**Molecular Epidemiology and Antibacterial Susceptibility of Streptococci Isolated from Healthy Children Attending Day Care Units**

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**ABSTRACT**

Objective: The aims of the study were to investigate antibacterial susceptibility and resistance mechanisms of streptococci isolated from healthy children attending day care units and to evaluate clonal relatedness of the strains.

Material and Methods: Antimicrobial susceptibilities of streptococci isolated from 212 children attending 3 different day care units were evaluated using the agar dilution method. Polymerase chain reaction and sequencing were used to investigate resistance mechanisms. Clonal relatedness was evaluated using pulsed field gel electrophoresis.

Results: Of 212 children, 11 (5.2%) carried Streptococcus pneumoniae, 17 (8.0%) S. pyogenes, and 42 (19.8%) erythromycin resistant viridans group streptococci. All S. pyogenes were susceptible to penicillin G and macrolides. Ten of 11 clonally unique S. pneumoniae were resistant to penicillin G. Three of 11 S. pneumoniae were macrolide resistant and carried erm(B). Among clonally unique 42 erythromycin resistant viridans group streptococci, 2 (4.8%) had erm(B), 33 (78.6%) had mef(A) and 7 (16.6%) had both erm(B) and mef(A) genes. All S. pyogenes from the first centre and three strains from the second centre were pulse-type A.

Conclusion: Among healthy children, colonization with penicillin resistant pneumococci and erythromycin resistant viridans group streptococci is quite high. Clonal spread of S. pyogenes is important for day care units.

Key Words: Streptococcus pneumoniae, Streptococcus pyogenes, viridans group streptococci, day care units, colonization, antimicrobial resistance

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**Introduction**

Viridans group streptococci comprise 30-60% of the human nasopharyngeal bacterial flora, together with other streptococci such as Streptococcus pneumoniae and S. pyogenes. Although S. pneumoniae and S. pyogenes are frequently involved in clinical infections, viridans group streptococci have a low infective potential except in infective endocarditis (1).

During the past ten years, increased rates of penicillin and macrolide resistance in clinical isolates of viridans group streptococci have been reported. These resistant strains are very important because of transmission of genes encoding antibiotic resistance from this kind of nonpathogenic bacteria to pathogenic bacteria (2, 3). It has been postulated that pneumococci originally acquired transposons coding for penicillin G resistance from viridans streptococci over 30 years ago. It has been shown, for example, that the proportion of pharyngeal carriers of commensal streptococci resistant to erythromycin is very high in Spain, similar to the prevalence of antibiotic resistant S. pneumoniae (4).

The incidence of drug resistant S. pneumoniae has increased worldwide. However, the prevalence of high level penicillin resistance is 3% in Turkey (5). The first penicillin resistant pneumococci was reported in 1977, (6) especially in Kayseri, a central city of Turkey, but no high level penicillin resistant clinical isolate of S. pneumoniae has been isolated (7). Erythromycin-resistant S. pneumoniae and erythromycin-resistant S. pyogenes strains have been reported in many countries (8-10). This resistance is mediated mainly by two resistance genes erm(B) and mef(A), and in rare strains by ribosomal mutation (9, 11).

Although there are some reports about S. pneumoniae and S. pyogenes isolated from healthy carriers, (12, 13) little is known about the susceptibility of viridans streptococci in Turkey. The aim of this study was to investigate the proportion, antibacterial susceptibility, resistance mechanisms and genetic relatedness of strains of S. pneumoniae, S. pyogenes and erythromycin-resistant viridans group streptococci found in pharyngeal flora of healthy children attending day care units in Kayseri, a city of one million inhabitants in Central Turkey.
Materials and Methods

Bacterial strains

Pharyngeal swabs were collected from 212 healthy children attending three different day care units in Kayseri. Swabs were inoculated on sheep blood agar (Diomed, Turkey) for S. pneumoniae and S. pyogenes strains, and on sheep blood agar supplemented with 0.25 mg/L erythromycin (Fako Laboratories, Turkey) to select erythromycin-resistant viridians streptococci. Plates were incubated at 35°C for 24-48 hours in 5% CO₂. Practically all healthy children harbour viridians streptococci in the throat as commensals. For this reason, the children from whom erythromycin resistant strains were not isolated on the selective agar were assumed as carrying susceptible strains in their throats.

Identification

Identification of S. pneumoniae and viridans streptococci was assessed by colony morphology, optochin susceptibility and bile solubility. Serotyping was performed by the Quellung reaction with Pneumotest (Statens Serum Institute, Denmark). Identification of S. pyogenes was done according to β haemolysis, presence of pyrrolidonyl arylamidase, and bacitracin susceptibility and confirmed by agglutination with group specific antisera (Binding Site, UK). Other streptococci were identified by rapid ID 32 STREP (bioMerieux, France). The test strips were read after 4 hour and identification was obtained using MINIAPI.

