Detection of *Mycoplasma pneumoniae* by Polymerase Chain Reaction in Lung Aspirates From Patients With Community-Acquired Pneumonia*

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**Study objective:** This study was designed to evaluate the usefulness of polymerase chain reaction (PCR) to detect *Mycoplasma pneumoniae* DNA in samples obtained by transthoracic needle aspiration (TNA).

**Design:** Prospective study of cases.

**Setting:** A university hospital in Lleida, Spain.

**Patients:** A total of 101 unselected patients, admitted between January 1993 and March 1994 in the emergency department, with a clinical and radiologic picture of community-acquired pneumonia, and without contraindications for TNA application.

**Interventions:** Patients were studied with conventional diagnostic techniques for community-acquired pneumonia. In addition, a sample obtained by TNA was processed by the following methods: culture in standard media, culture in selective media for Legionella, detection of capsular antigens for *Streptococcus pneumoniae* and *Haemophilus influenzae*, and detection of *M pneumoniae* specific genome by PCR.

**Results:** Serologic data were not available in eight patients and were excluded from this analysis. *M pneumoniae* PCR amplification was possible in eight cases, well correlated with serologic responses indicating current infection. Samples from ten additional patients, negative by PCR, were found to be demonstrative of recent *M pneumoniae* infection by serologic study. Finally, in all the remaining 75 cases, including the 59 patients for whom a different microbial diagnosis was established, *M pneumoniae* PCR test gave negative results.

**Conclusion:** This study indicates that PCR, applied to samples obtained by TNA, appears to be a moderately sensitive and highly specific method for rapid detection of *M pneumoniae* lung infection.

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**Key words:** community-acquired pneumonia; *Mycoplasma pneumoniae*; polymerase chain reaction; transthoracic needle aspiration

**Abbreviations:** PCR=polymerase chain reaction; TNA=transthoracic needle aspiration

*Mycoplasma pneumoniae* is a frequent pathogen causing community-acquired pneumonia.1-3 Laboratory evidence of this infection has been found in 5 to 30% of pneumonias in the general population, and up to 50% of cases that appear in epidemic periods. Certainly, most of them are relatively mild,4 but more severe and even fatal cases can also occur.5,6 In addition, mycoplasmal infections are readily treatable. Therefore, the development of rapid, sensitive, and specific microbiologic diagnostic techniques is necessary.

Isolation of the organism by culture and detection by serologic procedures are currently available methods, but both have some limitations.2 Thus, although cultures, in experienced hands, are sensitive tests, isolation of *M pneumoniae* is difficult, available in only a few laboratories, and too slow to be clinically relevant.7

However, the diagnosis made by serologic study is only retrospectively available, requiring paired serum samples to demonstrate a significant increase in antibody titer; in addition, false-negative results among immunocompromised hosts have been found frequently.8,9

Recently, polymerase chain reaction (PCR) tests for the detection of *M pneumoniae* have been developed.10-12 These techniques seem to be useful for a rapid diagnosis and they have high sensitivity and specificity. However, if they are to be used on a routine basis, these promising results need validation by
clinical studies; unfortunately, insufficient information of such data has been published to date.

The purpose of this study was to evaluate, in comparative study with serologic data, the usefulness of PCR for detecting M pneumoniae infection applied to samples obtained directly from lung parenchyma of patients with community-acquired pneumonia.

**Materials and Methods**

This study was performed at Arnau de Vilanova Hospital, a 500-bed teaching hospital in Lleida, Catalonia (Spain) that serves a predominantly rural population of approximately 400,000. Over a 15-month period (January 1993 to March 1994), all adult patients admitted to the emergency service with a clinical and radiologic diagnosis of primary community-acquired pneumonia were studied prospectively.

