CHAPTER IV

MATERIALS AND METHODS

1. Reference strains

Mycoplasma pneumoniae Mac strain, obtained from Serum Institute, Copenhagen, Denmark was used. It was grown on modified Hayflick's medium (18).

2. Patients and controls

Clinical specimens were obtained from patients with symptoms of a respiratory tract infection from Chulalongkorn Hospital and Pramongkutklao College of Medicine hospitals between December, 1998 and February 1999. One hundred patients consisted of 23 children, ranging in age 2 months to 12 years, and 77 adults, ranging in age 19 to 65 years.

Serum samples for serological testing and throat swab were obtained on admission. Sample of convalescent–phase sera were taken 30 to 45 days after the onset of disease in a follow-up visit. A cotton-tipped was used to obtain material from the space between the palatine archer for PCR and culture of *M. pneumoniae*. The throat swab was

placed into 2 ml of modified Hayflick broth (92) as the transport medium. All samples were sent to the laboratory within 24 hours.

Controls were collected from 100 healthy volunteers consisting of 40 Medical Technology students and 60 health care workers. The ages of controls ranged from 19 to 45 years.

3. Serology for *M. pneumoniae*

A commercially available microparticle agglutination assay (MAG assay; Serodia Myco II kit; Fujirebio, Inc., Tokyo, Japan) was used to detect antibodies to *M. pneumoniae* (22,28,33). The test was performed manually according to the manufacturer's recommendations. A single antibody titer of < 1:40 was regarded as negative; a single titer of ± 1:40 as indeterminate; and a single titer ≥ 1:40 or fourfold rise in titer of paired serum specimens was interpreted as positive results. For patients who were PCR positive, second sera were collected and tested by serological method.

4. Culture (99)

M. pneumoniae Mac strain was grown in modified Hayflick broth (77). After a color change, 0.2 ml of broth was subcultured on modified Hayflick agar and incubated at 37°C in 5% CO_2 for 2 weeks. Throat swabs were vortexed in the transport medium, 200 µl was taken for PCR and 0.2 ml was inoculated into 1.8 ml of Hayflick broth. Broths were subcultured on agar upon color change. The swabs were also streaked immediately onto Hayflick agar plate. They were incubated for

3 weeks at 37°C in 5% CO_2. During the 3 weeks of incubation, the plates were checked twice weekly under a microscope for presence of *Mycoplasma* colonies.

5. DNA extraction

5.1 DNA extraction from *M. pneumoniae* (104)

M. pneumoniae Mac strain was cultured in modified Hayflick medium. A total of 250 ml of a late-exponential phase culture was centrifuged for 30 min. at 10,000xg. The pellet was suspended in 5 ml of STE buffer (10mM NaCl, 20mM Tris hydrochloride [pH 8.0], 1mM EDTA) and incubated overnight at 37°C with 1% sodium dodecyl sulfate and 50μg of proteinase K per ml. DNA was extracted three times with an equal volume of phenol saturated with TE (10mM Tris hydrochloride [pH 7.5], 1mM EDTA), once with phenol-chloroform-isoamyl alcohol(10:9.6:0.4; vol/vol/vol), and once with chloroform-isoamyl alcohol (9.6:0.4; vol/vol). The aqueous phase was made to 0.3M sodium acetate and the DNA was precipitated with 2 volumes of absolute ethanol at room temperature or –20°C or -70°C. The nucleic acid were suspended in TE buffer and were treated with DNase-free RNase A (50μg/ml) at 37 °C for 30 min. After another extraction with phenol and ethanol precipitation, the DNA was suspended in TE buffer. The sample was checked for purity and determined for the amount of DNA by measuring the optical density at wavelengths of 260 nm and 280 nm

5.2 DNA extraction from clinical specimens

DNA was extracted by proteinase K treatment (99), 200 µl of the sample was centrifuged for 30 min, 12,000xg at 4°C. The pellet was resuspended in 40 µl of Tris HCl (10mM). Lysis buffer containing 500 µg of proteinase K per ml, 0.45% Nonidet P-40, 0.45% Tween 20, 100 mM KCl, 20 mM Tris HCl, and 3 mM $MgCl_2$ was added to a final volume of 100 µl. This solution was incubated overnight at 37.5°C, and the samples were subsequently boiled for 10 min to inactivate the proteinase K. The direct PCR was performed on 5 µl of this product.

