

The Prevalence of 23S rRNA Mutations in ML-Resistant *M. pneumoniae* Isolates to Clarithromycin in Patients with Respiratory Infections

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Abstract

Background: *Mycoplasma pneumoniae* is one of the widespread causes of community-acquired pneumonia (CAP). Over recent years, the widespread use of macrolides has led to the emergence of macrolide-resistant *M. pneumoniae* (MRMP) resulted from mutations at specific positions of domain V of the 23S rRNA gene.

Methods: We collected 100 samples of throat swabs from patients with respiratory infections. After extraction of DNA from bacterial cell cultured in PPLO broth media using Roche kit (Germany), the PCR was performed on specific samples of *M. pneumoniae* using specific primers for 23S rRNA gene. Afterwards, for positive samples, minimum inhibitory concentration (MIC) was determined using the broth microdilution with Clarithromycin. Finally, the PCR product was sequenced to detect mutations related to macrolide resistance in domain V of 23S rRNA.

Results: According to the analysis of the sequenced PCR product of *M. pneumoniae* 23S rRNA gene using Clustalw2 online software, one of the samples were shown to have a mutation at A2431G and G2491A positions. The MIC measurement also revealed that all isolates were sensitive to Clarithromycin, and there was no macrolide resistance to Clarithromycin in all isolates.

Conclusions: Sequence analysis of the 23S rRNA gene in *M. pneumoniae*, revealed no macrolide resistance of *M. pneumoniae* to Clarithromycin. Thus, the use of these antibiotics should be restricted to prevent the development of macrolide-resistant *M. pneumoniae* in Iran.

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

Introduction

Mycoplasma pneumoniae (*M. pneumoniae*) is one of the most leading causes of community-acquired pneumonia (CAP) (1–3). *M. pneumoniae* Infections occur endemically worldwide, with the highest prevalence among children aged 5-15 years (4). In recent years, pneumonia caused by *M. pneumoniae* has shown an increasing trend (5). Previous studies have shown that *M. pneumoniae* accounts for up to 5% of CAP cases (6). Although

M. pneumoniae is a self-limiting disease, some patients complain of progressive disease despite courses of antibiotic therapy (7-9). Macrolides (MLs) are used as the first-line treatment of *M. pneumoniae* infection (1-10). The duration of treatment of infections with ML-resistant *M. pneumoniae* (MRMP) is longer compared to treatment-sensitive infections (11-12). It was first reported in Japan, and then in other parts of the world, including the United States, Europe, East

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Asia, and the Middle East, respectively. The rate of MRMP has shown an annual increasing trend, especially in East Asian countries, including Japan, China, and Korea (12-13). In recent years, MRMP has become rampant in Asia, and has attracted the attention of scientists (1-3). The widespread use of macrolides has led to a rapid global emergence of MRMP as > 1% in Asia and > 2% in Europe (1). The resistance of *M. pneumoniae* to MLs has been shown to be caused by point mutations, nucleotide displacement, in domain V of the *23S rRNA* gene (1-3). According to the results of previous studies over the past few years, *M. pneumoniae* strains with mutations in domain V of the *23S rRNA* have been shown to have high levels of resistance to ML antibiotics in minimum inhibitory concentration (MIC) assay. Analysis of V domain sequence of *23S rRNA*, revealed point mutations at some positions, such as A2063G, A2064G, and A2064C (14-15). As a result, studies have shown that *M. pneumoniae* have macrolide resistance and that all macrolide-resistant *M. pneumoniae* strains isolated from patients with atypical pneumonia displayed point mutations in domain V of *23S rRNA*. The present study aimed to determine the prevalence of resistance of *M. pneumoniae* strains isolated from patients with respiratory infections to Clarithromycin due to *23S rRNA* mutation.

Materials and methods

Subjects

Totally, 100 samples of throat swab were collected from patients with atypical pneumonia admitted to Mostafa Khomeini and Khatamol-

Anbia hospitals of Tehran province in 2018. All patients were diagnosed with clinical symptoms of respiratory infections by a lung specialist, including weakness, lethargy, fatigue, persistent headache, dry cough, shortness of breath, diarrhea, sputum production, and muscle pain. The samples were taken from who did not have antibiotic therapy during the past month and filled up questioners and signed consent form.