**Microbiological Techniques**

The following microbiological tests were used routinely. (1) Two blood specimens were obtained before treatment and cultured, aerobically and anaerobically, in standard methods. (2) When it was possible, a fresh spontaneous or induced sputum specimen was collected and cultured in standard and selective media for Legionella species. (3) In patients with pleural effusion, a sample of pleural fluid was obtained by thoracentesis and cultured in standard methods. (4) Serum samples were collected on days 1 and 30. Both were simultaneously assayed for antibody detection to influenza virus (complement fixation test), Chlamydia pneumoniae (micro-immunofluorescence test), Chlamydia psittaci (complement fixation test), Coxiella burnetii (complement fixation test), Legionella pneumophila (immunofluorescence test), and M pneumoniae (immunofluorescence test). (5) When no contraindications (coagulation disturbances, PaO₂ <55 mm Hg, presence of bullae, or untreatable cough) were appreciated, transthoracic needle aspiration (TNA) by using an ultrathin needle (25G) was performed as described by Manresa and Dorca. Briefly, during suspended respiration, the spinal needle was inserted through the thoracic skin and advanced the distance estimated necessary to reach the infiltrate. A 20-mL syringe containing 10 mL of normal saline solution was attached firmly to the needle and 8 mL was expelled. Thereafter, firm negative pressure was applied and maintained over 30 to 60 s while the needle was jigged in and out several times; the 2.5- to 3-mL sample recovered was sent immediately to the laboratory and processed by the following methods: culture in standard bacterial media, aerobically and anaerobically (0.5 mL); culture in selective media for Legionella (0.5 mL); detection of capsular antigens of Streptococcus pneumoniae and Haemophilus influenzae by latex agglutination (0.5 mL); and detection of M pneumoniae-specific genome by PCR (0.5 mL). In some cases, a fraction of sample (0.5 mL) was also processed for detection of Mycobacterium tuberculosis or opportunistic agents. Prior informed consent was obtained from each patient.

**Diagnosis of M pneumoniae Infection**

The diagnosis of M pneumoniae infection was based on the following test results. (1) Serology: determination of M pneumoniae-specific antibody was performed by using a commercial IgG immunofluorescent assay (Boehringer Ingelheim), according to the manufacturer’s recommendations. When it was available, paired serum specimens were simultaneously tested. Antibody titers of 1:64 or less were recorded as negative while a fourfold or greater rise in titers or standing titers of 1:128 or greater were regarded as evidence of present M pneumoniae infection. (2) PCR: a total of 0.5 mL of TNA sample was taken and washed three times with distilled water to obtain a total lysis of RBCs. Subsequently, the sample was centrifuged and the pellet was resuspended in 300 μL of lysis buffer (0.01 mol/L Tris HCl [pH, 7.8], 0.005 mol/L EDTA) and incubated for 1 h at 37°C with 0.5% sodium dodecyl sulfate and 1.5 μL of protease K. Then, the sample was mixed with 400 μL of phenol-chloroform and centrifuged at 12,000 rpm for 5 min to separate organic and aqueous phases. This was placed in a 1.5-mL new tube and 0.6 volumes of isopropyl alcohol was added. The nucleic acid was precipitated by the addition of 0.3 mol/L sodium acetate and 2.0 mol/L sodium chloride and incubation for 10 min at room temperature. Further, the tube was centrifuged for 10 min, and the sample was decanted and washed with 70% ethanol; it was newly centrifuged for 10 min and the supernatant was removed. Finally, DNA was resuspended in 100 μL of distilled water.

For DNA amplification, we used the primer set MP5-1 (GAACCTTATGGTACAGGTTGG) and MP5-2 (ATTACCATCCTTTGTTAAGG) (purchased from Cruachem Ltd; Glasgow, UK), described by Bernet et al, which resulted in an amplification product of 144 base-pairs. The reaction mixture was prepared following the indications previously described. The amplification was performed in a thermocycler (model IHB2024; Cherlyn Electronics Ltd; Cambridge, UK), heating the sample at 94°C for 4 min for a cycle, and then 40 cycles of amplification were performed as follows: 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min. An additional incubation at 72°C for 10 min was added at the end to complete the elongation.

