Survey of Mycoplasmal Bacteremia Detected in Children by Polymerase Chain Reaction

Mitsuo Narita, Yoshihiro Matsuzono, Osamu Itakura, Takehiro Togashi, and Ilideaki Kikuta

To determine whether mycoplasmal bacteremia occurs during ordinary or complicated diseases due to M. pneumoniae (and if so, how frequently), we used polymerase chain reaction (PCR) to detect M. pneumoniae in serum samples. The PCR primers used were modified for nested amplification. The genome of this organism was detected in 1 of the 25 patients with pneumonia and 10 of the 17 patients without pneumonia (P < .001, χ² test). The genome was detected more frequently in patients who had encephalitis of which the neurological onset was within 7 days of the onset of fever rather than later. We hypothesize that mycoplasmal bacteremia occurs more frequently than previously appreciated, specifically in the absence of pneumonia, and that certain types of complications (e.g., encephalitis of early onset) are associated with its occurrence.

Materials and Methods

The diagnosis of mycoplasmal infection was made when at least one of the following criteria was met: (1) a fourfold or greater rise or fall in titer of antibody to Mycoplasma pneumoniae, as measured by CF or microparticle agglutination test [8]; and (2) a single or sustained high titer of ≥256 (by CF) or ≥320 (by microparticle agglutination). (Hereafter in this article, a fall in titer means a fall from the high titers mentioned in this second criterion.) No attempts were made to isolate the organism because of the inconvenience of this method in general practice.

In addition, the following conditions were required: (1) bacterial cultures yielded no growth of pathogenetic bacteria from any site, and (2) no serological evidence of viral infection was observed in the tests used. The viruses tested for included herpes simplex virus types 1 and 2; varicella-zoster virus; cytomegalovirus; Epstein-Barr virus; influenza A and B viruses; paramyxovirus types 1, 2, 3, and 4; echovirus types 3, 7, 11, and 12; coxsackievirus types A9, B1, B2, B3, B4, B5, and B6; measles virus; mumps virus; and rubella virus, although not all of these were tested for in every patient.

With the exception of a few cases, viral cultures could not be performed, because in Japan physicians who request such procedures from a commercial laboratory must shoulder the expense themselves. Cases in which simultaneous involvement of other bacterial or viral pathogens was suspected were excluded from study.

Consequently, 42 patients (aged 1–17 years; 26 males and 16 females) were included in this study. From each patient (except for a few with encephalitis), at least one acute-phase serum sample was obtained within 7 days of the onset of any symptom (for a total of 57 samples). The numbers of patients whose mycoplasmal infections were diagnosed on the basis of the established serological criteria are presented in table 1.

Thirty-eight (90%) of the 42 patients' mycoplasmal infections were diagnosed by microparticle agglutination. This proportion is a reflection of the fact that most of the clinical laboratories in Japan have now adopted this test, presumably because of its simplicity. The number of patients in each disease category is presented in table 2.

Pneumonia was diagnosed when dense infiltration and/or opaque consolidation was observed roentgenographically. Except for a few cases, pneumonia was recognized at the first presentation. Liver dysfunction was considered significant when the alanine aminotransferase level was ≥100 U/L, and cases in which such levels were lower were not included in this category.

Meningitis was diagnosed when signs and symptoms of meningeal inflammation were observed as well as pleocytosis. Encephalitis was diagnosed when some of the symptoms—such
Table 1. Numbers of patients with mycoplasmal infection, classified according to the diagnostic criteria and their status with regard to pneumonia.

<table>
<thead>
<tr>
<th>Serological test, criterion (titer*)</th>
<th>With pneumonia</th>
<th>Without pneumonia</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microparticle agglutination</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥4-Fold rise or fall</td>
<td>17</td>
<td>6</td>
<td>23</td>
</tr>
<tr>
<td>Single or sustained high titer:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>320–640</td>
<td>1</td>
<td>3</td>
<td>15</td>
</tr>
<tr>
<td>1,280–2,560</td>
<td>2</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td>&gt;2,560</td>
<td>4</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>Complement fixation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥4-Fold rise or fall</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Single or sustained high titer:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>256</td>
<td>0</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>&gt;512</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>25</td>
<td>17</td>
<td>42</td>
</tr>
</tbody>
</table>

* Titer of antibodies to *Mycoplasma pneumoniae*.

Results of PCR for the study subjects are presented in table 2. For none of the 24 control subjects did PCR yield a positive result. In cases in which the sham control or the buffer control yielded a positive band, the results of the run were excluded. Since the use of 8-methoxypsoralen/ultraviolet decontamination is now a standard part of our protocol [9, 11], such sporadic positives among the controls were substantially diminished.