Sample collection

Samples were taken from the upper throat of patients with respiratory infections using sterile cotton swabs, then were transferred to the laboratory in the transport PPLO broth medium. Then one ml of the transport medium was passed through the 0.45 µm filter in the main Glucose PPLO broth medium in 5-10% CO₂, and then incubated at 35 °C for 3 weeks. In this study, *M. pneumoniae* (ATCC: 29342), was used as a control provided by the Molecular Biology Research Center of Baqiyatallah University of Medical Sciences.

DNA extraction

The Roche Co. kit (High Pure PCR Template Preparation Kit, Germany) was used to extract DNA from the samples were grown on the Glucose PPLO broth medium.

Polymerase chain reaction (PCR)

Following the extraction of genomic DNA, the Specific Primer-Polymerase Chain Reaction (SSP-PCR) was used to identify the genus Mycoplasma (*23S rRNA* gene) and *M. pneumoniae* strain (*PI* gene) (Table 1).

Table 1. Sequences of primers used in this study.

Gene	Sequence	Product Size (Reference)
<i>PI</i>	FP: 5'- AAAGGAAGCTGACTCCGACA	450 bp (16)
	RP: 5'-TGGCCTTGCCTACTAAGTT	
<i>23S rRNA</i>	FP: 5'- TAACTATAACGGTCCTAAGG	793 bp (17)
	RP: 5'- CGCTACAACCTGGAGCATAAGA	

After Primer-BLAST on designated primers, the PCR was conducted at a final volume of 25 μ l, including an initial denaturation at 94 °C for 5 min, 35 cycles comprised of denaturation at 94 °C for 35 seconds, binding primer 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 at a final volume of 25 μ l, constituted of an initial denaturation at 94 °C for 4 min, 33 cycles comprised of denaturation at 94 °C for 45 seconds, binding primer 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. The kit was obtained from Roche Co. (Germany) was used to extract the 23S rRNA. The PCR products (ATCC: 29342) were at last electrophoresed. Eventually, PCR products that were positive for 23S rRNA were sequenced with next-generation sequencing (NGS) to detect point mutation of domain V in 23S rRNA, and the sequences of the 23S rRNA gene were analyzed.

Minimum inhibitory concentration (MIC) determination using microdilution

The MIC was carried out by micro-dilution of Glucose PPLO broth, enriched with horse serum, and D-glucose in 96 wells of microplates on *M. pneumoniae* positive samples, following

preparation of macrolide suspension of Clarithromycin. Then, the microplates containing colonies of *M. pneumoniae* were incubated at 37 °C for 5-6 days to determine the MIC of Clarithromycin. The MIC of Clarithromycin was measured and recorded for all *M. pneumoniae* positive samples. As such, the MIC of Clarithromycin was determined based on color changes of Glucose PPLO broth enriched with horse serum and D-glucose.

Statistical analysis

Data analysis was performed by using SPSS, version 20.0.

Results

Demographic

Of 100 assigned patients with respiratory infections, 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.

Molecular detection of *P1* and 23S rRNA

The SSP-PCR showed 450 bp amplification of *P1* in the standard strain and six samples (6%) using the *P1*-specific primer of *M. pneumoniae* (Fig. 1A). Using 23S rRNA-specific primers revealed a 793 bp - amplicon in the standard strain and six samples (Fig. 1B).