Susceptibility testing

Minimal inhibitory concentrations (MIC) of telithromycin (Aventis Pasteur, France), oxithromycin (Hoechst-Marion-Roussel, France) erythromycin (Sigma, UK), azithromycin (Pfizer Inc, NY, USA), clarithromycin (Abbott Laboratories, IL, USA), clindamycin (Sigma, UK), levofloxacin (Aventis Pasteur, France), gentamicin (Sigma, UK), chloramphenicol (Sigma, UK), penicillin G (Sigma, UK) and tetracycline (Sigma, UK) were determined by the Clinical Laboratory Standards Institute (14). The MIC values of erythromycin-resistant viridians streptococci in the throat as commensals. For this reason, the children from whom erythromycin resistant strains were not isolated on the selective agar were assumed as carrying susceptible strains in their throats.

Antimicrobial susceptibility results

MICS of penicillin G, erythromycin, azithromycin, clarithromycin, roxithromycin, telithromycin, clindamycin, levofloxacin, gentamicin, chloramphenicol, and tetracycline for S. pneumoniae, S. pyogenes and erythromycin-resistant viridians group streptococci isolates are shown in Table 1. When the MIC values of S. pneumoniae strains for penicillin G were evaluated according to the CLSI (CLSI, 2009) breakpoints for meningal isolates, 10 of 11 S. pneumoniae isolates, serogroups/serotypes were found to be 1 (n=4), 3 (n=2), 19 (n=2), 4 (n=1), 5 (n=1), and 6 (n=1). Of the 42 isolates of erythromycin-resistant viridans group streptococci examined in the study, 31 (73.8%) were identified as S. mitis, 10 (23.8%) as S. oralis, and 1 (2.4%) as S. sanguis.

Results

Proportions of streptococci in pharyngeal flora

Of 212 healthy children, 11 (5.2%) carried S. pneumoniae, 17 (8%) S. pyogenes, and 42 (19.8%) erythromycin-resistant viridians group streptococci. Among the 11 S. pneumoniae isolates, serogroups/serotypes were found to be 1 (n=4), 3 (n=2), 4 (n=1), 5 (n=1), and 6 (n=1). Of the 42 isolates of erythromycin-resistant viridans group streptococci examined in the study, 31 (73.8%) were identified as S. mitis, 10 (23.8%) as S. oralis, and 1 (2.4%) as S. sanguis.

Detection of resistance determinants

To determine the mechanism of macrolide resistance erm(B) and mef(A) genes and to determine tetracycline resistance, tet(M) and tet(K) genes were amplified by polymerase chain reaction (PCR). The PCR conditions and the specific primers were used as described previously (16, 17). PCR was used to amplify quinolone resistance determinant regions (QRDR) in gyrA, gyrB, parC and parE genes using primers and cycling conditions described previously (18). Template DNA for PCR was prepared using InstaGen Matrix, as recommended by the manufacturer (Bio-Rad Laboratories, Hercules, CA). After amplification, PCR products were purified from excess primers and nucleotides using QIAquick PCR Purification kit (Qiagen, Valencia, CA) and sequenced directly using CEQ8000 Genetic Analysis System (Beckman Coulter, Fullerton, CA).

Genetic relatedness

The genetic relatedness of the strains was investigated by pulsed-field gel electrophoresis (PFGE) using Smal-digested fragments as described by Soares et al. (19). The electrophoretic patterns were analysed visually and distinct patterns were assigned an arbitrary PFGE designation. Interpretation was done according to standard criteria (20).
Among four tetracycline-resistant *S. pneumoniae* isolates, one had tet(M), two had tet(M) and tet(K), and one was negative for both tet(M) and tet(K), while all tetracycline-resistant *S. pyogenes* isolates (n=4) carried only tet(M) gene. Tet(M) was the predominant gene among tetracycline and erythromycin-resistant viridans streptococci. Of 22 tetracycline-resistant strains, 18 (81.8%) had tet(M), two (9%) had both tet(M) and tet(K) and two (9%) had none of these.

No mutation was found in *gyrA*, *gyrB*, *parC*, and *parE* of the one levofloxacin-intermediate viridans group streptococcus. The detected resistance mechanisms were summarized in Table 2.

