More complications occurred in macrolide-resistant

*Mycoplasma pneumoniae* pneumonia

Running title: More complications occurred in MR MPP

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Abstract

Objectives: To understand the situation of macrolide-resistant genotypes of *Mycoplasma pneumoniae* (MP), and analyze the relationship between macrolide-resistant genotypes and clinical manifestations of *Mycoplasma pneumoniae* pneumonia (MPP).

Methods: Full-length sequencing of the 23S rRNA gene of MP was performed in 235 nasopharyngeal aspirates (NPAs) from children with MPP. We also retrospectively compared the clinical characteristics of macrolide-resistant (MR) MP infections and macrolide-sensitive (MS) MP infections.

Results: A total of 206 patients had point mutations in the 23S rRNA gene of MP, and they were defined as MR patients. The remaining 29 patients without point mutations were defined as MS patients. Among 206 MR patients, 199 (96.6%) had A2063G mutations, 6 with A2063T mutations, and the remaining one with an A2064G mutation. Concerning clinical manifestations, we found the median fever duration was 8 (range 0~42) days and 6 (0~14) days (P<0.01), the median hospitalization duration was 8 (2~45) days and 6 (3~16) days (P<0.01), and the median fever duration after macrolide therapy was 5 (0~42) days and 3 (0~10) days (P<0.01), respectively in the MR and MS group. We also found the incidence of extra-pulmonary complications in the MR group was significantly higher than that in the MS group (P<0.05). And radiological findings were more serious in the MR group than those in the MS group (P<0.05).

Conclusions: The increasing prevalence of MR MP has become a significant clinical issue in the pediatric patients, which may lead to more extra-pulmonary complications, and severe clinical features and radiological manifestations.
**Introduction**

*Mycoplasma pneumoniae* (MP) is one of the most prevalent pathogens causing community-acquired respiratory tract infections in children and young adults [1, 2]. *Mycoplasma pneumoniae* pneumonia (MPP) is usually a benign self-limited disease. However, sometimes it may cause various extra-pulmonary complications and progress to a severe life-threatening pneumonia [3-8]. These cases might show clinical and radiological deterioration despite of macrolide antibiotic therapy for 7 days or longer [9]. Severe MP infections may be related to occurrence of macrolide-resistant (MR) MP.

For children, macrolides are the first-choice agents for MP infections. However, in recent years, many isolates of MP from clinical samples showed resistance to macrolides with a high prevalence more than 90% in China [10]. The main mechanism of resistance is proved to be due to mutations in the domain V of 23S rRNA of MP [10-13]. The mutations that induce a high-level of macrolide resistance are an A to G transition at position 2063 and an A to G transition at position 2064, whereas low-level resistance is induced by an A to G transition at position 2617 and A to T transition at position 2063 [13]. Several studies have indicated that macrolide resistance in MP may have clinical significance in terms of diminishing response to treatment with drugs [14-16]. The presence of MR MP has been reported to be mainly associated with the persistent clinical symptoms such as fever causing prolonged hospital stay and elevated antibiotic change rate; however no increase in the incidence of complications has been reported to our knowledge.

In our study, we characterized macrolide resistance directly on MP-positive NPAs taken from hospitalized children with MPP in our hospital by analyzing the DNA sequence...
of domain V of 23S rRNA. Furthermore, we retrospectively compared the different clinical characteristics of MR MP infections and MS MP infections, including symptoms, extra-pulmonary complications and radiological findings.
Patients and methods

Study population and sample collection

A total of 235 nasopharyngeal aspirates (NPAs) were collected from children with MPP hospitalized in Children’s Hospital, Zhejiang University School of Medicine, China, from April 1, 2009 to March 31, 2010. All patients had signs and symptoms indicative of pneumonia on admission, including fever, cough, abnormal lung auscultation and a new infiltrate on chest radiograph. The MP infection was confirmed by serologic test (detecting MP IgM by ELISA) and/or MP PCR tests of NPA. All the 235 children had positive result for above-mentioned MP tests. Furthermore, other microbiologic tests were done to exclude other respiratory tract infections and tuberculosis, including protein purified derivative (PPD) test, blood cultures, nasopharyngeal aspirate/swab for common respiratory tract virus antigens (respiratory syncytial virus, influenza virus, adenovirus, and parainfluenza virus), and serology for Chlamydia pneumoniae and Legionella pneumophila. No other pathogens were found by these tests.

