RESEARCH ARTICLE / ARAŞTIRMA

DOI: 10.4274/mjima.galenos.2021.2020.19
Mediterr J Infect Microb Antimicrob 2021;10:19
Erişim: http://dx.doi.org/10.4274/mjima.galenos.2021.2020.19

Association of Erythromycin Resistance with the *mefA* and *ermB* Genes among Clinical Isolates of *Streptococcus pneumoniae* in Tehran, Iran

İran, Tahran'daki *Streptococcus pneumoniae* Klinik İzolatları Arasında Eritromisin Direncinin *mefA* ve *ermB* Genleri ile İlişkilendirilmesi

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Abstract

Introduction: *Streptococcus pneumoniae* is one of the major pathogens responsible for respiratory tract infections, meningitis, and bacteremia. In most countries around the world, an increase in the emergence of macrolide-resistant *S. pneumoniae* isolates has become a serious problem. Therefore, this study was conducted to investigate the association of erythromycin resistance with the *mefA* and *ermB* genes among clinical isolates of *S. pneumoniae* in Tehran City (Tehran Province, Iran).

Materials and Methods: In this cross-sectional study, 62 erythromycin-resistant *S. pneumoniae (*ERSP) isolates were obtained from patients in four hospitals in Tehran city during 2013-2015. The macrolide resistance genes *mefA* and *ermB* were detected in ERSP isolates using polymerase chain reaction (PCR) assays. Furthermore, the copy number and expression level of the *mefA* gene were determined by real-time PCR.

Results: In the present study, the overall resistance to erythromycin in *S. pneumoniae* isolates was 84.93% (62 isolates out of 73). Either *mefA* or *ermB* was identified in 10 (16.13%) and 36 (58.06%) ERSP isolates, respectively, and 7 (11.29%) ERSP isolates harbored both genes. Comparison of the mean relative expression of the *mefA* gene in ERSP isolates with different erythromycin minimal inhibitory concentration (MIC) values indicated that the expression level was increased approximately twofold for each doubling of the MIC value.

Conclusion: Our findings highlighted the dominance of ribosomal methylation encoded by *ermB* as the most common mechanism of macrolide resistance in clinical *S. pneumoniae* isolates from Iran. Furthermore, in the present study, the expression levels of the *mefA* gene showed a significant increase in the ERSP isolates with higher erythromycin MIC values.

Keywords: *Streptococcus pneumoniae*, erythromycin resistance, resistance genes, polymerase chain reaction, Iran

Öz

Giriş: *Streptococcus pneumoniae*, solunum yolu enfeksiyonları, menenjit ve bakteremiden sorumlu başlıca patojenlerden biridir. Dünya ülkelerinin çoğunda makrolide dirençli *S. pneumoniae* izolatlarının ortaya çıkışındaki artış ciddi bir sorun haline gelmiştir. Bu nedenle, bu çalışma Tahran'daki (Tahran Şehri, İran) *S. pneumoniae'*nin klinik izolatları arasında eritromisin direncinin *mefA* ve *ermB* genleri ile ilişkisini araştırmak için yapılmıştır.

Cite this article as: Fallah F, Tabatabaei SR, Yousefi M, Hashemi A, Nazari-Alam A, Saadat A. Association of Erythromycin Resistance with the *mefA* and *ermB* Genes among Clinical Isolates of *Streptococcus pneumoniae* in Tehran, Iran. Mediterr J Infect Microb Antimicrob. 2021;10:19.

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Received/Geliş Tarihi: 28.10.2020 **Accepted/Kabul Tarihi:** 19.03.2021 **ORCID ID:** orcid.org/0000-0003-4770-5836