### Table 1. MICs (mg/L) of 11 antibiotics for the isolates of 11 *S. pneumoniae*, 17 *S. pyogenes* and 42 erythromycin-resistant viridans group streptococci (ERVGS)

<table>
<thead>
<tr>
<th></th>
<th><em>S. pneumoniae</em></th>
<th><em>S. pyogenes</em></th>
<th>ERVGS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MIC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>MIC&lt;sub&gt;90&lt;/sub&gt;</td>
<td>range</td>
</tr>
<tr>
<td>Penicillin G</td>
<td>0.25</td>
<td>1</td>
<td>&lt;0.016-4</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>0.03</td>
<td>&gt;64</td>
<td>&lt;0.03-&gt;64</td>
</tr>
<tr>
<td>Azithromycin</td>
<td>0.06</td>
<td>&gt;64</td>
<td>&lt;0.03-&gt;64</td>
</tr>
<tr>
<td>Clarithromycin</td>
<td>&lt;0.03</td>
<td>&gt;64</td>
<td>&lt;0.03-&gt;64</td>
</tr>
<tr>
<td>Roxithromycin</td>
<td>0.25</td>
<td>&gt;64</td>
<td>0.06-&gt;64</td>
</tr>
<tr>
<td>Telithromycin</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01 (all)</td>
</tr>
<tr>
<td>Clindamycin</td>
<td>0.06</td>
<td>64</td>
<td>&lt;0.03-&gt;64</td>
</tr>
<tr>
<td>Levofloxacin</td>
<td>1</td>
<td>2</td>
<td>0.5-2</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>16</td>
<td>32</td>
<td>1-32</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>2</td>
<td>4</td>
<td>1-8</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>0.5</td>
<td>64</td>
<td>0.25-64</td>
</tr>
</tbody>
</table>

Among four tetracycline-resistant *S. pneumoniae* isolates, one had tet(M), two had tet(M) and tet(K), and one was negative for both tet(M) and tet(K), while all tetracycline-resistant *S. pyogenes* isolates (n=4) carried only tet(M) gene. Tet(M) was the predominant gene among tetracycline and erythromycin-resistant viridans streptococci. Of 22 tetracycline-resistant strains, 18 (81.8%) had tet(M), two (9%) had both tet(M) and tet(K) and two (9%) had none of these.

No mutation was found in *gyrA*, *gyrB*, *parC*, and *parE* of the one levofloxacin-intermediate viridans group streptococcus. The detected resistance mechanisms were summarized in Table 2.

### Genetic relatedness

Each *S. pneumoniae* isolate had a different PFGE pattern. Erythromycin-resistant viridans group streptococci were also clonally unique. Although there were six different clones among *S. pyogenes* isolates, genetically related groups were identified from each day care unit. Seven of 130 children from day care unit 1; 9 of 56 children from day care unit 2; and 1 of 26 children from day care unit 3 were positive for *S. pyogenes*. All *S. pyogenes* from the first centre and three strains from the second centre were pulse-type A, which was the most common pulse-type. Three pulse-type B strains were from the second centre and 3 pulse-type D strains were from centre 2 and 3 (Figure 1).

### Discussion

Antibiotic resistance among *S. pneumoniae* isolates has increased all over the world (21). Previous studies have reported the prevalence of *S. pneumoniae* resistant to macrolides to be approximately 8%, (5) and to penicillin G 0-3% in Turkey (5, 7). In the light of these low resistance rates, the findings of our study are disturbing, since 91% of the colonizing *S. pneumoniae* isolates were found to be resistant to penicillin G and 27.2% to erythromycin. These findings suggest that clinical antibiotic resistance will soon become a threat in Turkey. According to Baquero’s threshold hypothesis (21) penicillin resistance among *S. pneumoniae* isolates in Kayseri may be in the cryptic phase.

A striking finding of this study was the low prevalence of colonization with *S. pneumoniae* in healthy children (5.2%). This rate is higher in the western part of Turkey (12, 13). It is known that conjugate vaccines are successful in reducing nasopharyngeal carriage of both *Haemophilus influenzae* and *S. pneumoniae* in children (22, 23). However, this is not the case for Kayseri, since vaccination with conjugate pneumococcal
The prevalence of penicillin-resistant colonizers in S. pneumoniae can be transferred between viridans group streptococci and are products of mosaic genes. These resistance determinants in one strain and that strain showed the MLS B phenotype. In same (4). In the latter study, both resistance genes were found erythromycin-resistant viridans group streptococci were the resistance among viridans group streptococci may serve as a detection of an increase in the local prevalance of penicillin resistance among viridans group streptococci (4). It could be suggested that the prevalence of antibiotic resistant S. pneumoniae, with a high prevalance of penicillin- and/or macrolide-resistant S. pneumoniae, the predominant resistance mechanism was found to be routine administrated in Turkey.

