J Infect Chemother (2004) 10:274–279
DOI 10.1007/s10156-004-0338-y

ORIGINAL ARTICLE

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Application of PCR for *Mycoplasma pneumoniae* detection in children with community-acquired pneumonia

Received: May 25, 2004 / Accepted: July 23, 2004

Abstract Between April 2002 and March 2003, to detect *Mycoplasma pneumoniae* by polymerase chain reaction (PCR), a primer set designed for the *16S* rRNA gene was used to examine clinical samples from 369 children with community-acquired pneumonia. Samples were collected from 12 Japanese institutions participating in a study group concerning acute respiratory infectious diseases. The sensitivity of primers – 2 CFU per reaction tube, using *M. pneumoniae* M129, a standard strain – was calculated to represent 1.1×10^3 *M. pneumoniae* organisms adherent to the tip of the swab used to collect clinical samples. Results for PCR were obtained within 2.6 h. Cases identified by PCR, cultures, and serologic tests were 68 (18.4%), 53 (14.4%), and 76 (20.6%) respectively. Among 57 PCR-positive patients tested serologically, 56 showed a significant elevation or rise in antibody titer. PCR positivity was high among patients prescribed β-lactam antibiotics (86.7%) or no antibiotic (87.0%) before PCR analysis, but was low among patients receiving macrolides, new quinolones, or tetracyclines (37.5%). We concluded that the PCR constructed by us had a high probability for confirming a diagnosis of *M. pneumoniae* pneumonia and for guiding antibiotic choice for patients not yet treated.

Key words *Mycoplasma pneumoniae* · PCR identification · Community-acquired pneumonia

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Introduction

Mycoplasma pneumoniae is one of the most common causes of atypical pneumonia among children[1–5] and young adults.[6,7] In cases of community-acquired pneumonia (CAP), this infection is provisionally diagnosed from chest radiographic abnormalities, cough, fever, white blood cell (WBC) counts, and C-reactive protein (CRP) concentration at the time of the first patient visit. The diagnosis is confirmed subsequently by a rising or high antibody titer.[2,5]

In several recent studies, *M. pneumoniae* detection by polymerase chain reaction (PCR) has been described as an alternative to the cultivation of the microorganism, which requires up to 1 week.[8–13] Multiplex PCR for simultaneous detection of *M. pneumoniae* and *Chlamydia pneumoniae* has also been reported.[14–16] However, these PCR techniques seemed to be complex, requiring excessive time for a routine clinical test.

Correlation between PCR and culture results for *M. pneumoniae* has been uncertain, although a relationship between PCR results and antibody titers has been reported by other investigators.[17–21]

We sought to identify *M. pneumoniae*, in clinical samples collected from pediatric patients with CAP, by PCR using new primers that we have designed, while also carrying out conventional examination by culture for *M. pneumoniae.* The PCR results were compared with those from cultures and from antibody titer determinations. We also examined the influence of oral antibiotics, prescribed before samples were taken for PCR, on the results obtained with this method.

Patients, materials, and methods

Patients and clinical samples

A study group concerning acute respiratory diseases (ARD) was organized by the authors, with the aim of increasing the accuracy of rapid definitive diagnosis, using

PCR. Participating pediatricians and medical institutions included Drs. Satoshi Iwata (head of the study group; National Tokyo Medical Center), Hiroko Endo (Tohoku Rosai Hospital), Tomohiro Oishi (Department of Pediatrics, Joetsu General Hospital), Shigeru Ohnari (Nakafukawa Pediatric Clinic), Naohisa Kawamura (Osaka Rosai Hospital, Osaka Medical College), Haruo Kuroki (Nagatsu-kai Saitoh Hospital), Masaaki Kobayashi (Kobayashi Pediatric Clinic), Kouta Saito (Saito Pediatric Clinic), Ritsuko Sakai (Sakai Clinic), Keisuke Sunakawa and Masato Nonoyama (Department of Infectious Diseases, Kitasato University School of Medicine), Takeshi Tajima (Hakujikai Memorial Hospital), and Masahiko Nitta (Osaka Medical College, Seikeikai Hospital).

After informed consent was obtained from the patients and/or parents or guardians, clinical samples were collected using a sterile swab (Nippon Becton Dickinson, Tokyo, Japan). Samples were collected from patients under 16 years old diagnosed with CAP, based upon chest radiography, physical findings, and symptoms when they were first evaluated at a participating medical institution.

