

Molecular detection and evaluation of resistance of *Mycoplasma Pneumoniae* to macrolide (Erythromycin) due to mutation in the 23S rRNA gene among Iranian Patients with respiratory infections

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Research note

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Abstract

Objective: *Mycoplasma pneumoniae* is a common cause of community-acquired pneumonia. The global increased resistance of *M. pneumoniae* strains to macrolide (ML) has become a worrisome health problem. The widespread use of these drugs has led to increased rate of reported ML-resistant *M. pneumoniae* (MRMP) throughout the world. Therefore, this study was aimed to evaluate the resistance of *M. pneumoniae* against erythromycin due to mutations in the *23S rRNA* gene of patients with respiratory infections in Iran.

Results: According to the findings of the present study, employing specific primers showed that 17 cases (17%) were positive for mycoplasma genus and 6 cases (6%) positive for *M. pneumoniae* species. Also, analysis of the sequence of *23S rRNA* gene, revealed that one of the samples had mutations at positions A2431G and G2491A. Measuring the minimum inhibitory concentration (MIC), revealed that all samples positive for *M. pneumoniae* with *23S rRNA* gene were sensitive to erythromycin, and no ML resistance was reported.

Introduction

Mycoplasma pneumoniae (*M. pneumoniae*) is one of the common causes of upper and lower respiratory tract infections, and is a major cause of community-acquired pneumonia (CAP) and atypical pneumonia which is responsible for mortality and morbidity among children and adults (1–4). Since *M. pneumoniae* lacks cell wall, the infection is primarily treated with macrolide antibiotics (MLs), tetracycline, and fluoroquinolones (5–7). Macrolides like erythromycin, azithromycin and clarithromycin are commonly considered as the first-line treatment of *M. pneumoniae* infections, because alternative antibiotics, such as tetracycline and fluoroquinolones have side effects (8, 9). In addition to antimicrobial activity against *M. pneumoniae*, MLs have anti-inflammatory effects against *M. pneumoniae* infections, and are pharmacologically inhibitors of cytokines, particularly IL-8 (10, 11). Because of limited therapeutic options, the global increase in resistance of *M. pneumoniae* to MLs is a major health concern worldwide. Moreover, the widespread application of these drugs has led to elevated rate of ML-resistant *M. pneumoniae* (MRMP) throughout the world (7, 12, 13). MRMP infection has been shown to cause prolonged treatment, persistent cough, and an elevated time of fever reduction compared with those infections sensitive to treatment (14, 15). Currently, the MRMP has been reported more than 90% in Asia and 26% in the Europe (7, 8, 16). Undoubtedly, the emergence of MRMP is a serious health threat due to the widespread use of macrolide antibiotics in the treatment of *M. pneumoniae* infections, especially in Asia in recent years. Thus, it has attracted the attention of many scientists (13). The mechanism of resistance of *M. pneumoniae* to ML antibiotics has been as a point mutation (nucleotide displacement) of domain V in certain positions in *23S rRNA* gene of MRMP (17, 18). In recent years, a number of previous studies, exposed strains of *M. pneumoniae* strains to macrolides, and proposed that all strains with resistance mutations displayed high levels of resistance to ML antibiotics in broth dilution experiments. After Specific Primer-Polymerase Chain Reaction (SSP-PCR) and analysis of the sequences of domain V of the 23S rRNA gene in some positions, such as A2063G, A2064G, and A2064C, a series of

A-to-G or A-to-G nucleotide displacements were identified. Therefore, resistance to MLs in *M. pneumoniae* can occur due to point mutations in the *23S rRNA* gene (10, 17, 19). Therefore, this study was aimed to evaluate the resistance of *M. pneumoniae* against erythromycin due to mutations in the *23S rRNA* gene of patients with respiratory infections in Iran.

