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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.

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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 viridans streptococci. Plates were incubated at 35°C for 24-48 hours in 5% CO_2. Practically all healthy children harbour viridans 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), roxithromycin (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) (CLSI) agar dilution method on Mueller-Hinton agar supplemented with 5% sheep blood at 35°C in ambient air. CLSI breakpoints for meningeal isolates were used to determine penicillin susceptibility status (15). For telithromycin, European breakpoints of ≤0.5 mg/L (susceptible), 1.0 mg/L (intermediate) and ≥2 mg/L (resistant) were used. *S. pneumoniae* ATCC 49619 and *Staphylococcus aureus* ATCC 29213 were used as quality control strains. All strains were tested twice and penicillin resistance was confirmed by using the E-test.

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 *gyr*A, *gyr*B, *par*C and *par*E 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 *Sma* I-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).

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 viridans group streptococci. Among the 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 eryhtromycin-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*.

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 viridans 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 meningeal isolates, 10 of 11 *S. pneumoniae* were resistant to penicillin G. There was only one strain susceptible to penicillin G. Three of 11 pneumococci, which were also resistant to clindamycin and tetracycline, were found to be resistant to both 14- and 15-membered macrolides. However, all of the *S. pneumoniae* isolates were susceptible to telithromycin and levofloxacin. There were four strains resistant to tetracycline.

All of the *S. pyogenes* isolates were susceptible to penicillin G, macrolides, levofloxacin and clindamycin. Four of 17 *S. pyogenes* were found to be resistant to tetracycline and one to chloramphenicol.

Among viridans streptococcal strains, 20 (48%) were resistant and 19 (45%) were intermediately resistant to penicillin G. Twenty-two of the isolates were resistant to tetracycline, 13 to chloramphenicol, and 10 to clindamycin. There was only one strain intermediately resistant to levofloxacin (MIC 4 mg/L).

Resistance mechanisms

PCR amplification of the *erm* and *mef* genes demonstrated that among 42 erythromycin-resistant viridans group streptococci, two (4.8%) had *erm*(B), 33 (78.6%) had *mef*(A), and 7 (16.6%) had both *erm*(B) and *mef*(A) genes when all of the erythromycin-resistant *S. pneumoniae* (n=3) isolates had *erm*(B).

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

	S. pneumoniae			*S. pyogenes*			ERVGS		
	MIC_{50}	MIC_{90}	range	MIC_{50}	MIC_{90}	range	MIC_{50}	MIC_{90}	range
Penicillin G	0.25	1	<0.016-4	<0.016	<0.016	<0.016 (all)	2	8	0.06->8
Erythromycin	0.03	>64	<0.03->64	0.06	0.12	<0.03-0.12	8	64	1->64
Azithromycin	0.06	>64	<0.03->64	0.12	0.25	0.06-0.5	4	>64	0.5->64
Clarithromycin	<0.03	>64	<0.03->64	0.06	0.12	<0.03-0.12	8	>64	0.5->64
Roxithromycin	0.25	>64	0.06->64	0.5	0.5	0.25-1	32	>64	4->64
Telithromycin	0.01	0.01	0.01 (all)	<0.01	0.03	<0.01-0.5	0.12	0.25	<0.016-0.5
Clindamycin	0.06	64	<0.03->64	0.06	0.06	<0.03-0.12	<0.03	64	<0.03->64
Levofloxacin	1	2	0.5-2	0.5	1	0.25-2	2	2	0.5-4
Gentamicin	16	32	1-32	4	8	2-32	8	32	2-32
Chloramphenicol	2	4	1-8	2	4	2-16	4	16	1-32
Tetracycline	0.5	64	0.25-64	0.25	64	0.25-64	8	64	0.25->64

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 *gyr*A, *gyr*B, *par*C, and *par*E 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).

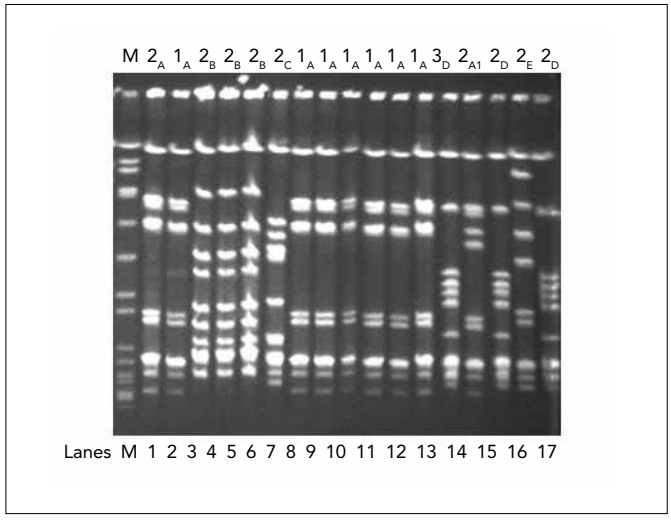

Figure 1. PFGE patterns of the *Streptococcus pyogenes* strains, isolated form children attending 3 day care units in Kayseri Numbers show the day care centers and letters in subscript the pulse-type. Lanes 7-12 strains isolated from day care center 1, strains 1, 3-6, 14-17 were from day care center 2 and 13 was from day care center 3. Pulse-type A was the most common clone. Nine strains isolated from centres 1 and 2 were pulse-type A. Pulse-type B strains were from center 2 and pulse-type D from center 2 and 3. *Staphlylococcus aureus* NCTC 8325 was used as moleculer size marker (M)

Discussion

Antibiotic resistance among *S. pneumoniae* isolates has increased all over the world (21). Previous studies have reported the prevalance 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

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

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

* Tetracyclin resistant strains negative for *tet*(M) and *tet*(K)

vaccine or capsular polysaccharide vaccine has recently started to be routinely 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 prevalance 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 prevalance 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 MLS_B 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-Avial, 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_{90}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 ß-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

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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