As shown in table 2, results of PCR were positive for only 1 of the 25 patients with pneumonia but 10 of the 17 patients without pneumonia. This difference reached statistical significance (*P* < .001). Among the 11 PCR-positive patients, myco-

Table 2. Results of PCR with use of serum samples from patients with mycoplasmal infections.

<table>
<thead>
<tr>
<th>Patient group</th>
<th>Total</th>
<th>Positive</th>
<th>Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients with pneumonia</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pneumonia alone</td>
<td>14</td>
<td>0</td>
<td>14</td>
</tr>
<tr>
<td>Pneumonia with liver dysfunction</td>
<td>3</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Pneumonia with CNS involvement</td>
<td>8</td>
<td>1</td>
<td>7</td>
</tr>
<tr>
<td>Meningitis</td>
<td>4</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>Encephalitis of early onset*</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Encephalitis of late onset</td>
<td>3</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Patients without pneumonia but with</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver dysfunction</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>CNS involvement</td>
<td>16</td>
<td>9</td>
<td>7</td>
</tr>
<tr>
<td>Meningitis</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Encephalitis of early onset*</td>
<td>10</td>
<td>7</td>
<td>3</td>
</tr>
<tr>
<td>Encephalitis of late onset</td>
<td>5</td>
<td>2</td>
<td>3</td>
</tr>
</tbody>
</table>

NOTE. The difference (for PCR-positive vs. PCR-negative) is statistically significant (*χ² = 13.02; *P* < .001).

* For details, refer to our previous report [9].

as mental alteration, loss of consciousness, convulsions, urinary disturbances, or cerebellar symptoms—were observed along with abnormal brain CT findings and/or electroencephalographic findings.

All of the patients with meningitis or encephalitis were included in our previous report [9], in which we demonstrated that the genome of *M. pneumoniae* was detectable in CSF. It was detected with significantly higher frequency in patients with encephalitis of early onset (i.e., CNS symptoms appeared within 7 days following the onset of fever) than in patients with encephalitis of later onset (at ≥8 days following fever). In this study, therefore, this classification system (i.e., early vs. late onset) was arbitrarily used.

Because of our preliminary observation that CNS complications associated with *M. pneumoniae* might not infrequently develop in the absence of pneumonia [7], many of our colleagues kindly provided us with clinical samples from patients with CNS manifestations who did not have pneumonia, so that we could survey for the presence of mycoplasmal DNA. This accounted for the accumulation of nonpneumonia cases in which there was serological evidence of mycoplasmal infection.

In addition, 24 acute-phase serum samples from 24 patients with respiratory symptoms (including 17 with CNS manifestations), who had no serologically evident mycoplasmal infection, served as negative controls.

A whole-blood sample was routinely centrifuged at 3,000g for 15 minutes to remove clots (this gravity is generally considered not to precipitate cell debris or free mycoplasmas), and the serum was stored at −20°C until use. The serum samples, usually 1–3 mL, were then centrifuged at 13,000g for 30 minutes to precipitate cell debris as well as free mycoplasma; in most cases, this resulted in the formation of a pellet 0.5–1.0 mm³ in size.

The pellet was dissolved in 120 µL of a proteinase solution, as previously described [7], and DNA was extracted and purified through phenol extraction and ethanol precipitation. The PCR procedures were performed as also detailed previously [7, 9], with use of the primer set described by Bernet et al. [10], which was modified for nested amplification.

For each run, distilled water was processed in parallel with clinical samples and served as a sham control to check for contamination during the DNA preparation process. In addition, distilled water was amplified instead of a DNA sample and served as a buffer control to check for contamination during the amplification process.

Categorical data were statistically analyzed by the χ² test with Yates's correction factor.

Results

For none of the 24 control subjects did PCR yield a positive result. In cases in which the sham control or the buffer control yielded a positive band, the results of the run were excluded. Since the use of 8-methoxypsoralen/ultraviolet decontamination is now a standard part of our protocol [9, 11], such sporadic positives among the controls were substantially diminished.

As shown in table 2, results of PCR were positive for only 1 of the 25 patients with pneumonia but 10 of the 17 patients without pneumonia. This difference reached statistical significance (*P* < .001). Among the 11 PCR-positive patients, myco-
plasmal infection was diagnosed in 3 by CF and in 8 by micro-
partical agglutination.

With regard to disease categories, it was remarkable that 15
of the 19 patients with encephalitis (of both early and late
onset) did not have pneumonia. It is notable that patients with
encephalitis of early onset (including 1 with pneumonia and
10 without pneumonia) were PCR-positive at a high rate (8 of
the 11 patients).

The distribution of PCR results is shown in figure 1. The
genome of \textit{M. pneumoniae}, when detected, remained in the
blood for $>20$ days.

