CHAPTER IV

MATERIALS AND METHODS

1. Reference strains

Chlamydia pneumoniae strain TWAR was provided by Virology Division, Department of Microbiology, Faculty of Medicine, Siriraj hospital, Mahidol University. Purified TWAR antigen was purchased from Washington Research Foundation, Seattle, Washington.

2. Study population

One hundred and fourteen patients with community-acquired pneumonia (CAP) attending at Lung Clinic, Division of Pulmonary Medicine, Department of Medicine, Pramongkutklao College of Medicine were enrolled in this study. The diagnosis was based on clinical signs and symptoms and new or progressive radiographic changes consistent with pneumonia. The clinical diagnosis of CAP included evidences of a new chest radiographic infiltrate, and at least one of the major and two of the minor criteria as follow. Major criteria were: fever >37.8 ^{o} C, cough, or sputum production; minor criteria were: dyspnea, pleuritic chest pain, and leukocytosis 10,000/mm^3 or leukopenia. One hundred and fourteen CAP patients consisted of 63 males ranging in age 17 to 91 years and 51 females ranging in age 17 to 85 years.

3. Clinical specimens

3.1 Throat swabs

Throat swab specimens were collected from all 114 patients with community-acquired pneumonia. The specimens were collected by rubbing the posterior wall of throat with a steriled cotton tipped swab up and down, then placed into 2.5 ml of Chlamydia transport medium (0.2 M sucrose phosphate [2SP]) and kept at -70°C prior to use in this study.

3.2 Serum

Blood specimens for MIF test were obtained from 114 patients in the acute stage of illness and after 30 days of convalescence. All sera were separated from the clotted blood and stored at -20 °C until tested.

4. Microimmunofluorescence (MIF) test

4.1 Preparation of MIF antigen slide

TWAR antigens were spotted with sharp pen onto wells of teflon-coated slides, each well containing 2 consecutive spots of TWAR antigen and one yolk sac control. Antigens were air dried for 30 min, fixed with acetone for 15 min at room temperature and stored at –70°C in the slide box in which they can be kept up to a year.

4.2 Procedure of MIF for specific Ig G or Ig M antibody.

For MIF test, frozen antigen slides were left at room temperature for 30 min before testing. Serum samples were diluted with PBS buffers pH 7.2 for IgG while sera samples for IgM were absorbed with anti-human IgG antibody. Approximately

25 µl of the diluted sample were added to an appropriate slide well. After incubation at -37 °C for 45 min in a moist chamber, the slides were rinsed and agitated first in PBS pH 7.2 for 10 min, then in distilled water and air dried for 30 min. Anti-human immunoglobulin (IgG or IgM) fluorescein conjugated (DAKO, Glostrop, Denmark) diluted to 1:30 in PBS pH 7.2, was applied onto each well. After incubation for 45 min, the slides were washed, air dried and finally mounted with mounting medium before examination under a fluorescent microscope. Respective positive and negative control were included in each test (3,7).

4.3 Interpretation of the results of MIF test

The slides were read from fluorescent intensity of elementary bodies, and graded the fluorescence as follow:

2^+ to 4^+ : moderate to intense apple-green fluorescence

1^+ : definite, but dim flluorescence

Negative: no fluorescence or fluorescence equal to that observed in the corresponding yolk sac control spot or in the negative control well

As serum dilutions 1:8 and 1:16 were used for initial screening of chlamydial antibodies, sera reactive to the *C. pneumoniae* antigens will be further titrated for final dilution.

5. Indirect immunofluorescent antibody (IFA) test

5.1 Preparation of samples for IFA test

Throat swabs in transport medium were vortex mixed and 1 ml of the suspension were centrifuged at 12,000 rpm for 15 minutes. The remainder suspension was kept at -70 °C for PCR. The supernatant was discarded and the pellet resuspended in 1 ml of distilled water. The procedure of votexing and centrifugation was repeated once, and the supernatant discarded to leave approximately 50 - 100 µl, which was again vortexed to yield a milky suspension. A 5 µl amount was placed on a single well of a teflon coated slide, air dried and fixed in acetone for 15 minutes, drained, and air dried before staining (96).

