J Microbiol Immunol Infect.
2007;40:507-512

Original Article

Rapid diagnosis of *Mycoplasma pneumoniae* infection in children by polymerase chain reaction

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Received: May 30, 2006 Revised: July 28, 2006 Accepted: August 10, 2006

Background and Purpose: Endemic atypical pneumonia was noted in central Taiwan during 2005. The serological response to *Mycoplasma pneumoniae* infection was usually poor in its early course; convalescent serum was needed in most cases, which was sometimes difficult to obtain in children. Empiric antimicrobial therapy was usually initiated before serological testing. A rapid test would be useful to define the etiology and initiate appropriate management. We studied the usefulness of polymerase chain reaction (PCR) analysis for diagnosis in this setting.
Methods: This 1-year prospective study conducted during 2005 in central Taiwan enrolled 307 hospitalized children (aged 3 months to 16 years) with respiratory tract infections, some complicated with systemic manifestations, such as encephalitis and skin rash. Fifty one patients were excluded due to unavailability of data or lack of consent. PCR analysis of samples using a primer set for the P1 gene of *M. pneumoniae* was compared to serological testing, including particle agglutinin test and enzyme-linked immunosorbent assay.
Results: 263 throat swabs from 256 patients were available for PCR tests, and serological tests were performed in 140 children (55%) with clinical suspicion of atypical pneumonia. Eighty two children (32%) were positive by the PCR method and 76 (30%) were serologically positive. Seventy one patients (87%) with duration of disease onset of 2 to 7 days had positive PCR results. The mean age of patients with *M. pneumoniae* infection was 5.2 years, with 27% of patients <2 years old and 73% of patients >2 years of age. The diagnoses were as follows: pneumonia (n = 44); pneumonia complicated with pleural effusion (n = 12); bronchitis and bronchopneumonia (n = 18); asthmatic bronchitis (n = 2); croup syndrome (n = 1); pharyngitis (n = 3); and herpangina (n = 2). Coinfection with bacteria or virus was found in 21% of patients with *M. pneumoniae* infection.
Conclusions: The PCR method could provide earlier diagnosis of *M. pneumoniae* infection and was useful to identify variable clinical features of infection, especially in younger children.

Key words: Child; *Mycoplasma pneumoniae*; Polymerase chain reaction; Respiratory tract infections; Taiwan

Introduction

Mycoplasma pneumoniae is an important cause of atypical pneumonia and other respiratory tract diseases, including common cold, pharyngitis and pneumonia. It was estimated that 15-20% of community-acquired pneumonia was associated with *M. pneumoniae*, an incidence of 2 cases per 1000 population annually [1]. A variety of extrapulmonary manifestations, involving the central nervous system, skin, gastrointestinal tract, and cardiovascular, musculoskeletal and renal systems, are believed to be associated with the invasion of *M. pneumoniae* or the post-infection immune response [2].The diagnosis of *M. pneumoniae* is difficult. The organism is a fastidious agent and culture is insensitive and time-consuming and impractical in clinical practice [3]. The cold agglutination test [4] is an easy, rapid and sensitive method; however, its low specificity has limited its usefulness in clinical practice [5]. Serological methods, including mycoplasma immunoglobulin M (IgM) [6] antibody, complement fixation, enzyme-linked immunosorbent assay (ELISA) [7] and microparticle agglutination test [8] are commonly

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used clinically. Commercially available complement fixation test and ELISA test were believed to offer improved sensitivity and specificity, however, the requirement for a second sampling and indeterminate time for seroconversion have proven to be major drawbacks [9,10].

Polymerase chain reaction (PCR) for amplification of specific short segments of nucleic acid sequence is the simplest and the most practical typing method [11], and is a good alternative for the rapid and sensitive detection of *M. pneumoniae* in throat swabs [12,13]. For epidemiological, clinical, economic and time considerations, PCR has been accepted as a valuable method for the diagnosis of *M. pneumoniae* infections [14].

