

Mutations in domain V of *Mycoplasma pneumoniae* 23S rRNA are not associated with clinical characteristics of *M. pneumoniae* pneumonia in children: a case control study

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Research

Keywords: *Mycoplasma pneumoniae*; refractory *Mycoplasma pneumoniae* pneumonia; mutation; clinical characteristics

Posted Date: April 3rd, 2020

DOI: <https://doi.org/10.21203/rs.2.23539/v2>

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Abstract

Background *Mycoplasma pneumoniae* (MP) is a common agent of community-acquired pneumonia in children and young adults that can lead to refractory or persistent *Mycoplasma pneumoniae* pneumonia (MPP). Macrolide-resistant MP harbors point mutations in domain V of 23S ribosomal Ribonucleic Acid (rRNA) with substitutions detected at positions 2063, 2064, 2067 and 2617. This study aims to investigate the prevalence and clinical characteristics of mutations in domain V of MP 23S rRNA.

Methods We sequenced the 23S rRNA domain V of MP strains collected from children with MPP. Clinical and laboratory data were also obtained, including gender, age, duration of fever, duration of fever after the start of macrolide therapy, MP-Deoxyribonucleic Acid (DNA) load at enrollment, leukocyte count, neutrophil, and lymphocyte count, immunomodulators treatment and pulmonary complications.

Results Of 276 strains, 255 (92.39 %) harbored A to G transition at the position 2063 (A2063G), and 21 (7.61 %) were not mutated. There were no significant differences in gender, age, duration of fever, duration of fever after the start of macrolide therapy, MP-DNA load at enrollment, hospitalization days, lymphocyte count and pulmonary complications when patients were stratified based on the presence or absence of domain V mutations. We also found that children with refractory MPP experienced higher MP-DNA load than the non-refractory MPP, but the prevalence of domain V mutations was no statistical difference.

Conclusions We found that clinical MP strains harbored very high mutation rate in 23S rRNA domain V, especially A2063G mutation. However, these mutations were not associated with clinical symptoms, laboratory results, pulmonary complications and development of refractory MPP. Instead, MP-DNA load was significantly different between refractory and non-refractory MPP.

Background

The prevalence of infection with *Mycoplasma pneumoniae* (MP) is widely underestimated, as most infected individuals are seldom symptomatic and rarely seek medical attention. MP is considered as a common agent of community-acquired pneumonia in children and young adults. MP epidemics tend to cycle every 3 to 7 years[1], and severe infection may lead to refractory or life-threatening pneumonia with pulmonary and extrapulmonary complications[2-4].

As MP lacks a true cell wall, it is intrinsically resistant to beta-lactams, glycopeptides, and fosfomycin antibiotics that target the cell wall. Therefore, macrolides, tetracyclines, and fluoroquinolones are used clinically instead. Macrolides are considered as first-line agents in children to avoid potential age-related side effects of other therapies (tetracyclines have possible adverse effects on enamel hypoplasia and bone, and fluoroquinolones may influence the growth of bone and articular cartilage) [5,6]. However, cases of refractory *Mycoplasma pneumoniae* pneumonia (MPP) have also steadily increased in recent years [2,3].

Indeed, because macrolides inhibit protein synthesis by binding domain II and/or domain V of 23S ribosomal Ribonucleic Acid (rRNA) in the 50S MP ribosomal subunit, several studies have demonstrated that genetic mutations in the 23S rRNA can result in macrolide resistance of MP[6-9]. In particular, point

mutations at nucleotide positions A2063, A2064, A2067 and C2617 in domain V, especially A to G transition at position 2063 (A2063G) and A to G transition at position 2064 (A2064G), confer strong resistance[10-12], whereas mutations at positions A2067 and C2617 confer lower levels of resistance[2,6,12]. Notably, since 2000, the prevalence of macrolide-resistant MPP has rapidly increased, especially in Asian countries [6]. Worryingly, one study suggested that macrolide resistance was found in more than 90% of Chinese MP isolates, which all harbored gene mutations [13]. Our previous research found that the morbidity of macrolide-resistant MP was even up to 92.45% [14]. The excessive use and misuse of macrolides may contribute to these mutations [15]. Refractory pneumonia is also increased, which shows no clinical or radiological response to macrolides, and could be easier to become severe or fatal pneumonia.