Methods

In this study, 100 samples of throat swab were collected from patients with atypical pneumonia referring to Mostafa Khomeini and KhatamolAnbiah hospitals in Tehran province in 2018. For sampling, individuals diagnosed with clinical symptoms of respiratory infections by a lung specialist, such as weakness, lethargy, fatigue, persistent headache and dry cough, shortness of breath, sputum production, muscle pain, and no antibiotic use during the past month were targeted for recruitment. Samples were stored in transport pleuropneumonia-like organisms (PPLo) broth medium were obtained from Merck (Germany) to transfer them to the laboratory. In the laboratory, 1 ml of the transport medium was transferred following passing through the 0.45 µm filter in the main Glucose PPLo broth medium, containing 20% horse serum, 0.3% yeast extract, .3% meat extract, 0.33 ml penicillin, 0.5 ml polymyxin B, and 0.5 ml amphotericin B in 5–10% CO₂, and were then incubated at 35 °C for 3 weeks. In this study, the standard strain was *M. pneumoniae* (ATCC: 29342) which was developed from the Molecular Biology Research Center of Baqiyatallah University of Medical Sciences. The kit was obtained from Roche Co. (Germany) was used to extract DNA from the Glucose PPLo broth medium in which clinical specimens were cultured and incubated at 37 °C for 3 weeks in 5–10% CO₂. After extraction of DNA samples, specific primers were used to identify *Mycoplasma* genus (*16S rRNA* gene) and *M. pneumoniae* strain (*P1* gene) as shown in Table 1.

Table-1: primers sequences used in this study

Gene	Primer Sequence	Product Size (bp) Reference
<i>16S rRNA</i>	GP01 MGSO	F primer: 5' -ACTCCTACGGGAGGCAGCAGT-3' R primer : 5'
<i>P1</i>	MPPIF	TGCACCATCTGTCACTCTGTAAACCTC-3'
<i>23S rRNA</i>	MPPIR Mp-F1 Mp-R1	F primer: 5'- AAAGGAAGCTGACTCCGACA-3' R primer: 5'-TGGCCTTGCGCTACTAAGTT-3' Mp-F1: 5'- TAACTATAACGGTCCTAAGG - 3' Mp-R1: 5'- CGCTACAACCTGGAGCATAAGA - 3'

The PCR reaction was performed at final concentration of 25 µl including an initial denaturation at 94 °C for 5 min, 35 cycles comprised of denaturation at 94 °C for 35 seconds, primer binding to the target DNA at 56 °C for 40 seconds, elongation at 72 °C for 45 seconds, as well as a final elongation at 72° C for 5 minutes, according to the protocol, for *M. pneumoniae*, and also another PCR reaction at final concentration of 25 µl, including an initial denaturation at 94 °C for 4 min, 33 cycles comprised of denaturation at 94 °C for 45 seconds, primer binding to the target DNA at 55 °C for 45 seconds,

elongation at 72 °C for 50 seconds, as well as a final elongation at 72° C for 5 minutes, according to the protocol for the *23S rRNA* gene. The PCR products (ATCC: 29342) were finally electrophoresed. Finally, the PCR products positive for *23S rRNA* gene were sequenced to detect mutations of domain V in *23S rRNA* gene of MRMP, and sequences of *23S rRNA* gene were analyzed. In order to prepare samples positive for *P1* gene of *M. pneumoniae*, the MIC was carried out by micro-dilution of Glucose PPLO broth containing 25% horse serum, 0.3% yeast extract, and 0.3% meat extract in 96 wells of microplates, after preparation of antibiotic macrolide suspension (erythromycin). Then, the microplates prepared for microscopic specimens were incubated for 5–6 days at 37 °C. Next, the MIC for erythromycin was determined based on color changes of Glucose PPLO broth enriched with horse serum, yeast extract, and meat extract. Glucose PPLO broth is a phenol red (a PH indicator) medium and *M. pneumoniae* growth causes the color of the medium to change from original purple to yellow.

Results

The present study recruited 100 patients with respiratory infections, of whom 48 (48%) were males and 52 (52%) were females. The average age of the participants was 53.62 years, ranging from 17 years to 85 years. According to the findings of our study, 14 subjects (14%) had *M. pneumoniae* colonies grown on PPLO agar. Figure 1 (A-B) indicates samples tested with SSP-PCR with specific primers of *M. pneumoniae*. The length of *16S rRNA* gene fragment of Mycoplasma strain is about 713 bp. Of 100 examined samples obtained from electrophoresis, this fragment was observed in the standard strain in 17 samples (17%). Also, among specimens positive for PCR, the 450 bp DNA fragment of *P1* gene was found in our standard strain among 6 subjects using *P1*-specific primer of *M. pneumoniae*. According to the results of the PCR test, 6 cases (6%) were infected with *M. pneumoniae*. Figure 1 (C), illustrates the electrophoretic evaluation of *23S rRNA* in samples positive for *M. pneumoniae* infection. Using the *23S rRNA*-specific primer, the 793 bp fragment was observed in the standard (ATCC: 29342) strain in 6 samples.

