CHAPTER III

MATERIALS AND METHODS

1. Selection of primers

Primers were selected from the published sequence of P1 gene(36). The selection of these primers were performed by using the "OLIGO" program of Rychlik & Rhoads (83).

2. Isolation of DNA

Mycoplasma pneumoniae Mac strain, obtained from Serum Institute, Copenhagen, Denmark, was cultured in modified Hayflick medium(84). 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 (10 mM Nacl, 20 mM Tris hydrochloride [pH 8.0], 1 mM EDTA) and incubated overnight at 37° C with 1% sodium dodecyl sulfate and 50 ug of proteinase K per ml. DNA was extracted three times with an equal volume of phenol saturated with TE (10 mM Tris hydrochloride [pH 7.5], 1 mM 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 0.3 M CH_2CooNa and the DNA was precipitated with 2 volumes of absolute ethanol at room temperature or -20°C or -70°C. The nucleic acids were suspended in TE buffer and were treated with DNase-free RNase A (50 ug/ml) at 37° C for 30 min. After another extraction

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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.

3. Amplification of *M. pneumoniae* DNA by PCR

3.1 Setting up the PCR reaction mixture

The amplification reactions were performed in a final volume of 50 ul. The standard reaction mixture contained 10mMTris-HCl (pH8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton-X100, 200 uM (each) deoxy-nucleoside triphosphates, 0.5 uM (each) primers, and 1.25 U of *Taq* polymerase. The reactions were performed in 0.5 ml tubes; the reagent mixture in each tube was covered with a drop of mineral oil. Since the components which effect the outcome of the amplification are $MgCl_2$, primers, and *Taq* polymerase, the optimal concentration of these components was determined for optimization of PCR reaction mixture with 0.5 uM of each primer. Varied concentration of $MgCl_2$ (0.5, 1.0, 1.5, 2.0, or 2.5 mM) and *Taq* polymerase (0.25, 0.5, 0.75, 1.0, 1.25, 1.5 or 2.0 U) was each tested.

3.2 Temperature, time, and the number of cycles

The PCR was performed by incubating the samples at three temperatures corresponding to the three steps in a cycle of amplification : the double-stranded DNA is denatured by heating the sample to 94° C for 1 min (DNA denaturation), the primers are allowed to anneal to their complementary sequences by cooling to 56°C

for 1 min (primer annealing), followed by heating to 72° C for 1 min to extend the annealed primers with the *Taq* polymerase (primer extension). This cycling was accomplished automatically with the DNA Thermal Cycler (Perkin-ElmerCetus, Norwalk, Conn.). The three-step reaction was repeated 25, 30, 35, 40, and 45 cycles in order to determine the optimal number of cycles. After repeating a three-step PCR reaction, the samples were heated 72° C for 10 min to complete extension.

4. Analysis of amplified DNA

4.1 Agarose gel electrophoresis

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

4.2 Dot blot hybridization (ECL; Amersham, Buckinghamshire, England)

4.2.1 Probe

A 342-bp DNA situated within the 466-bp fragment was used as probe. The probe was prepared by amplifying *M. pneumoniae* DNA with nested primers selected by "OLIGO" program. The 342-bp products were precipitated and labelled with fluorescein using ECL random prime system protocols as follows :

The 342-bp products was diluted to a concentration 2-25 ng/ul in either distilled water or TE buffer. The sample was

denatured by heating for 5 minutes in a boiling water bath and then chilled on ice. It is advisable to denature in a volume of at least 20 ul. The reagent comprised of Nucleotide mix, Primers, Denatured DNA (minimum), Enzyme solution (Klenow 4 unit/ul) and ddw. Each reagent was added to 1.5 ml microcentrifuge tube, mixed and incubated at 37°C for 1 hour. The reaction was terminated by the addition of EDTA to a final concentration of 20 mM and the probe can then be stored in a freezer at -20 °C for at least 3 months.

4.2.2 Dot blotting

Prewet nylon membranes in water followed by 10XSSC. Samples can either be applied directly to the wet membrane or the membrane can be allowed to air dry before sample application. Denature DNA samples by heating in a boiling water bath for 2-5 minutes. Chill rapidly on ice, then centrifuge briefly to collect sample at the bottom of the tube. Carefully apply 1 ul samples of denature DNA to the membrane, avoiding pressure on the membrane by the pipette tip. Leave filter to air dry. Fix DNA to nylon membranes by baking in a vacuum oven at 80°C for 2 hours.

4.2.3 Hybridization

Prehybridization (1 hr) was carried out at 60°C in a solution of 5XSSC, 0.5% blocking agent, 5% dextran sulphate, 0.1% SDS, and 100 ug/ml of denatured sheared heterologous DNA. Hybridization was conducted for 16 hr in the same mixture at 60°c but containing 342-bp fluorescein-labelled probes. After hybridization, the filters used for dot blot hybridization were washed at 60°C as follow : once for 15 min in 1XSSC-0.1% (w/v) SDS, once for 15 min in 0.5XSSC-0.1% (w/v) SDS. The membranes were used directly for detection of

hybridized DNA by anti-fluorescein HRP conjugate according to instructions of the manufacturer. Results were visuallized on film.

