

Molecular Detection and Evaluation of ML-Resistance *M. Pneumoniae* Associated with Mutation in 23S rRNA Gene among Iranian Patients with Respiratory Infections

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Abstract

Background: *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 medications has led to increased rate of reported ML-resistant *M. pneumoniae* (MRMP) throughout the world. 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 from a patient with respiratory problems were collected. After the cultured of all samples in *M. pneumoniae*-specific PPLO medium, PCR technique was performed with specific primers. Afterwards, the broth micro-dilution MIC assay was employed. Finally, the PCR product of the 23S rRNA gene was sequenced to detect mutations of domain V in 23S rRNA gene of MRMP.

Results: It was found that 17 cases (17%) were positive for mycoplasma genus and six 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. All positive samples *M. pneumoniae* with 23S rRNA gene were sensitive to erythromycin.

Conclusions: These use of these antibiotics should be limited to prevent the emergence of MRMP in Iran.

Keywords: Erythromycin, Macrolide resistance, *Mycoplasma pneumoniae*, 23S rRNA gene.

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 a 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

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Received: 18 May, 2020; Accepted: 21 Jun, 2020

health concern worldwide. Moreover, the widespread application of these drugs has led to an 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 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 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.

Materials and methods

This study was approved by the ethics committee

of Shahed University of Medical Sciences, Tehran, Iran.

Sampling

One hundred samples of throat swab were collected from patients with atypical pneumonia referring to Mostafa Khomeini and Khatamol-Anbia hospitals in Tehran province in 2018. All included patients were informed orally of the purpose and experimental procedures of the study and signed the consent form. 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 (Merck, Germany) and moved to the laboratory.

Culturing

One 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, 0.3% 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 (20).

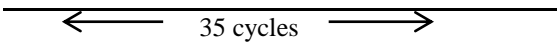
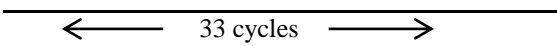
DNA extraction and PCR

The *M. pneumoniae* (ATCC: 29342) used as standard strain and was provided by Molecular Biology Research Center of Baqiyatallah University of Medical Sciences. DNA was extracted using a Kit (Roche, Germany) from the cultures and were subjected to PCR to identify *Mycoplasma* genus and *M. pneumoniae* strain with 16S rRNA and *P1* primers, respectively (Table 1).

Table 1. Primers sequences used in this study

Gene	Sequence	Product Size (bp)	Reference
16S rRNA	F primer: 5'-ACTCCTACGGGAGGCAGCAGT-3'	713b	(21)
	R primer: 5'-TGACCATCTGTCACTCTGTAAACCTC-3'		
P1	F primer: 5'-AAAGGAAGCTGACTCCGACA-3'	450bp	(21)
	R primer: 5'-TGGCCTTGCCTACTAAGTT-3'		
23S rRNA	Mp-F1: 5'-TAACTATAACGGTCCTAAGG-3'	793bp	(22)
	Mp-R1: 5'-CGCTACAACCTGGAGCATAAGA-3'		

Table 2. Temperature protocol relates to the cycle of the thermocycler for the P1 gene and the 23S rRNA gene.

Gene	Pre Denaturation	Denaturation	Annealing	Extension	Final Extension
Genus P1 <i>M. pneumoniae</i>	94 °C	94 °C	56 °C	72 °C	72 °C
	5 min	35 seconds	40 seconds	45 seconds	5 min
					
Genus 23S rRNA <i>M. pneumoniae</i>	94 °C	94 °C	55 °C	72 °C	72 °C
	4 min	45 seconds	45 seconds	50 seconds	5 min
					

The PCR reaction with a final volume of 25 µl was performed according to the protocol shown in Table 2 for the P1 and 23S rRNA genes in *M. pneumoniae*. Finally, PCR reaction products were electrophoresed. At the end of the PCR step, the samples that were positive for 23S rRNA gene were sequenced to evaluate for the presence or absence of mutations in domain V of 23S rRNA associated with ML resistance, and was analyzed the sequences of 23S rRNA genes.

Minimum inhibitory concentration (MIC)

The MIC was carried out using micro-dilution of Glucose PPLO broth contains phenol red as a PH indicator, and *M. pneumoniae* growth change the color of the medium purple to yellow. This medium was containing 25% horse serum, 0.3% yeast extract, and 0.3% meat extract. We used macrolide class antibiotics (erythromycin). Antibiotic susceptibility test was conducted to distinguish between sensitive and clinically resistant strains according to the Clinical and Laboratory Standards Institute (CLSI) criteria (23). The assay was run in 96-well plates. Then, plates were incubated for 5-6 days at 37 °C and the MIC for erythromycin was determined based on color changes.

Results

One hundred patients with respiratory infections were recruited in this study, 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. 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, in positive specimens in 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 (B), 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 (Fig. 2).

Measuring the MIC in all positive samples for *M. pneumoniae* using broth micro-dilution method, showed that all specimens were sensitive to erythromycin. Moreover, no ML resistance was reported (Table 3). Therefore, mutations in domain V of 23S rRNA gene at A2431G and G2491A were not associated with resistance of *M. pneumoniae* against erythromycin.