Between April 2002 and March 2003, samples from a total of 369 patients, including 349 nasopharyngeal secretion samples, 8 throat swabs, and 12 sputum samples were sent by participating physicians to our laboratory (Infectious Agents Surveillance, Kitasato Institute for Life Sciences), within 24 h of collection, for PCR analysis.

Preparation of samples before PCR

Samples suspended in 1.5 ml of pleuropneumonia-like organism (PPLO) broth (Difco Laboratories, Detroit, MI, USA) containing 0.7% glucose were centrifuged at 4°C at 5000 rpm for 5 min to collect ciliated epithelial cells from samples. Pelleted material (150 μl) remaining after discarding the supernatant was mixed gently, and 5 μl was added to a 0.5 ml microtube containing 30 μl of lysis solution.[22] The tubes were placed in a thermal cycler (Gene Amp PCR System 9600-R; Perkin-Elmer Cetus, Norwalk, CT, USA) for lysis at 60°C for 20 min and 94°C for 5 min to obtain template DNA.

PCR

PCR was performed using a new set of primers that we have designed, based on the *16S* rRNA gene[23] of *M. pneumoniae,* according to the methods described previously.[24]

The sense primer was Mpn-S: 5′-G_{77}TAATACTTTA GAGGCGAACG$_{97}$-3′, while the reverse primer was Mpn-R: 5′-T_{301} ACTTCTCAGCATAGCTACAC$_{281}$-3′.

Each reaction tube contained 30 μl of reaction mixture, consisting of 100 μl of × 10 reaction buffer (100 mM Tris-HCl at pH 8.9, 800 mM KCl, 15 mM MgCl, 2.1% Triton X-100, 1% sodium cholate, and 5 mg/ml bovine serum albumin), 100 μl of 25 mM dNTP mixture, a 600-ng concentration of each primer, and 40 U *Tth* DNA polymerase per ml (Toyobo, Osaka, Japan). A DNA sample (2 μl) was added to each tube of reaction mixture. Conditions for 35 PCR cycles were 15 s at 94°C, 15 s at 53°C, and 15 s at 72°C; initial denaturation of DNA and final extension were carried out for 2 min at 94°C and for 2 min at 72°C, respectively. The PCR products were separated by 3% agarose gel electrophoresis. The theoretical length of the DNA fragment obtained by PCR was 225 bp, and the total time required for PCR was 2.6 h. The sensitivity of the primers was 2 CFU (colony-forming units) per reaction tube for *M. pneumoniae* M129, a standard strain, which was calculated to correspond to 1.1×10^3 organisms per swab in a clinical sample, as previously reported.[25]

Cultivation of *M. pneumoniae*

M. pneumoniae was cultured using PPLO broth supplemented with 20% horse serum, 2.5% yeast extract, 0.5% glucose, 0.002% phenol red, 0.025% thallium acetate, penicillin G (1000 units/ml), and cefotaxime (150 μg/ml) in accordance with conventional culture methods. Each sample described above under "Preparation of samples before PCR" was cultured in PPLO broth and PPLO agar for 12 to 13 days at 37°C in a humid atmosphere. PPLO agar was used for counting *M. pneumoniae* colonies. Then broth cultures suspected to show *Mycoplasma* positivity, based on a change of broth color to yellow, were spread directly on PPLO agar plates and cultured for colony isolation. Colonies were stored at −80°C for subsequent identification of *M. pneumoniae*, according to the *Manual of clinical microbiology*, *8th edition*.[26]

Cultivation of *Streptococcus pneumoniae* and *Haemophilus influenzae*

S. pneumoniae and *H. influenzae* were cultured routinely with biological methods, using sheep blood agar (Nippon Becton Dickinson, Tokyo, Japan) and chocolate agar plates (Nippon Becton Dickinson) at 37°C in an atmosphere containing 5% CO_2.

Serology

Antibody titers against *M. pneumoniae* in serum samples from each patient were determined by complement fixation (CF) or passive agglutination (PA) tests performed routinely at each institution. Infection with *M. pneumoniae* was confirmed serologically by a fourfold rise in the titer for paired sera, and titers of at least 1:64 by CF or 1:320 by PA for single sera.

