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A study on molecular characterization of macrolide resistance mechanism among isolates of *Streptococcus pneumoniae* from the southern Marmara region of Turkey, as well as resistance to macrolides and penicillin in these isolates

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Aim: To determine the distribution of macrolide resistance genes as well as resistance rates in isolated *Streptococcus pneumoniae* strains from the southern Marmara region of Turkey.

Materials and methods: Antimicrobial resistance rates and MIC values were determined by the E-test method in 300 *Streptococcus pneumoniae* isolates that were isolated from clinical samples. Resistance genes were determined by the polymerase chain reaction (PCR) method in erythromycin-resistant strains.

Results: It was found that 11.4% of *Streptococcus pneumoniae* isolates were resistant to macrolides. The penicillin MIC value was ≥0.12 µg/mL in 23% of the strains and 2 µg/mL in 2% of the strains. The *erm*(B) genotype was observed in 58.8% of all macrolide-resistant strains, 38.2% were of the *mef*(A) genotype, and 3% were a combination of both genotypes.

Conclusion: Based on the data from this study, it was concluded that the local resistance to antibiotics is not as high as that observed in other countries, and the *erm*(B) genotype was dominant in macrolide-resistant strains. Therefore, it is suggested that macrolide-group antibiotics still be included in treatment protocols.

Key words: *mef*(A), *erm*(B), PCR, *S. pneumoniae*

Türkiye'nin Güney Marmara Bölgesi'nden izole edilen *Streptococcus pneumoniae* suşlarında makrolid ve penisilin direnci ve makrolid direncinin moleküler özelliği

Amaç: Son yıllarda *Streptococcus pneumoniae* suşlarında makrolid ve diğer antibiyotiklere giderek artan bir direnç oranı mevcuttur. Bu çalışmada Türkiye'de Güney Marmara Bölgesi'nden izole edilen *S. pneumoniae* suşlarında penisilin ve makrolidler için minimum inhibitör konsantrasyonlarının ve makrolid direnç mekanizmasının genetik dağılımının tespit edilmesi amaçlanmıştır.

Yöntem ve gereç: Çalışmada klinik örneklerden izole edilen 300 adet *S. pneumoniae* izolatında E test yöntemi ile antimikrobiyal direnç oranları ve MIK değerleri ve eritromisine dirençli izolatlarda ise PCR yöntemiyle direnç genleri tespit edildi.

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Bulgular: Çalışmada makrolid direnci % 11,4 oranında bulundu. Penisilin MIC değeri 0,12 µg/mL ve üzeri olan suşlar % 23 oranında, penisilin MIC değeri 2 µg/mL olan suşlar % 2 oranında bulundu. Makrolid dirençli izolatların % 58,8'inde *erm*(B) genotipi, % 38,2'sinde *mef*(A) genotipi, ve % 3'ünde her iki direnç genotipi birden tespit edildi.

Sonuç: Bölgemizde izole edilen suşlarda antibiyotik direncinin yüksek olmadığı ve makrolid direnci olan suşlarda *erm*(B) tipi direnç geninin hakim olduğu görüldü. Bölgemizde direnç oranlarının yüksek olmaması nedeniyle tedavi protokolünde makrolid grubu antibiyotikler kullanılabilir.

Anahtar sözcükler: *mef*(A), *erm*(B), PCR, *S. pneumoniae*

Introduction

Streptococcus pneumoniae is the major bacterial pathogen in community-acquired respiratory tract infections and meningitis, both in prevalence and in its ability to cause systemic infection with a high level of morbidity and mortality (1-3). Macrolide antibiotics exhibit strong antimicrobial activity against streptococci and atypical respiratory pathogens. Therefore, they are among the drugs that can be used for chemotherapy of infections caused by *Streptococcus pneumoniae* (1,2,4).

Macrolides and beta-lactams are commonly used for the treatment of pneumococcal infections (1-4). Since macrolides are largely prescribed for the empiric chemotherapy of community-acquired respiratory tract infections and may be useful in cases of intolerance to β-lactams as well as pneumococcal resistance to other antimicrobial agents, the macrolide resistance of this organism has increased rapidly worldwide (1-5). Macrolide resistance in pneumococci has been detected at variable rates in different epidemiological studies, and increasing resistance has been reported by several investigators (1,2,4).