NPAs were obtained from the patients on admission. An aliquot of DNA was extracted from NPAs and stored at -80°C for determination. DNA was extracted using the TIANcombi DNA Lyse&Amp PCR kit (Tiangen, Hangzhou, China) in accordance with the manufacturer’s instructions.

Amplification of macrolide resistance genes

A PCR assay followed by direct amplicon sequencing was developed to detect point mutations conferring resistance to macrolides in the MP 23S rRNA gene. Domain V of the 23S rRNA gene was amplified using primers (5'-CCTAGTCGGGTAAATTCCGT-3',...
5'-CCTAGTCGGGTAAATTCCGT-3') (TaKaRa, Shanghai, China). The primers were used to specifically amplify a 244 bp region of MP including positions 2063 and 2064.

The PCR was carried out in a volume of 50µl containing 1µl of total DNA, 5µl of 10×buffer, 3µl of 10mM dNTP, 1µl of 10µM of each primer, 38.7µl of ddH₂O and 0.3µl of Taq DNA Polymerase (Invitrogen, Milan, Italy). The reaction mixture then underwent denaturation for 5 min at 94°C and 35 PCR cycles, each consisting of 1 min at 94°C, 1 min at 55°C and 1 min at 72°C, followed by a final extension step of 5 min at 72°C. The amplified products were analyzed by electrophoresis on a 2% agarose gel (Invitrogen, Milan, Italy) and visualized by Bio-Rad gel-imaging analysis system.

DNA sequencing

All PCR products of 244 bp were sequenced (Yingweijie Co., Ltd., Shanghai, China), and the DNA sequences were compared to the sequence of an MP reference strain M129 (GenBank accession No. X68422) by using BLAST. MP strain M129 (Type Culture collection, USA) was acted as a positive control.

Evaluation of clinical characteristics

Clinical information was retrospectively collected from the medical records of the patients. Complete information about the antimicrobial agents prescribed and clinical symptoms, radiologic findings was reviewed. The patient demographics, clinical symptoms, the incidence of extra-pulmonary complications and radiological findings were compared between MR MPP patients and MS MPP patients when discharged.

In our study, all patients were treated with 10mg/ (kg·d) of azithromycin on the first three days, then ceased for 4 days, followed by another 3-day duration of azithromycin, or with
15-30 mg/ (kg-d) of erythromycin lasting for 7-14 days. Temperature and respiratory signs and symptoms were examined at study entry and every 8 hr thereafter. A febrile day was defined as a day during which the body temperature exceeded 38.0°C at least once \(^{[16]}\). Total febrile days, febrile days after macrolide treatment and hospitalized days were assessed. All patients underwent chest X-ray on admission, and if the patient had large pulmonary lesions in chest radiograph, chest X-ray was done again about 7 days after admission. Large lesion was defined when the extent of infiltration on chest radiography was more than 1/3 of the lung \(^{[18]}\). The severity of pneumonia was evaluated according to the diagnostic standard of pneumonia advocated by British Thoracic Society (BTS) \(^{[19]}\).