This prospective study was performed to evaluate the diagnostic value of PCR for *M. pneumoniae* in children admitted with respiratory tract infections. The endemic nature of *M. pneumoniae* infection during 2005 in central Taiwan provided the opportunity to re-evaluate and compare the usefulness of serological and molecular methods in defining the etiology of respiratory tract infections.

Methods

Patients and specimens

A 1-year prospective study was done at the Department of Pediatrics, Taichung Veterans General Hospital in central Taiwan during 2005. The clinical manifestations of these 256 patients were as follows: pneumonia (n = 143); bronchitis and bronchopneumonia (51); asthmatic bronchiolitis (11); croup syndrome (3); pharyngitis (29); upper respiratory infections (7); and herpangina (12). If serial specimens were collected from the same patient, they were collected at first visit to the hospital before initiation of therapy (day 0) and after therapy (day 1-10). All specimens were stored at –70°C until analyzed.

PCR amplification of *M. pneumoniae*

DNA was extracted from clinical samples by guanidine isothiocyanate technique [15]. PCR was performed using a *M. pneumoniae* attachment protein P1 Primer Set Kit (Maxim Biotech, Inc., CA, USA) targeted at the P1 gene of *M. pneumoniae* [12]. Samples to be amplified were incubated in a 50-μL reaction volume containing 10 μL of extracted DNA, 40 μL of premixed primers and optimized PCR buffer and 0.2 μL *Taq* DNA polymerase. Samples were amplified for 35 cycles by using a PCR processor (GeneAmp PCR system 2700; Applied Biosystems, CA, USA), starting with a 1-min pre-denaturing step at 96°C. In the optimized PCR, each cycle consisted of the following steps: denaturation at 94°C for 1 min; annealing at 60°C for 1 min; and elongation at 72°C for 1 min. After 35 cycles, a temperature delay step of 10 min at 72°C was done to complete the elongation. Ten μL of the PCR product was separated by electrophoresis on a 2% agarose gel. A band of 375 base pairs stained with ethidium bromide under ultraviolet light was considered to be a positive result.

Serological test for *M. pneumoniae*

A commercially available microparticle agglutination assay (MAC assay; Serodia Myco II; Fujirebio Inc., Taiwan) was used for determination of antibody titers (immunoglobulin G and IgM) against *M. pneumoniae*. ELISA (Sero MP; Savyon Diagnostics Ltd, Israel) was used for the semiquantitative detection of specific IgM antibodies to *M. pneumoniae* in human serum. The assays were performed according to the instructions of the manufacturer.

Definition of active *M. pneumoniae* infection

The diagnosis of active infection with *M. pneumoniae* was based on an at least four-fold mycoplasma particle agglutinin titer elevation in paired sera or the presence of anti-mycoplasma IgM above the cut-off value. The results of mycoplasma PCR were compared with those of serological tests for *M. pneumoniae*. The presence of a positive PCR for *M. pneumoniae* in the absence of a positive serological response was interpreted as possible carriage. In order to see the influence of oral antibiotics, 7 patients with clinically suspected *M. pneumoniae* infection were also evaluated for the presence of *M. pneumoniae* in their throat, before and after the antimicrobial therapy.

Potential pathogens responsible for pneumonia

Viral culture from throat and nasopharyngeal specimens was performed routinely if possible for all patients with suspected pneumonia. Bacteria isolated from blood and pleural effusion in patients with pneumonia were considered to be significant pathogens. The presence of a positive urine antigen test for *Streptococcus pneumoniae* with concomitant pneumonia was considered indicative of the probable etiology. *Chlamydia pneumoniae* infection was defined as a four-fold immunoglobulin G titer rise or the presence of IgM antibody in sera.

Statistical analysis

The result of mycoplasma PCR and the serological response to *M. pneumoniae* were compared using the

chi-squared test with Yate's correction of contingency. A p value <0.001 was defined as clinically significant.