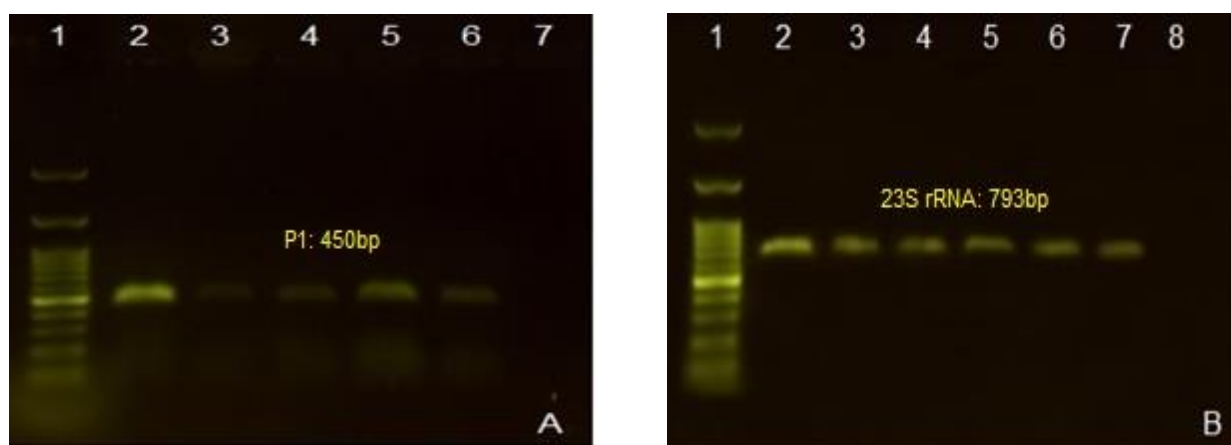


Fig. 1. A) PCR amplification of the *P1* gene. Lane 1: Ladder (100 bp), lane 7: negative control, lane 2: positive control (450 bp); lane 3-6: positive samples. B) PCR amplification of the 23S rRNA gene. Lane 1: Ladder (100 bp), lane 8: negative control, lane 2: positive control (793 bp); lane 3-7: positive samples.

Sequencing and Determination of minimum inhibitory concentration (MIC) in *M. pneumoniae* isolates

With analysis of the 23S rRNA gene sequence, only one sample was found with a point mutation at A2431G and G2491A positions

(Fig. 2). Also, the measurement of MIC in all *M. pneumoniae*-positive samples using the broth micro-dilution method, indicated that all specimens were sensitive to Clarithromycin, and no ML resistance was reported (Table 2).



Fig. 2. A schematic representation of a mutation in 23S rRNA genes in online clustalW2 software. One of the mutations is shown at position 2491.

Table 2. Determination of MIC of Clarithromycin in *Mycoplasma pneumoniae*-positive samples.

Clinical isolate	MIC	Mutation
A	0.00025	NO
B	0.000125	Yes
C	0.001	NO
D	0.00025	NO
E	0.00025	NO
F	0.00025	NO
Reference strain	0.0039	NO

Discussion

Mycoplasma Pneumoniae is a major cause of community-acquired pneumonia (5). Macrolide antibiotics are recommended as the first-line treatment for *M. pneumoniae* (1, 10). Extensive use of these antibiotics resulted in the emergence of ML-resistant isolates all over the world. Sharifi et al. conducted a study in Tabriz city on 200 patients and reported the prevalence of *M. pneumoniae* as 6%, which was in accordance with our study. However, the prevalence of *M. pneumoniae* reported in this study was significantly higher than the rate of *M.*

pneumoniae reported in Ahvaz city and (3%), Rasht city (1%) (19). Lucier et al. found a number of ML-resistance *M. pneumoniae* at different positions in the V domain of the 23S rRNA (2063 and 2064) with the displacement of A-G (14, 18). The prevalence of MRMP displays various patterns in different countries, which is attributed to the excessive use of MLs and the age of the patients (20).

Macrolides resistance is linked with a point mutation in domain V of the 23S rRNA gene (nucleotide displacement at specific positions of

domain V of 23S rRNA). Mutation of 23S rRNA at A2063G is the commonest mutation, followed by A2064G, which is mostly responsible for high ML resistance in *M. pneumoniae* (18, 21, 22).

Here, the analysis of the sequence of 23S rRNA demonstrated point mutations at A2431G and G2491A only in one specimen. In addition, the broth micro-dilution MIC method indicated that all *M. pneumoniae*-positive samples, were sensitive to Clarithromycin, and no ML resistance was reported. The frequency of MRMP amongst the participants was 0.6%. Accordingly, the prevalence of MRMP was shown as 2% (1/50) in Switzerland, 87.1% (176/202) in Japan, 97% (32/33) in China, 1.2% (2/167) in Germany, and 9.8% (5/51) in France, which were inconsistent with the results of our study (2). Hong and colleagues reported two cases of MRMP, and proposed that MRMP-positive cases had point mutation at A2063G of domain V of 23S rRNA (18). A study conducted in England (2015), reported mutations associated with ML resistance in *M. pneumoniae*-positive samples between September 2014 and September 2015. They stated that 43 of the total 60 *M. pneumoniae*-positive samples presented mutations in domain V of 23S rRNA, with ML resistance in four samples (9.3%). Those with point mutations in domain V of 23S rRNA were identified as ML-resistant isolates (23), which was not in accordance with the results of our study. Haruki and colleagues recruited hospitalized children in Japan (2014) and detected 33 cases of *M. pneumoniae* by PCR, of whom 31 cases displayed resistance to MLs, and all cases were shown to have point mutation at 2063 and 2064 in domain V of 23S rRNA (24). The contradiction in these