During the hospitalization, we also evaluated the extra-pulmonary complications of patients. Liver function abnormalities were defined as at least 2-fold increase in glutamic pyruvic transaminase (GPT) value. Myocarditis was defined as patients with cardiovascular signs and symptoms (such as chest distress, weak, palpitation, pale) with elevated myocardial enzymogram (troponin or creatine kinase cardiac isoenzymes) or abnormal electrocardiographic (ECG) findings (diffuse T wave inversions, ST-segment elevation). Encephalitis was defined when patients had various neurological signs and symptoms (such as convulsions, paresis) with positive MP nucleic acid of cerebrospinal fluid (CSF) by PCR, and any other common pathogens excluded \(^{[3]}\). Proteinuria was defined as a urine protein/creatinine ratio greater than 0.2 \(^{[20]}\). Hemolytic anemia was defined as patients with hemolytic signs and symptoms with a positive direct Coombs test in the presence of cold agglutinins \(^{[20]}\). Arthritis was diagnosed on clinical symptoms (joint pain and/or swelling). In some cases, we found erythematous rash scattering in the body.
The study was approved by the ethics committee of the Children's Hospital, Zhejiang University School of Medicine. Written informed consent was obtained from at least one guardian of each patient before enrollment.

Statistical Analysis

Statistical analyses were performed using SPSS software (version 15.0). Skewed distribution data were expressed as median values (range from minimum to maximum). The comparisons were made by the Mann-Whitney U-test. Chi-squared tests were used to compare categorical data. Statistical significance was defined as P<0.05.
Results

Clinical Characteristics

On admission, all patients had symptoms and signs indicative of pneumonia, including fever (>38°C per axilla), cough and abnormal breath sounds on auscultation. No patients were transferred to the intensive care unit or received mechanical ventilation during hospitalization. All patients were PCR positive, and for 199 patients, there were positive serological results. The median age of the 235 children was 4 (range 0~14) years. 149 patients were males.

Analysis of macrolide-resistant genotypes

The 244bp bands were detected in all samples. Among 235 patients with MPP, 206 (87.7%) showing point mutations in domain V of 23S rRNA were defined as MR patients. The remaining 29 patients (12.3%), who showed no point mutations, were defined as MS patients. Of the MR patients, 199 (96.6%) were positive for an A-to-G transition mutation at position 2063 in domain V of 23S rRNA gene (A2063G), 6 showed an A-to-T transition mutation at position 2063 (A2063T), and the remaining one an A-to-G transition mutation at position 2064 (A2064G). In general, the 2063 mutation rate reached 99.5% (205/206).

Comparison of the clinical characteristics between MR and MS patients

Patients were categorized in one of two groups, MR or MS, on the basis of presence or absence of 23S rRNA gene mutations. 206 patients were in the MR group (132 males, 74 females), with a median age of 4 (range 0~14) years. 29 patients were in the MS group (17 males, 12 females), with a median age of 5 (range 0~11) years. No significant difference between the two groups was shown in age and sex distribution (Table 1).
Clinical characteristics in the MR and MS patients, including incidence of complications, radiologic findings, clinical course, are summarized in Table 1. Of the 206 MR patients, extra-pulmonary complications were found in 61 cases (29.6%), including liver function abnormalities in 27, myocarditis in 15, rash in 11, encephalitis in 4, proteinuria in 2, hemolytic anemia in 1, and arthritis in 1. In the MS group, only 3 patients (10.3%) had extra-pulmonary complications, who were involved with liver function abnormalities. There was significant difference of the incidence of extra-pulmonary complications between the two groups (P<0.05). We also evaluated the severity of MPP in all patients. The incidence of severe MPP was 18.4% in the MR group and 3.4% in the MS group, with a significant difference (P<0.05). In addition to clinical symptoms, radiological findings were more severe in the MR group than that in the MS group, 61.7% of the patients in the MR group showed large lesions versus 41.4% in the MS group (P<0.05).

Concerning clinical course, we found that the median duration of fever was 8 (0~42) days in the MR group and 6 (0~14) days in the MS group (P<0.01). The median hospitalization duration was 8 (2~45) days and 6 (3~16) days (P<0.01), respectively. The median fever duration after macrolide therapy was significantly longer in the MR patients than in the MS patients (median of 5 days versus 3 days, P<0.01).
Discussion

Although MP infection was traditionally thought to be a self-limited process, more and more severe cases even fatal cases of MP infections were reported in recent years [3-8]. The reasons why more severe MP infections occurred remain unclear, but resistance to macrolides does have a close association with the emergence and increase of MR strains.

