Amplification of 16S rDNA by nested PCR for measurement of *Mycoplasma pneumoniae* DNA over time: clinical application

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*Mycoplasma pneumoniae* (MP) is the most common atypical pathogen that causes respiratory infections in children. Such infections are typically treated by macrolide antibiotics, but the duration of treatment is variable. In this study, we used nested PCR to amplify the 16S rDNA (16S rRNA gene) of MP at different stages of MP pneumonia (MPP) in 100 children who were admitted for lower respiratory tract infections and diagnosed with MPP. Our results indicate that the median duration of MP-DNA positivity was 5 weeks, and 78% of cases tested positive for 3–6 weeks. Patients with severe disease were positive for MP-DNA for a significantly longer time (median of 6 weeks) than those with mild disease (median of 4 weeks). Thirty-one patients with severe disease who received intravenous immunoglobulin were MP-DNA positive for significantly less time than patients with severe disease who did not receive this treatment. The duration of MP-DNA positivity was prolonged when MP antibody levels were high and treatment was started at a later stage. Therefore, nested PCR can be used for early diagnosis of MP and the duration of MP-DNA reflects the clinical stage of MPP. Early treatment of MPP and the administration of intravenous immunoglobulin during the acute phase of severe MPP shorten the duration of MP-DNA positivity.

INTRODUCTION

*Mycoplasma pneumoniae* (MP) is a common pathogen in paediatric patients with respiratory tract infections and is responsible for nearly 40% of community-acquired pneumonia in children (Lee, 2008). MP pneumonia (MPP) and extrapulmonary complications caused by MP infection have serious health consequences in children (Okazaki et al., 2007). Treatment with macrolides, primarily erythromycin and azithromycin, for at least 2–3 weeks is recommended for children infected with MP (Waites & Talkington, 2004). However, the specific course of treatment for an individual paediatric patient is usually based on a clinician’s personal experience, and this may lead to inappropriate treatment. In recent years, many strains of MP have developed resistance to macrolide antibiotics (Okazaki et al., 2007; Waites et al., 2008), and this is presumably related to the inappropriate use of antibiotics.

Serological testing is the most widely used method for detection of MP in clinical practice (Daxboeck et al., 2003). However, this method only provides a retrospective diagnosis, and the results can be affected by the phase of antibody production, patient age and immunity status (Yuan & Xue, 2002). Moreover, antibodies are typically present even after the patient is cured (asymptomatic), so this method cannot be used to assess disease status.

The MP 16S rRNA gene (16S rDNA) has species-specific repeated sequences and its copy number accurately indicates the *in vivo* level of MP (Sung et al., 2006). Additionally, previous research indicated that detection of 16S rDNA by nested PCR is more sensitive than that of the P1 adhesin gene (Nour et al., 2005). An elevated concentration of MP is present in the nasopharynx after MP infection (Zhang, 2004), so infection can be readily diagnosed by measurement...
of MP-DNA in the nasopharyngeal epithelial cells. MP is only rarely present in the pharynx of healthy patients (Hou et al., 2002).

In the present study, we collected throat swabs of children with MP infection at different stages of disease and used nested PCR to amplify and measure MP 16S rDNA. Based on MP-DNA, all children diagnosed with MPP were given early treatment. The duration of macrolide antibiotic treatment was guided by detection of MP-DNA and clinical symptoms over time, thereby preventing recurrence of MPP due to inadequate treatment or drug resistance due to unnecessarily long treatment.

METHODS

Subjects and experimental grouping. A total of 110 children with pneumonia who were hospitalized in the Department of Respiratory Care at our hospital from November 2008 to August 2009 were examined. There were 57 males and 53 females and the mean age was 5.3 years (range 3 months to 14 years). All hospitalized children tested positive for MP 16S rDNA or had mycoplasma antibody (MP-Ab) titres of at least 1 : 160, and all were diagnosed with MPP. All enrolled parents and children were cooperative and exhibited good treatment compliance, with patients coming to scheduled follow-up visits as requested. Children with no evidence of MPP, poor compliance (defined as inability to come in for follow-up visits), or congenital diseases (heart disease, immunodeficiency, pulmonary dysplasia, bronchial stenosis or mediastinal abnormalities) were excluded.

All 16S rDNA- and MP-Ab-positive patients were classified as having either severe MPP or common MPP. The diagnostic criteria for severe MPP were (Zhang, 2004): (i) persistently high fever for more than 10 days after normative macrolide antibiotic treatment, and severe coughing that interfered with sleep; (ii) large areas of high-density lobe consolidation with moderate or large amounts of pleural effusion; (iii) consolidation with moderate or large amounts of pleural effusion; (iv) severe MPP (defined as inability to come in for follow-up visits), or congenital diseases (heart disease, immunodeficiency, pulmonary dysplasia, bronchial stenosis or mediastinal abnormalities) were excluded.