Results

PCR results

Figure 1 shows the results of PCR performed, using our primer set, to detect *M. pneumoniae* in clinical samples. Lanes 5 and 7 show a 225-bp DNA fragment corresponding

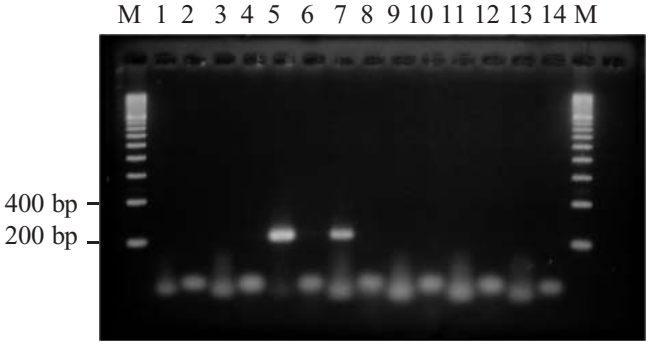

Fig. 1. Positions of DNA fragments amplified by polymerase chain reaction (PCR). *M*, size markers

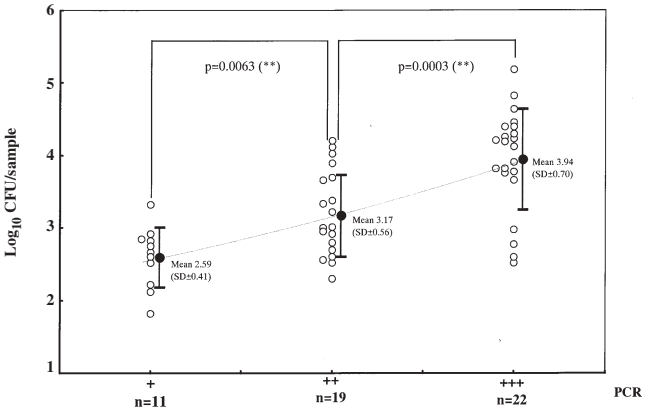

Fig. 2. Correlation between results of PCR and pleuropneumonia-like organism (PPLO) agar cultures for *Mycoplasma pneumoniae* (*black filled circles*, means; *bars*, SD)

Table 1. Results of PCR and PPLO broth cultures for *Mycoplasma pneumoniae*

PCR	Culture		Total
	Positive	Negative	
Positive	52 (14.1%)	16 (4.3%)	68 (18.4%)
Negative	1 (0.3%)	300 (81.3%)	301 (81.6%)
Total	53 (14.4%)	316 (85.6%)	369

PCR, polymerase chain reaction; PPLO, pleuropneumonia-like organism

to the *16S* rRNA gene of *M. pneumoniae*. The sensitivity of the primers was described in the "PCR" section above. No amplification of nonspecific DNA fragments was observed to suggest Gram-positive or -negative bacteria causative of respiratory tract infections (RTI).

Correlation between PCR and cultures for *M. pneumoniae*

Table 1 shows the PCR and conventional culture results in clinical samples from 369 children diagnosed with CAP. Of these, 68 cases (18.4%) were PCR-positive and 53 (14.4%) were culture-positive. Only 1 case was PCR-negative and culture-positive.

In considering the relationship between approximate DNA amount amplified by PCR and the number of CFUs in the 52 culture-positive samples, the density of the amplified DNA fragments was categorized, based on comparison with the 200-bp DNA marker, as follows: +++, density greater than that of the marker; ++, density less than that of the marker; and +, identified with certainty, but with only slight density. Numbers of *M. pneumoniae* cultured from samples were expressed in CFUs. As shown in Fig. 2, a strong positive correlation was obtained between CFU and DNA fragment density by PCR ($r = 0.9996$).

It was shown that PCR could detect *M. pneumoniae* with a very high probability if $2.59 \log_{10}$ (4×10^2) CFU or more *M. pneumoniae* adhered to the tip of the sample collection swab.

Effect of antibiotics prescribed before the sampling for PCR

Table 2 shows the antibiotics prescribed for patients before they presented to member institutions, together with the PCR results and antibody titers. Serologic tests were performed in a total of 77 patients, including 20 in whom there was a strong suspicion of acute infection with *M. pneumoniae*, based on clinical symptoms despite being PCR-negative. Seventy-six of these 77 patients showed significant antibody titer elevation for *M. pneumoniae*.

Patients who had received no antibiotics, or those who had been prescribed oral β-lactam antibiotics, were highly likely to be PCR-positive (20/23; 87.0% and 26/30; 86.7%, respectively). However, patients who had received macrolides, new quinolones, or tetracyclines between 1 to 5 days before sampling for PCR were much less likely to be positive (6/16; 37.5%).