4.3 Nested PCR

Nested primers were used to amplify the product from the first PCR. Ten microliter of the product was added to 50 ul of standard PCR reaction solution mixture, containing 50 mM KCl, 0.1% Triton-X100, 10 mM Tris-HCl (pH 8.4), 200 uM of each dNTP, 1.5 mM $MgCl_2$, 0.5 uM of each of nested primer, and 1.25 unit of *Taq* polymerase. The mixture was subjected to 35 cycles of amplification with denaturation at 94° C 1 min, annealing at 58° C 1 min, and extension at 72°C 1 min by means of a thermal cycler.

5. Determination of the sensitivity of PCR

Serial dilutions of purified *M. pneumoniae* DNA containing between 1 pg and 0.1 fg of DNA were used in a PCR amplification. The amplified products were detected by gel electrophoresis and dot blot hybridization. For negative control, double distilled water (ddw) was used as the sample for PCR.

6. Determination of the specificity of PCR

The specificity of the PCR detection was studied in *Mycoplasma* species (Table I), other bacterial isolates from respiratory tract infected patients from Chulalongkorn hospital (Table II), and also human leukocytes. To extract the DNA from

Mycoplasma, 1 ml of 5-7 days late exponential phase culture were centrifuged for 10 min at 10,000xg. The sediment was washed once with 0.01 M Tris-HCl(pH 8.0), then resuspended in residual buffer (approximately 50 ul). The samples were subsequently boiled for 15 min to disrupt the cells and remove DNA for amplification(31). To amplify the DNA, 5 ul of this preparation was added to the reaction mixture.

For bacterial DNA preparation, the 0.5 Mac Farland turbidity of the fresh culture from blood agar plate were suspended in 0.01 M Tris-HCl (pH 8.0). Subsequently methods will be the same as those of *Mycoplasma* DNA preparation. Human leukocytes DNA provided by Chatsuwan, T., Department of Microbiology, Chulalongkorn University, which were extracted by followed the procedure of Frederick, M.A.(85).

7. <u>PCR protocols to prevent amplicon contamination</u>

The optimal concentration of dUTP and $MgCl_2$ were determined by varying the concentration of dUTP from 200 uM to 2.0 mM and the concentration of $MgCl_2$ from 1.5 mM to 4.0 mM.

To ensure that uracil-N-glycosylase (UNG) can break down contaminating amplicons, the method described by Kox. et al.(86) was used as follows : the amplicon solution was prepared by using 1 pg of *M. pneumoniae* DNA as a template in a PCR. Following, the 1,000-fold serial dilution of15 ng/ul of 466-bp amplicon (estimated by gel electrophoresis) were prepared. Ten ul of this solution were amplified in the absence and the presence of 0.1 U of UNG.

8. Evaluation of the PCR in simulated samples.

Simulated specimens were prepared from throat swabs taken from healthy volunteers. The swabs were transported in Trypticase soy broth containing 0.5% bovine serum albumin. The transport medium was extracted as described and tested for *M. pneumoniae* by PCR. Twenty microliters of each extract were pooled together after mixing throughly on a vortex mixer. The pooled specimens were then redistributed in small quantities and seeded with known amounts of *M. pneumoniae* DNA (31).

<u>Table I.</u> *Mycoplasma* species used for evaluation of the specificity of the PCR detection method

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- *Mycoplasma pneumoniae* (ATCC 15293, ATCC 29085, ATCC 15531, ATCC 15377, and ATCC 29342)
- *Mycoplasma hominis* (ATCC 27545)
- *Mycoplasma orale* (ATCC 15544)
- *Mycoplasma genitalium* (ATCC 33530)
- *Mycoplasma buccale* (ATCC 23636)
- *Mycoplasma fermentans* (ATCC 15474)
- *mycoplasma salivalium* (ATCC 14277)
- *Mycoplasma faecium* (ATCC 25293)
- *Acholeplasma laidlawii* (ATCC 29804) and
- *Ureaplasma urealyticum* (ATCC 27613)

Table II. Bacterial species used for evaluation of the specificity of the PCR detection method

- *Klebsiella* species
- *Enterobacter* species
- *Pseudomonas* species
- *Haemophilus influenzae*
- *Haemophilus parainfluenzae*
- *Acinetobacter* species
- *Corynebacterium* species
- *β-Streptococcus* group A & others group
- *Streptococcus pneumoniae* & others α- Streptococcus
- *Neisseria* species
- *Staphylococcus aureus*
- *Staphylococcus* coaggulase negative