Results

During the 12-month study period, 307 hospitalized children (age range, 3 months to 16 years) with acute respiratory tract infections were available for inclusion in the study. Samples were not available for 51 children, mainly due to family refusal or clinical suspicion of complicated pneumonia at transferal. Thus, a total of 263 throat swabs were obtained from 256 patients (126 females and 130 males) at admission and subjected to PCR analysis. Paired throat specimens were collected from 7 patients, in order to evaluate the influence of antimicrobial therapy. Throat virus culture was done in 164 (64%) of 256 patients. Serological tests were performed in a total of 140 children (55%) and paired sera were obtained from 41 of them.

Table 1 shows the PCR and serological results from 256 patients. Of these, 82 cases (32%) were PCR-positive and 76 (30%) were serology-positive. In 51 cases (20%), seroconversion or positive IgM antibody in acute serum were concordant with PCR results. Discrepancies were observed in 38 children. In children (5.1%), positive PCR results were not accompanied by detection of antibodies in paired sera or in acute phase serum. The sensitivity and specificity of the PCR method was 67% and 80%, respectively.

The results for correlation of duration of disease onset with the laboratory tests are shown in Table 2. Among 82 PCR-positive cases, 71 children (87%) had throat swabs obtained less than 7 days after the onset of symptoms. Among 76 patients with serologically proven *M. pneumoniae* infections, 12 children (15.7%) had no antibodies against *M. pneumoniae* in acute phase sera obtained 2-7 days after the onset of symptoms. There were statistically significant correlations between disease onset days and the 2 diagnostic methods (p<0.0001). Fourteen throat swabs from 7 patients were obtained, in order to evaluate the influence of oral antimicrobial therapy. *M. pneumoniae* DNA was detected in 3 patients who had received macrolides for 1-4 days. PCR results became negative in 4 patients whose throat swabs samples were collected more than 7 days after the initiation of macrolide therapy.

Table 1. Comparison of polymerase chain reaction (PCR) and serological testing for *Mycoplasma pneumoniae*

Item	Serology[a]			Total
	Positive	Negative	Not done	
PCR-positive	51	13	18	82
PCR-negative	25	51	98	174
Total	76	64	116	256

[a]Serology: immunoglobulin M-positive or ≥4-fold rise in *M. pneumoniae* particle agglutinin titer in convalescent sera.

Table 2. Relation between disease onset and laboratory tests

Disease onset (days)	PCR-positive (n = 82)	Serology-positive (n = 76)	p
<7	71	12	<0.0001[a]
≥7	11	64	

Abbreviation: PCR = polymerase chain reaction
[a]Yate's correction of contigency.

Clinical diagnoses of the 82 patients with positive PCR results are shown in Table 3. In patients less than 2 years of age, we detected 18 patients (72%) with acute bronchitis and bronchopneumonia, 2 with pneumonia, 1 with asthmatic bronchiolitis, 1 with pharyngitis, 1 with croup syndrome and 2 with herpangina. In patients aged >5 years, 35 children (95%) had pneumonia and in 11 this was complicated by pleural effusion. Three patients (3.7%) had complicating extrapulmonary symptoms during illness: 1 was diagnosed as erythema multiforme and 2 were diagnosed as encephalitis and acute stroke syndrome.

Etiologies were identified in 137 (53.5%) of 256 children (Table 4). On the basis of serological and PCR results, 107 children (41.6%) had *M. pneumoniae* infection. Mixed infection occurred in 22 patients and 20 patients had superinfection with 2 organisms and 2 patients with 3 organisms. The infecting organisms (including concurrent infections) were as follows: adenovirus in 8 patients; *C. pneumoniae* in 5; respiratory syncytial virus in 3; *S. pneumoniae* in 2; Coxsackie B3 in 2; and influenza virus, herpes zoster virus, varicella-zoster virus, and *Haemophilus influenzae* in 1 patient each. Respiratory viruses were isolated in 30 patients (11.7%) from throat swabs, and adenovirus was the most common pathogen from this source.