Age distribution at diagnosis with *M. pneumoniae* pneumonia

Table 3 shows the age distribution of the 76 patients diagnosed with *M. pneumoniae* pneumonia based on a rising or high antibody titer for *M. pneumoniae* together with PCR results, considering all 369 cases of CAP.

The incidence of *M. pneumoniae* CAP increased gradually with age, and half of the CAP cases in children over 6 years old (mean, 6.7 years) were caused by *M. pneumoniae*.

WBC count and C-reactive protein (CRP) in patients with *M. pneumoniae* pneumonia

Figure 3 shows the mean WBC count and CRP values in 76 patients when they first visited member institutions. The median WBC count and median CRP concentration were $6600/mm^3$ and 1.8 mg/dl. The values of the 25th percentile and 75th percentile for the WBC count were $5188/mm^3$ and

Table 2. Antibiotics used before sampling, PCR results, and antibody titers for *M. pneumoniae* determined by PA or CF in pneumonia patients

Antibiotics prescribed before PCR	No. of patients tested	PCR (+)		PCR (−)
		Antibody titer (+)	Antibody titer (−)	Antibody titer (+)
(−)	23	20 (87.0%)	1 (4.3%)	2 (8.7%)
β-Lactam	30	26 (86.7%)	0	4 (13.3%)
Macrolide / other[a]	16	6 (37.5%)	0	10 (62.5%)
Unknown	8	4	0	4
Total	77	56	1	20

[a] Includes new quinolones and tetracyclines
PA, passive agglutination; CF, complement fixation

Table 3. Age distribution of patients with *M. pneumoniae* infection diagnosed using PCR and serologic testing

Age (years)	No. of patients	Antibody titer (+)		Total
		PCR (+)	PCR (−)	
<1	26	0	0	0
1	74	3	1	4 (5.4%)
2	60	4	0	4 (6.7%)
3	43	4	3	7 (16.3%)
4	36	3	5	8 (22.2%)
5	34	7	0	7 (20.6%)
6	21	8	2	10 (47.6%)
7–14	75	27	9	36 (48.0%)
Total	369	56	20	76

8125/mm^3, and those for CRP were 0.88 mg/dl and 3.54 mg/dl. Five patients showing a WBC count of 10000/mm^3 or more were found to have coinfection with other pathogens (*S. pneumoniae* in 4, *H. influenzae* in 1).

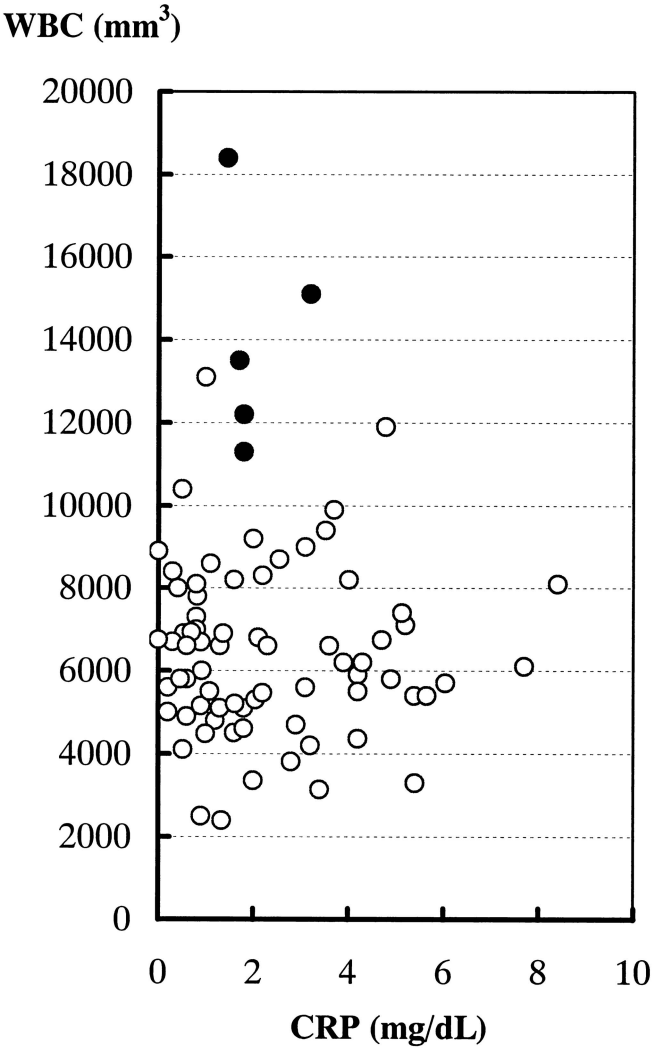

Fig. 3. Early *M. pneumoniae* infection and clinical inflammatory markers. Correlation between white blood cell (*WBC*) count and C-reactive protein (*CRP*) ($n = 76$). Filled circles represent patients with coinfection