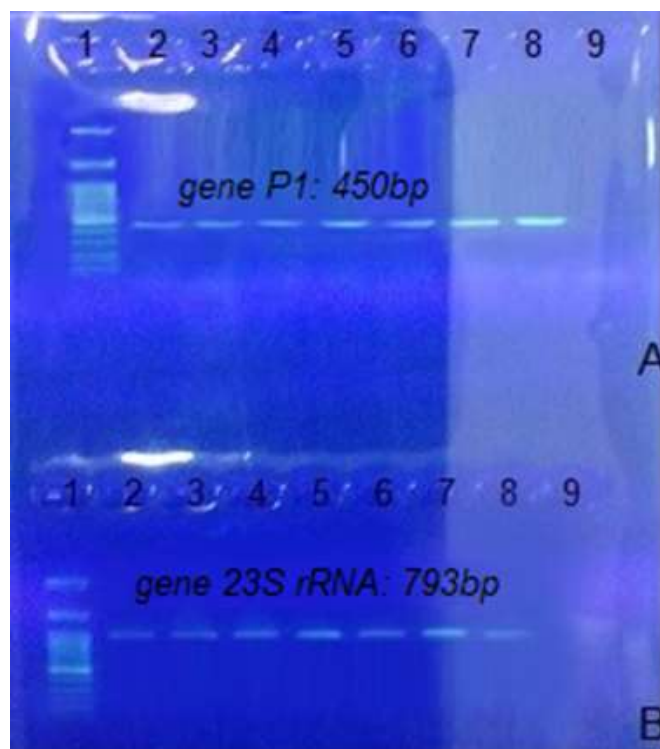


Fig. 1. A) PCR amplification of the *PI* gene. Lane 1: Ladder (100 bp), lane 9: negative control, lane 2: positive control (450bp); lane 3-8: positive results. **B)** PCR amplification of the *23S rRNA* gene. Lane 1: Ladder (100 bp), lane 9: negative control, lane 2: positive control (793bp); lane 3-6, 7, and 8: positive results.



Fig. 2. Schematic representation of a mutation in *23S rRNA* genes in online clustalW2 software. The image of both mutations is shown in positions 2431 and 2491.

Table 3. MIC ranges ($\mu\text{g/ml}$) for Erythromycin antimicrobial against *M. pneumoniae*.

Clinical isolate	MIC	Mutation
A	0.0039	NO
B	0.0039	Yes
C	0.0156	NO
D	0.0078	NO
E	0.0039	NO
F	0.0156	NO
Reference strain ATCC: 29342	0.0039	NO
MIC range for all isolates	0.0039– 0.0156	

Discussion

Macrolides are recommended for the treatment of *M. pneumoniae* which are usually considered as the first selective agent for the treatment of *M. pneumoniae* infection (24). With widespread use of these drugs, ML resistant isolates have been reported worldwide (24, 25). In 1970, the first clinical case of erythromycin-resistant *M. pneumoniae* was reported in a girl (13). 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* (13, 26). The present study did not show the presence of MRMP in patients with respiratory diseases caused by *M. pneumoniae* in Tehran and also A2063G mutation, which is the most common cause of high macrolide resistance in patients with *M. pneumoniae* pneumonia. In this study MRMP in patients with respiratory diseases (0%) were reported. This is very low compared to recent cases in children in Japan (87.1%) and China (97.0%) (27). The study groups are different thus limiting the comparison. High levels of ML drug resistance in *M. pneumoniae* in East Asia are associated with widespread use of macrolides (28). A study by Waites et al in the United States found that *M. pneumoniae* ML resistant (MRMP) was associated with clinical symptoms. Between 2015 and 2018, hospitals in eight states collected and stored respiratory samples which were positive for the presence of *M. pneumoniae* PCR test was performed to detect mutations in the 23SrRNA gene of these samples These mutations were associated with macrolide resistance During this study, MRMP was reported in 27 strains of 360 samples (7.5%). The prevalence of MRMP was significantly higher in the south and east (18.3%) than in the west (2.1%). And A2063G were identified as the dominant mutation in the 23S rRNA gene (29). In the study, Zeng Zhang et al., Out of 55 positive samples, 25 (45%) were hospitalized. Most of these patients were male (58%) aged 7-8 years. 72% (25/18) of hospitalized patients had radiographic findings consistent with pneumonia and some cases were

hospitalized for up to 4 weeks. The results of pathogen diagnosis showed that *M. pneumoniae* (P1 gene) was the causative agent of the disease in this outbreak and all strains had an A to G point mutation at position 2063 (30). In the study of Dongxing Guo et al., Out of 178 collected samples, 164 (90.85%) cases of *M. pneumoniae* positive were reported, and in 149 of 164 positive cases of ML-resistant *M. pneumoniae* (MRMP) using allele-specific real- time PCR was detected, while 153 cases of *M. pneumoniae* were reported positively using nested PCR method, in 110 cases (110/153) of these samples MRMP was detected (31). In a study by Brown et al. (2015) in England ML related mutations were reported in positive samples of *M. pneumoniae* found that 43 samples of the total 60 positive specimens for *M. pneumoniae* had mutations in domain V of 23S rRNA gene, and ML resistance was found in four samples (9.3%) (26). The results of this study are not consistent with the present study. 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, (87.1%, 202/176) in Japan, (97%, 33/32) in China, (1.2%, 167/2) in Germany, and (9.8%, 51/5) in France. As a result, in order to control the emergence of ML resistance among *M. pneumoniae* strains, the use of these antibiotics should be restricted in Iran and even should be avoided widespread use.

Acknowledgment

The authors would like to thank all staff members of the Department of Microbiology of Shahed University of Medical sciences. The authors declare that there are no competing interests. The results described in this paper were part of a thesis. This study was supported by Department of Medical Microbiology, Faculty of Medicine, Shahed University, Tehran, Iran.

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