A growing body of researches has characterized the molecular epidemiology of MP infection. However, to our knowledge, rarely large clinical research has been conducted to characterize clinical strains of MP, and investigate the clinical significance of mutations. Therefore, we amplified and sequenced the 23S rRNA domain V of a large number of clinical MP strains and then compared with the sequence in MP standard strain (M129). The aim was to investigate genotypes of clinical MP strains on a large scale, determine the prevalence of domain V mutations, and evaluate the association between domain V mutations and clinical characteristics of MPP, especially refractory MPP.

Methods

1. Study Population

We enrolled patients diagnosed with MPP into the Department of Respiratory, Children's Hospital Affiliated to Nanjing Medical University from March 1st, 2014 to May 31st, 2015. Diagnosis of pneumonia was based on symptoms at admission, including fever, cough, productive sputum, chest pain, dyspnea, abnormal breathing sounds, and radiographic pulmonary abnormalities that were at least segmental. In addition, acute MP infection was confirmed by Polymerase Chain Reaction (PCR) and serology (detecting MP IgM by enzyme-linked immunosorbent assay in acute phase), as well as by the absence of other pathogens (bacteria, other viruses, chlamydia pneumoniae, legionella pneumophila and so on) and the ineffectiveness of cephalosporin. All patients had both positive results for the above-mentioned MP tests and were diagnosed with pneumonia. We excluded convalescent patients, as well as patients with immunosuppressive illness, asthma, chronic lung disease, or other systematic diseases. Complete medical records as below were obtained: fever, hospitalization days, blood tests, MP-DNA load and chest radiography, along with pulmonary complications including pulmonary atelectasis, pleuritis, and hydrothorax.

2. Sample Preparation

Bilateral nasopharyngeal aspirates and serum samples were collected at admission. A portion of nasopharyngeal aspirates was assayed for MP-DNA load using the MP-DNA PCR kit (Acon Biotechnology, Zhejiang, China), following the manufacturer's instructions. The other remaining nasopharyngeal

aspirates were vigorously mixed with 4 volumes of 0.9 % normal saline, incubated overnight at 4 °C, and centrifuged at 13,500 rpm and 4 °C for 20 min. The supernatant was discarded, and the pellet was resuspended in 40 µl lysis solution (1 % Triton-100, Amresco, USA), and shaken for 10 min in a water bath at 100 °C. Finally, lysates were clarified by centrifugation at 14,500 rpm and 4 °C for 6 min, and supernatants were retained.

3. Gene Amplification

23S rRNA domain V was amplified by nested PCR (Table 1). Amplification products, with expected size of 690 bp, were resolved on 1 % agarose, visualized by ribonucleic acid staining, and imaged using a gel-imaging and analysis system. Amplification products were then shipped at 4 °C within 2 days for sequencing at Yingweijie, Shanghai, China. Finally, DNA sequences were compared using BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) to strain M129 (ATCC 29342).

4. Clinical Data, Treatment, and Diagnosis of Refractory MPP

Clinical data were collected from medical records, including gender, age, fever, hospitalization days, immunomodulators, radiological findings, laboratory data, and MP-DNA load. Macrolide therapy strategies were 10 mg azithromycin/kg/day for the first three days in seven days, or with 30 mg erythromycin/kg/day. According to clinical manifestations, some drugs were selective used, such as expectorant, cough medicine, inhaled corticosteroid, bronchodilator and immunomodulators (systemic corticosteroids and/or intravenous gamma immunoglobulin). Interventional therapeutic fiber bronchoscopy or thoracentesis could be used in the treatment of atelectasis or hydrothorax. Refractory MPP was defined as prolonged high degree fever (>38.5°C or 103.3°F) , worsening cough and (or) increasing infiltrates on chest radiographs after administration of macrolide antibiotics for 7 days or more.

