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Phenotypes and Genotypes of Macrolide-Resistant *Streptococcus Pneumoniae*

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Background: Macrolide resistance in Streptococcus pneumoniae *(S. pneumoniae)* is a worldwide problem.
Aims: The aim of this work was to analyze the phenotypes, genotypes, and clonal relatedness among macrolide-resistant *S. pneumoniae* strains isolated from various clinical specimens in our hospital.
Study Design: Cross-sectional study.
Methods: 80 non-duplicate *S. pneumoniae* strains were analyzed by polymerase chain reaction for both the *erm (B)* and *mef (A)* genes.
Results: Macrolide resistance was observed in 22.5% (18 strains) of strains. Two (11.2%) isolates possessed *mef (A)*, eight possessed *erm (B)* (44.4%) and eight strains (44.4%) were positive for both *erm (B)* and *mef (A)* genes. Although BOX-PCR of 18 macrolide-resistant strains revealed 11 band patterns, they clustered as seven clones with a genetic distance >10% to each other. Eight isolates possessed both *erm (B)* and *mef (A)* genes and belonged to a single clone (44.44% of all macrolide-resistant strains).
Conclusion: Increased positivity rates for both resistance genes have also been reported from other hospitals in Turkey, but this is the first study from Turkey showing the clonal dissemination of both resistance genes.
Keywords: erm (B), BOX-PCR, Macrolide resistance, mef (A), *Streptococcus pneumoniae*

Streptococcus pneumoniae (S. pneumoniae) is a major pathogen of bacterial pneumonia, meningitis, sinusitis and otitis media. Antimicrobial resistance in *S. pneumoniae*, including macrolide resistance, has been a growing problem in recent years due to the increasing numbers of cases with treatment failures of infections caused by macrolide-resistant pneumococci (1).

Macrolide resistance in *S. pneumoniae* is mediated by: the *erm* (B) gene encodes methylation of the ribosomal macrolide target sites and *mef* (A) encodes the drug efflux. Deterioration of riboproteins L4 and L22 and mutations in the 23S rRNA genes are involved in the other less common mechanisms (2).

In this study, we analyzed the distribution of the phenotypes, genotypes, and clonal relatedness of macrolide-resistant *S. pneumoniae* strains isolated in our hospital.

MATERIALS AND METHODS

Eighty *S. pneumoniae* strains were collected between January 2008 and September 2009 in the Department of Microbiology of the Cerrahpaşa Faculty of Medicine. Ethics committee approval was received for this study from the Ethics Committee of Cerrahpaşa Faculty of Medicine. Strains were isolated mostly from sputum. The remaining isolates were from tracheal aspirates, bronchoalveolar lavage fluid, nasal swab and blood culture.

S. pneumoniae isolates were identified based on conventional microbiological methods. Antibiotic susceptibility testing was performed with the disc diffusion method (3).

Macrolide resistance phenotypes were determined by Montanari et al. (4) method in 2001.

This study was presented as a poster at the 34th Congress of Turkish Microbiology Society, 7-11 November 2010, Kyrenia, Cyprus and 10th International Meeting on Microbial Epidemiological Markers, 2-5 October 2013, Paris, France.

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MICs of macrolides (erythromycin, clarithromycin and azithromycin), lincosamides (clindamycin), streptogramins (quinupristin-dalfopristin) and penicillin G were measured by E-test (AB Biodisk; Solna, Sweden). The strain *S. pneumoniae* ATCC 49619 was used as a control strain.

Detection of erythromycin-resistance genes

A PCR method was used for the detection of erythromycin-resistance genes (5). Bacterial DNA was isolated using a DNA isolation kit (Roche Diagnostic; Mannheim, Germany). All PCR mixtures contain a volume of 25 µL with 5µL of target DNA, 0.5 µL of each primer (25 mM), 1.5 µL of 25 mM $MgCl_2$, 0.5 µL of dNTP mixture (25 mM each), 0.125 µL of Taq DNA polymerase (5 U/µL) and 2.5 µL of 10X buffer (MBI; Fermentas, Lithuania). PCR for both genes was performed on PTC-200 (Peltier Thermal Cycler; MJ Research, USA). Cycling conditions for amplification were: 5 min at 94°C, followed by 40 cycles of 1 min at 94°C, 1 min at 48°C and 1.5 min at 72°C, with a final 10 min incubation at 72°C. PCR products were separated on ethidium bromide stained 1.5% agarose gels. The expected sizes of amplification products for *erm* (B) and *mef* (A) were 639 bp and 348 bp, respectively.