Published: 25 March 2021

Gereç ve Yöntem: Bu kesitsel çalışmada, 2013-2015 yılları arasında Tahran'daki dört hastanedeki hastalardan 62 eritromisine dirençli *S. pneumoniae* izolatı elde edilmiştir. Makrolid direnç genlerinden *mefA* ve *ermB*, eritromisine dirençli *S. pneumoniae* (ERSP) izolatlarında polimeraz zincir reaksiyonu (PCR) testlerini kullanarak tespit edilmiştir. Ayrıca *mefA* geninin kopya sayıları ve ekspresyon düzeyi real-time PCR yöntemi ile belirlenmiştir.
Bulgular: Bu çalışmada, *S. pneumoniae* izolatlarında eritromisine genel direnç %84,93 (73 izolattan 62'si) olarak bulunmuştur. Sonuçlara göre *mefA* ve *ermB* genlerinden biri sırasıyla 10 (%16,13) ve 36 (%58,06) ERSP izolatında tanımlandı. Yedi (%11,29) ERSP izolatı hem *mefA* hem de *ermB* genlerini barındırdı. Farklı eritromisin minimal inhibitör konsantrasyon (MIC) değerlerine sahip ERSP izolatlarında *mefA* geninin ortalama nispi ekspresyonunun karşılaştırılması; ekspresyon seviyelerinin, MIC değerlerinin her iki katına çıkması için yaklaşık iki kat arttığını gösterdi.
Sonuç: Bulgularımız, İran'daki klinik *S. pneumoniae* izolatlarında makrolid direncinin en yaygın mekanizması olarak *ermB* tarafından kodlanan ribozomal metilasyonun baskınlığını vurguladı. Ayrıca, bu çalışmada *mefA* geninin ekspresyon seviyeleri, daha yüksek eritromisin MIC değerlerine sahip ERSP izolatlarında anlamlı bir artış göstermiştir.
Anahtar Kelimeler: *Streptococcus pneumoniae*, eritromisin direnci, direnç genleri, polimeraz zincir reaksiyonu, İran

Introduction

Streptococcus pneumoniae (*Pneumococcus*) is an ordinary resident of the human upper respiratory tract, but it may cause a wide variety of infections in children and adults, ranging from pneumonia, meningitis, otitis media, septicemia, sinusitis, and septic arthritis to relatively benign soft-tissue infections[1-3].

Macrolides are commonly used as first-line agents for the empirical treatment of community-acquired pneumonia (CAP), and their combination with a beta-lactam is recommended for severe pneumonia. However, treatment of pneumococcal infections has become challenging due to the ever-increasing emergence of macrolide-resistant *S. pneumoniae*[4-6].

Resistance of *S. pneumoniae* isolates to macrolides was identified in the late 1980s. Two main macrolide resistance mechanisms have been described in *S. pneumoniae*: target-site modification and active efflux of the drug out of the bacterium. Target-site modification is mediated by an rRNA-methylating enzyme and is coded by the *ermB* gene, resulting in coresistance to macrolide, lincosamide, and streptogramin B antibiotics (MLSB resistance)[7,8]. The macrolide efflux pump (M phenotype) is encoded by the *mefA* gene, and bacterial isolates with this phenotype are resistant to erythromycin, clarithromycin, and azithromycin. High-level macrolide resistance is commonly associated with the *ermB* gene, whereas the *mefA* gene usually results in a lower level of resistance[9,10]. It is noteworthy that the prevalence of the two main macrolide resistance mechanisms in pneumococci varies depending on the local epidemiology; thus, *ermB*-mediated ribosomal methylation commonly occurs in European countries, while macrolide-specific efflux pump-mediated resistance is clinically relevant in the USA[8,11].

Molecular epidemiological information on antibiotic resistance patterns and mechanisms of resistance can help to prevent and treat infectious diseases, such as multidrug-resistant *S. pneumoniae* infections[12,13]. Given that widespread distribution of macrolide and multidrug resistance in clinical *S. pneumoniae* isolates is a major concern, the need for extensive research is greater than ever. Accordingly, this study was performed to investigate the association of erythromycin resistance with the *mefA* and *ermB* genes among clinical isolates of *S. pneumoniae* in Tehran City, Iran.

Materials and Methods

Study Design and Bacterial Isolates

In this cross-sectional study, 62 non-duplicate erythromycin-resistant *S. pneumoniae* (ERSP) isolates were collected from patients in four hospitals in Tehran City from December 2015 to July 2017. The isolates were obtained from various clinical samples including cerebrospinal fluid (CSF), blood, sputum, eye swabs, nasal discharge, bronchoalveolar lavage (BAL), and other body fluids (brain abscesses, synovial fluid, throat swabs, and abdominal fluid). The study was approved by the Executive Board of the Shahid Beheshti University of Medical Sciences (Tehran Province, Iran). This study was a retrospective, laboratory based study; therefore, there was no need to obtain informed written consent from the patients.

The *S. pneumoniae* isolates were identified by conventional microbiological methods and biochemical testing (hemolysis, Gram staining, susceptibility to optochin disc [MAST, UK], and solubility in bile)[14]. Polymerase chain reaction (PCR) assays were used to confirm diagnosis of *S. pneumoniae* infection in isolates by detecting the *cpsA* target gene.