The results of PCR, which were classified according to the
presence or absence of pneumonia and the serological diagno-
sic criteria, are shown in table 3. Although there was a slight
tendency for patients with pneumonia to have a rise or fall in
titer of antibody (17 cases) rather than sustained high titers (8
cases)—in contrast with the patients who did not have pneumo-
nia, of whom more had sustained high titers (10 cases) than a
rise or fall in titer (7 cases)—this did not reach statistical
significance ($P > .05$).

In addition, the rate of PCR positivity increased according
to the diagnostic criteria, i.e., a rise in titer (17%), a high titer
(28%), or a fall in titer (50%). However, this finding may not
be conclusive because of the limited number of PCR-positive
patients.

Because of the retrospective nature of this study, the manage-
ment of the cases, specifically those involving encephalitis,
was not controlled. The timing, selection, and route of adminis-
tration of antibiotics, as well as the use of corticosteroids,
varied from patient to patient. Nevertheless, overall the out-
comes were favorable. All but one patient (who had encephali-
tis of late onset, did not have pneumonia, and had PCR-negative
serum and CSF) recovered within 4 weeks of the onset of CNS
manifestations and had no major sequelae.

**Discussion**

It is a rather unexpected finding that the genome of \textit{M. pneu-
moniae} was detected more frequently in patients without pneumo-
nia than in patients with pneumonia, although there are two issues
to be concerned about with regard to this observation.

First, the distribution of patients was uneven. The group of
patients with encephalitis of early onset consisted of 10 of the
17 patients without pneumonia, and 7 of these 10 had positive
PCR results. In sharp contrast, only one PCR-positive patient
with such encephalitis was from the group of patients with
pneumonia. This uneven distribution of the patients with en-
cephalitis of early onset might have biased the conclusion.

Second, the PCR did not always detect viable organisms.
Nevertheless, a positive PCR result must indicate that the total
amount of free mycoplasmal cells or human cell debris that
contained mycoplasmas [12] was increasing in the blood in
these patients.

**Table 3. Results of PCR for patients with mycoplasmal infection, according to the diagnostic criteria.**

<table>
<thead>
<tr>
<th>Patients</th>
<th>No. of PCR-positive (%) and PCR-negative patients per diagnostic (titer) group*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rise</td>
</tr>
<tr>
<td>With pneumonia</td>
<td>0 (…)</td>
</tr>
<tr>
<td>Without pneumonia</td>
<td>3 (60)</td>
</tr>
<tr>
<td>Total</td>
<td>3 (17)</td>
</tr>
</tbody>
</table>

NOTE. Pos = PCR-positive; Neg = PCR-negative.

* Rise and fall refer to a $\geq 4$-fold rise or fall in titer of antibody to \textit{Mycoplasma pneumoniae}, as measured by CF or micropartical agglutination (MPA); Sustained refers to a single or sustained high titer of $\geq 256$ (by CF) or $\geq 320$ (by MPA).
This finding may have significant implications concerning the development of complications associated with mycoplasmal infection. It can be speculated that pneumonia, which has long been appreciated as the hallmark of mycoplasmal infection, might be only a part (albeit a major part) of the complications associated with such infection.

Similarly, Thomas et al. [13] found in a study of neurological disease associated with mycoplasmal infection that only one of 13 patients with encephalitis and/or other symptoms had pneumonia. This finding and our findings suggest that a number of patients with encephalitis due to M. pneumoniae may have been missed because of the absence of pneumonia.

Moreover, patients with encephalitis of early onset had a high frequency of serum-PCR positivity, the finding of which is compatible with our previous finding that the genome of M. pneumoniae was detected in CSF predominantly in cases of this type of CNS involvement [9]. The occurrence of mycoplasmal bacteremia, often without pneumonia, may be an underlying factor leading to encephalitis of early onset.

Whether or not there is a causal relationship between the absence of pneumonia and the occurrence of mycoplasmal bacteremia is uncertain. Webster et al. suggested that immunocompetent patients would produce enough antibody at the mucosa to prevent systemic spread of the infection [12]. From this point of view, it can be speculated that if the host immune response against the organism is strong enough to elicit pneumonia, the organism can no longer penetrate into blood or may be eliminated rapidly from blood.

Our limited data provided no further information concerning a wide variety of other complications associated with M. pneumoniae. To determine further the localization of the genome or the viability of the organism in the blood is a problem for future investigation. More studies of a prospective nature are needed to characterize patients without pneumonia to further our understanding of the pathophysiology of extrapulmonary complications associated with mycoplasmal infection. Such studies will provide information useful for the treatment of patients, such as which patients should be treated with antibiotics and which with corticosteroids.

Acknowledgments

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References