5.2 Procedure of IFA test

A 15 µl amount of 1:40 diluted *C. pneumoniae* monoclonal antibody (Washington Research Foundation, Seattle, Washington) was added to each specimen well. Slides were incubated in a moist chamber at 37°C for 30 minutes, then washed in PBS pH 7.4, for 10 minutes, rinsed with distilled water and dried. Fifteen microliters of 1:60 diluted FITC conjugated anti-mouse IgG (Sigma, Deisenhofen, Germany) was applied to the well. The slide was incubated at 37 °C for 30 minutes in a moist chamber, washed in PBS pH 7.4 for 10 minutes, rinsed with distilled water and air dried. A drop of mounting fluid was added to the slide before applying a coverslip. The slide was then examined by fluorescent microscope with an oil immersion lens at x1000 magnification.

The control slide was used to check the morphology of the elementary bodies. The entire area of the smear was examined. Findings on slides were considered positive when four or more elementary bodies, appearing as bright apple-green fluorescent disc shaped bodies about 300 nm in diameter were seen. Positive and negative control slides were included in each test (96).

6. Polymerase chain reaction (PCR)

6.1 Construction of the control DNA

6.1.1 Positive control for *omp1*-based PCR

Positive control was constructed by the protocol previously described (107) and shown in Fig. 4. A DNA fragment with a deleted 60 bp sequence of the *omp1* regions was constructed by amplification of *C. pneumoniae* strain TWAR DNA with composite primer CPC-Del and CP2. The composite primer contains the CPC sequence and a 19 bp sequence termed Del, which is 60 bp from 3' to the CPC sequence.

Sequence of composite primers used:

CPC-Del : 5' TTATTAATTGATGGTACAATATTACGTGCTGGATTTTAC G 3'

CP1-CPC : 5' TTACAAGCCTTGCCTGTAGGTTATTAATTGATGGTACAAT 3'