Erythromycin-Resistant *S. pneumoniae* Isolates

Erythromycin-resistant *S. pneumoniae* isolates [minimal inhibitory concentration (MIC) ≥1 µg/ml] were identified using the broth microdilution method according to the clinical and laboratory standards institute guidelines[15]. The MIC values were read visually as the lowest concentration of the antimicrobial agent that prevented visible growth. *S. pneumoniae* ATCC 49619 was used as a standard strain.

Detection of the *mefA* and *ermB* Genes

Polymerase chain reaction assays were used to detect the *mefA* and *ermB* genes in ERSP isolates with specific primers (Table 1).

The primers were designed in the present study using Primer-BLAST [National Center for Biotechnology Information (NCBI), USA] and primer3 software. Genomic DNA was extracted from pure cultures of the strains using the High Pure PCR Template Preparation Kit according to the manufacturer's instructions.

Polymerase chain reaction was conducted in a final volume of 25 µl using the HotStar Taq Master Mix kit containing 12.5 µl of 2× HotStar Taq Master Mix (containing 3 mM $MgCl_2$, 0.4 mM of each dNTP, and 0.08 U/µl Taq DNA polymerase in reaction buffer), 1 µl of the DNA template, 1 µl of each primer (20 pmol), and 9.5 µl of ddH2O. DNA amplification was performed in a thermocycler with an initial denaturation step at 95 °C for 5 minutes; 30 amplification cycles of 30 seconds at 95 °C, 30 seconds at different temperatures for different genes, and 40 seconds at 72 °C; followed by an additional extension step of 7 minutes at 72 °C. The amplified products were separated by electrophoresis through 1% agarose gel and visualized by RedSafe staining.

DNA sequencing of nine *S. pneumoniae* isolates randomly selected from each of the resistance genotypes (*mefA*, *ermB*, and *mefA*+*ermB*) was performed by Bioneer Corporation (South Korea), and the obtained sequence data were aligned using the BLAST program and compared with the GenBank database in the NCBIational CenterBiotechnology Information.

Expression Levels of the *mefA* Gene

The copy number and expression level of the *mefA* gene were determined in ERSP isolates by real-time PCR.

RNA extraction from the isolates was performed using the High Pure RNA Isolation Kit according to the manufacturer's instructions. The RNA was treated with DNase using DNase I to remove any remaining genomic DNA, and complete removal of contaminating DNA was confirmed by PCR. Reverse transcription was performed using the PrimeScript RT reagent Kit for 15 minutes at 37 °C, the enzyme was inactivated for 5 seconds at 85 °C, and then the reaction was cooled.

Synthesized complementary DNA was amplified by quantitative real-time PCR (qPCR) using a Rotor-Gene 6000™ Real-Time PCR System. Real-time PCR amplification was performed in a volume of 25 µl containing 12.5 µl of Power SYBR® Green PCR Master Mix, 1 µl of each primer (50 pmol/µl), 2 µl of cDNA, and 8.5 µl of DNase free ddH2O. The amplification conditions for real-time PCR assays were as follows: initial denaturation at 95 °C for 30 seconds, followed by 40 cycles of 95 °C for 7 seconds, 53 °C for 20 seconds, and 60 °C for 25 seconds. Triplicates of a negative control were included in all qPCR runs, and 16s rRNA was used as an endogenous control. The primers used in this study are described in Table 1. Amplification data were analyzed using the software.

Statistical Analysis

The data were entered and analyzed using SPSS statistical software. Frequencies and percentages were calculated, the Pearson chi-square test and analysis of variance were performed as appropriate, and p values of less than 0.05 were regarded as statistically significant.

Results

Erythromycin-Resistant *S. pneumoniae* Isolates

In the present study, 62 ERSP isolates (MIC ≥1 µg/ml) were collected from various clinical samples including CSF (13 isolates, 21%), blood (12 isolates, 19.4%), sputum (13 isolates, 21%), eye swabs (3 isolates, 4.8%), nasal discharge (2 isolates, 3.2%), BAL (5 isolates, 8.1%), and other body fluids (14 isolates, 22.6%). It is noteworthy that the overall prevalence of resistance to erythromycin in *S. pneumoniae* isolates was 84.93% (62 isolates out of 73).

The MIC values of erythromycin for the ERSP isolates in the susceptibility test were determined using the broth microdilution method as shown in Table 2.