Since 2000, the emergence of macrolide resistant has been reported mainly in Asia [21]. In China, prevalence of MR MP isolated in pediatrics has increased rapidly. In 2009, Xin et al. [22] reported that 46/50 (92%) MP isolates from pediatric patients were resistant to macrolides. In 2010, Liu et al. [10] reported that 90/100 (90%) MP isolates were resistant to macrolides. In our study, we found that 206 (87.7%) NPAs from patients with MPP showed point mutations in domain V of 23S rRNA, which were considered as MR. The high prevalence of MR was similar to the data reported.

Macrolide-resistant genotypes are defined by specific point mutations in the V domain of the 23S rRNA gene of MP. Several potential transition mutations in the complete sequence of the 23S rRNA were found: A2063G, A2064G, A2063T. A2063G and A2064G mutations are responsible for the high-level macrolide resistance in MP [23]. Morozumi et al. [24] found that among 380 MP isolates from 3,678 pediatric patients with community-acquired pneumonia, 50 MR strains had an A2063G transition in domain V of the 23S rRNA, whereas 5 had an A2064G transition. In our study, we amplified the full-length of the 23S rRNA fragment from 235 NPAs of patients with MPP, and compared with the sequence of M129. We found that among the 206 MR patients 199 (96.6%) possessed an A2063G transition in domain V of the 23SrRNA, 6 cases showed A2063T mutation, and
one A2064G mutation. Furthermore, the 2063 point mutations in domain V of the 23S rRNA gene accounted for 99.5% (205 cases) of all mutations, which was well consistent with that reported in the literature. The other less frequent point mutation, 2617 also had been detected, but no positive results were found (data not shown).

As to current situation of macrolide resistance of MP, it is important to evaluate the clinical significance of MR MP. Suzuki et al. found the total febrile days and the numbers of febrile days during macrolide administration were longer in the MR patients than in the MS patients. Ensuing clinical studies were reported with similar results. In our study, MR MP-infected patients showed much longer median duration of fever, longer median hospitalization duration and longer median fever duration after macrolide therapy than those of patients with MS MP. These results implied that MR MP was refractory to treatment and could result in prolonged clinical course.

More interestingly, our study found that the incidence of extra-pulmonary complications in the MR patients were higher than that in the MS patients. 29.6% (61/206) of MR patients had extra-pulmonary complications versus 10.3% in the MS group. Moreover, only one patient (3.4%) demonstrated severe pneumonia in the MS group, and 38 of 206 MR patients (18.4%) were diagnosed as severe MPP. In addition to clinical symptoms, 61.7% of patients in the MR group showed large lesions of radiological manifestation versus 41.4% in the MS group. To our knowledge, this is the first report showing severe clinical course and more extra-pulmonary complications in the MR patients.

Several reasons may explain the difference between previous reports and our study. Firstly, the MPP children enrolled in our study reflected a natural clinical course. Because of
potential side effects, fluoroquinolones are contraindicated in all children and tetracyclines, such as doxycycline and minocycline can only be used in older children aged 8 years or above; there are almost no alternatives for macrolide-resistant MP infection children especially preschool-aged children. Therefore, all the cases in our study received only macrolides. Local MP loads 48 hours after macrolide therapy in the MR patients were reported to be much higher than those in the macrolide-sensitive patients [14]. The higher and persistent MP stimulation may induce much stronger host response especially IL-8 and IL-18-associated inflammation, which is related to the severity of MPP in children [27]. While in previous studies, initial treatment of macrolides were usually switched to other antibiotics such as levofloxacin, ciprofloxacin, minocycline or doxycycline within 2 to 3 days after macrolide medication with an antibiotic change rate of 63.6%–85.7% [14, 16, 28]. Secondarily, our hospital is a tertiary hospital and the enrolled patients had much longer duration before hospitalizing into our hospital than previous studies. Again, the persistent MP antigen stimulation and/or invasion greatly increased the possibilities of severe lung lesions, pulmonary and extra-pulmonary complications. Thirdly, our study had the largest numbers of MPP children and we focused on the incidence of extra-pulmonary complications. Until now, no other study focusing on the real state of extra-pulmonary complications in the MR children was reported. Our study firstly demonstrated a higher incidence of extra-pulmonary complications in the MR patients as compared with the MS patients.