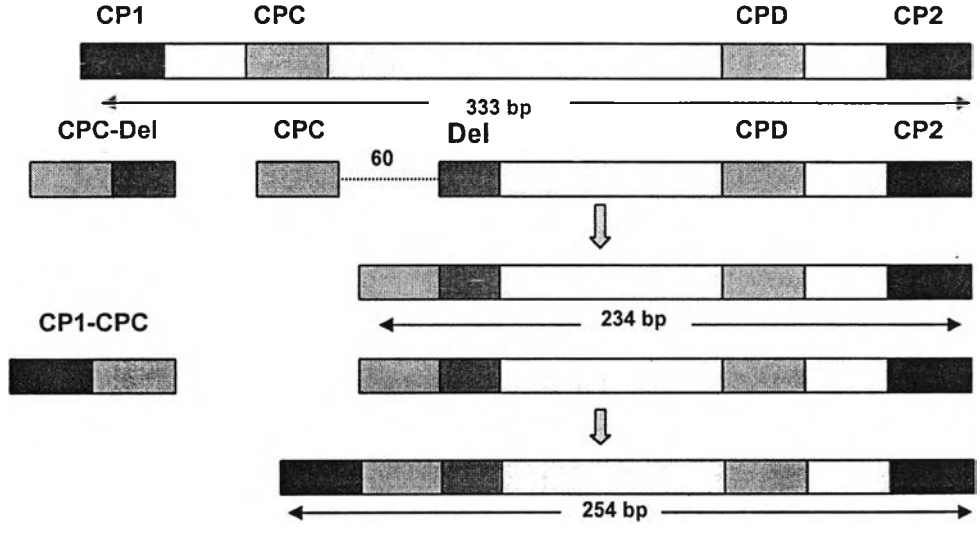

Fig 4. Diagram of construction of *omp1*-based PCR positive control

Amplification reaction contained 0.5 μM of primer CPC-Del and CP2, 200 μM deoxyribonucleotide triphosphate, 10 mM Tris-HCl (pH8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 1.25 U of Taq polymerase (Gibco, BRL), 5 μl of treated *C. pneumoniae* strain TWAR. Amplification was performed in a DNA Thermal cycler (Perkin-Elmer Cetus, Norwalk, Connecticut). Amplification of CPC-Del/CP2 fragments using the technique of "Touchdown" PCR similar to amplification of primers CP1 and CP2. Amplification with primers CPC-Del and CP2 yielded 234 bp products in contrast to 333 bp products of the primer CP1 and CP2. Then, the 254 bp construct was amplified from the 234 bp products with composite primer CP1-CPC and CP2. The mixture and the condition of amplification was the same as the first step PCR. The amplification yielded a 254 bp fragments, which was ligated into the pGEM®-T cloning vector by using the pGEM®-T Easy Vector System (Promega, Madison, WI, USA). The test was performed manually according to the manufacturer's recommendations. After transformation into *E.coli* JM109, the plasmid control were purified. These plasmid control were named "pKW2". The concentration of the plasmid control was determined by measurement of optical density at 260 nm . This information and the molecular weight of the plasmid control were used for calculation of the copy number as followed (107);

$$1\mu g \text{ of dsDNA} \left(\frac{1 \text{ DNA molecule}}{\text{length (nt)}} \right) \left(\frac{6.02 \times 10^{23} \text{ nt}}{660 \text{ g}} \right) \left(\frac{1 \text{g}}{10^6 \mu g} \right) = \text{no. of molecules}$$

6.1.2 Positive control for 16S rDNA-based PCR

A positive control for the 16S rRNA gene primers was constructed by ligating the 463 bp amplicon generated by primers CpnA and CpnB into the pPCR-Script

Amp SK(+) cloning vector with PCR-Script™ Amp Cloning Kit (Stratagene, USA) following the supplier's instructions. The cloned DNA plasmid was purified and named "pKW3". The plasmid control pKW3 was digested at restriction site located within the 463 bp amplicon with restriction enzyme *Hpa1* and *Pml1*, then religated and propagated into *E. coli* JM 109 to generate a DNA target truncated by 63 bp. After transformation, the plasmid control were purified and named "pKW4". The concentration of the plasmid control pKW4 was determined by measurement of optical density at 260 nm . This information and the molecular weight of the plasmid control were used for calculation of the copy number.

6.1.3 Determination of the amount of plasmid control used for PCR

The plasmid control pKW2 and pKW4 were diluted ten-fold dilution starting from $2x10^3$ copies. Five microliters of diluted plasmid control from each dilution were used for PCR. The amount of plasmid used for spiking in the sample was determined from the final concentration of plasmid control pKW2 and pKW4 that showed a single band of 254 bp fragment for *opm1*-based PCR and 400 bp fragment for 16S rDNA-based PCR, respectively.

6.2 DNA extraction from *C. pneumoniae*

C. pneumoniae strain TWAR elementary bodies (EBs) were quantitated by IFA staining. One microliter aliqouts of purified EBs at varying dilutions in normal yolk sac were smeared onto wells of teflon-coated slide. Slides were air dried for 30 min, fixed with acetone for 15 min at room temperature, examined and counted under fluorescent microscope after staining with IFA. EBs were diluted ten-fold dilution

starting from 200 IFU/μl by using lysis buffer containing 0.5% nonidet P-40, o.5% tween- 20 and 20 mg/ml of proteinase K. The suspension was incubated at 58°C for 1 hr and boiled for 10 min to inactivate proteinase K. Five microliters of an extracted DNA from each dilution were used for the PCR.

6.3 DNA extraction from throat specimens

One milliliter of the processed throat swab specimens kept at -70°C were thawed and pelletted by centrifugation at 12,000 rpm at 4°C for 30 min. The supernatant was removed, the pellet was resuspended in 100 μl of lysis buffer containing 0.5% nonidet P-40, o.5% tween 20 and 20 mg/ml of proteinase K. The suspension was incubated at 58°C for 1 hr and boiled for 10 min to inactivate proteinase K. Five microliters of the treated specimens were used for the PCR.

