ISOLATION AND POLYMERASE CHAIN REACTION DETECTION OF *MYCOPLASMA PNEUMONIAE* IN MALAYSIAN PATIENTS WITH RESPIRATORY TRACT INFECTIONS

Tay ST, Habsah MY, Tan SC and Rohani MY

Bacteriology Division, Institute for Medical Research, Jalan Pahang, 50588 Kuala Lumpur, Malaysia

Abstract. Isolation and polymerase chain reaction (PCR) were performed for detection of *Mycoplasma pneumoniae* from respiratory tract specimens obtained from 200 adult and 200 pediatric patients. *M. pneumoniae* was isolated from bronchoalveolar lavage fluid of 1(0.5%) adult patient and 4(2.0%) tracheal aspirates of pediatric patients. PCR was positive for only one (0.5%) broncoalveolar lavage fluid of an adult patient and fifteen (7.5%) tracheal aspirates of pediatric patients. This study suggested that *M. pneumoniae* was more frequently detected in pediatric patients and PCR appears to have advantages over isolation, in terms of rapidity and sensitivity.

INTRODUCTION

Mycoplasma pneumoniae is an important cause of human respiratory disease, accounting for 15-20% of community acquired pneumonia. Symptomatic disease is typically mild and is characterized by nonproductive cough, fever, malaise and pharyngitis, with 3-13% of patients infected with *M. pneumoniae* developing pneumonia (Clyde, 1993). Extrapulmonary symptoms due to *M. pneumoniae* disease particularly central nervous system manifestations are also increasingly reported (Lind, 1983; Narita *et al*, 1992; Abele-Horn *et al*, 1998a).

Clinical diagnosis of *M. pneumoniae* pneumonia is difficult because many viral and other pneumonias present clinically similar pictures. The cultivation of *M. pneumoniae* is time-consuming. *M. pneumoniae* grows slowly, with colonies taking a week to more than a month to become visible microscopically (Kenny *et al*, 1990). Serological procedures are the most widely used and require the demonstration of a rise in antibody titer. However, it takes too long for results of isolation and serological methods to be obtained to allow for the rapid application of an effective treatment. To render a more rapid and specific means of detecting *M. pneumoniae*, different attempts have been made by using molecular techniques such as DNA probes (Hyman *et al*, 1987; Kleemola *et al*, 1990) and polymerase chain reaction (PCR) (Bernet *et al*, 1989; Skakni *et al*, 1992; Abele-Horn *et al*, 1998b; Dorigo-Zetsma *et al*, 1999).

In Malaysia, an average of 23.3% cases with respiratory infections were seropositive to *M. pneumoniae* by a microparticle agglutination test, with seroprevalence highest among the pediatric patients and lower in adult patients (Tay and Cheong, 1995). The need for an improved detection method for *M. pneumoniae* is evident. PCR for amplification of specific short segments of nucleic acid sequences is a promising rapid diagnostic test and this technique is proposed for detection of *M. pneumoniae*. This study compared the use of PCR with isolation for the detection of *M. pneumoniae* in respiratory tract specimens of adult and pediatric patients.

MATERIALS AND METHODS

Patients and specimens

Patients were those with symptomatic respiratory infections admitted to Kuala Lumpur Hospital from 1995-1997. Specimens analyzed in this study included 200 bronchoalveolar lavage specimens obtained from adult patients and 200 tracheal aspirates from pediatric patients.

Isolation and antibiotic sensitivity of *M. pneumoniae*

Glucose agar and diphasic medium as described by Velleca *et al.* (1980) were used for the primary isolation of *M. pneumoniae*. For diphasic medium, the mycoplasmal broth was composed of 70 volumes of pleuropneumonia-like organismal broth (PPLO, Difco), 20 volumes of horse serum (Gibco), 10 volumes of 25% (w/v) freshly prepared yeast extract supplemented with 0.0125%

