Aminoglycoside-modifying enzyme activity. Strains 531, 859, and MGH-167 were studied and found not to possess adenylylating, phosphorylating, or acetylating activity against streptomycin.

Curing experiments. No strain tested was cured of its streptomycin resistance by novobiocin. This implied that the resistance marker was not carried on a plasmid.

Ribosomal protein synthesis. The effect of streptomycin and gentamicin on inhibition of protein (polyphenylalanine) synthesis is demonstrated in Fig. 3 for strain 531. The ribosome preparation was much more resistant to inhibition by streptomycin than gentamicin over a wide range of concentrations. Similar experiments run in duplicate also demonstrated ribosomal resistance to streptomycin in strains 859 and 167. In both of these strains, there was less of a drop in counts per minute between 0 and 0.1 μg of streptomycin and gentamicin per ml.

DISCUSSION

Colonization of the oropharynx by penicillin-resistant streptococci is a well-recognized occurrence in patients receiving chronic penicillin therapy as prophylaxis against rheumatic fever

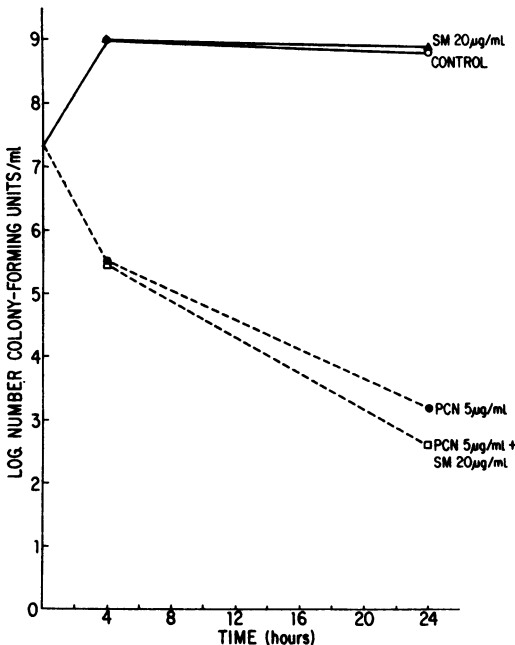


FIG. 1. Effect of penicillin (PCN) and streptomycin (SM) alone and in combination against *Streptococcus mitis* 531.

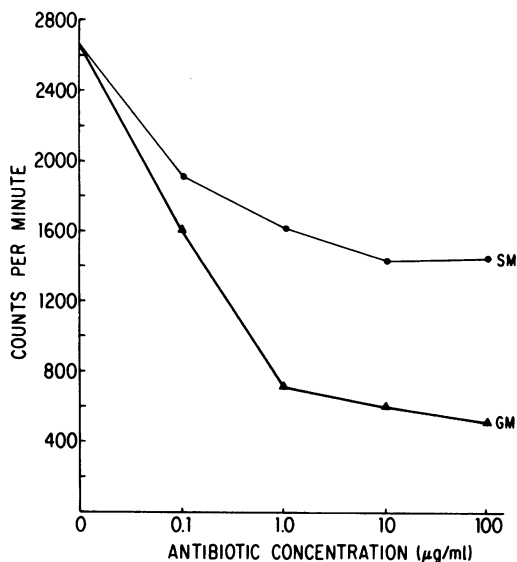


FIG. 3. Effect of streptomycin (SM) and gentamicin (GM) on polyphenylalanine synthesis in cell-free extracts of *S. mitis* 531.

(14). Such organisms have occasionally caused endocarditis which has not responded to conventional therapy (13). In addition, penicillin-resistant viridans streptococci may colonize and cause serious infections in immunosuppressed children (B. C. Weiner, A. E. Brown, G. Bell, F. Edwards, O. Quesada, T. E. Kiehn, and D. Armstrong, Abstr. Intersci. Conf. Antimicrob. Agents Chemother. 21st, Chicago, Ill., abstr. no. 803, 1981).