The amplified product was analyzed by 3% agarose gel electrophoresis and ethidium bromide staining, and examined by UV transillumination. Identification of the size of the band was performed by comparison with standard molecular weights (100 base pairs [bp] DNA ladder; Gibco BRL).

To prevent contamination, a strict spatial separation of the different technical steps involved in PCR was maintained during the process, and the recommendations of Kwok and Higuchi were followed. In addition, positive and negative control was added in each assay for avoiding false PCR results.

**Diagnosis of the Remaining Etiologies**

For the other pathogens, the etiologic diagnosis was made by using one or more of the following criteria: (1) organisms isolated by culture of blood, TNA, or pleural fluid; (2) isolation of Legionella species in selective media from sputum or TNA samples; (3) detection of capsular antigens of S pneumoniae or H influenzae in TNA samples; and (4) a fourfold or greater rise in antibody titers or, alternatively, stable titers of 128 or greater for immunofluorescence serologic tests, 256 or greater for microimmunofluorescence serologic test, or 20 or greater for complement fixation serologic tests.

**Results**

One hundred twenty-six consecutive patients with community-acquired pneumonia were enrolled in the study; 18 of them were immunocompromised. Twenty-five patients were subsequently considered not evaluable in this study for the following reasons: 3 did not accept the study, 10 had contraindications for TNA (PaO₂ <55 mm Hg; 5; coagulation disturbances, 3; and untreatable cough, 2), and 12 had a final diagnosis of infection by M tuberculosis (2 cases) or opportunistic microorganisms (Pneumocystis carinii, 8; cytomegalovirus, 1; and Cryptococcus neoformans, 1). In addi-
tion, serologic samples were missing in 8 patients; thus, 93 patients were finally eligible for analysis.

We were able to make a diagnosis, according to the criteria described above, in 77 (83%) cases. Frequently, etiologic diagnosis was based on simultaneous positivity of more than one diagnostic test. For a few patients, tests were positive for two different pathogens. Specific etiologies are shown in Table 1. Adverse effects of TNA were infrequently observed: two developed a minimal pneumothorax and treatment was not necessary; seven experienced mild hemoptysis; and three required analgesia after the procedure.

For serologic data, *M pneumoniae* was considered responsible for 18 episodes of pneumonia, and no cases of mixed infection with other pulmonary pathogens were observed. No significant epidemic pattern was found during the study. Most of these patients were young adults (11 were younger than 45 years), without underlying disease (no patients were immunocompromised), having a prodromal illness and a clinical picture characterized by fever and nonproductive cough, and frequently showed signs of pulmonary consolidation.

### Table 1—Microbial Diagnosis of 93 Cases of Community-Acquired Pneumonia

<table>
<thead>
<tr>
<th>Microorganisms</th>
<th>No. of Patients*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Streptococcus pneumoniae</td>
<td>28</td>
</tr>
<tr>
<td>Mycoplasma pneumoniae</td>
<td>18</td>
</tr>
<tr>
<td>Chlamydia pneumoniae</td>
<td>9</td>
</tr>
<tr>
<td>Influenza virus</td>
<td>6</td>
</tr>
<tr>
<td>Chlamydia species</td>
<td>6</td>
</tr>
<tr>
<td>Haemophilus influenzae</td>
<td>5</td>
</tr>
<tr>
<td>Chlamydia psittaci</td>
<td>4</td>
</tr>
<tr>
<td>Coxiella burnetii</td>
<td>2</td>
</tr>
<tr>
<td>Streptococcus viridans</td>
<td>1</td>
</tr>
<tr>
<td>Enterococcus faecium</td>
<td>1</td>
</tr>
<tr>
<td>Etiology unknown</td>
<td>16</td>
</tr>
</tbody>
</table>

*Included three patients with dual diagnoses: *S pneumoniae*—*C pneumoniae* (2); and *S viridans*—*C pneumoniae* (1).