Specimen collection. For all patients, specimen collection was completed within 24 h after admission. After rinsing the mouth, a sterile cotton-tip throat swab moistened with sterile saline was used to wipe the posterior pharyngeal wall with moderate force. Samples were stored at ~80 °C. At the same time, a 1 ml blood sample was collected for measurement of MP-Ab. Identical collection methods were used at 1 week intervals until the MP-DNA was negative. As a positive control, standard strains of MP, provided by Guizhen Wang (Microbiology Lab, Chinese Medical University, Shenyang, China), were used.

Amplification of MP 16S rDNA. DNA was extracted from the throat swab specimens. Briefly, the specimen in the swab was dissociated by soaking the swab in 1 ml sterile saline. The sample was vortexed, transferred to a 1.5 ml Eppendorf tube and centrifuged at 8500 g for 10 min. The pellet was resuspended in 50 µl DNA lysis buffer (Fuxing Biotechnology Co.) and heated at 100 °C for 10 min. The lysate was then centrifuged at 8500 g for 10 min, and the supernatant was subjected to nested PCR.

Gene amplification was performed using nested PCR as previously described by Nour et al. (2005). The PCR was performed in a total volume of 50 µl (reaction mix comprised 50 mM KCl, 10 mM Tris/ HCl pH 8.8, 1.5 mM MgCl₂, 200 µM dNTPs, 25 mM forward and reverse primers, 2 U Taq polymerase and 10 µl DNA template). The first round of PCR for the 16S RNA gene was performed using outer primers 5'-AAGGACCTGCAAGGGTTGT-3' and 5'-CTCTGACC- ATTACCTGTCAA-3', which amplified a fragment of 277 bp. PCR was performed by denaturation at 95 °C for 5 min, 30 cycles of denaturation at 94 °C for 30 s and renaturation at 54 °C for 30 s, and a final extension at 72 °C for 1 min. Thereafter, extension was continued for 9 min. The second round of PCR was done with 10 µl product from the first-round PCR as the template, using inner primers 5'-CT- CTAGCCATTACCTGTCAA-3' and 5'-ACTCTCAGGGAGCAG- AGTA-3', which amplified a fragment of 141 bp. The amplification conditions were identical to the first round except that the denaturation temperature was 56 °C. One positive control (cultured MP extract) and one negative control (MP-DNA negative sample from a normal control) were included in each round and PCR products were analysed on 2 % agarose gels in Tris/borate/EDTA buffer. The microorganism was simultaneously amplified as a control in order to rule out false-negative PCR results (if the throat swab did not contain endothelial cells). The β-globin primers were 5'-ACAGAATCTTGGTTCATAGC-3' and 5'- CATCAGATGCGACATCC-3', and the amplified fragment size was 326 bp (Gullsby et al., 2008). MP-DNA was considered positive when gel electrophoresis of the second-round PCR products indicated the presence of the 141 bp fragment of the 16S rDNA and the 326 bp fragment of the β-globin gene.

Measurement of mycoplasma antibody (MP-Ab). The micro-particle agglutination method was used to measure MP-Ab (Serodia Myco II kit, Fujirebio Inc.). All procedures were performed according to the kit instructions, and total MP-Ab, which included IgG and IgM, was measured.

Statistical analysis. Categorical data are presented in contingency tables by frequency. Comparisons were performed using Fisher’s exact test. Continuous data are presented in median and interquartile range and were compared with the Mann–Whitney U or Kruskal–Wallis test, unless otherwise stated. The kappa value (κ) was used to assess agreement between the MP-DNA and MP-Ab results. This agreement was interpreted as poor (κ = 0), slight (κ = 0.0–0.2), fair (κ = 0.21–0.40), moderate (κ = 0.41–0.60), substantial (κ = 0.61–0.80) or almost perfect (κ = 0.81–1.00). Data were analysed using SPSS 15.0 (SPSS Inc.). A P-value <0.05 was considered statistically significant.
RESULTS

We measured MP-DNA in throat swabs using nested PCR and measured serum MP-Ab using conventional particle agglutination. The MP-DNA and serum MP-Ab were both positive in 100 of the 110 patients. The kappa value for agreement of the two methods was 0.7371, indicating substantial agreement (Table 1). All 20 healthy children in the control group were negative for the presence of MP-DNA, although nine of these children were positive for the presence of MP-Abs. Four children with MPP tested negative for MP-DNA but positive for MP-Ab. The medical histories of these four children indicated MP infection 3 months previously, based on detection of MP-Ab at that time. Our measured antibody titres of these four children were significantly lower than the previously measured titres, also indicating previous rather than current MP infection.