Discussion

M. pneumoniae is a one of the most common causes of atypical pneumonia among children and young adults [16]. The incidence of *M. pneumoniae* infection increases gradually with age. Longitudinal data found that the peak age of endemic *M. pneumoniae* infection was 5-9 years, followed by 10-14 years [17]. *M. pneumoniae* infection is endemic in most areas of the

Table 3. Clinical diagnosis among 82 polymerase chain reaction-positive patients

Clinical diagnosis	Patient age (years)				
	0-2 (n = 25)	2-5 (n = 20)	5-10 (n = 29)	≥10 (n = 8)	Total (n = 82)
Pneumonia	1	15	22	6	44
Pneumonia with pleural effusion	1	4	5	2	12
Croup	1	0	0	0	1
Pharyngitis	1	1	1	0	3
Asthmatic bronchitis	1	0	1	0	2
Bronchitis/bronchopneumonia	18	0	0	0	18
Herpangina	2	0	0	0	2

world, but it can be epidemic at 4- to 7-year intervals [17]. Annual rates of infection vary between 1.3% in endemic periods and 50% in epidemic periods. The prevalence of *M. pneumoniae* infection varies greatly from study to study [10,11,17], and a recent large surveillance study [18] found that the prevalence in children with community-acquired pneumonia was 12.2% in Asian and 30 to 40% in Taiwanese groups. In our study, *M. pneumoniae* infection was identified in 41.6% of our patients; compared with 2004, increased numbers of atypical pneumonia with *M. pneumoniae* infection were found during 2005.

The diagnosis of *M. pneumoniae* infection is difficult. Culture requires 10 to 14 days, which limits its clinical usefulness. Serology is the most frequently used method, with diagnosis confirmed by a four-fold increase in antibody titer, the presence of specific IgM or high antibody titers. In Taiwan, a particle agglutination test was widely used [18] and evidence of infection was based on a four-fold increase in titer, or a titer of 1:160

Table 4. Etiology of respiratory tract infection among 256 hospitalized patients with respiratory disease

Etiology	Patients (n = 256) No. (%)
Mycoplasma pneumoniae alone[a]	85 (33.2)
Mixed (two pathogens)	20 (7.8)
Mycoplasma pneumoniae + *Chlamydia pneumoniae*[b]	3 (1.2)
Mycoplasma pneumoniae + *Streptococcus pneumoniae*[c]	1 (0.4)
Mycoplasma pneumoniae + *Haemophilus influenzae*[c]	1 (0.4)
Virus[d] + *Mycoplasma pneumoniae*	15 (5.9)
Adenovirus + *Mycoplasma pneumoniae*	7 (2.7)
RSV + *Mycoplasma pneumoniae*	3 (1.2)
Influenza virus + *Mycoplasma pneumoniae*	1 (0.4)
Coxsackie B3 + *Mycoplasma pneumoniae*	2 (0.8)
VZV + *Mycoplasma pneumoniae*	1 (0.4)
HSV-1 + *Mycoplasma pneumoniae*	1 (0.4)
Mixed (more than 2 pathogens)	2 (0.8)
Mycoplasma pneumoniae + adenovirus + *Chlamydia pneumoniae*[b]	1 (0.4)
Mycoplasma pneumoniae + *Chlamydia pneumoniae* + *Streptococcus pneumoniae*	1 (0.4)
Virus alone[d]	30 (11.7)
RSV	2 (0.8)
Adenovirus	22 (8.6)
Coxsackie B3	1 (0.4)
Influenza virus	3 (1.2)
Parainfluenza virus	2 (0.8)
Not identified	119 (46.5)

Abbreviations: RSV = respiratory syncytial virus; VZV = varicella-zoster virus; HSV = herpes simplex virus

[a]Diagnosis of acute *M. pneumoniae* infection was based on serology testing with 4-fold rise in antibody titers in paired sera or positive result for immunoglobulin M (IgM) antibody in enzyme-linked immunosorbent assay (ELISA).

[b]The diagnosis of *C. pneumoniae* infection was based on the positive serological test of IgM antibody by ELISA.