### Table 2—Clinical and Laboratory Features of 18 Patients With *M pneumoniae* Infection

<table>
<thead>
<tr>
<th>Data</th>
<th>Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, yr, mean±SD</td>
<td>41±18</td>
</tr>
<tr>
<td>Underlying disease, No. of patients (%)</td>
<td>7 (39)</td>
</tr>
<tr>
<td>Duration of symptoms before hospital admission, d, mean±SD</td>
<td>5±3</td>
</tr>
<tr>
<td>Temperature ≥38°C, No. (%)</td>
<td>18 (100)</td>
</tr>
<tr>
<td>Chills, No. (%)</td>
<td>3 (17)</td>
</tr>
<tr>
<td>Cough, No. (%)</td>
<td>17 (94)</td>
</tr>
<tr>
<td>Expectoration of purulent material, No. (%)</td>
<td>5 (28)</td>
</tr>
<tr>
<td>Pleuritic chest pain, No. (%)</td>
<td>8 (44)</td>
</tr>
<tr>
<td>Signs of consolidation, No. (%)</td>
<td>12 (67)</td>
</tr>
<tr>
<td>Leukocyte count ≥15,000/mm³, No. (%)</td>
<td>4 (22)</td>
</tr>
<tr>
<td>Increased percentage of immature granulocytes ≥5%, No. (%)</td>
<td>2 (11)</td>
</tr>
<tr>
<td>Po₂ ≥60 mm Hg, No. (%)</td>
<td>3 (17)</td>
</tr>
</tbody>
</table>

(Table 2). Chills, chest pain, and dyspnea, as well as leukocytosis, were unusual findings. Radiologically, all patients had a well-defined infiltrate and only one developed pleural effusion. Mainly, these patients had a nonsevere pneumonia; although 10 (55.5%) patients required hospitalization, none required ICU admission and no patients died.

Both serologic samples were obtained from 17 of these patients; however, in 1 patient, only the first determination was available. A fourfold increase in the serologic titer was observed in 15 (88.2%) patients; the initial test result was negative in 8 of them. In the remaining 2 patients, both serologic tests give similar results, 1:128 or greater. However, 8 (44.4%) of the 18 lung aspiration samples were found to be positive by *M pneumoniae* PCR. All of the patients with a positive PCR result showed significant increases in the serologic data. Among the ten PCR-negative patients, seven experienced seroconversion, two had standing high titers in both samples, and in one patient only the first sample was available. All the remaining patients, with pneumonia due to alternative pathogens, had *M pneumoniae* PCR-negative results. In Table 3, results of serologic and PCR tests are compared.

### Table 3—Comparison Between Serologic and PCR Data

<table>
<thead>
<tr>
<th>PCR Data</th>
<th>Serologic Data</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>Positive*</td>
</tr>
<tr>
<td>Negative</td>
<td>Negative†</td>
</tr>
<tr>
<td>Positive</td>
<td>8</td>
</tr>
<tr>
<td>Negative</td>
<td>10</td>
</tr>
</tbody>
</table>

*†Titers ≤1:64 were considered as negative.

Discussion

In our study, evaluating unselected patients with community-acquired pneumonia, we could establish the etiologic diagnosis in 77 of 93 evaluable cases. We found evidence of recent *M pneumoniae* infection in 18 subjects; among these, detection of *M pneumoniae* by PCR, performed in samples obtained directly from lung parenchyma, was possible in 8 cases, showing, in comparison with serologic tests, a sensitivity of 44%. However, no cases with pneumonia caused by other microorganisms were positive in the *M pneumoniae* PCR test; consequently, no false-positive results were found.

One of the most important problems that clinical studies investigating the usefulness of new diagnostic tests for community-acquired pneumonia have presented is the difficulty in making a certain etiologic diagnosis in a high percentage of cases by alternative methods. Consequently, we carefully selected the microbiologic tests used in our study. We did not accept
Gram's stain and culture of sputum as diagnostic tool, except for obligate respiratory pathogens; instead, we used samples obtained by invasive techniques as valuable and specific specimens.