6.4 Primers and probes

The 16S rRNA gene and the major outer membrane protein gene (*ompl*) were used as targets for amplification of *C. pneumoniae* DNA (33,35). Oligonucleotide primers were purchased from Gibco BRL. DNA probes were generated by the nested primers which located within the first PCR product. The oligonucleotides sequences used in this study were shown in Table 2.

Table 2 : Oligonucleotides used in this study and size of PCR Product

Oligonucleotide	sequence	size of PCR product (bp)
omp1 gene		
CP1	5'-TTACAAGCCTTGCCTGTAGG-3'	333
CP2	5'-GCGATCCCAAATGTTTAAGGC-3'	
CPC	5'-TTATTAATTGATGGTACAATA-3'	207
CPD	5'-ATCTACGGCAGTAGTATAGTT-3'	
16S rRNA gene		
CpnA	5'-TGACAACTGTAGAAATACAGC-3'	463
CpnB	5'-CGCCTCTCTCCTATAAAT-3'	
pTW50	5'-TTAAGTCCCGCAACGAGCGCA-3'	270
pTW51	5'-GCTGACACGCCATTACTA-3'	

6.5 Amplification of *C. pneumoniae* DNA in clinical specimens by PCR

6.5.1 *omp1* gene amplification

The reaction volume for the amplification reaction was 50 µl with 0.5 µM of primer CP1 and CP2, 200 µM deoxyribonucleotide triphosphate (including dUTP instead of dTTP), 10 mM Tris-HCl (pH8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 2 U of Taq polymerase (Gibco), 0.1 U uracil-N-glycosylase (UNG)(Gibco, BRL), 5 µl of treated sample. Amplification was performed in a DNA Thermal cycler (Hybaid, OmniGene). To degrade contaminating amplification products from previous PCR with UNG, reaction volumes were first incubated at 37°C for 10 min and then heated

at 94°C for 10 min to inactivate the UNG. Amplification of *ompl* using the technique of "Touchdown" PCR in which the annealing temperature was lowered 1°C every two cycles from 65°C until touching down to 55°C at which temperature 25 more cycles were performed. The denaturation temperature and the extension temperature were constant at 94°C and 72°C, respectively. The holding time at each temperature was 1 min. After the last cycle, samples were incubated for 10 min at 72°C. Nested amplification was performed by using CPC and CPD primers and 10 μl of the first round PCR product. The mixture was the same as the first round PCR but without UNG. Prior to amplification, the reaction mix was heat at 94°C for 5 min. The amplification conditions for 45 cycles consisted of 94°C for 1 min, 50°C for 1 min, 72°C for 1 min, and complete extension at 72°C for 10 min.

6.5.2 16S rRNA gene amplification

The PCR was done 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 of each primer, 2 U of Taq DNA polymerase (Gibco, BRL), and 5 μl of treated samples. Primer CpnA and CpnB were used and 0.1 U of UNG was added in the PCR reaction. Amplification were carried out in a DNA Thermal cycler (Perkin-Elmer Cetus, Norwalk, Conecticut). The reaction volumes were first incubated at 37°C for 10 min and then heat at 94°C for 10 min. Forty five amplification cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C followed. After the last cycle, samples were incubated for 10 min at 72°C. Nested PCR was performed by using pTW50 and pTW51 primers and 10 μl of the first round PCR product. The mixture was the same as the first round but without

UNG. The reaction mixture was heat at 94°C for 5 min, followed by 45 cycles of 94°C for 1 min, 53°C for 1 min, and 72°C for 1 min with the final extension at 72°C prolonged to 10 min.

6.6 Analysis of amplified products by agarose gel electrophoresis

Ten microliter of amplified products from the first round and nested PCR were analysed by electrophoresis in 1.5 % Tris-acetate agarose gel containing 50 μg/ml ethidium bromide, in Tris-acetate buffer at 80 volts for 40 min and visualized under UV light. The positive result of first round PCR showed a single band of 333-bp fragment for *ompl*-based PCR and 463 bp fragment for 16S rDNA-based PCR. The positve result of Nested PCR showed a single band of 207-bp fragment for *ompl* gene and 270 bp fragment for 16S rRNA gene compared with the 100-bp molecular size marker.