[c]Sterile site (including cerebrospinal fluid, blood, pleural fluid) culture-positive.

[d]Virus was identified by throat swab culture.

or greater. Serology can only provide a retrospective diagnosis, and has the drawbacks of lack of antibody response in the acute phase and unknown time for seroconversion.

PCR methods have been developed over the last decade. Over the years, various primers have been investigated including the P1 virulence gene [12,19], the 16S rRNA gene [20] and the elongation factor gene [21]. The P1 adhesin gene primers have proved more sensitive than the 16S RNA primers in the detection of *M. pneumoniae* infection [12,22]. The greatest difficulty involving PCR results is that they can be influenced by antimicrobial therapy or the immune response of the host; asymptomatic colonization is another problem.

In our study, PCR did not detect *M. pneumoniae* in 3 of 7 patients whose throat swabs were obtained 7 days after completion of macrolide therapy. In a study by Skakni et al, PCR did not detect *M. pneumoniae* in a sample obtained 2 months after the initiation of erythromycin therapy [15]. Another study in Japan suggested that samples for PCR must be collected during acute infection, prior to antibiotic therapy [23]. No data were available to allow estimation of the time to eradication, and more studies will be needed. Thirteen of our patients (5.1%) were positive by PCR results, but had no detection of antibody against *M. pneumoniae* in sera obtained 2 to 7 days after symptom onset. These discrepancies between serology testing and PCR results may be partly explained by the timing of the serum samples: the interval between symptom onset and serology testing was too short for a serological response to develop. The presence of the carriage state in patients was another possible cause.

M. pneumoniae could be one of the causes of pneumonia in school-age children and was estimated to be responsible for approximately 5% of bronchiolitis in young children [24]. Clinical features in children of a younger age were variable, and included pneumonia, acute bronchitis, bronchial asthma, acute pharyngitis and acute otitis media [21]. Our study showed the main clinical features in children less than 2 years of age to be acute bronchiolitis and bronchopneumonia**.** PCR was useful for confirmation of diagnosis in younger children [25].

In this study, we detected unusual clinical features of infection: herpangina in 2 children and croup syndrome in 1. No previous case report has shown these symptoms associated with *M. pneumoniae* infection. Contamination by the carryover of PCR products might have lead to false-positive results. However, special care was taken to avoid carryover in every PCR experiment and negative controls were randomly included in each reaction set, to monitor contamination. Another explanation for these unusual cases is that PCR may detect a mild infection, a carrier state [26] or the persistence of *M. pneumoniae* in the respiratory tract after a recent infection [15]**.**

In this study, *M. pneumoniae* was one of the most important respiratory tract pathogen causing hospitalization in children (n = 107, 41.6%), followed by adenovirus (30, 11.7%), respiratory syncytial virus (5, 2.0%), influenza virus (4, 1.6%), Coxsackie virus (3, 1.2%) and parainfluenza virus (2, 0.8%). Twenty one percent of our patients with demonstrable *M. pneumonaie* infection had evidence of co-infection with other pathogens. Possible explanations for the high rate of coinfection are that *M. pneumoniae*, like viruses, may facilitate respiratory tract pathogens [27], and that PCR can detect *M. pneumoniae* in circumstances other than acute infection, such as colonization in the respiratory tract or asymptomatic infection [1]. Gnarpe et al [26] found *M. pneumoniae* colonization of the nasopharynx in 4.6-13.5% of normal healthy personnel.

In conclusion, PCR provided earlier and more rapid demonstration of *M. pneumoniae* infection in the acute stage, while the serological response was still suboptimal. It allowed us to identify variable pulmonary or extrapulmonary infections by *M. pneumoniae*, particularly in children less 2 years of age, and lead to a better understanding of the epidemiology involved. However, detection of asymptomatic carriers was the main problem when the PCR method was used alone for the diagnosis of *M. pneumoniae* infection [1,25]. Careful interpretation of PCR results in combination with clinical symptoms and serology can provide more precise diagnosis of *M. pneumoniae* infections.

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