In *S. pneumoniae*, macrolide resistance can be mediated by 2 mechanisms: ribosomal modification (6) or active drug efflux (7). Both resistance effectors are encoded by acquired determinants: the *erm*(B) gene encoding the ribosome-modifying enzyme and the *mef*(A) gene for the efflux system (6,7). The gene *erm*(B), which causes the modification of a specific adenine residue on the 23S rRNA, is dimethylated by rRNA methylases (6). The isolates with the *erm*(B) gene show high-level resistance to macrolides, lincosamides, and streptogramin B (MLSB-type resistance pattern), whereas the isolates carrying the m*ef*(A) gene only express resistance to 14- and 15-membered ring macrolides (M-type pattern resistance) by removing them from the cell (7). Macrolides are efficient at high doses in *mef*(A)-type resistant strains (3,4,8). In *S. pneumoniae* isolates, rates of resistance and genotypic distribution of resistance show geographic differences (9-12).

As the data related to the genotypic distribution and epidemiology of resistance in pneumococci in Turkey are limited, we aimed to determine the genotype of erythromycin-resistant *S. pneumoniae* isolates by the polymerase chain reaction (PCR) method and to define the minimum inhibitory concentration (MIC) values of macrolides and penicillin by the E-test method in *S. pneumoniae* isolates isolated from the southern Marmara region.

Materials and methods

Included in the study were 300 *S. pneumoniae* isolates isolated from culture samples taken from patients who were residents of the southern Marmara region of Turkey. Samples were collected in the bacteriology laboratory of the Microbiology and Infectious Diseases Department at the Uludağ University Faculty of Medicine over a 7-year period (1999 to 2005). Repeated isolates from the same individual were not included. Of the 300 samples, 73 were taken from sputum; 66 from endotracheal aspirates; 65 from blood; 19 from bronchoalveolar lavage; 18 from middle-ear aspirates; 10 from cerebrospinal fluid; 7 each from conjunctivas, acid fluid, and pleura fluid; 5 from wounds; 16 from nasopharyngeal aspirates; 2 each from joint fluids and vitreous; and 1 each from isolated operation material taken from cistern aspiration fluid and an abscess. The identification of isolates was performed according to standard procedures (13). All strains were stored at –80 °C in skim milk with glycerin at a volume of 500 µL in sterile microtubes (Cryobank Mixed, Mast Group Ltd., Merseyside, UK), applying 1 colony passage routinely.

The isolates that were used in this study were vitalized by performing 1 colony passage to Columbia agar with 5% sheep blood from storage microtubes. After the confirmation of identification of the vitalized isolates, in vitro susceptibility to erythromycin, clarithromycin, azithromycin, and penicillin were examined by E-test (AB Biodisk, Solna, Sweden) on Mueller-Hinton agar supplemented with 5% sheep blood. Isolates were incubated with 5% CO_2 at 35 ±2 °C for 20-24 h before reading the MICs (14).

S. pneumoniae ATCC 49619 was used as a quality control organism for susceptibility tests.

Of the 300 pneumococcal isolates, 34 (11.4 %) were found to be resistant to erythromycin (≥0.5 μg/mL) as a result of the E-test and were further investigated for macrolide resistance mechanisms. For PCR, all strains kept in incubation in Columbia agar with 5% sheep blood for a period of 18-24 h were used. From each isolate, 5-6 colonies were taken and suspended in a microcentrifuge tube containing 500 μL of RNAse- and DNAse-free distilled water, and then suspensions were frozen and kept at –80 °C until studied. The samples that dissolved thoroughly were boiled at 99 °C for 10 min in a thermocycler (T3 thermocycler, Biometra, Germany). After centrifugation for 3 min at 11,000 × *g* in a refrigerated centrifuge (HERMLE Z233 MK-2, ALYS Labware, Lausanne, Switzerland), 10 μL of the supernatant was used in the PCR studies as a DNA source. The amplification was performed in a T3 thermocycler (Biometra). The denaturation phase of the amplification was done at 94 °C for 15 s, the annealing phase at 52 °C for 15 s, the extension phase at 72 °C for 15 s (37 cycles), and the postextension phase at 72 °C for 7 min (15-17). PCR was carried out with primers *erm*(B) 1, (5'-GAA AAG GTA CTC AAC CAA ATA-3'); *erm*(B) 2, (5'-AGT AAC GGT ACT TAA ATT GTT TAC-3'); *mef*(A) 1, (5'-AGT ATC ATT AAT CAC TAG TGC-3'); and *mef*(A) 2, (5'-TTC TTC TGG TAC TAA AAG TGG-3'), which were designed on the basis of previously published sequences (18).