(w/v) thallium acetate (Sigma), 0.5% (w/v) glucose, 0.0001% methylene blue and 0.002% (w/v) phenol red. The glucose agar medium was prepared as for mycoplasmal broth except that PPLO agar (Difco) was added to a final concentration of 1.5%. Three hundred microliters of the specimens was inoculated into glucose diphasic media and 0.1 ml was inoculated onto glucose agar medium. These media were incubated aerobically at 37ºC. The diphasic medium was examined daily for a shift to acid pH as demonstrated by a color change from blue or red to yellow. When a color change was apparent, subculture to another fresh glucose agar medium was performed. The isolates were identified by colonical morphology, glucose fermentation and guinea pig hemadsorption test. For guinea pig hemadsorption test, a 0.4% suspension of guinea pig erythrocytes was flooded on the suspected colonies of *M. pneumoniae* on glucose agar surface at 37ºC for 30 minutes with occasional rotation. The agar surface was then washed three times with 3 ml of PPLO broth and the remaining washing fluid was removed. The colonies were observed at 100X magnification. Colonies with red blood cells adsorbed onto the surface were identified as *M. pneumoniae* and these were further confirmed by PCR. The antibiotic sensitivity of the isolates was determined using the Pneumofast kit (International MYCOPLASMA, France) according to the manufacturer's recommendations. The kit contains both reagents for the preparation of solid agar plates and Pneumofast trays for broth culture. The trays contain 10 separate wells, allowing semiquantitative determination of colony counts, biochemical identification of growing organisms and antimicrobial resistance testing. The plates and trays were cultured at 37ºC for 12 days and were examined daily for the presence of colonies with a granular morphology and a color change in the tray wells. Positive cultures resistant to ampicillin (40 μg/ml) and lincomycin (1 μg/ml) but sensitive to erythromycin (8 μg/ml) were identified as *M. pneumoniae*.

Sample preparation for PCR

Approximately 1 ml of respiratory tract specimen (bronchoalveolar lavage fluid from adult patient and tracheal aspirate from pediatric patient) was first centrifuged at 13,000 rpm for 10 minutes following which the pellet was resuspended in lysis buffer [50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100 and 200 μg/ml proteinase K]. The mixture was incubated at 55ºC for 2 hours before subjected to heating at 95ºC for 30 minutes.

PCR

For *M. pneumoniae*-specific amplification, primer set MP5-1 (5′-GAAGCTTATGGTACAGG TTGG-3′) and MP5-2 (5′-CGTAAGCTATCA GCTACATGGAGG-3′) as described by Bernet *et al* (1989) were used. A total volume of 10 μl DNA sample was incubated in a 50 μl reaction volume containing 0.1 μM each primer, 200 μl each deoxynucleoside triphosphate, 10 mM Tris HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, and 1 U of *Taq* polymerse (Boehringer Mannheim). Amplification was carried out for 30 cycles (1 minute at 95ºC, 2 minutes at 55ºC and 1 minute at 70ºC) in a thermal cycler (ThermojeT, Belgium). Positive control (DNA extracted from the *M. pneumoniae* ATCC 10119) and negative control (H_2O) were run simultaneously in each PCR experiment. A positive signal was defined by a 144-bp fragment visualized in ethidium bromide-stained agarose gel.

RESULTS

Typical colonies of *M. pneumoniae* on glucose agar media were obtained with respiratory tract specimens of 1 adult and 4 pediatric patients. The isolates were confirmed by guinea pig hemadsorption test and PCR. These isolates were resistant to ampicillin (40 μg/ml), sulfamethoxazole-trimethroprim (4 μg/ml) and lincomycin (1 μg/ml) but sensitive to erythromycin (0.1 and 8 μg/ml) and ciprofloxacin (2 μg/ml).

Table 1 shows the demographic data, other clinical findings and isolation of bacteria from cases with positive PCR findings. Only one bronchoalveolar lavage of adult patient and 15 (7.5%) of tracheal aspirates of pediatric patients were positive by PCR amplification. *M. pneumoniae* was isolated from the bronchoalveolar lavage fluid of a 34-year-old male patient who required ventilation in the intensive care unit. Culture was negative for other bacterial agents and *Mycobacterium tuberculosis*. Most pediatric patients with positive PCR findings were below 5 years old except one (10 years old). Seven patients were female and eight were male. Of the 15 pediatric patients with positive PCR findings, 6 had central nervous system manifestations, 1 had myocarditis and 1 had acute lymphoblastic leukemia. Methicillin resistant *Staphylococcus aureus* (MRSA), *Enterococcus* and *Klebsiella*, spp were also isolated together with *M. pneumoniae* from three tracheal aspirates.

