Macrolide and Tetracycline Resistance and *emm* Type Distribution of *Streptococcus pyogenes* Isolates Recovered from Turkish Patients

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The aims of this study were to determine the susceptibilities to macrolide and tetracycline antibiotics and *emm* type distribution of *Streptococcus pyogenes* strains isolated in the Kocaeli University Hospital, Turkey. A total of 127 *S. pyogenes* clinical isolates were tested. Eleven (9%) isolates were resistant to erythromycin, and 23 (18%) isolates were resistant to tetracycline. Ten of the erythromycin-resistant isolates were also resistant to tetracycline. By the triple-disk test, all erythromycin-resistant isolates showed the inducible macrolide-lincosamide-streptogramin-C phenotype and harbored *erm*(TR) gene. *tet*(O) was the most common tetracycline resistance gene. Among erythromycin-tetracycline coresistant isolates, seven harbored the *tet*(O) gene. *emm* 4, *emm* 1, *emm* 2,114, and *emm* 89 were the most common *emm* types. These isolates were more susceptible to erythromycin. There was considerable *emm* type heterogeneity in macrolide or tetracycline resistant isolates. According to our knowledge, this is the first study in which *emm* type distribution is investigated in Turkey. More comprehensive studies are needed to obtain true information about the epidemiology of macrolide and tetracycline resistance and *emm* type distribution in Turkey.

Introduction

STREPTOCOCCUS PYOGENES (group A β -haemolytic streptococcus) is a common human pathogen that causes a wide spectrum of diseases, and penicillins remain the drugs of choice for their treatment. Macrolides are also used for patients who are allergic or nontolerant to penicillins. The increasing rates of macrolide resistance in *S. pyogenes* have been reported from different parts of the world.²⁶

There are two main phenotypes of macrolide resistance in *S. pyogenes*: the M phenotype (efflux-mediated), mediated by the *mef* genes, which confers low levels of resistance to 14- and 15-membered macrolides but not to 16-membered macrolides, lincosamides, or streptogramin B; and the macrolide-lincosamide-streptogramin B (MLS) phenotype, mediated by the *erm* genes, which confers resistance to macrolides, lincosamides, and streptogramin B antibiotics. This latter phenotype can be constitutive (cMLS), which is generally mediated by the *erm*(B) gene, or inducible (iMLS), which is generally mediated by the *erm*(A) subclass TR gene.^{24,36,38} iMLS strains show susceptibility to lincosamides but turn to high-level resistance after induction.¹⁸

There are several reports about the association between erythromycin, and tetracycline resistance has been consid-

ered to be an important cofactor in the selection of erythromycin resistance.^{5,28}

The M protein is a major virulence factor of *S. pyogenes*. The *emm* gene encodes the M protein. *emm* gene sequencing is presently used for routine typing of *S. pyogenes*.⁶

The aims of this study were to determine the susceptibilities to macrolide and tetracycline antibiotics of *S. pyogenes* isolates and in the case of resistant isolates, to ascertain their patterns and genetic mechanisms of resistance. We also determined the prevalence of *emm* types among *S. pyogenes* isolates. According to our knowledge, this is the first study about *emm* typing of *S. pyogenes* in Turkey.

Methods

Bacterial isolates

A total of 127 clinical isolates of *S. pyogenes* isolated between October 2005 and July 2008 in the Clinical Microbiology Laboratory of Kocaeli University Hospital were tested. A total of 103 (81%) isolates were cultured from pharyngeal specimens, and 24 (19%) were cultured from other sources (18 from wound, 3 from sputum, 2 from urine, and 1 from pericardial fluid). Duplicates were excluded.

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The isolates were identified as *S. pyogenes* by colony morphology, β -hemolysis on sheep blood agar, bacitracin disc test (0.04 U), and pyrrolidonyl arylamidase test. The strains were stored at -70°C and then subcultured twice on blood agar before susceptibility testing.