Analysis of the *23S rRNA* gene sequence, revealed that only one of the samples had point mutation at A2431G and G2491A positions. Measuring the MIC in all samples positive for *M. pneumoniae* using broth micro-dilution method, showed that all specimens were sensitive to erythromycin. Moreover, no ML resistance was reported (Table 2). Therefore, mutations in domain V of *23S rRNA* gene at A2431G and G2491A were not associated with resistance of *M. pneumoniae* against erythromycin.

Table 2
MIC ranges ($\mu\text{g/ml}$) for Erythromycin antimicrobial against *M. pneumoniae*.

Clinical isolate	Antimicrobial Agent	MIC	Mutation
1	Erythromycin	0/0039	NO
2	Erythromycin	0/0039	Yes
3	Erythromycin	0/0156	NO
4	Erythromycin	0/0078	NO
5	Erythromycin	0/0039	NO
6	Erythromycin	0/0156	NO
Reference strain <i>ATCC: 29342</i>	Erythromycin	0.0039	NO
MIC range for all isolates	Erythromycin	0.0039– 0.0156	

Discussion

Pneumonia caused by *M. pneumoniae* is a self-limiting illness, occurring by direct damage of the lung airways and inflammatory response (22). Lucier et al. reported a number of ML-resistance *M. pneumoniae* each at different positions in the V domain of the *23S rRNA* gene (2063 and 2064) with displacement of A-G, which appeared following exposure to erythromycin (10, 17). The prevalence of MRMP follows a diverse pattern in different countries, which is attributed to the excessive use of macrolides and age of the patients (12). ML resistance is associated with a point mutation in domain V of *23S rRNA* gene and ribosomal proteins L22 and L14. Mutation affecting *23S rRNA* gene at A2063G position is the most common mutation, followed by A2064G position, which is mostly responsible for significant ML resistance in *M. pneumoniae* (10, 23). Results of the analysis of the sequence of *23S rRNA* gene showed that point mutations at A2431G and G2491A positions were reported only in one specimen. In addition, the broth microdilution MIC method revealed that all samples positive for *M. pneumoniae*, were sensitive to erythromycin, and no ML resistance was reported in specimens. The rate of MRMP among study participants was 0.6%, 0%. The prevalence of MRMP was reported as 2%, 50/1 in Switzerland, 1.5%, 87.1%, 202/176 in Japan, 97%, 33/32 in China, 1.2%, 167/2 in Germany, and 9.8%, 51/5 in France (7). Brown and colleagues conducted a study in England (2015), and reported mutations associated with ML resistance in samples positive for *M. pneumoniae*. They found that 43 samples of the total 60 specimens positive for *M. pneumoniae* had mutations in domain V of *23S rRNA* gene, with ML resistance in four specimens (9.3%), which was not consistent with the results of our study (23). Haruki and colleagues investigated hospitalized children in Japan (2014) and detected 33 cases of *M.*

pneumoniae by PCR, of which 31 isolates were resistant to macrolide, and all isolates displayed point mutation at 2063 and 2064 in domain V of *23S rRNA*, which was not in accordance with the present study. This difference in the results of these two studies may be due to the older age of the patients in our study (24). Liu and colleagues (2014) examined 580 samples of throat swab suspected for *M. pneumoniae*, and reported that 70 cases were resistant to erythromycin. They also revealed point mutation at 2063 and 2064 in domain V of 23SrRNA in all specimens positive for *M. pneumoniae*, which was not in accordance with our study (21). Zhou et al. (2015) also examined 650 samples of throat swab, and used PCR method. All sequences of the domain V of the 23SrRNA were investigated. They showed that 100 percent (71/71) of *M. pneumoniae* isolated from patients with (CAP) were resistant to erythromycin, clarithromycin, and azithromycin. In addition, all ML-resistant strains of *M. pneumoniae* had point mutation at A2063G in the domain V of *23S rRNA* (25), which was not consistent with the results of our study, and it can be attributed to the larger sample size of this study. This was one of the first studies conducted in Iran. Results of the analysis of the sequence of the *23S rRNA* gene, showed that only one strain displayed point mutations at A2431G and G2491A. The broth microdilution MIC showed that all specimens positive for *M. pneumoniae* were resistant to erythromycin, and no ML resistance was observed. Besides, all point mutations affecting domain V of *23S rRNA* at A2431G and G2491A were not associated with ML resistance to erythromycin. In conclusion, in order to control the emergence of ML resistance among *M. pneumoniae* strains, the use of these antibiotics should be restricted in Iran and reduce the widespread use.