6. Primers

Two sets of primers were used for amplify *M. pneumoniae* DNA. The first primer set was directed against the P1 adhesion gene (42,82). The second set was directed against a species-specific sequence of the 16S ribosomal gene (40). The nucleotide sequences of the different primers used in the *M. pneumoniae* PCR are given in Table 1. The specificity of the *M. pneumoniae* PCR has been tested extensively, as described by Sumanee Sirilertpanrana (104) and Van Kuppeveld et al (40). The inner primers for 16S rDNA PCR were selected from the sequence of 16S rRNA gene. The selection of these primers were performed by using the "OLIGO" program of Rychlik & Rhoads (105).

Table 1 : Sequences of primers and size of PCR products

Target primer	Nucleotide sequence	Size of PCR Product (bp)
P1 gene		
MP-1	5′-GTGAATGGGTTGTTGAATC-3′	466
MP-2	5′-TTGTTGCGGTGGTGGTAGA-3′	
MP-1S	5′-TCCAACACGACCAGTTCACC-3′	342
MP-2S	5′-CCCCTTCAAATCCCACACAC-3′	
16S rDNA		
16S-1	5′-AAGGACCTGCAAGGGTTCGT-3′	277
16S-2	5′-CTCTAGCCATTACCTGCTAA-3′	
16S-1S	5′-AAGGCAATGACGTGTACGT-3′	205
16S-2S	5′-TCTAGCCATTACCTGCTAA-3′	

7. Determination of sensitivity of PCR

In order to determine the PCR sensitivity for detection of *M. pneumoniae*, serial dilutions of purified *M. pneumoniae* DNA containing between 1pg and 0.01 fg of DNA were perform in PCR amplification with two sets of primer. After the nested-PCR, amplified products were detected by gel electrophoresis. For negative control, double distilled water (DDW) was used as the template for PCR.

8. Amplification of *M. pneumoniae* DNA in clinical specimens by PCR

8.1 P1 gene amplification

The reaction volumes for the first and second rounds of amplification were 50 µl with 0.5 µM of each primer, 200 µM (each) deoxynucleoside triphosphate (including dUTP instead of dTTP), 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 1.25U of Taq polymerase (AmpliTaq; Perkin-Elmer, Langen, Germany), 5 µl of sample, and 2 drops of mineral oil. For the first round, primer, MP-1 and MP-2, were used and 0.1U of uracil-N-glycosylase (UNG) was added. Nested amplification was performed by using MP-1S and MP-2S primers and 5 µl of PCR product from the first round of amplification. The mixture was as same as the first round PCR but without UNG. Amplification was performed in a DNA Thermal cycler (Perkin-Elmer Cetus, Norwalk, Connecticut). The temperature program was as follows:

The first round PCR: 94°C 5 min for initiation denaturation
35 cycles of
94 °C 1 min for denaturation
56 °C 1 min for annealing
72 °C 1 min for extension
and final extension 72 °C 10 min

Nested PCR: 94 °C 10 min
35 cycles of
94 °C 1 min

	58 °C	1 min
	72 °C	1 min
and final extension	72 °C	10 min

8.2 16S rDNA amplification

The PCR was performed in a total volume of 50 µl containing 50 mM KCl, 10 mM Tris HCl (pH 8.3), 2.5 mM $MgCl_2$, 200 µM (each) deoxynucleoside triphosphates (including dUTP instead of dTTP), 0.5 µM of each primer, 1U of Taq DNA polymerase, and sample. Primer, 16S-1 and 16S-2, were used and 0.1U of UNG was added in the first round PCR. Nested PCR was performed by using 16S-1S and 16S-2S primers and 5µl of the first PCR product. The mixture was as same as the first step but no added UNG. Samples were amplified for 40 cycles by using a PCR processor (Hybrid, OmniGene). Conditions of the amplification for the first and second round were as follows:

Initiation denaturation	94 °C	5 min
	40 cycles of	
	94 °C	1 min
	52 °C	1 min
	72 °C	2 min
and final extension	72 °C	10 min

8.3 Amplification control

Positive control : *M. pneumoniae* DNA 100 fg was added to test the amplifiability in the samples. When inhibition occurred, a 1/20 dilution of the sample was retested.

Negative control : culture medium and DDW were used. Culture medium was treated as same as the specimen. Nested PCR was considered valid when all negative controls were negative in both round of PCR.

9. Analysis of amplified DNA

Ten microliter of amplified products were analysed by agarose 1.5% gel electrophoresis, consisted of 50 µl/ml Ethidium bromide, in Tris-acetate buffer at 80 volts for 35 min and visualized under UV light.

10. Interpretation

Samples were defined as positive if culture was positive for *M. pneumoniae*. If culture was negative, positive result required agreement of at least two different methods for detection, i.e. serological test and PCR for one or both primers pairs, or PCR was positive for both P1 and 16S rDNA primers.