Antimicrobial susceptibility testing and macrolide resistance phenotype

Penicillin, vancomycin, levofloxacin, chloramphenicol, tetracycline, erythromycin, clarithromycin, azithromycin, telithromycin, and clindamycin susceptibilities were investigated by disk diffusion method. Minimum inhibitory concentrations (MICs) were determined using Etest (AB Biodisk) for isolates demonstrating intermediate susceptibility or resistance to erythromycin and/or tetracycline. Interpretation of the results was basically as outlined in the Clinical and Laboratory Standards Institute (CLSI) guidelines.¹¹ Since CLSI telithromycin breakpoints for group A β -haemolytic streptococcus are not available, the results were interpreted according to the breakpoints proposed by the French Society for Microbiology.¹³ *Streptococcus pneumoniae* ATCC 49616 was used as the quality control strain.

The erythromycin resistance phenotypes were classified by triple-disk test with erythromycin (15 μg), clindamycin (2 μg), and spiramycin (100 μg) disks, as previously described.¹⁸

Detection of erythromycin and tetracycline resistance genes

Erythromycin-resistant and intermediate-susceptible isolates were screened for the *erm*(B), *erm*(TR), and *mef*(A) genes, and tetracycline-resistant and intermediate-susceptible isolates were screened for the *tet*(K), *tet*(L), *tet*(M), and *tet*(O) genes by real-time polymerase chain reaction (PCR) (iCycler IQ; v3.0a-BioRad Laboratories). Total genomic DNA was isolated from streptococcal strains with QIAamp[®] DNA Mini Kit (Qiagen). DNA in the reaction mixture (25 μl) was denatured at 93°C for 3 min, and the amplification cycles consisted of elongation at 72°C for 60 sec, denaturation at 93°C for 60 sec, and annealing at 52°C for 60 sec. After 35 amplification cycles, the last elongation step was performed at 72°C for 5 min.³⁶ Primer sets specific for the several genes were used as seen in Table 1.^{3,16} Two reference *Escherichia coli* strains carrying *erm*(A) and *erm*(B) genes and one *S. pyogenes* strain positive for *mef*(A) (kindly provided by Dr. Ziya Cibali Acikgoz) were used as positive PCR controls.

emm typing

A total of 91 *S. pyogenes* isolates were *emm* typed. *S. pyogenes* *emm* sequence database (National Centers for Disease Control Biotechnology Core Facility Computing Laboratory, Blast 2.0) was used for Streptococci Group A Subtyping. The *emm* type-specific sequence databases have been downloaded from ftp://ftp.cdc.gov/pub/infectious_diseases/biotech/tsemm/ (for DNA). All PCR products were purified using the High Pure PCR Product Purification Kit[®] (Roche Diagnostics GmbH) and directly sequenced with the ABI PRISM 310 Genetic Analyzer[®] equipment using the DYEnamic ET Terminator Cycle Sequencing Kit[®] (Amersham Pharmacia Biotech, Inc.). The electropherogram-obtained sequences were assembled using Vector NTI[®] v5.1 (InforMax[™] Invitrogen[™] life science software).

Statistical analysis was performed by chi-square test using Epi Info (TM) 3.5.1 program.

Results

Among 127 isolates tested, 11 (9%) were resistant to erythromycin, 7 (6%) were resistant to clarithromycin, and 23 (18%) were resistant to tetracycline. All of the erythromycin-resistant isolates were also resistant to azithromycin. Among 11 erythromycin-resistant isolates, 10 were also resistant to tetracycline, and 7 were also resistant to clarithromycin. All of the seven clarithromycin-resistant isolates were also resistant to erythromycin, azithromycin, and tetracycline. All 127 isolates were susceptible to other tested antibiotics including telithromycin.

By the triple-disk test, all of the erythromycin-resistant isolates were assigned to the iMLS-C phenotype. None of the isolates had M, cMLS, iMLS-A, or iMLS-B resistance phenotypes.