One limitation of our study is that the MIC values were not measured because MP was not isolated from all these patients. However, it is widely acknowledged that the presence of 2063 and 2064 mutations is usually associated with macrolide resistance.
In conclusion, we amplified and analyzed the full-length sequence of the 23S rRNA gene from 235 NPAs, and compared with that of an MP reference strain M129. It showed that the increasing prevalence of MR MP has become a significant clinical issue in pediatric patients, and it may lead to more extra-pulmonary complications, severe clinical features and radiological manifestations. The impact of the macrolides resistance on clinical outcome of children with MPP is needed to be further investigated.
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Conflicts of Interest: None.
References


Matsubara K, Morozumi M, Okada T, Matsushima T, Komiyama O, Shoji M, Ebihara


Table 1—Clinical information of MR and MS patients

<table>
<thead>
<tr>
<th>Clinical information</th>
<th>MR Group (n=206)</th>
<th>MS Group (n=29)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median age (years)(^a)</td>
<td>4 (0–14)</td>
<td>5 (0–11)</td>
<td>NS</td>
</tr>
<tr>
<td>Sex (male/female)</td>
<td>132/74</td>
<td>17/12</td>
<td>NS</td>
</tr>
<tr>
<td>Severe MPP</td>
<td>18.4% (38/206)</td>
<td>3.4% (1/29)</td>
<td>0.042</td>
</tr>
<tr>
<td>Chest radiography (large lesions)(^**)</td>
<td>61.7% (127/206)</td>
<td>41.3% (12/29)</td>
<td>0.038</td>
</tr>
<tr>
<td>Median fever duration (days)(^a)</td>
<td>8 (0–42)</td>
<td>6 (0–14)</td>
<td>0.001</td>
</tr>
<tr>
<td>Median hospitalization duration (days)(^a)</td>
<td>8 (2–45)</td>
<td>6 (3–16)</td>
<td>0.007</td>
</tr>
<tr>
<td>Median fever duration after macrolide therapy (day)(^a)</td>
<td>5 (0–42)</td>
<td>3 (0–10)</td>
<td>0.007</td>
</tr>
<tr>
<td>Extra-pulmonary complications</td>
<td>29.6% (61/206)</td>
<td>10.3% (3/29)</td>
<td>0.029</td>
</tr>
<tr>
<td>Digestive system (liver function abnormalities)</td>
<td>44.3% (27/61)</td>
<td>100% (3/3)</td>
<td></td>
</tr>
<tr>
<td>Cardiovascular system (myocarditis)</td>
<td>24.6% (15/61)</td>
<td>0% (0/3)</td>
<td></td>
</tr>
<tr>
<td>Rash</td>
<td>18.0% (11/61)</td>
<td>0% (0/3)</td>
<td></td>
</tr>
<tr>
<td>Nervous system (encephalitis)</td>
<td>6.6% (4/61)</td>
<td>0% (0/3)</td>
<td></td>
</tr>
<tr>
<td>Urinary system (proteinuria)</td>
<td>3.3% (2/61)</td>
<td>0% (0/3)</td>
<td></td>
</tr>
<tr>
<td>Hematological system (hemolytic anemia)</td>
<td>1.6% (1/61)</td>
<td>0% (0/3)</td>
<td></td>
</tr>
<tr>
<td>Joint system (arthritis)</td>
<td>1.6% (1/61)</td>
<td>0% (0/3)</td>
<td></td>
</tr>
</tbody>
</table>

*NS not significant; **Large lesion: the extent of infiltration on chest radiography was more than 1/3 of the lung. \(^a\)data shown as median (range).