results may be due to the older age of the patients in our study. Liu and colleagues (2014) evaluated 580 samples of throat swab suspected for *M. pneumoniae*, and reported that 70 cases were ML-resistant. They also revealed mutation at 2063 and 2064 in domain V of 23S rRNA in all specimens positive for *M. pneumoniae* (17). Zhou et al. (2015) also assessed 650 samples of throat swab and used the PCR. All sequences of the domain V of the 23S rRNA gene were investigated. They proposed that 100 percent (71/71) of *M. pneumoniae* isolated from patients with community-acquired pneumonia were resistant to erythromycin, Clarithromycin, and azithromycin. Furthermore, all ML-resistant strains of *Mycoplasma pneumoniae* displayed point mutation at A2063G in the domain V of 23S rRNA (25). These contradictory results can be attributed to the larger sample size of this study.

According to the analysis of the sequence of the 23S rRNA gene, all positive samples for *M. pneumoniae* were not resistant to MLs. Besides, all point mutations in domain V of 23S rRNA at A2431G and G2491A were not linked with Clarithromycin resistance. In conclusion, in order to control or prevent the emergence of ML resistance among *M. pneumoniae* strains, the application of these antibiotics should be limited in Iran.

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References

1. Eshaghi A, Memari N, Tang P, Olsha R, Farrell DJ, Low DE, et al. Macrolide-resistant *Mycoplasma pneumoniae* in humans, Ontario, Canada, 2010-2011. *Emerg Infect Dis*. 2013;19(9).
2. Meyer Sauteura P, Bleischa B, Voith A, Maurerb F, Relya C, Berger C, et al. Survey of macrolide-resistant *Mycoplasma pneumoniae* in children with community-acquired pneumonia in Switzerland. *Swiss Med Wkly*. 2014;144:w14041.
3. Zhao F, Liu G, Wu J, Cao B, Tao X, He L, et al. Surveillance of Macrolide-Resistant *Mycoplasma pneumoniae* in Beijing, China, from 2008 to 2012. *Antimicrob Agents Chemother*. 2013;57(3):1521-1523.
4. Babaei Z, Pouladi I, Ashtari A, Azimi G, Niakan M. The Prevalence of Atypical Pneumonia Caused by *Mycoplasma pneumoniae* (P1 gene) in Patients with Respiratory Infections by Culture and Molecular PCR Methods in Tehran, Iran.