Limitations

Non-application and evaluation of other macrolide group antibiotic resistance in the study can be mentioned as one of the main limitations of the present study.

Declarations

Ethics approval and consent to participate

Administrative or ethical permissions were required to use the patient swabs. (IR.SHAHED.REC.1397. 039). The study protocol was approved by the Department of Medical Microbiology, Faculty of Medicine, Shahed University, Tehran, Iran The study used bacteria isolated from clinical samples in the clinical microbiology laboratory.

Consent for publication

Not applicable

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

MN, RM, conceived, designed and supervised the study and MI revised the manuscript; SR, MH, and MI: collected and analyzed the data and drafted the manuscript. All authors read and approved the final manuscript.

Abbreviations

ML:macrolide, PPLO: pleuropneumonia-like organisms; PCR: polymerase chain reaction; MIC:minimum inhibitory concentration, MRMP: ML-resistant *M. pneumoniae*.

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Figures

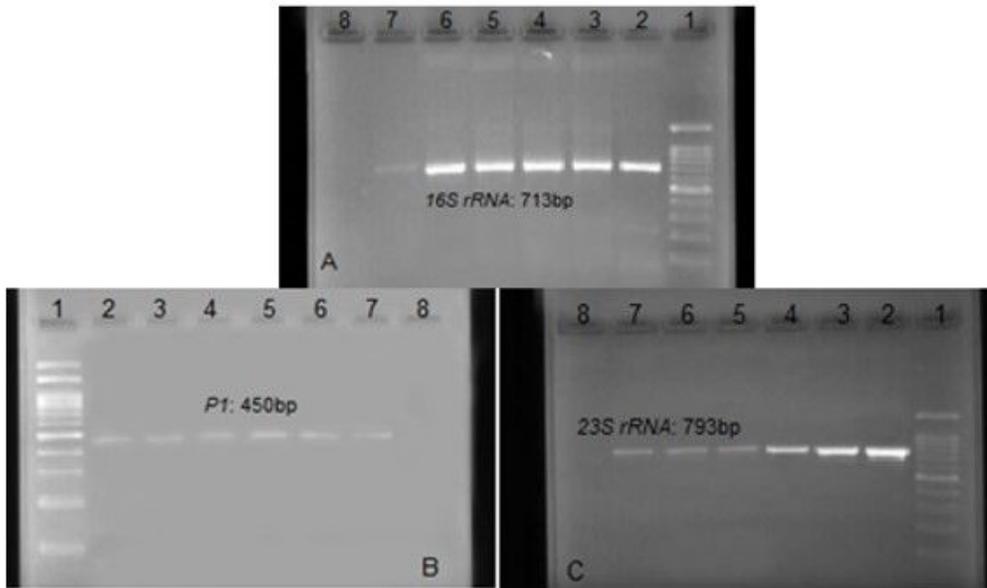


Figure 1

A) PCR amplification of the 16S rRNA gene. Lane 1: Ladder (100 bp), lane 8: negative control, lane 2: positive control (ATCC: 29342-713bp); lane 3-7: positive results. B) PCR amplification of the P1 gene. Lane 1: Ladder (100 bp), lane 8: negative control, lane 2: positive control (450bp); lane 3-7: positive results. C) PCR amplification of the 23S rRNA gene. Lane 1: Ladder (100 bp), lane 8: negative control, lane 2: positive control (793bp); lane 3-5, 6, and 7: positive results.