Table 1
Demographic, other clinical findings and isolation of bacteria from cases with respiratory tract infections with positive PCR findings of *M. pneumoniae.*

Patient	Age (years)	Race	Sex	Other clinical findings	Bacterial isolation
Adult					
1	34	Malay	M		*M. pneumoniae*
Pediatric					
1	5	Malay	F	Encephalopathy	*M. pneumoniae*
2	7/12	Chinese	F	Meningitis	
3	5/12	Malay	M	Myocarditis	*Klebsiella* spp, *Pseudomonas* spp
4	10	Malay	M	Cerebral palsy	
5	5/12	Chinese	F		
6	10/12	Malay	F		*Pseudomonas aeruginosa*
7	2/12	Malay	M		*M. pneumoniae*, MRSA
8	3	Malay	F	Meningitis	
9	NA	Chinese	F	Acute lymphoblastic leukemia	*M. pneumoniae*, *Enterococcus* spp
10	2	Chinese	M	Hydrocephalus	
11	5/12	Malay	M		
12	3	Malay	M	Status epilepticus	*M. pneumoniae*, *Klebsiella* spp
13	NA	Malay	M		
14	1	Chinese	F		
15	NA	Chinese	M		

NA = not available

DISCUSSION

M. pneumoniae is probably under-recognized as a cause of disease because the clinical presentation is nonspecific and currently available diagnostic techniques have many shortcomings. A positive respiratory culture for *M. pneumoniae* in association with a compatible clinical syndrome should be considered diagnostic. In a study conducted by the Ministry of Health, Malaysia for children below 5 years of age (Anonymous, 1986), *M. pneumoniae* was identified as the second most common cause of acute respiratory infection, with an isolation rate of 2.9%. In this study, there was an isolation rate of 2.0% from 200 pediatric patients with symptomatic respiratory tract infections. Despite serological findings (Tay and Cheong, 1995), there were no reports on the isolation of *M. pneumoniae* from adult patients in this country.

The direct isolation of *M. pneumoniae* are generally considered as insensitive and require a week or more for recovery of the microorganisms on complex media and thus are not practical in most laboratories (Kenny *et al*, 1990). Much emphasis has therefore been placed on rapid diagnosis of *M. pneumoniae* infection by PCR (Bernet *et al*, 1989; Skakni *et al*, 1992; Abele-Horn *et al*, 1998b; Dorigo-Zetsma *et al*, 1999). PCR is sufficiently sensitive to detect presumably low numbers of the organisms present in samples and requiring no viable cells. The technique has been applied successfully for detection of *M. pneumoniae* in a variety of clinical samples such as sputum, nasopharyngeal aspirates, throat swabs, bronchoalveolar lavages, pleural fluids, serum and cerebrospinal fluid (Narita *et al*, 1992; Abele-Horn *et al*, 1998b; Dorigo-Zetsma *et al*, 1999). In this study, PCR detected more *M. pneumoniae* cases compared to conventional cultural method. Only five *M. pneumoniae* isolates were obtained from 400 respiratory samples in this study (1 from adult and 4 from pediatric patients) compared to 16 cases by PCR. These results emphasize the value of PCR for the rapid diagnosis of *M. pneumoniae* infections, especially in pediatric patients with respiratory tract infections.

Using amplification of a 144 bp sequence

from *M. pneumoniae*, Bernet *et al* (1989) were able to detect between 10^2 and 10^3 organisms in an initial experiments on artificial seeded human bronchoalveolar lavages. The PCR assay did not amplify human DNA, DNA from species generally found in the respiratory tract and *M. genitalium* DNA, thus allowed for the specific and sensitive detection of *M. pneumoniae* DNA. The discrepancy between the results of the PCR assay and those of culture in this study might arise from difficulties encountered in culturing *M. pneumoniae*. An extended delay between the moment of sampling and culturing may explain the absence of culture. A low quantity of inoculum can be another reason. It appears that 10-100 cfu is required to initiate growth in the cell monolayer/agar culture system (Harris *et al*, 1988). Several host factors, such as lysolecithin, lysosomal enzymes, or other mycoplasmacidal substance release from tissue homogenates, antibody, residual antibiotics might also influence the success of isolation (Tully *et al*, 1979). Therefore although isolation of microorganism from clinical specimen is considered as gold standard, it may not be true for *M. pneumoniae*.