The positive control strains were ATCC 700673 (Hungary^{19A}-6) for *erm* (B) and ATCC 51916 (Tennessee^{23F}-4) for *mef* (A).

BOX-PCR

DNA amplifications for BOX-PCR were performed in 25 µl final volumes with 5 µL of purified bacterial DNA, 1.5 µL of primer AR1 (CTACGGCAAGGCGACGCTGACG) (25 mM), 1.5 µL of $MgCl_2$ (25 mM), 0.75 µLof dNTPs (25 mM), 0.125 µL of Taq DNA polymerase (5 U/µL) and 2.5 mL of 10X reaction buffer (6).

After the initial denaturation at 95°C for 5 min, this step was followed by 30 cycles of 1 min at 90°C, 1 min at 52°C, and 2 min at 72°C, with a final extension at 72°C for 5 min (7). Amplification products were run on 1.5% agarose gels and detected by staining with ethidium bromide. The band patterns were converted to series of 1s and 0s where 1s indicates the presence and 0s the absence of any band.

The analysis of BOX-PCR results and the phylogenetic analysis were performed using FreeTree and TreeView software packages (8, 9).

RESULTS

Of the total 80 isolates, 22.5% (18/80) were resistant to erythromycin, azithromycin and clarithromycin. Twenty percent of strains were clindamycin-resistant and four (5%) strains had quinupristin-dalfopristin intermediate resistance. According to Clinical Laboratory Standards Institute (CLSI) criteria, penicillin resistance in *S. pneumoniae* isolates was found to be 7.5% (6/80) and the intermediate resistance rate was 20% (16/80) for oral penicillin criteria. Penicillin intermediate resistance was found to be 2.5% (2/80) for non-meningitis parenteral penicillin criteria. No meningeal isolate was included in the study. Tetracycline, levofloxacin and trimethoprim-sulphomethoxazole resistance rates were as follows: 18.75%, 1.25% and 62.5%, respectively.

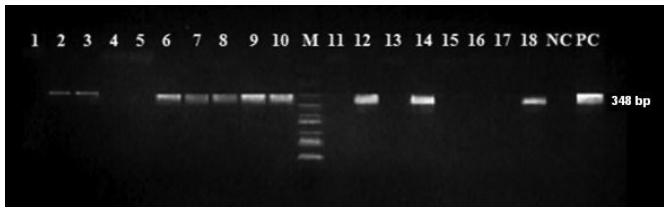

FIG. 1. PCR results for *mef* (A)/(E) genes. Lanes 1, 4, 5, 11, 13, 15, 16, 17 negative strains; Lanes 2, 3, 6, 7, 8, 9, 10, 12, 14, 18 positive strains; NC, Negative control; PC, Positive control; M, DNA size marker

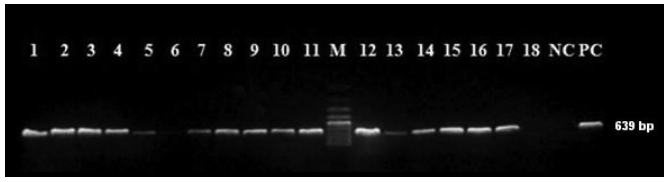

FIG. 2. PCR results for *erm* (B) genes. Lanes 1, 2, 3, 4, 5, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17 positive strains; Lanes 6 and 18 negative strains; NC, Negative control; PC, Positive control; M, DNA size marker

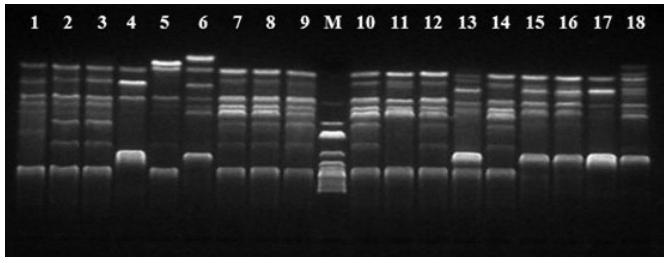

FIG. 3. BOX-PCR results of erythromycin-resistant *S. pneumoniae* isolates. The numbers above the lanes correspond to the strain numbers. M, DNA size marker

According to the erythromycin-clindamycin double-disk test, 16 (88.8%) of the 18 test strains had the $cMLS_B$ phenotype and two (11.2%) strains possessed the M phenotype.