5. Statistical Analyses

Data were analyzed in IBM SPSS statistics 20.0. For data with skewed distribution, median values and range are reported. Student's t-test was used to compare continuous variables, while χ^2 test and Fisher's exact test were used for categorical variables. A *P* value < 0.05 was considered statistically significant.

Results

1. Demographics

A total of 276 patients were enrolled, of whom 137 (49.64 %) were male and 139 (50.36 %) were female. The median age was 4.93years.

2. MP 23S rRNA genotypes

Of 276 strains, 255 (92.39 %) harbored the A2063G mutation in domain V, while the remaining 21 (7.61 %) did not contain mutations. One A2063G MP isolate strain contained an additional G insertion between position 2586 and 2587. And another one strain contained three additional mutations: G-to-C transition at position 2601, T insertion between position 2589 and 2590, T insertion between position 2612 and 2613 (Table 2).

3.Clinical Characteristics of infection with Mutant and Non-mutant MP strains

Patients were stratified into two groups based on the presence or absence of mutations in the 23S rRNA domain V of MP. The mean age was 4.84 in 255 patients (124 males, 131 females) infected with mutant MP, and 6.07 in 21 patients (13 males, 8 females) infected with non-mutant MP strains. There were no significant differences in age and sex distribution, pulmonary complications, duration of fever, duration of fever after the start of macrolide therapy, MP-DNA load at enrollment, hospitalization days and immunomodulators treatment. However, leukocyte and neutrophil count were significantly higher in patients infected with mutant strains, although lymphocyte count was comparable between groups (Table 3).

4.Clinical Characteristics of Refractory and Non-refractory MPP

Based on the clinical and radiologic findings, 64 patients (29males, 35 females) were deemed to have refractory MPP, while 212 (108 males, 104 females) were considered to have non-refractory MPP. There was no significant difference in gender between groups. Notably, the mutation was found with comparable frequency between non-refractory (93.87%) and refractory cases (87.5%), $P=0.092$. Moreover, MP-DNA load at enrollment and age were significantly higher in patients with refractory MPP (Table 4).

Discussion

Although MP infection shows typically self-limited course even without antibiotics, macrolides are still recommended as the first-line therapeutics. However, a steadily increasing number of recent cases progress to refractory, severe, life-threatening MP pneumonia [1,2]. Extensive use and misuse of macrolides may cause the rapid emergence of macrolide resistance [15]. Macrolides inhibit protein synthesis by binding to specific nucleotides of the 23S RNA in the 50S MP ribosomal subunit. Mutations at domain V of 23S RNA reduce the affinity of the macrolides for the ribosome, which develop macrolide resistance [6-9]. Since 2000, some studies have confirmed the increase of this microbiological problem throughout the world, the highest prevalence has been especially observed in East Asia. The published rate was reported to be 87.2% in Korea, 81.6% in Japan, and up to 90% in China [6,13]. In this study, we found that 92.39% of clinical MP strains harbored A2063G mutation from children with MPP. Notably, we didn't detect mutations at positions 2064, 2067, or 2617. However, several other novel mutations were found, including a G to C transition at position 2601, a T insertion between positions 2589-2590 and 2612-2613, and a G insertion between positions 2586-2587. Further studies are required to test whether these new mutations contribute to macrolide resistance.