- Jundishapur Journal of Microbiology. 2019; 12(11):e84174.
5. Song Q, Xu B, Shen K. Effects of bacterial and viral co-infections of *Mycoplasma pneumoniae* pneumonia in children: analysis report from Beijing Children's Hospital between 2010 and 2014. *Int J Clin Exp Med*. 2015;8(9):15666-74.
 6. Zhang Y, Mei S, Zhou Y, Yang D, Pan T, Chen Z, et al. TIPE2 negatively regulates *Mycoplasma pneumoniae*-triggered immune response via MAPK signaling pathway. *1SciEntIfIc RepoRts*.2017;7(1):13319.
 7. Zhou Y, Zhang Y, Sheng Y, Zhang L, Shen Z, Chen Z. More complications occur in macrolide-resistant than in macrolide-sensitive *Mycoplasma pneumoniae* pneumonia. *Antimicrob Agents Chemother*. 2014;58(2):1034-8.
 8. Youn YS, Lee SC, Rhim JW, Shin MS, Kang JH, Lee KY. Early Additional Immune-Modulators for *Mycoplasma pneumoniae* Pneumonia in Children: An Observation Study. *Infect Chemother*. 2014;46(4):239-47.
 9. Meyer Sauteur P, Unger W, Nadal D, Berger C, Vink C, Rossum A. Infection with and Carriage of *Mycoplasma pneumoniae* in Children. *Front Microbiol*.2016;7:329.
 10. Kawai Y, Miyashita N, Kubo M, Akaike H, Kato A, Nishizawa Y, et al. Nationwide Surveillance of Macrolide-Resistant *Mycoplasma pneumoniae* Infection in Pediatric Patients. *Antimicrob Agents Chemother*.2013;57(8):4046-4049.
 11. Saraya T. *Mycoplasma pneumoniae* infection: Basics. *J Gen Fam Med*. 2017;18(3):118-125.
 12. Izumikawa K, Izumikawa K, Takazono T, Kosai K, Morinaga Y, Nakamura S, et al. Clinical features, risk factors and treatment of fulminant *Mycoplasma pneumoniae* pneumonia: a review of the Japanese literature. *J Infect Chemother*. 2014;20:181-5.
 13. Valentine-King MA, Cisneros K, James MO, Huigens RW 3rd, Brown MB. Turning the Tide against Antibiotic Resistance by Evaluating Novel, Halogenated Phenazine, Quinoline, and NH125 Compounds against Ureaplasma Species Clinical Isolates and Mycoplasma Type Strains. *Antimicrob Agents Chemother*. 2019;63(3):e02265-18.
 14. Lucier TS, Heitzman K, Liu SK, Hu PC, et al. Transition mutations in the 23S rRNA of erythromycin-resistant isolates of *Mycoplasma pneumoniae*. *Antimicrob Agents Chemother*. 1995;39(12): 2770-3.
 15. Liu Y, Ye X, Zhang H, Xu X, Li W, Zhu D, et al. Antimicrobial Susceptibility of *Mycoplasma pneumoniae* Isolates and Molecular Analysis of Macrolide-Resistant Strains from Shanghai, China. *Antimicrob Agents Chemother*. 2009;53(5):2160-2.
 16. Golmohammadi R, Atae R A, Alishiri G H, Mirnejad R, Najafi A, Tate M, et al. Molecular Diagnosis of *Mycoplasma pneumoniae* in Synovial Fluid of Rheumatoid Arthritis Patients. *Iran J Med Microbiol*. 2014;8(1):1-8.
 17. Liu X, Jiang Y, Chen X, Li J, Shi D, Xin D. Drug Resistance Mechanisms of *Mycoplasma pneumoniae* to Macrolide Antibiotics. *Biomed Res Int*. 2014;2014:1-7.
 18. Hong JH, Chun JK, Uh Y, Oh KJ, Kim J, Yoon KJ. Two Cases of *Mycoplasma pneumoniae* Pneumonia with A2063G Mutation in the 23S rRNA Gene in Siblings. *Ann Lab Med* 2013; 33(1):65-8.
 19. Sharifi S, Ghotaslou R, Aki MT, Soroush MH, Ansarian Kh, Shabanpour J, et al. Identification of respiratory infections caused by *Mycoplasma pneumoniae* with three methods of cultivation, ELISA and PCR. *Medical Journal of Tabriz University*. 2011; 33(3): 36-41.
 20. Morozumi M, Iwata S, Hasegawa K, Chiba N, Takayanagi R, Matsubara K, et al. Increased macrolide resistance of *Mycoplasma pneumoniae* in pediatric patients with community-acquired pneumonia. *Antimicrob Agents Chemother* 2008;52(1):348-50.
 21. Lin C, Li S, Sun H, Zhao H, Feng Y, Cao L, et al. Nested PCR-linked capillary electrophoresis and single-strand conformation polymorphisms for detection of macrolide-resistant *Mycoplasma pneumoniae* in Beijing, China. *J Clin Microbiol*. 2010;48(12):4567-72.
 22. Morozumi M, Takahashi T, Ubukata K. Macrolide-resistant *Mycoplasma pneumoniae*: characteristics of isolates and clinical aspects of community-acquired pneumonia. *J Infect Chemother*. 2010;16(2):78-86.

23. Brown RJ, Macfarlane-Smith L, Phillips S, Chalker VJ, et al. Detection of macrolide resistant *Mycoplasma pneumoniae* in England, September 2014 to September 2015. Euro Surveill. 2015;20(48):30078.

24. Haruki K, Tomoyuki T, Ayano I, Tsuyoshi S, Tomoo F. Characteristics of hospitalized children

infected with macrolide-resistant *Mycoplasma pneumoniae* braz j infectdis.2014;18(3):294-299.

25. Zhou Z, Li X, Chen X, Luo F, Pan C, Zheng X, et al. Macrolide-Resistant *Mycoplasma pneumoniae* in Adults in Zhejiang, China Antimicrob Agents Chemother. 2015;59(2):1048-51.