Mycoplasmal pneumonia primarily affects young people between 5 and 25 years of age (Denny *et al*, 1971). In this study, significantly more children (7.5%) than adults (0.5%) were found to be positive by the PCR. This finding is also reflected in the results of serodiagnosis performed previously in this laboratory, in which the seropositive rate of *M. pneumoniae* was highest amongst patients of 6-20 years old but lower amongst adult patients (Tay and Cheong, 1995). Cases of *M. pneumoniae* infection in adults (Cassell *et al*, 1991) and immunocompromised patients (Parides *et al*, 1989) were also reported. The isolation and PCR detection of *M. pneumoniae* from an adult patient in this study is the first report in this country. The finding suggests that *M. pneumoniae* should be considered as a cause of severe pneumonia requires hospitalization among adult patients.

A rapid specific diagnosis is important because treatment of *M. pneumoniae* infection with β-lactam antibiotics is ineffective, whereas treatment with macrolides or tetracyclines may markedly reduced the duration of the illness (Ruuskanen *et al*, 1992; Hammerschlag, 1995). All *M. pneumoniae* isolated in this study were sensitive to erythromycin, therefore macrolide antibiotics such as erythromycin remain the drug of choice in mycoplasmal infections. PCR detected *M. pneumoniae* far more frequently than culture and was able to detect DNA from *M. pneumoniae* in samples from patients for whom cultures were negative (Bernet *et al*, 1989; Skakni *et al*, 1992, de Barbeyrac *et al*, 1993). The specificity, the exquisite sensitivity and the relative rapidity of PCR make this technique promising for the rapid diagnosis of *M. pneumoniae* infections.

In the past mycoplasmal infections used to be considered mild and self-limiting. While the majority of these infections appear to be upper respiratory tract infections or relatively mild cases of pneumonia, more severe infections, such as pneumonia requiring hospitalization (White *et al*, 1981) or lung abscess (Siegler, 1973), can also occur. In addition, increasing number of recent reviews have also documented complications of Mycoplasmal pneumonia including adult respiratory distress syndrome, myocarditis, pericarditis, the Stevens Johnson syndrome, hemolytic anemia, arthritis and central nervous system manifestations (Lind, 1983; Clyde, 1993). It was not known whether the central nervous system manifestations among the 6 pediatric patients in this study were due to *M. pneumoniae* infections. Direct confirmation of the role of *M. pneumoniae* could be obtained by detection of the organism in the cerebrospinal fluids of these patients. The detection of *M. pneumoniae* DNA has been reported in the sera and cerebrospinal fluid of patients with clinical central nervous system infections (Narita *et al*, 1992).

PCR was also proven useful in the diagnosis of seronegative patients with immunological impairment and in patients with a past infection (Skakni *et al*, 1992; Williamson *et al*, 1992). The fact that there is no single "gold standard" diagnostic test for *M. pneumoniae* infection makes it difficult to assess both established and new diagnostic methods. The poor sensitivity of culture in this study and the possibility of impaired serological responses make the PCR assay promising for the diagnosis of *M. pneumoniae* infections.

ACKNOWLEDGEMENTS

We thank the Director of the Institute for Medical Research for the permission to publish this paper. We also wish to thank the staff of the Microbiological Division, Hospital Kuala Lumpur and Bacteriological Division, Institute for Medical Research for their technical assistance. This study was funded by grant no. IMR-95-96.

REFERENCES

Abele-Horn M, Franck W, Busch U, Nitschko H, Roos R, Heesemann J. Transverse myelitis associated with *Mycoplasma pneumoniae* infection. *Clin Infect Dis* 1998a; 26: 909-12.

Abele-Horn M, Busch U, Nitschko H, *et al.* Molecular approaches to diagnosis of pulmonary diseases due to *Mycoplasma pneumoniae*. *J Clin Microbiol* 1998b; 36: 548-51.

Anonymous. Hospital-based study of acute respiratory infection in children (Sept 1984-Aug 1985). Kuala Lumpur: Ministry of Health Malaysia, 1986.

Bernet C, Garret M, de Barbeyrac B, Bebear C, Bonnet J. Detection of *Mycoplasma pneumoniae* by using the polymerase chain reaction. *J Clin Microbiol* 1989; 27: 2492-6.

Cassell GH, Drnec J, Waites KB, *et al*. Efficacy of clarithromycin against *Mycoplasma pneumoniae*. *J Antimicrob Chemother* 1991; 27: 47-59.