Frequency of the *mefA* and *ermB* Genes

In this study, the *mefA* and *ermB* genes were detected in ERSP isolates by PCR assays. Either of the *mefA* and *ermB* genes were identified in 10 (16.13%) and 36 (58.06%) ERSP isolates, respectively. It is noteworthy that 7 (11.29%) isolates harbored

Table 1. Polymerase chain reaction (PCR) and real-time PCR primers used in this study

Gene	Primer sequence (5′-3′)	Annealing (°C)	Products sizes (bp)	Ref.
cpsA- F	GCAGTACAGCAGTTTGTTGAACTGACC	50	160	Tabatabaei et al.[5]
cpsA- R	GAATATTTTCATTATCAGTCCCAGTC			
ermB-F	CGACGAAACTGGCTAAAATA	58	331	This study
ermB-R	AATTGCTGAATCGAGACTTG			
mefA-F	GGTGTGCTAGTGGATCGTC	53	188	This study
mefA-R	GTAACCGCATTGAGAGCCG			
16s rRNA-RT-F	CTGTGGCTTAACCATAGTAG	55	91	Al-Yassari[16]
16s rRNA-RT-R	CTACGCATTTCACCGCTACA			

both genes simultaneously, and 9 (14.52%) isolates had neither of the genes (Table 3).

Finally, the nucleotide sequence data reported here have been submitted to the GenBank sequence database, and the accession numbers KU739787, KU739788, KU739789, and KU739790 for the *mefA* gene and KT758056 for the *ermB* gene have been assigned. Statistical analysis showed that there was no significant relationship between the presence of the *mefA* and *ermB* genes and the MIC values for erythromycin in clinical ERSP erythromycin-resistant *S. pneumoniae* isolates (p value = 0.616) (Table 3).

Expression Levels of the *mefA* Gene

Comparison of mean relative expression of the *mefA* gene in ERSP isolates with different erythromycin MIC values (ranging between 8 and 32 µg/ml) indicated that expression levels were increased by approximately twofold for each doubling of MIC values ($p<0.05$) (Figure 1).

Discussion

Macrolides are currently recommended as the mainstay of treatment for empirical therapy of CAP. However, the emergence of macrolide-resistant *S. pneumoniae* isolates has increased over the past decade, and clinical failure of macrolide treatment in pneumococcal infections has been reported in various parts of the world. Notably, studies have shown that macrolide resistance in pneumococcal infections has remained a serious problem in many Asian countries[11-13].

Target modification by a ribosomal methylase encoded by the *ermB* gene and drug efflux encoded by the *mef* gene are two major mechanisms mediating resistance to macrolides in *S. pneumoniae*. The macrolide-specific efflux mechanism is clinically relevant in macrolide-resistant pneumococci in North America, whereas *ermB*-mediated ribosomal methylation has been found in more than 80% of ERSP isolates in most European countries[11,17].

In the present study, 62 ERSP isolates (MIC≥1 µg/ml) were collected from various clinical samples. The distribution rates of the *mefA* and *ermB* genes in ERSP isolates were 16.13% and 58.06%, respectively. These results are consistent with many reports from Asian countries such as China, Taiwan, Sri Lanka, and Korea, as well as most other studies conducted in Iran, showing that ribosomal methylation encoded by *ermB* is the most common mechanism of resistance in ERSP isolates[11,18]. In contrast, in some studies from Hong Kong, Singapore, Thailand,

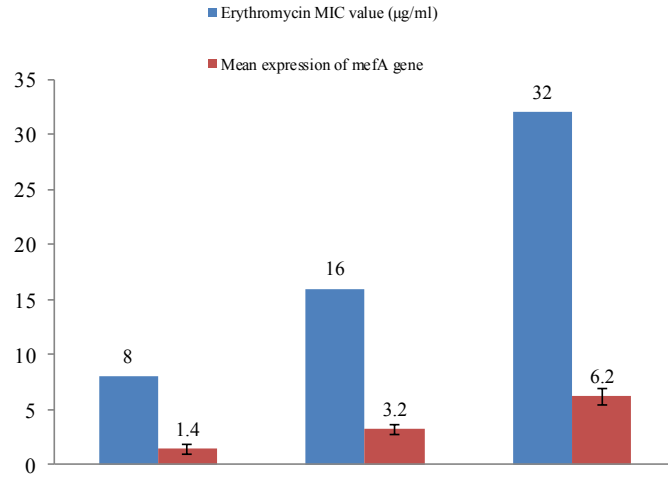

Figure 1. Mean relative expression of the *mefA* gene in clinical erythromycin-resistant *S. pneumoniae* isolates with different erythromycin MIC values ($p<0.05$). 16s rRNA was used as an endogenous control