S. pneumoniae ATCC 49619, ATCC 700673, and ATCC 51919 strains were used as negative, *erm*(B)-positive, and *mef*(A)-positive controls, respectively.

The Pearson chi-square test was employed for statistical analysis.

Results

Of the 300 pneumococcal isolates evaluated, 34 (11.4%) were found to be resistant to erythromycin, azithromycin, and clarithromycin. For 23% of isolates, the penicillin MIC value was 0.12-1 μg/mL, whereas the MIC value was ≥2 μg/mL in only 2% (Table 1). When the strains grouped and evaluated as isolates from sterile fluids and respiratory tracts were compared, it was found that the MIC_{90} values of macrolide-group antibiotics in the strains isolated from the respiratory tract samples (erythromycin and azithromycin, 4 μg/mL; clarithromycin, 2 μg/mL) and the macrolide resistance rates (11.4%) were higher than those of the strains isolated from sterile fluids (6.4%) (Table 1). However, these rate differences were not statistically significant ($P > 0.05$). On the other hand, when the strains with a penicillin MIC of ≤0.06 μg/mL were compared to those with a penicillin MIC of ≥0.12 μg/mL, the macrolide resistance rate was seen to increase from 4.5% to 32% (Tables 2 and 3). The difference in macrolide resistance rates between the strains susceptible to and resistant to penicillin was noted to be statistically significant ($P < 0.001$).

Of the 300 strains, 34 (11.4%) were studied by the E-test method and found to be macrolide resistant. Genotypic evaluation of these strains demonstrated that 20 (58.8%) were *erm*(B)-positive, 13 (38.2%) were *mef*(A)-positive, and 1 (3%) was positive for both *erm*(B) and *mef*(A).

When comparing *mef*(A)-positive strains with *erm*(B)-positive strains, it was observed that the number of strains with a penicillin MIC value of ≥0.12 μg/mL in the group of *erm*(B)-positive strains (n = 15) was much greater than the number of *mef*(A)-positive strains (n = 8). Additionally, the MIC_{50} and MIC_{90} values of penicillin were higher in *erm*(B)-positive strains than *mef*(A)-positive strains. While the MIC values of macrolide-group antibiotics were determined to be at lower levels in *mef*(A)-positive strains (n = 13), MIC values of macrolide-group antibiotics were observed to be high in *erm*(B)-positive strains (n = 20). In *erm*(B)-positive strains, the rate of strains with a penicillin MIC of ≥0.12 μg/mL was 75%; it was 61.5% in the *mef*(A)-positive strains (Tables 4 and 5). In both the *erm*(B)- and *mef*(A)-positive strains, MIC values were 256 μg/mL for macrolide and 2 μg/mL for penicillin.

Table 1. The MIC_{50} and MIC_{90} values and resistance rates of macrolides and penicillin in *S. pneumoniae* strains isolated from clinical samples.

Antibiotic	MIC_{50} (μg/mL)	MIC_{90} (μg/mL)	Susceptible		Intermediate		Resistant	
All strains (n = 300)			n	(%)	n	(%)	n	(%)
Erythromycin	0.023	3	266	(88.6)	0		34	(11.4)
Azithromycin	0.064	3	266	(88.6)	0		34	(11.4)
Clarithromycin	0.023	2	266	(88.6)	0		34	(11.4)
Penicillin (before 2008)	0.023	0.75	224	(74.6)	70	(23.3)	6	(2.1)
Penicillin parenteral (non-*menengitidis*)	0.023	0.75	300	(100)	0		0	
Penicillin oral	0.023	0.75	223	(74.3)	71	(23.6)	6	(2.1)
Respiratory tract samples (n = 158)								
Erythromycin	0.023	4	140	(88.6)	0		18	(11.4)
Azithromycin	0.064	4	140	(88.6)	0		18	(11.4)
Clarithromycin	0.023	2	140	(88.6)	0		18	(11.4)
Penicillin (before 2008)	0.023	0.5	116	(73.4)	39	(24.6)	3	(2)
Penicillin parenteral (non-*menengitidis*)	0.023	0.5	158	(100)	0		0	
Penicillin oral	0.023	0.5	114	(72.2)	41	(25.9)	3	(1.9)
Sterile fluids (n = 94)								
Erythromycin	0.023	0.047	88	(93.6)	0		6	(6.4)
Azithromycin	0.047	0.25	88	(93.6)	0		6	(6.4)
Clarithromycin	0.016	0.047	88	(93.6)	0		6	(6.4)
Penicillin (before 2008)	0.023	0.5	70	(74.5)	23	(24.5)	1	(1)
Penicillin parenteral (*menengitidis*)	0.023	0.5	72	(76.6)	0		22	(23.4)
Penicillin parenteral (non-*menengitidis*)	0.023	0.5	94	(100)	0		0	