MIC ranges were between 0.75 and 1 $\mu\text{g}/\text{ml}$ in erythromycin-resistant (and intermediate) isolates and 4–32 $\mu\text{g}/\text{ml}$ in tetracycline-resistant (and intermediate) isolates.

Genotype analysis of erythromycin resistance revealed that 8 of the 11 erythromycin-resistant isolates carried the *erm*(TR) gene. In the other three isolates, macrolide resistance genes were not detected. None of the erythromycin-resistant isolates had the *erm*(B) and *mef*(A) genes. *erm*(TR) was strongly correlated to *tet*(O).

Seven of 23 tetracycline-resistant isolates had the *tet*(O) gene, 3 isolates had the *tet*(L) gene, 2 isolates had the *tet*(K) gene, 1 isolate had the *tet*(M) gene, 2 isolates had both *tet*(K) and *tet*(L) genes, and 1 isolate had both *tet*(K) and *tet*(O) genes. Seven isolates did not have any of these *tet* genes. Among erythromycin-tetracycline coresistant isolates, seven carried the *tet*(O) gene, one carried both *tet*(K) and *tet*(O) genes, and two did not have any of these *tet* genes (Table 2).

A total of 37 different *emm* types were found. The most prevalent *emm* types were *emm*4 ($n = 10$, 11%), *emm*1 ($n = 9$, 10%), *emm*2,114 ($n = 8$, 9%), and *emm*89 ($n = 7$, 6%). There was no predominant *emm* type among erythromycin-resistant and tetracycline-resistant isolates. However, *emm*1, *emm*4, *emm*89, and *emm*2,114 were detected more in erythromycin-susceptible isolates ($n = 8$, $n = 10$, $n = 6$, $n = 6$, respectively) than in erythromycin-resistant isolates ($n = 1$, $n = 0$, $n = 0$, $n = 0$, respectively) ($p < 0.05$) (Table 3). Further, *emm*2,114 was observed more in pharyngeal isolates ($n = 7$) than in the other isolates ($n = 0$) ($p < 0.05$) (data not shown). There were no differences in distribution of *emm* types among pharyngeal and nonpharyngeal isolates with the exception of *emm*2,114, which was found to be more frequent among pharyngeal isolates ($n = 7$ vs. $n = 0$) ($p < 0.05$) (data not shown).

Discussion

Macrolide resistance and the distribution of the macrolide resistance genes of *S. pyogenes* vary widely in different regions of the world. In this study, erythromycin resistance rate (9%) was lower compared with that in many countries (21.3%–45%).^{8,29,33,39} Low erythromycin resistance rates (0.6%–6.8%) were reported in some countries.^{14,15,21,23,25,28,30,37} Our erythromycin resistance rate was similar to rates in the United States (9.6%),¹⁹ Germany (12.7%),⁴ and Greece

TABLE 1. LIST OF PRIMERS USED IN REAL-TIME POLYMERASE CHAIN REACTION

Gene	Primer target	Sequence (5'-3')	Product size (bp)
<i>erm</i> (A)	TR-up	ATAGAAATTGGGTCAGGAAAAGG	530
	TR-rev	TTGATTTTGTAGTAAAAAG	
<i>erm</i> (B)	ERMB-up	GAAAAGGTACTCAACCAAATA	639
	ERMB-rev	AGTAACGGTACTTAAATTGTTTAC	
<i>mef</i> (A)	<i>mef</i> A-up	AGTATCATTAATCACTAGTGC	348
	<i>mef</i> A-rev	TTCTTCTGGTACTAAAAGTGG	
<i>tet</i> (M)	<i>tet</i> M-up	ACAGAAAGCTTATTATATAAC	171
	<i>tet</i> M-rev	TGGCGTGTCTATGATGTTTAC	
<i>tet</i> (O)	<i>tet</i> O-up	ACGGARAGTTTATTGTATACC	171
	<i>tet</i> O-rev	TGGCGTATCTATAATGTTGAC	
<i>tet</i> (K)	<i>tet</i> K-up	TATTTTGGCTTTGTATTCTTTCAT	1,159
	<i>tet</i> K-rev	GCTATACCTGTTCCCTCTGATAA	
<i>tet</i> (L)	<i>tet</i> L-up	ATAAATTGTTTCGGGTCGGTAAT	1,077
	<i>tet</i> L-rev	AACCAGCCAACTAATGACAATGAT	