PCR of the 18 macrolide-resistant *S. pneumoniae* strains showed that 44.4% (n=8) strains harbored only the *erm* (B) gene and 11.2% (n=2) only the *mef* (A) gene. Eight (44.4%) strains were positive for both the *erm* (B) and *mef* (A) genes (Figure 1 and 2). All *erm* (B) and *erm* (B)+*mef* (A) positive strains were resistant to erythromycin, clarithromycin, azithromycin and clindamycin, while *mef* (A) positive strains had lower erythromycin, clarithromycin and azithromycin MICs and remained susceptible to clindamycin (Table 1).

TABLE 1. Genotypic and phenotypic data of 18 macrolide resistant *S. pneumoniae* isolates

			MIC (µg/mL)					
Number of isolates	Phenotype	Genotype	E	Cd	C	A	P	Q/D
1	cMLSB	*erm* (B)	>256	>256	>256	>256	0.016	0.5
2	cMLSB	*erm* (B)+*mef* (A)/(E)	>256	>256	>256	>256	0.5	2
3	cMLSB	*erm* (B)+*mef* (A)/(E)	>256	>256	>256	>256	1	0.5
4	cMLSB	*erm* (B)	>256	>256	>256	>256	1	0.75
5	cMLSB	*erm* (B)	>256	>256	>256	>256	1	1
6	iMLSB+M	*mef* (A)/(E)	4	0,125	6	24	0.75	0.38
7	cMLSB	*erm* (B)+*mef* (A)/(E)	>256	>256	>256	>256	1	1.5
8	cMLSB	*erm* (B)+*mef* (A)/(E)	>256	>256	>256	>256	1	2
9	cMLSB	*erm*(B)+*mef* (A)/(E)	>256	>256	>256	>256	1	0.75
10	cMLSB	*erm* (B)+*mef* (A)/(E)	>256	>256	>256	>256	2	1.5
11	cMLSB	*erm* (B)	>256	>256	>256	>256	0.23	2
12	cMLSB	*erm* (B)+*mef* (A)/(E)	>256	>256	>256	>256	2	2
13	cMLSB	*erm* (B)	>256	>256	>256	>256	1	1
14	cMLSB	*erm* (B)+*mef* (A)/(E)	>256	>256	>256	>256	1.5	1.5
15	cMLSB	*erm* (B)	16	>256	6	>256	0.016	0.5
16	cMLSB	*erm* (B)	>256	>256	>256	>256	1	0.75
17	cMLSB	*erm* (B)	>256	>256	>256	>256	2	0.75
18	M	*mef* (A)/(E)	24	0,094	24	>256	1.5	0.38

E: erythromycin; Cd: clindamycin; C: clarithromycin; A: azithromycin; P: penicillin; Q/D: quinupristin/dalfopristin

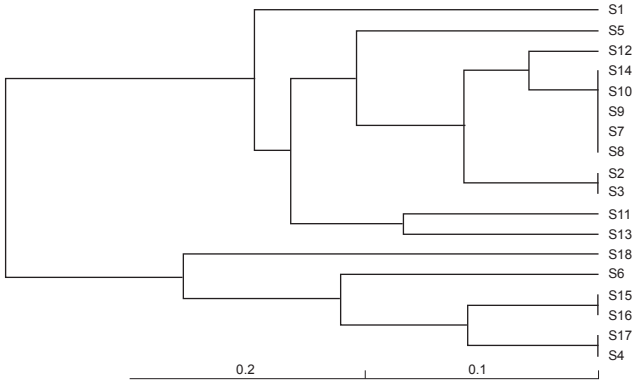

FIG. 4. Dendrogram depicting the phylogenetic relatedness of macrolide-resistant *S. pneumoniae* containing *ermB* and/or *mefA* genes (by FreeTree program, UPGMA cluster analysis with Nei and Li/Dice algorithm)

BOX-PCR

Through DNA fingerprint analysis by BOX-PCR, seven clones were identified among 18 macrolide-resistant strains. All of the *erm* (B) and *mef* (A) positive isolates were shown to belong to a single clone. The eight strains carrying *erm* (B) were grouped into four clones. Two *mef* (A) carrying strains were unrelated (Figure 3 and 4).

Among erythromycin-susceptible *S. pneumoniae* strains, the penicillin resistance rate was very low (5 isolates were intermediate and only one strain was fully resistant). In contrast, only two of the 18 erythromycin-resistant isolates were penicillin susceptible. All *erm* (B) and *mef* (A) positive strains were penicillin-resistant according to oral criteria; two of these strains showed intermediate resistance according to non-meningitis parenteral penicillin criteria. Three of the four quinupristin-dalfopristin intermediate resistance isolates were positive for *erm* (B)+*mef* (A) genes.