MIC: Minimal inhibitory concentration

Table 2. MIC values of erythromycin for 62 clinical erythromycin-resistant *S. pneumoniae* isolates

ERSP isolates	MIC (µg/ml)					
	1	2	4	8	16	32
No. of isolates (%)	10 (16.13)	7 (11.29)	4 (6.45)	11 (17.74)	16 (25.81)	14 (22.58)

ERSP: Erythromycin-resistant *Streptococcus pneumoniae*, MIC: Minimal inhibitory concentration

Table 3. Relationship between the presence of the *mefA* and *ermB* genes with the MIC values for erythromycin in clinical erythromycin-resistant *S. pneumoniae* isolates

Erythromycin resistance genes	ERSP isolates		p value
	Number (%)	Erythromycin MICs (µg/ml) (mean±SD)	
ermB positive	36 (58.06)	12.13±11.41	0.616
mefA positive	10 (16.13)	15.6±9.69	
ermB+*mefA* positive	7 (11.29)	17.71±14.16	
ermB+*mefA* negative	9 (14.52)	12.33±12.61	

ERSP: Erythromycin-resistant *Streptococcus pneumoniae*, MIC: Minimal inhibitory concentration, SD: Standard deviation

and Malaysia, the macrolide-specific efflux encoded by *mefA* was more common in erythromycin-resistant isolates[11]. Overall, the dominance of each of the two major macrolide resistance mechanisms in *S. pneumoniae* isolates from Asian countries seems to differ on the basis of geographical area. Notably, in ERSP isolates lacking the *mefA* and *ermB* genes, other genes involved in macrolide resistance may have induced erythromycin resistance.

As mentioned earlier, active macrolide efflux is one of the major mechanisms of macrolide resistance in *S. pneumoniae* in many parts of the world. The use of relative real-time PCR assay allows for the characterization of macrolide-specific efflux gene expression in macrolide-resistant *S. pneumoniae* isolates[19,20]. In this study, the expression level of the *mefA* gene in ERSP isolates was determined by real-time PCR. A comparison of mean relative expression of the *mefA* gene in ERSP isolates with different erythromycin MIC values indicated that expression levels were increased approximately twofold for each doubling of the MIC value. Similar findings have also been reported in rare studies conducted elsewhere. Wierzbowski et al.[21] indicated that higher expression levels of the *mefE* gene were associated with higher MICs of erythromycin in ERSP isolates. Although these results highlight the association between increased expression of macrolide-specific efflux genes and higher levels of macrolide resistance in *S. pneumoniae*, there is an apparent need for further studies in this area. Considering the sample size limit, one of the limitations of this study was the lack of investigation of high-level erythromycin-resistant isolates (with MICs for erythromycin usually ≥256 µg/ml).

Conclusion

Our findings revealed a dominance of ribosomal methylation encoded by the *ermB* gene as the most common mechanism of macrolide resistance in clinical *S. pneumoniae* isolates from Iran. Furthermore, in the present study, increased expression of the *mefA* gene was associated with higher levels of macrolide resistance in macrolide-resistant *S. pneumoniae* isolates. Finally, it is suggested that further studies be conducted in order to investigate the role of macrolide-specific efflux pumps in increasing levels of macrolide resistance in *S. pneumoniae*.

Ethics

Ethics Committee Approval: The study was approved by the executive board of Shahid Beheshti University of Medical Sciences (decision date-number: 2015/10-28).

Informed Consent: Since this was a retrospective laboratory based study, informed consent was not received.

Peer-review: Externally and internally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: F.F., S.R.T., M.Y., A.H., A.N.A., A.S., Concept: F.F., S.R.T., M.Y., A.H., A.N.A., A.S., Design: F.F., S.R.T., M.Y., A.H., A.N.A., A.S., Data Collection or Processing: F.F., S.R.T., M.Y., A.H., A.N.A., A.S., Analysis or Interpretation: F.F., S.R.T., M.Y., A.H., A.N.A., A.S., Literature Search: F.F., S.R.T., M.Y., A.H., A.N.A., A.S., Writing: F.F., S.R.T., M.Y., A.H., A.N.A., A.S.

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

Financial Disclosure: This study was a part of the Ph.D. thesis of the first author and was supported and approved by Shahid Beheshti University of Medical Sciences (grant number: 420).

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