Discussion

M. pneumoniae is a common cause of community-acquired atypical pneumonia, as are *C. pneumoniae* and *Legionella pneumophila*.[14–16] Conventional PPLO cultures, which require several days, are performed only infrequently; more often, a clinical diagnosis is confirmed subsequently by serologic tests with paired sera.[2,5] The chemotherapeutic agents used against *M. pneumoniae* differ from those used against *S. pneumoniae* and *H. influenzae*, so accurate diagnosis is needed in the acute phase.

To accomplish timely diagnosis, detection of *M. pneumoniae* by PCR has been attempted by some authors,[9,18–21] but routine clinical application requires more rapid and simple techniques. We are currently seeking to establish PCR methods that can simultaneously detect the main causative bacteria of RTI at the time of initial outpatient medical examination. These bacteria include six microorganisms: *S. pneumoniae*, *H. influenzae*, *Streptococcus pyogenes*, *M. pneumoniae*, *C. pneumoniae*, and *L. pneumophila.* Reaction mixtures and procedures for these agents were set up using the same conditions. For convenience and high PCR sensitivity and specificity, *Tth* polymerase was used instead of *Taq* polymerase in our method; the sensitivity of PCR using *Taq* polymerase was found to be lower than that with *Tth* polymerase in preliminary experiments with a standard strain (data not shown).

It is desirable to have a minimal number of steps for obtaining PCR results. We successfully devised a four-step

PCR method with a total time requirement that was within 2.6 h.

Sensitivity using our new primers for *M. pneumoniae* was 2 CFU per reaction tube for the standard strain when PCR was carried out under the same conditions as for standard strains for other RTI pathogens. Sensitivity corresponded to about 3 $\log_{10}$ CFU of *M. pneumoniae* adherent to the tip of a swab used to collect clinical samples.[25] This positivity also corresponded to 2.59 $\log_{10}$ CFU/sample in cultures of *M. pneumoniae* described in this study.

When correlation between PCR and culture or serologic testing was evaluated, the percentages showing positivity for *M. pneumoniae* in the 369 cases of CAP were: 20.6% for serologic testing; 18.4% for PCR; and 14.4% for culture.

Two serologic assay methods were used at individual member institutions: PA for most cases, and CF for the remainder. Antibody titers were high or rose significantly in all but one PCR-positive patient.

Reported rates of *M. pneumoniae* among cases of CAP in children and adults have ranged from as high as 15% or 29.5%[1,3,4] to as low as 1% or 3%.[27,28] These differences in relative frequency are likely to reflect the presence or absence of outbreaks during specific study periods. However, a comparatively high percentage was obtained in this study, which was conducted when no epidemic of childhood CAP cases caused by *M. pneumoniae* was observed.

The greatest difficultly involving PCR results is that they can be influenced by a variety of antibiotics given before clinical sampling. *M. pneumoniae* was likely to be shown by PCR when outpatients had been treated with oral β-lactam antibiotics (86.7%) or had been untreated (87.0%).

However, PCR was far less likely to disclose *M. pneumoniae* (37.5%) in patients who had been treated with a macrolide or a new quinolone agent. Accordingly, samples must be collected during acute infection, prior to antibiotic therapy.

In accordance with previous reports,[17,29] elevations of WBC and CRP at the first medical examination were absent or modest in patients with *M. pneumoniae* pneumonia, except for a few patients in whom WBC counts of 10000/mm^3 or more were associated with *S. pneumoniae* or *H. influenzae* coinfection.

Identification of *M. pneumoniae* by PCR as described here fulfills the need for early, rapid identification of this pathogen to guide antimicrobial chemotherapy for CAP, whereas PPLO culture involves undesirable delays.

Acknowledgments We are extremely grateful for the assistance given by Reiko Kobayashi and Etsuko Suzuki.

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