Table 2. The MIC_{50} and MIC_{90} values and resistance rates in *S. pneumoniae* strains in which penicillin MIC values were ≤0.06 μg/mL.

Antibiotic (n = 224)	MIC_{50} (μg/mL)	MIC_{90} (μg/mL)	Susceptible		Intermediate		Resistant	
			n	(%)	n	(%)	n	(%)
Erythromycin	0.023	0.047	214	(95.5)			10	(4.5)
Azithromycin	0.064	0.19	214	(95.5)			10	(4.5)
Clarithromycin	0.032	0.047	214	(95.5)			10	(4.5)

Table 3. MIC_{50} and MIC_{90} values and resistance rates in *S. pneumoniae* strains in which penicillin MIC values were ≥0.12 μg/mL.

Antibiotic (n = 76)	MIC_{50} (μg/mL)	MIC_{90} (μg/mL)	Susceptible		Intermediate		Resistant	
			n	(%)	n	(%)	n	(%)
Erythromycin	0.032	256	52	(68)			24	(32)
Azithromycin	0.094	256	52	(68)			24	(32)
Clarithromycin	0.032	256	52	(68)			24	(32)

Table 4. MIC_{50} and MIC_{90} values and resistance rates of antibiotics in *S. pneumoniae* strains in which *erm*(B) genotypes were determined.

Antibiotic (n = 20)	MIC range (μg/mL)	MIC50 (μg/mL)	MIC90 (μg/mL)	Susceptible		Intermediate		Resistant	
				n	(%)	n	(%)	n	(%)
Erythromycin	96-256	256	256					20	(100)
Azithromycin	192-256	256	256					20	(100)
Clarithromycin	64-256	256	256					20	(100)
Penicillin (before 2008)	0.012-2	0.5	1.5	5	(25)	13	(65)	2	(10)
Penicillin parenteral (non-*menengitidis*)	0.012-2	0.5	1.5	20	100)	0		0	
Penicillin oral	0.012-2	0.5	1.5	5	(25)	13	(65)	2	(10)

Table 5. MIC ranges, MIC_{50} and MIC_{90} values, and resistance rates of antibiotics in *S. pneumoniae* strains in which *mef*(A) genotypes were determined.

Antibiotic (n = 13)	MIC range (μg/mL)	MIC_{50} (μg/mL)	MIC_{90} (μg/mL)	Susceptible		Intermediate		Resistant	
				n	(%)	n	(%)	n	(%)
Erythromycin	2-256	4	24					13	(100)
Azithromycin	3-256	4	24					13	(100)
Clarithromycin	2-256	3	12					13	(100)
Penicillin (before 2008)	0.008-2	1	2	5	(38.5)	6	(46)	2	(15.5)
Penicillin parenteral (non-*menengitidis*)	0.008-2	1	2	13	(100)	0		0	
Penicillin oral	0.008-2	1	2	5	(38.5)	6	(46)	2	(15.5)

Discussion

The rate of macrolide resistance in *S. pneumoniae* ranges from 4% to 70% in worldwide surveillance studies (5). In a study titled "Prospective Resistant Organism Tracking and Epidemiology for the Ketolide Telithromycin" (PROTEKT), of 3362 pneumococcal isolates collected from 69 centers in 25 countries, including Turkey, macrolide resistance was determined at rates of 57.6%, 42.9%, and 28.6% in the western European countries of France, Italy, and Spain, respectively. Slightly lower rates of resistance were determined in Germany (15.7%), England (13.2%), and Sweden (4.7%). In the same study, the macrolide resistance rate of the pneumococcal strains from Turkey was 15.6% (10).