DISCUSSION

Macrolide resistance rates among pneumococci have increased over the last two decades. Reported resistance rates were high among strains isolated in the Far East (80%) and South Africa (54%). Low rates were reported from South America (15%), Australia (18%) and Northern Europe (18%). In Europe, the highest resistance rate was reported in France and Greece (10). In Turkey, macrolide resistance among *S. pneumoniae* has increased from 7% to 29% (11-15). Although Telli et al. (16) reported higher percentages (40%) in 2007-2009, the macrolide resistance rate in this study was 22.5%, which is similar to the result of EARRSS, but higher than the rates reported previously in our hospital (13, 14).

The percentage of isolates positive for *erm*(B)+*mef*(A) genotype is increasing and this is a serious public health problem

(17). In Germany, Bley et al. (18) reported the presence of combined *erm* (B)+*mef* (A) genotype in 4.1% of pneumococcal isolates. Similar results have been increasingly reported in North America, South Asia and South Africa during the last decade (17, 19). In Russia, dual resistance genes among isolates collected between 2003 and 2005 were found in 30.3% (20). In China, McGee et al. (21) found both the *erm* and *mef* genes in 6% of isolates. In South Africa, the percentage of strains possessing the resistance genes *erm* and *mef* was 30.5% in 2001 and 30 (83%) of them belonged to a single, multi-resistant clone (19). In the USA, Di Persio et al. (22) reported that 71% of macrolide-resistant pneumococci possessed dual resistance genes. Among these strains, two predominant and possibly related clones were detected. A study in Arizona revealed that 23.6% of 592 clinical isolates were macrolide-resistant from 1999 to 2008. More than 50% of the macrolide-resistant population were *erm* (B)+*mef* (A) positive (23). In Canada, *erm* (B) and *mef* (A) positive isolates represent 4% in 1998 and 12% in 2004 and one major cluster containing 36 (72%) of the 50 isolates was described (24).

In Turkey, Şener et al. (25) studied 669 *S. pneumoniae* strains isolated in Ankara, Turkey, between 1994 and 2002. They found that 57 (62.6%) of the 91 erythromycin-resistant pneumococci had the $cMLS_B$ phenotype, 19 (20.9%) had the $iMLS_B$ phenotype and 15 (16.5%) had the M phenotype. Overall, 83.5% of isolates had the *erm* (B) and 16.5% the *mef* (A) genotype. Gulay et al. (15) analyzed a total of 151 randomly selected *S. pneumoniae* isolates from seven centers in Turkey between 1998 and 2002. The erythromycin resistance rate was 26.4%. Only one strain was found to possess both *erm* (B) and *mef* (A) genes (2.5%) and a clonal relationship could not be demonstrated by BOX-PCR. Sağıroğlu et al. (26) analyzed 50 erythromycin-resistant pneumococci in Istanbul; 86% had the $cMLS_B$ and 14% had the M phenotype. Overall, 42% of strains were *erm* (B) and *mef* (A) positive . Clonal dissemination wasn't studied. Telli et al. (15) analyzed 89 *S. pneumoniae* strains isolated between 2007 and 2009 in Aydın, Turkey; of these, 74%, 14% and 9% were the $cMLS_B$ phenotype, the M phenotype and the $iMLS_B$ phenotype, respectively. In addition, 20% were found to be *erm* (B)+*mef* (A) positive. No clonal dissemination was found according to PFGE analysis.

In conclusion, this study show that the macrolide resistance of *S. pneumoniae* isolates is relatively high and the $cMLS_B$ phenotype is the most prevalent resistance mechanism in our hospital. The percentage of isolates harboring both *erm* (B) and *mef* (A) genes is the highest reported from the studies conducted in our country to date. Dual *erm* (B) and *mef* (A) positive isolates tended to have higher resistance rates against penicillin and quinupristin-dalfopristin. In this study, we also showed the clonal relationship among macrolide-resistant *S. pneumoniae* strains possessing both *erm* (B) and *mef* (A) in Turkey for the first time.

Ethics Committee Approval: Ethics committee approval was received for this study from the ethics committee of İstanbul University Cerrahpaşa Faculty of Medicine.

Informed Consent: N/A.

Peer-review: Externally peer-reviewed.

Author contributions: Concept - N.G.; Design - N.G., K.M., L.S.; Supervision - N.G.; Resource -L.S., M.K.; Materials - L.S., M.K.; Data Collection&/or Processing - L.S.; Analysis&/or Interpretation - M.K., S.S.; Literature Search - L.S.; Writing -L.S., N.G.; Critical Reviews - N.G., K.M.

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