Clyde WA Jr. Clinical overview of typical *Mycoplasma pneumoniae* infections. *Clin Infect Dis* 1993; 17: S23-6.

de Barbeyrac B, Bernet C, Febrer F, Renaudin H, Dupon M, Bebear C. Detection of *Mycoplasma pneumoniae* and *Mycoplasma genitalium* in clinical samples by polymerase chain reaction. *Clin Infect Dis* 1993; 17: S83-9.

Denny FW, Clyde WA Jr, Glezen WP. *Mycoplasma pneumoniae* disease: clinical spectrum, pathophysiology, epidemiology, and control. *J Infect Dis* 1971; 123: 74-92.

Dorigo-Zetsma JW, Zaat SAJ, Wertheim-van Dillen PME, *et al.* Comparison of PCR, culture, and serological tests for diagnosis of *Mycoplasma pneumoniae* respiratory tract infection in children. *J Clin Microbiol* 1999; 37: 14-7.

Hammerschlag MR. Atypical pneumonias in children. *Adv Pediatr infect Dis* 1995; 10: 1-39.

Harris R, Marmion BP, Varkanis G, *et al.* Laboratory diagnosis of *Mycoplasma pneumoniae* infection. 2: comparison of methods for the direct detection of specific antigen or nucleic acid sequences in respiratory exudates. *Epidemiol Infect* 1988; 101: 685-94.

Hyman HC, Yogev D, Razin S. DNA probes for detection and identification *of Mycoplasma pneumoniae* and *Mycoplasma genitalium*. *J Clin Microbiol* 1987; 25: 726-8.

Kenny GE, Kaiser GG, Cooney MK, Foy HM. Diagnosis of *Mycoplasma pneumoniae* pneumonia: sensitivities and specificities of serology with lipid antigen and isolation of the organism on soy peptone medium for identification of infections. *J Clin Microbiol* 1990; 28: 2087-93.

Kleemola SRM, Karjalainen JE, Raty RKH. Rapid diagnosis of *Mycoplasma pneumoniae* infection: clinical evaluation of a commercial probe test. *J Infect Dis* 1990; 162: 70-5.

Lind K. Manifestations and complications of *Mycoplasma pneumoniae* disease: a review. *Yale J Biol Med* 1983; 56: 461-8.

Narita M, Matsuzono Y, Togashi T, Kajii N. DNA diagnosis of central nervous system infection by *Mycoplasma pneumoniae*. *Pediatrics* 1992; 90: 250-3.

Parides GC, Bloom JW, Ampel NM, Ray CG. *Mycoplasma* and *Ureaplasma* in bronchoalveolar lavage fluids from immunocompromised hosts. *Diagn Microbiol Infect Dis* 1989; 9: 55-7.

Ruuskanen O, Nohynek H, Ziegler T, *et al.* Pneumonia in childhood: etiology and response to antimicrobial therapy. *Eur J Clin Microbiol Infect Dis* 1992; 11: 217-23.

Siegler DIM. Lung abscess associated with *Mycoplasma pneumoniae* infection. *Br J Dis Chest* 1973; 67: 123.

Skakni L, Sardet A, Just J, *et al.* Detection of *Mycoplasma pneumoniae* in clinical samples from pediatric patients by polymerase chain reaction. *J Clin Microbiol* 1992; 30: 2638-43.

Tay ST, Cheong YM. A review of the serological results obtained in a routine diagnostic laboratory for *Mycoplasma pneumoniae* infections. *Mal J Pathol* 1995; 17: 35-8.

Tully JG, Rose DL, Whitcomb RF, Wenzel RP. Enhanced isolation of *Mycoplasma pneumoniae* from throat washings with a newly modified culture medium. *J Infect Dis* 1979; 139: 478-82.

Velleca WM, Bird BR, Forrester FT. Laboratory diagnosis of *Mycoplasma* infections. US Department of Health and Human Services, Public Health Service Centers for Disease Control, 1980: 137.

White RJ, Blainey AD, Harrison KJ, Clarke SKR. Causes of pneumonia presenting to a district general hospital. *Thorax* 1981; 36: 566-70.

Williamson J, Marmion BP, Worsick DA, *et al.* Laboratory diagnosis of *Mycoplasma pneumoniae* infection. 4. Antigen capture and PCR-gene amplification for detection of the *Mycoplasma*: problems of clinical correlation. *Epidemiol Infect* 1992; 109: 519-37.