(11.9%).³⁴ The low erythromycin resistance rates (%3–7) were reported from different parts of Turkey.^{1,2,12} Previously, we found low-level erythromycin resistance in the European section of Turkey.² Moreover, all resistant isolates had low-level resistance to erythromycin (MIC ranges: 0.75–1 µg/ml) in the present study.

In our study, iMLS phenotype was detected in all erythromycin-resistant isolates. iMLS strains are susceptible to lincosamides but turn to high-level resistance after induction.¹⁸ In the previous studies performed in Turkey, the M phenotype was the most common phenotype followed by the iMLS phenotype.^{1,2} The M phenotype is the predominant phenotype in many countries,^{7,15,19,34,37} whereas iMLS is the most common phenotype in some countries^{4,14,25,30}. Differences among the countries could be due to different antibiotic using patterns. In our study, 3 of 11 erythromycin-resistant isolates did not have *erm*(A), *erm*(B), and *mef*(A) genes. This may be due to the ribosomal mutations.²⁸

Telithromycin resistance has particularly been reported in *erm*(B)-positive (cMLS) *S. pyogenes* isolates, although telithromycin retained activity against strains possessing the other macrolide resistance genotypes, such as *erm*(A) and *mef*(A).^{17,20,30} This explains the full susceptibility to telithromycin in all of the isolates we studied, in which erythromycin resistance was due to *erm*(TR).

In gram-positive bacteria, tetracycline resistance is mediated mainly by two mechanisms: ribosomal protection (*tet* M and *tet* O gene) and efflux (*tet* K and *tet* L gene).^{3,10} In some studies, tetracycline resistance in *S. pyogenes* was found to be associated with the *tet*M gene and, less frequently, with the *tet*O gene.^{16,29,34} Since tetracycline resistance genes can reside in mobile genetic elements that carry macrolide resistance genes, the cooccurrence of resistance to both classes of drugs is possible.⁵ Particularly, a linkage between *erm*(B) and *tet*(M) is well known.⁹ In many studies, *erm*(B) or both *erm*(B) and *mef*(A) were found to be associated with the *tet*(M) gene.^{5,9,25,28} Further, *tet*(M) can be found in a silent form in tetracycline-susceptible isolates.⁹ In the present study, we investigated the resistance genes in only resistant isolates. In this study, *tet*(O) was the most common tetracycline resistance gene. However, the most of the *tet*(O) positive isolates were also *erm*(TR) positive. There are several studies that reported the *erm*(TR)-*tet*(O) linkage.^{25,28} Seven tetracycline-resistant isolates did not have *tet*(M), *tet*(O), *tet*(K), and *tet*(L) genes. These isolates warrant further investigation on the other resistance genes.

This is the first study where in which *mm* types were investigated in *S. pyogenes* isolates from Turkey. *emm* 4, *emm* 1, *emm* 2,114, and *emm* 89 were the most common *emm* types, which accounted for 37% of all isolates. In a review that