The erm(B) gene determining constitutive or inducible cross-resistance to macrolides, lincosamides, and streptogramin was found in all erythromycin-resistant S. pneumoniae isolates. This is the predominant resistance mechanism in Turkey (24), as in other southern European countries (25-27). All S. pneumoniae strains were clonally unique. Prevalence of colonization with erythromycin-resistant viridans group streptococci was 19.8% and of these strains, approximately 93% were non-susceptible to penicillin G. Countries, such as Spain, with a high prevalence of penicillin- and/or macrolide-resistant S. pneumoniae, have a high prevalence of antibiotic resistant viridans group streptococci (4). It could be suggested that the detection of an increase in the local prevalence of penicillin resistance among viridans group streptococci may serve as a sentinel for the appearance of resistant pneumococci.

Penicillin resistance in viridans group streptococci as well as in S. pneumoniae results from alterations in the PBPs. In highly penicillin-resistant S. pneumoniae isolates, altered PBPs are products of mosaic genes. These resistance determinants can be transferred between viridans group streptococci and S. pneumoniae (2). This is also the case for macrolide resistance (3). The prevalence of penicillin-resistant colonizers in the pharyngeal flora of children living in Kayseri is high. This finding suggests that the appearance of high-level penicillin resistance among S. pneumoniae will soon appear in Kayseri.

Among erythromycin-resistant viridans group streptococci, the predominant resistance mechanism was found to be active efflux encoded by mef(A) gene (78.6%). Although this finding is similar to the finding from Greece (74%) (28), it was found that the percentages of mef(A) and erm(B) among erythromycin-resistant viridans group streptococci were the same (4). In the latter study, both resistance genes were found in one strain and that strain showed the MLSB phenotype. In the current study the percentage of this duplicity of genes was 16.6%.

Tetracycline resistance was found in 55% of erythromycin-resistant viridans group streptococci. Resistance to macrolides, lincosamides, streptogramin B, tetracycline, kanamycin and chloramphenicol has been linked to carriage of the conjugative transposon Tn 1545 (29). Seral et al. (30) found the prevalence of erythromycin-resistant S. pneumoniae strains carrying tet(M) determinants as 82% in Spain. In the present study, 3 of the 4 tetracycline-resistant S. pneumoniae strains were also resistant to erythromycin and clindamycin. One strain which was susceptible to erythromycin and clindamycin carried neither tet(M) nor tet(K). There were also two strains of erythromycin-resistant viridans group streptococci negative for both genes. Rodriguez-Avil, et al. (31) found that erm(B) and tet(M) determinants were associated in viridans group streptococci. However, we could not find such an association in our erythromycin-resistant viridans group streptococci.

MIC$_{50}$/s of levofloxacin for both S. pyogenes and erythromycin-resistant viridans group streptococci were similar (2 mg/L) to those in other studies (28, 32). There was only one strain of erythromycin-resistant viridans group streptococci intermediately-resistant to levofloxacin, but it did not have mutations in type II topoisomerase. Other portions of these genes could be implicated in resistance, or active efflux of the drug could be involved (33). These aspects were not investigated in our strain.

Antimicrobial resistance is one of the most important public health problems worldwide. Clinical microbiology laboratories, especially in countries like Turkey where macrolide and β-lactam antibiotics are frequently overprescribed, must perform periodic surveillance of antimicrobial susceptibility among various species of streptococci.

According to the results of PFGE which was used to evaluate clonal relatedness among the strains, all S. pyogenes from

### Table 2. The rates of antimicrobial resistance and resistance mechanisms among of S. pneumoniae, S. pyogenes and erythromycin-resistant viridans group streptococci (ERVGS), isolated from healthy children

<table>
<thead>
<tr>
<th>Organism (Number of strains)</th>
<th>Antibiotic (Number of nonsusceptible strains)</th>
<th>Detected genes (Number of strains)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. pneumoniae (11)</td>
<td>Penicillin G (10) Erythromycin (3) Tetracyclin (4)</td>
<td>erm(B) (3) Tet(M) (1), tet(M)+tet(K) (2), *unknown (1)</td>
</tr>
<tr>
<td>S. pyogenes (17)</td>
<td>Penicillin G (0) Erythromycin (0) Tetracyclin (4)</td>
<td>tetM (4)</td>
</tr>
<tr>
<td>ERVGS (42)</td>
<td>Penicillin G (intermediate 19, resistant 20) Erythromycin (42) Tetracyclin (22) Levofloxacin (intermediate 1)</td>
<td>erm(B) (2), mef(A) (33), erm(B)+mef(A) (7) Tet(M) (18), tet(M)+tet(K) (2), *unknown (2) No mutation in gyrA, gyrB, parC, and parE</td>
</tr>
</tbody>
</table>

* Tetracyclin resistant strains negative for tet(M) and tet(K)
the first centre and three strains from the second centre were pulse-type A, which showed that the pulse-type A strain was spread between the children attending the same day care unit and also between day care units.

Conflict of Interest
No conflict of interest was declared by the authors.

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