Previous studies focus on the minimum inhibitory concentrations of macrolides *in vitro*, and explore point mutations of MP clinical strains [10-12]. However, the clinical relevance of these mutations has not been clearly characterized and large sample studies is fewer. In this study, the clinical, laboratory and radiologic characteristics of MPP patients with mutant strains were similar with those patients without mutations. These results hint the clinical symptoms, laboratory and radiologic characteristics of MPP are generally similar between mutations and without mutations. Previous studies on the comparisons of clinical manifestations between the groups also reported no significant differences [14,16,17]. As we all know, refractory MPP is characterized by long duration of fever, and severe pulmonary inflammatory response. In this study, the prevalence of mutations in non-refractory and refractory MPP was 93.87% and 87.5% respectively, which suggested that the infection of mutant strains does not increase the refractoriness of MPP in children. In addition, some children infected with mutant strain were also cured by macrolides, may be on the account of anti-inflammatory property of macrolides. And the use of timely and effective immunomodulators is beneficial to improve prognosis [18,19]. Based on previous researches, the pathogenesis of MP consists of direct damage mechanisms, immune damage and inflammatory damage [20]. While there is some value in investigating the clinical significance of genetic mutations in MP, it is probably necessary to consider other risk factors that may trigger refractory, severe, or life-threatening pneumonia, such as a more robust host immune response to inflammatory cytokines, interleukins(IL), alexin, CD⁴⁺ T cell and so on [21-23]. In refractory MPP, immunomodulators such as systemic corticosteroids or intravenous gamma immunoglobulin are considered to be an effective treatment option by reducing host inflammatory response [18,19]. Based on previous researches, MP infection enhances mucin production, neutrophil recruitment, and excretes inflammatory factors [20]. Mucus cell hypersecretion, especially goblet cell hyperplasia, has been shown in airways of MP infected mice [24]. Additionally, in children infected with MP, the levels of tumor necrosis factor- α (TNF- α), IL-1 β , IL-6, IL-10, C1q, C3, C4 in serum increase to varying degrees [21,22]. Those cytokines, ILs and alexin may participate in some classical or bypass activation pathways, mediate inflammatory reaction and immune responses, and have various biological activities.

More interestingly, our study and Wang et al found that MP-DNA load at enrollment was significantly different between refractory pneumonia and non-refractory pneumonia groups [18], which may be a risk factor of refractory MPP because of direct damage caused by increased MP load. The direct damage mechanisms of MP infection include adhesion damage, destruction of membrane fusion, invasive damage, and toxic damage [20]. These suggest that the higher and more persistent MP stimulation may induce a much stronger direct damage. To sum up, the occurrence of refractory MPP in children may largely depends on the interaction between MP and host immune response, regardless of mutations.

Our study is significant because it has compared the manifestations of MPP in children in a high macrolide resistance period for MP. But it also had several limitations. Firstly, our hospital is a tertiary hospital, the enrolled population may have included some very severe MPP cases and have a much longer period prior to hospitalization at our hospital than in previous studies. Secondarily, our sample size

was relatively small, and there was no multicenter research. Lastly, minimum inhibitory concentration values were not measured.

Conclusions

This study presents the infected MP strain in 92.39 % of our patients harbor genetic mutations in the 23S rRNA domain V. The A2063G mutation has been found in all mutant strains, and those new mutations need further study. Moreover, this study demonstrated that there are no significant differences in clinical features, laboratory results, pulmonary complications and development of refractory pneumonia among patients infected by non-mutant MP strains and those mutant strains. This finding suggests that MP mutation may be not related to clinical MPP severity. Instead, we found that MP DNA load and host immune response may contribute to the development of refractory pneumonia.

List Of Abbreviations

MP: *Mycoplasma pneumoniae*; MPP: *Mycoplasma pneumoniae* pneumonia; rRNA: ribosomal Ribonucleic Acid; DNA: Deoxyribonucleic Acid; A2063G: A to G transition at position 2063; A2064G: A to G transition at position 2064; PCR: Polymerase Chain Reaction; TNF- α : tumor necrosis factor- α ; IL: interleukin;

Declarations

Ethics approval and consent to participate: The study protocol was approved by the ethics committee of Children's Hospital Affiliated to Nanjing Medical University. Participant consent was written by the legal representatives of patients and informed consent forms were achieved.

Consent for publication: The study has obtained consent to publish from children's parents or legal guardians.

Availability of data and materials: All data generated or analyzed during this study are included in this published article and available from the corresponding author on reasonable request.

Competing interests: The authors declare that they have no conflict of interest.

Funding: This study is supported by a grant from .