TABLE 2. DISTRIBUTION OF RESISTANCE GENES AMONG 127 *STREPTOCOCCUS PYOGENES* ISOLATES

Macrolide susceptibility	Susceptible to tetracycline	No. of isolates						Tetracycline resistant, gene unknown	Total
		<i>tet</i> (K)	<i>tet</i> (L)	<i>tet</i> (M)	<i>tet</i> (O)	<i>tet</i> (K)+ <i>tet</i> (L)	<i>tet</i> (K)+ <i>tet</i> (O)		
Susceptible to erythromycin	103	2	2	1	0	2	0	6	116
<i>erm</i> (TR)	1	0	1	0	6	0	0	0	8
<i>erm</i> (B)	0	0	0	0	0	0	0	0	0
<i>mef</i> (A)	0	0	0	0	0	0	0	0	0
Macrolide resistant, gene unknown	0	0	0	0	1	0	1	1	3
Total	104	2	3	1	7	2	1	7	127

TABLE 3. *emm* TYPE DISTRIBUTION OF 91 *STREPTOCOCCUS PYOGENES* ISOLATES

<i>emm</i> type	Fully sensitive	Erythromycin-resistant	Tetracycline-resistant	Erythromycin and tetracycline-resistant	Total
1	8	—	—	1	9
4	10	—	—	—	10
5	3	—	1	—	4
6	3	—	—	—	3
8	1	—	—	—	1
9	1	—	—	—	1
11	—	—	1	—	1
12	4	—	—	—	4
14	1	—	—	—	1
17	—	—	1	1	2
18	1	—	—	—	1
19	2	—	—	1	3
22	—	—	—	1	1
29	2	—	—	—	2
49	1	—	—	—	1
52	—	—	2	—	2
73	—	—	—	1	1
79	1	—	—	1	2
81	2	—	—	—	2
82	1	—	1	—	2
87	3	—	—	—	3
89	6	—	—	—	6
98	—	—	1	—	1
99	—	—	1	—	1
100	1	—	—	—	1
102	1	—	—	2	3
118	1	—	—	—	1
2, 114	6	—	1	—	7
17, 41	—	—	1	—	1
22, 78	1	—	—	—	1
33, 41	—	—	1	—	1
41, 75	3	—	—	—	3
44, 65	2	—	—	—	2
65, 69	1	—	—	—	1
77, 102	—	1	—	2	3
79, 87	1	—	—	—	1
sts 104	2	—	—	—	2
Total	69	1	11	10	91

investigated the global *emm* type distribution, *emm1*, *emm12*, *emm28*, *emm3*, and *emm4* were reported to be the most common *emm* types. However, significant differences in *emm* type distribution by region and clinical disease state were also reported.³⁵

In the present study, *emm1*, *emm4*, *emm89*, and *emm2,114* typed isolates were more susceptible to erythromycin ($p < 0.05$). However, we did not detect any significant distribution in macrolide or tetracycline-resistant isolates. The heterogeneity of *emm* types among macrolide-resistant and tetracycline-resistant isolates in the present study is compatible with some reports³⁷ and contrasts with the others^{14,19,26,27,30,39}. Montes *et al.* reported an association between *emm77* and *tet(M)*. They also reported that the *emm22* isolates were the most frequent clone among the inducible resistant isolates in Spain.²⁷ Green *et al.* reported that *emm75* and *emm12* accounted for more than half of the macrolide-resistant isolates.¹⁹ Fluctuations in the serotype distribution over time were also reported.^{22,31,32}

In conclusion, prevalence of macrolide and tetracycline resistance in *S. pyogenes* isolates is low in our region and

mainly due to *erm*(TR) and *tet*(O) genes, respectively. Although the number of the isolates is low in this study, a high frequency of macrolide-tetracycline coresistance is found. Therefore, tetracycline resistance can be considered as a co-factor in selection of macrolide-resistant strains. This is the first study in which *emm* type distribution is investigated in Turkey. Detected considerable *emm* type heterogeneity mainly in macrolide and tetracycline resistant isolates is probably due to the limited number of the studied isolates. More comprehensive and multicentre surveillance studies are needed to obtain true information about the epidemiology of macrolide and tetracycline resistance and *emm* type distribution in Turkey.

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Disclosure Statement

No competing financial interests exist.

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