Importantly, 50 patients were negative for the presence of early MP-Abs, but positive for the presence of MP-DNA (Table 1).

Among all 110 patients, 64 children sought medical help at an early stage of disease (within 1 week of onset). For these 64 children, the MP-DNA test was positive in 56 patients (83.3 %) and the MP-Ab test was positive in eight patients (13.3 %) (Table 1). The kappa value for agreement of these two methods was 0.1134, indicating slight agreement.

We further analysed the data of the 100 patients with positive MP-DNA and MP-Ab results (Fig. 1). The average hospital stay was 16 days, and the duration of MP-DNA positivity was correlated with the length of hospital stay ($r = 0.4538$, $P < 0.0001$). In particular, the duration of MP-DNA positivity was less for patients diagnosed with the early stage of the disease (4.5 ± 1.5 weeks, $n = 54$) than for those diagnosed with the late stage of the disease (5.9 ± 1.8 weeks, $n = 46$, $P < 0.01$). In addition, the length of hospital stay was less for early diagnosis (15.1 ± 3.3 days for early diagnosis vs 16.5 ± 4.2 days for later diagnosis, $P < 0.05$). These results indicate a correlation of the duration of MP-DNA positivity with clinical outcome, and suggest that there may be a clinical advantage for early diagnosis and treatment.

Table 1. Presence of MP-Abs within 1 week of disease onset (early) and after 1 week of onset (late) in paediatric patients with MPP

<table>
<thead>
<tr>
<th>MP-DNA</th>
<th>Early and late MP-Ab*</th>
<th>Early MP-Ab†</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>100</td>
<td>4</td>
</tr>
<tr>
<td>−</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>Total</td>
<td>100</td>
<td>10</td>
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</table>

*κ = 0.7371.
†κ = 0.1134.

Our results indicate that the median duration of MP-DNA positivity was 5 weeks and that 82 % of children had MP-DNA positivity for more than 3 weeks (Table 2). In 77 children (77%), the throat swab was positive for MP-DNA at 3–6 weeks after disease onset. MP-Ab titre was persistently 1:160 or greater in all the 100 children in the period of MP-DNA positivity.

We classified 60 patients as having severe MPP and 40 patients as having common MPP (Table 3). Patients were diagnosed with severe MP only when they met all the four criteria described in Methods. Some of the patients with severe MP experienced liver damage, which was thought to be due to MP infection. The common MPP group was positive for MP-DNA for a median of 4 weeks, and the severe MPP group was positive for MP-DNA for a median of 6 weeks ($P = 0.002$). In addition, the odds ratio for severe MPP increased with increasing time of MP-DNA positivity.

After 3 days of hormone therapy (methylprednisolone), 31 patients who had severe MPP and persistent high fever received a 3 day course of IVIG. Of these 31 patients, 15 were male and 16 were female; 16 belonged to the early treatment group and 15 belonged to the late treatment group. However, these distributions were not significantly different from the severe MPP patient group that did not receive IVIG. The ages of severe MPP patients with
and without IVIG treatment were 5.8 ± 3.2 [median (IQR): 5 (3–8)] and 5.3 ± 3.06 [median (IQR): 5 (3–7)] years respectively (P = 0.66). The median duration of MP-DNA positivity was 5 weeks in patients treated with IVIG, but was 6 weeks in patients not treated with IVIG (P = 0.001).

We classified 58 patients as having high levels of MP-Abs and 42 patients as having low levels of MP-Abs (Table 3). The duration of MP-DNA positivity was not significantly different in these two groups (P = 0.179). There were more patients with high levels of MP-Abs among those who were MP-DNA positive for 7 weeks or more (19 of 39) than in those who were MP-DNA positive for 4 weeks or less (16/20, P = 0.048). The duration of MP-DNA positivity was not significantly dependent on gender (P = 0.889) or age (P = 0.852). There was no significant difference in the age and gender of children belonging to the early and late treatment groups, although the age of the children belonging to the group receiving early treatment was generally lower than that of the children receiving late treatment (5.3 ± 3.2 vs 5.8 ± 2.8 years, respectively, P = 0.428). Fifty-two per cent (28/54) and 70% (32/46) of children in the early and late treatment groups, respectively, were diagnosed with severe MPP (P = 0.10).