Among these techniques, TNA with an ultrathin needle was chosen. This procedure, used by experienced hands, has proved to be a safe technique, acceptable for the patients. Culture of TNA samples has a very high specificity and a sensitivity of between 40 and 70%. The inclusion of additional and also specific diagnostic methods, such as detection of antigens by latex agglutination or specific genomes by PCR, may notably enhance this sensitivity; furthermore, these results do not appear to be modified by prior antibiotic therapy. This selected method allowed us to evaluate more precisely the sensitivity and specificity of *M. pneumoniae* PCR diagnostic method performed on TNA samples.

The PCR has already been demonstrated to be useful for detection of *M. pneumoniae* for several years. This technique, which has proved to be more sensitive than classic culture or DNA probe methods, is highly specific. Thus, Bernet et al., studying samples obtained from experimentally infected animals, were able to detect between 100 and 1,000 organisms. This level of sensitivity was reduced to one to ten organisms in the study of Buck et al., using simulated clinical specimens. Comparable results, under experimental conditions, have been found by others.

Unfortunately, experience about this technique applied to clinical samples is still reduced and studies have some limitations. Thus, in comparison with culture and/or serologic tests, PCR seems to have a sensitivity between 70 and 90%. However, while specificity appeared excellent in experimental studies, positive results of PCR in culture-negative and seronegative patients, even among people without respiratory symptoms, suggests lower specificity. This finding has been attributed to persistence of *M. pneumoniae* in the respiratory tract after acute infection or maybe to the existence of asymptomatic carriers. In front of these studies that used samples collected from the upper respiratory tract, TNA has an additional advantage because the specimen is obtained directly from the infectious locus. It can be hypothesized that this fact could contribute to the elimination of false-positive results, although persistence of *M. pneumoniae* in the lungs from guinea pigs inoculated experimentally with Mycoplasma has also been reported.

Several target sequences for *M. pneumoniae* amplification have been explored showing similar efficacy. In our study, we selected the primers MR5-1 and MP5-2 that had been evaluated extensively by Bernet et al. Their specificity was well demonstrated and no cross-reactivity was detected when the primer set was tested with other members of Mycoplasma species or with other organisms that are likely to be found in the human respiratory tract. Furthermore, these primers are easily available from a commercial supplier.

In our study, discrepancies between both techniques, PCR and the serologic test, were found in ten patients who had serologic evidence of *M. pneumoniae* infection but negative PCR results. This apparent insensitivity of PCR could be attributable to different interpretations. Certainly, a sample error is possible because of the reduced volume obtained usually by TNA, particularly in those patients with deeper or smaller infiltrates. Moreover, PCR could still be an imperfect technique, giving negative results because of the presence of unrecognized inhibitors, as some reports showed recently. Alternatively, false-negative results of serologic tests could also be a possibility. Thus, among those patients who already had high titers at hospital admission, serologic test results could reflect recent but not current clinical infection, with *M. pneumoniae* already absent from the lung parenchyma. In addition, cross-reactivity among different mycoplasmal species may also provide false-positive serologic results. Finally, despite the high rate and the high specificity of etiologic diagnosis obtained in our study, an upper respiratory tract infection due to *M. pneumoniae*, concomitantly with pneumonia caused by another microorganism, cannot be absolutely excluded. Indubitably, the use of serologic data as the exclusive gold standard has some limitations. It can be suggested that additional information could be obtained from processing TNA specimens for *M. pneumoniae* culture; however, we did not perform it. The reasons are multiple: TNA provides a very reduced sample (2 to 3 mL) to be processed simultaneously for many different techniques; sensitivity of cultures appears to be lower than serologic data, particularly if paired serum samples are collected; cultures for *M. pneumoniae* are difficult, and wide experience in this field is needed.

In summary, the PCR test performed on samples obtained directly from lung parenchyma by TNA appears to be a useful and rapid diagnostic method for *M. pneumoniae* infection. Although it may have minor sensitivity than that reported for upper respiratory tract samples, the specificity could be higher by avoiding false-positive results from nasopharyngeal carriers.

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