In our study, macrolide resistance (11.4%) was determined to be at a lower level than rates in the United States (30.9%), the Far East (71.4%-87.6%), and European countries such as France (57.6%), Italy (42.9%), and Spain (28.6%); it was higher, however, than the levels in Sweden (4.7%) and the Netherlands (7.8%). In many countries worldwide, macrolide and penicillin resistance rates are similar. For example, in the PROTEKT study conducted in the United States from 2000 to 2001, macrolide resistance was 13.4%, 20.8%, and 65.8% in *S. pneumoniae* strains with a penicillin MIC of ≤0.06 μg/mL, 0.12-1 μg/mL, and ≥2 μg/mL, respectively (19). Similarly, in the Alexander Project conducted from 1998 to 2000, macrolide resistance rates in strains with a penicillin MIC of ≤0.06 μg/mL, 0.12-1 μg/mL, and ≥2 μg/mL were determined to be 9.9%, 41.8%, and 66.8%, respectively (20). In our study, the rate of macrolide resistance was 4.5% (n = 10) in the strains with a penicillin MIC of ≤0.06 μg/mL, 27.1% (n = 19) in strains with a penicillin MIC of 0.12-1 μg/mL, and 83.4% (n = 5) in strains with a penicillin MIC of ≥2 μg/mL. In a study conducted by Özalp et al. in Turkey, the erythromycin resistance rate in *S. pneumoniae* was 5%, whereas the rates of intermediate and high levels of resistance to penicillin were 29.6% and 2%, respectively (12). A previous study from Turkey reported that, of 283 *S. pneumoniae* isolates, only 6 (2.1%) were resistant to azithromycin (11). In our study, the rate of erythromycin resistance in pneumococcal strains was 11.4%, whereas resistance rates with penicillin MIC values of 0.12-1 μg/mL and ≥2 μg/mL were 23% and 2%, respectively. Several studies from different regions of Turkey reported that the erythromycin resistance rate was between 13.6% and 26.4% (21-23).

Among isolates of *S. pneumonia*, 2 principal mechanisms of erythromycin resistance are known to exist: a ribosomal methylase encoded by *erm*(B) and an efflux pump encoded by *mef*(A) (6,7). In all genotypic analysis studies, the *erm*(B) genotype was determined to be widespread in the Far East and in European countries such as Italy and Spain, where macrolide resistance is higher; the *mef*(A) genotype was widespread in North America (4). Likewise, the rate of *erm*(B) genotype-positive strains was higher in our study. The PROTEKT study conducted from 1999 to 2000 in 25 countries, including Turkey, reported the genotypic distribution results of 1043 macrolide-resistant *S. pneumoniae* isolates as follows: 56.2%, *erm*(B); 35.3%, *mef*(A); 6.8% for both *mef*(A) and *erm*(B); 0.2%, *erm*(A) subclass *erm*(TR); and 1.5% negative for all mechanisms tested (9). The same study noted that the mechanisms of macrolide resistance showed geographical variability, and the *mef*(A) genotype prevailed in countries such as Sweden, Germany, Hong Kong, and the United States; the *erm*(B) genotype prevailed in other European countries, Turkey, and in the Far East in countries such as South Korea and Japan. It was also reported that *erm*(B) and *mef*(A) genotypes coexist at a high rate (38.3%) in South Korea. In our study, it was observed that the rates of *erm*(B), *mef*(A), and both genotypes together were 58.8%, 38.2%, and 3%, respectively. Similarly, previous studies reported from Turkey demonstrated that the genotype leading to macrolide resistance is usually *erm*(B) (21-23).

In conclusion, in an era in which treatment protocols are prepared according to profiles of resistance, macrolide resistance rates have been gradually increasing. Based on the data from our study, it may be suggested that, in Turkey, macrolide resistance and *erm*(B) genotype rates are not higher

than those in other countries. Including macrolide-group antibiotics in treatment protocols should be suitable because of the lower resistance rate. In Turkey, data collected and obtained from multicenter studies are needed for follow-up about resistance to penicillin, macrolides, and other antibiotics in *S. pneumoniae* strains, using suitable methods.

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