**DISCUSSION**

Although the presence of MP-specific Abs is a reliable indicator of MP infection (McDonough et al., 2005), because the immune systems of infants and young children are not fully developed and the rate of detection for patients with early stage-disease is low (Youn et al., 2010), MP infection cannot be ruled out by negative MP-Ab results, especially in children. However, PCR is a highly specific and sensitive method for detection of MP (Beersma et al., 2005; Dumke & Jacobs, 2009; Gullsby et al., 2008). In this study, we used nested PCR to detect presence of the MP 16s rDNA and measured serum MP-Abs. In general, although these two tests yielded consistent results, PCR is more sensitive. The sensitivity of PCR over serology is emphasized by the low kappa value seen when MP-DNA positivity is compared with the presence of early MP-Abs, which may be due to limited immune response or time required for seroconversion. However, MP antibodies can remain long after an infection has subsided. Our data showed that four patients were negative for the presence of MP-DNA, but positive for early MP-Abs. Since these patients were all known to have had an MP infection 3 months previously, our data suggest that it is possible for PCR to differentiate between current and past infections. However, it should be noted that, in addition to indicating a current infection, the presence of MP DNA could also indicate the presence of sequestered DNA from a previous infection.

The presence of MP-DNA over time was monitored in this study. All patients tested positive for MP-DNA for at least 3 weeks, and none were positive for more than 11 weeks. The mean duration of MP-DNA positivity was 5 weeks, and MP-DNA disappeared in 80% of children within 6 weeks after disease onset. A previous study of a Swedish population (median age of 41 years) reported that the duration of MP-DNA positivity decreased over time and

### Table 3. Presence of MP-DNA positivity over time for different patient groups

<table>
<thead>
<tr>
<th>Patients</th>
<th>MP-DNA disappearance time (weeks):</th>
<th>Total</th>
<th>P-value*</th>
<th>Length of stay</th>
<th>Median (IQR)</th>
<th>P-value</th>
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<tr>
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<td>7.3</td>
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<td>IVIG treatment</td>
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<tr>
<td>Age (years)</td>
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<tr>
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<td>5</td>
<td>8</td>
<td>6</td>
<td>6</td>
<td>32</td>
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</table>

*Fisher’s exact test, P = 0.001.
with the number of pathogens (Nilsson et al., 2008). The authors also reported that the duration of MP-DNA positivity was 2 days to 7 months, and that the median duration of MP-DNA positivity was 7 weeks. These results are very different from ours, possibly because of the very different study populations.

The present study also showed that the median duration of MP-DNA positivity was longer in children with severe MPP (6 weeks) than in those with common MPP (4 weeks). Currently, many clinicians in China recommend a 3–4 week course of treatment for mild cases and a 4–6 week course of treatment for severe cases. The results of this study provide clinicians with a biological basis for determining the duration of macrolide treatment of MP infection.

IVIG treatment affects immune replacement and immune regulation (Negi et al., 2007), and can be used as an immune-modulator in the treatment of MP. In the present study, 31 children with severe MPP received IVIG treatment, and the duration of MP-DNA was significantly reduced compared to those without IVIG treatment. We also found that the duration of MP-DNA positivity was longer in patients with high levels of MP-Abs.

Our results also indicate that the duration of MP-DNA positivity and number of days in the hospital were less for patients who received therapy in the early stage of disease (within 1 week of onset) and that the duration of MP-DNA was positively correlated with the duration of stay in the hospital. Duration of hospital stay was also influenced by severity of MPP and by IVIG treatment. These data indicate that the duration of MP-DNA positivity accurately reflects pathogen activity and clinical stage of MPP.

Although the difference was not statistically significant, our data showed that the age of children receiving early treatment was generally lower than that of the children who received later treatment, and that early treatment correlated with a shorter duration of MP-DNA positivity and less occurrence of severe MPP. These data could be reflective of younger children receiving earlier medical care. However, we did not find a correlation between age and the duration of MP-DNA positivity.

Among the limitations of this study is the fact that we do not have data on the effect of different durations of antibiotic treatment on symptoms and development of resistance in MP. We also did not study the survival of MP at different levels of 16S rDNA in response to treatment.

In summary, we have demonstrated that nested PCR can be used for early diagnosis of MP and that the duration of MP-DNA presence reflects the clinical stage of MPP. Early diagnosis and treatment of MPP and the administration of IVIG during the acute phase of severe MPP shorten the duration of MP-DNA positivity.

REFERENCES