Authors' contributions: Huan Deng and Yifan Zhu participated in the design of the study and the statistical analysis. Huan Deng wrote and submitted the manuscript. Jiamin Zhang and Yao Quan participated in the sample preparation, gene magnification and sequence alignment. Qiangquan Rong participated in the design of the study and performed the statistical analysis. Heng Tang conceived of the study, participated in its design and helped to draft the manuscript. Deyu Zhao participated in the design of the study. All authors read and approved the final manuscript.

Acknowledgements: Not applicable.

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Tables

Table 1—Primers for PCR and Sequencing

Primer	Sequence (5' to 3')	Position	Amplicon size (bp)
Forward-1	GCAGTGAAGAACGAGGGG	1758-1775	942
Reverse-1	CACACTTAGATGCTTTCAGAG	2680-2700	
Forward-2	CGGTCCTAAGGTAGCGAAAT	1963-1982	690
Reverse-2	AACACTCTTCAATCTTCCTACGG	2631-2653	

Table 2—Mutations in MP 23S rRNA domain V

Mutation	Value (n = 276)
Only A2063G	253 (91.67 %)
A2063G and G insertion between 2586 and 2587	1 (0.36 %)
A2063G, G2601C, T insertion between 2589 and 2590, T insertion between 2612 and 2613	1 (0.36 %)
No mutation	21 (7.61 %)

Table 3—Clinical Characteristics of Infection with Mutant and Non-mutant MP Strains

Variable	Mutant (n = 255)	Non-mutant (n = 21)	<i>P</i> value
Male/female	124/131	13/8	0.242
Mean Age ± SD (y)	4.84±2.99	6.07±3.49	0.073
With/without pulmonary complications ^a	53/202	4/17	0.85
With/without immunomodulators	56/199	7/14	0.233
Duration of fever (d)			
Median (range)	6(0-15)	7(0-14)	
Mean ± SD	6.23±3.76	7.05±3.90	0.342
Duration of fever after macrolide therapy (d)			
Median (range)	3(0-11)	3(0-11)	
Mean ± SD	3.65±2.83	4.52±3.41	0.184
MP-DNA load at enrollment ^b			
Median (range)	5.78(4.03-8.15)	6.31 (4.3-7.78)	
Mean ± SD	5.96±1.08	6.25±1.13	0.233
Hospitalization days			
Median (range)	8 (5-15)	8 (6-15)	
Mean ± SD	8.78±2.48	8.95±2.66	0.233
Mean leukocyte ± SD (× 10 ⁹ /L)	10.19±4.22	8.14±2.56	0.002
Mean neutrophil ± SD (× 10 ⁹ /L)	5.73±3.55	4.14±1.54	<0.001
Mean lymphocyte ± SD (× 10 ⁹ /L)	3.46±2.23	2.92±1.89	0.278

^apulmonary complications including pulmonary atelectasis, pleuritis, and hydrothorax. ^bLogarithm of MP- DNA load at enrollment.

TABLE 4—Clinical Characteristics of Non-refractory and Refractory MPP

Variable	Non-refractory (n = 212)	Refractory (n = 64)	<i>P</i> value
Male/female	108/104	29/35	0.43
Mean Age ± SD (yr)	4.36±2.89	6.82±2.81	< 0.001
With/without mutations	199/13	56/8	0.092
With/without pulmonary complications ^a	13/199	44/20	< 0.001
With/without immunomodulators	15/197	48/16	< 0.001
Duration of fever (d)			
Median (range)	5(0-14)	10(3-15)	
Mean ± SD	5.00±3.05	10.58±2.61	< 0.001
Duration of fever after macrolide therapy (d)			
Median (range)	2(0-10)	7.5(3-11)	
Mean ± SD	2.61±2.05	7.39±2.06	< 0.001
MP-DNA load at enrollment ^b			
Median (range)	5.64(4.03-8.15)	6.43(4.61-7.96)	
Mean ± SD	5.83±1.10	6.47±0.87	<0.001
Hospitalization days			
Median (range)	7(5-15)	12(7-15)	
Mean ± SD	7.86±1.74	11.88±2.06	<0.001

^apulmonary complications including pulmonary atelectasis, pleuritis, and hydrothorax. ^bLogarithm of MP-DNA load at enrollment.