In the current report we describe a unique group of streptococci isolated in South Africa. All were highly resistant to penicillin and most of the beta-lactam antibiotics (2a). The organisms were found in close temporal and physical proximity to the well-described outbreak of multiply resistant pneumococci (6). Although the exact relationship between the two remains speculative, a common mechanism of resistance seems most likely. Penicillin resistance among pneumococci has been intensively studied and appears to be chromosomally mediated (4). The lack of detectable β -lactamase and uniform resistance to a broad range of β -lactam antibiotics suggest that the mechanism of penicillin resistance in the viridans streptococci may be similar to that of pneumococci.

High-level streptomycin resistance among viridans streptococci has only recently been reported (5). Horodniceanu and co-workers (5) also found several organisms from various species of streptococci, including viridans streptococci, with streptomycin, kanamycin, and penicillin

resistance. Attempts to pass the resistance into a susceptible recipient were successful at a very low frequency or were unsuccessful. Since transfer of aminoglycoside resistance occurred without detectable plasmid transfer, their study suggested that resistance was mediated by chromosomal elements. The authors suggest that these elements might code for modifying enzymes. The isolation of penicillin- and streptomycin-resistant organisms from Paris, South Africa, and Boston implies that the frequency of this phenomenon may be increasing or that the phenomenon is being recognized more frequently. Although these organisms have not been known to cause endocarditis, they have been isolated from blood cultures.

Most of the penicillin-resistant pneumococci isolated in South Africa have also been highly resistant to streptomycin (MIC, $\geq 2,000$ $\mu\text{g/ml}$) (6). The mechanism of this resistance has not been studied. However, the occurrence of streptomycin resistance among pneumococci does not have the same significance as it does among viridans streptococci.

Our initial curing and enzyme studies of viridans streptococci produced no evidence of plasmid-mediated aminoglycoside-modifying enzymes and suggested either a permeability barrier or a ribosomal site of resistance. Further studies confirmed that the ribosomes of three strains were more resistant to the effects of streptomycin than gentamicin. This is the first time that ribosomal resistance to streptomycin has been reported in clinical streptococcal isolates. We cannot rule out a simultaneously occurring permeability barrier, but this seems unlikely in view of the susceptibility of strains 531 and 859 to relatively low concentrations of gentamicin (16).

The isolation of multiply resistant viridans streptococci may have important clinical consequences. Presently, there are three accepted regimens used to treat endocarditis due to viridans streptococci (2). These include penicillin alone for 4 weeks, penicillin for 4 weeks plus streptomycin for 2 weeks, and penicillin plus streptomycin for 2 weeks. The use of combination therapy is based on the repeated observation that penicillin plus streptomycin is synergistically active against strains of viridans streptococci. In 1974, Wolfe and Johnson studied 48 strains of viridans streptococci isolated from patients with endocarditis (19). None of the organisms had an MIC of penicillin of ≥ 0.4 $\mu\text{g/ml}$ or an MIC of streptomycin of ≥ 200 $\mu\text{g/ml}$. In addition, 47 of 48 strains were synergistically killed by the combination of penicillin plus streptomycin. Whether such combination therapy improves the clinical outcome is still unclear; all regimens are currently being used empirically.

Our studies clearly demonstrate that high-level streptomycin resistance among viridans streptococci predicts resistance to synergy between penicillin plus streptomycin but not penicillin plus gentamicin. This finding is analogous to a similar phenomenon with enterococci (10). Enterococci resistant to high levels of streptomycin now account for at least 45% of clinical isolates in Boston (10). It is conceivable that a similar phenomenon could occur with other streptococci. Our observations suggest that detailed susceptibility testing of all clinically significant viridans streptococcus isolates might be required. If high-level streptomycin and penicillin resistance is found, then the combination of penicillin plus gentamicin or another bactericidal antibiotic should be used. A final note of caution regarding the combination of penicillin plus gentamicin is in order. Although the combination was synergistic against our ribosomally resistant organisms, we cannot be certain that strains resistant by other mechanisms would also be synergistically killed by this combination. Strains resistant to aminoglycosides by enzymatic modification or by alterations in permeability might not be killed synergistically.

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