6.7 Analysis of amplified products by dot blot hybridization

PCR products from the first amplification were subjected to detection by dot blot hybridization. The hybridization and detection were performed by using ECL direct nucleic acid labelling and detection system (Amersham, England)

6.7.1 Preparation of probe

The 207 bp DNA fragments from *ompl*-based PCR and the 270 bp DNA fragments from 16S rDNA-based PCR were used as probes. The DNA fragments was diluted to 10 ng/μl, then denatured 100 ng (10 μl) of the DNA sample by heating for 5 min in boiling water bath. The DNA was cooled immediately on ice for 5 min, spined briefly. An equal volume of DNA labelling reagent (10 μl) was added to the cooled DNA and they were mixed gently. An equivalent volume of glutaraldehyde

solution to the volume of labelling reagent (10 μl) was added, mixed throughly and spined briefly, then incubated at 37°C for 20 min.

6.7.2 Generation of hybridization dot blot

The whatman 3 mm paper was cut fit to dot blot manifold and a nylon membrane was cut to a desired surface area. The whatman 3 mm paper and the nylon membrane were soaked in distilled water, then whatman 3 mm was placed on the manifold and the nylon membrane was placed on the whatman 3 mm paper. Five microliters of each PCR product were suspend in 50 μl of denature solution for 10 min, then chilled the sample on ice immediately. With a dot blotter apparatus, the samples were applied onto the nylon membrane and washed with 100 μl of TE buffer. The sample were suctioned until dry and the dot blotter apparatus was disassembled. The nylon membrane was baked at 80°C for 1 – 2 hr in an oven in order to fix nucleic acid to the membrane.

6.7.3 Setting up hybridization

The nylon membrane was placed in a plastic hybridization bag and prehybridized in 250 μl prehybridization solution per cm^2 of membrane surface area for 30 min at 42°C. The prehybridization solution was removed from the hybridization bag and the labeled probe solution was added. The bag was incubated at 42 °C overnight with gentle agitation. The membrane was washed twice with 2 ml per cm^2 of 0.1x SSC, 0.4 % (w/v) SDS at 55°C for 10 min. The membrane was washed twice with 2 ml per cm^2 2x SSC at room temperature for 5 min.

6.7.4 Detection of hybridization

The detection of hybridization was performed by using ECL nucleic acid detection system. An equal volume of detection reagent 1 and detection reagent 2 were mixed to give sufficient solution of 0.125 ml per cm^2 to cover the blot. Drain the excess secondary wash buffer from the blots and place them in fresh containers, DNA side uppermost. The detection reagent was added directly to the blots, incubated for 1 min at room temperature. Excess detection reagents was drained off. The blots then were wrapped with SaranWrap. Air pockets were gently smoothed out. The blots DNA side up were placed in the film cassette. A sheet of Hyperfilm was placed on the top of the blots in the dark. The cassette was closed and exposed for 2 hr. The film was then removed and developed.

6.8 Determination of PCR sensitivity

The sensitivity of the PCR was determined by adding known amount of purified EB of *C. pneumoniae* strain TWAR DNA to the reaction. The starting amounts of EB was 200 IFU per µl. The DNA was diluted 10-fold serially to 0.002 IFU per µl. Five microliters of each dilution was tested by the PCR. The results were analyzed by agarose gel electrophoresis. The products of first amplification were analyzed by re-amplification with nested primers and dot blot hybridization.

6.9 Positive and negative control for the PCR

Positive control : Part of each sample was spiked with 1000 copies of plasmid control pKW2 and pKW4 correspond to *omp1*-based PCR and 16S rDNA-based PCR as a control to detect the presence of amplification inhibitor in each sample.

Negative control : Five microliters of DDW was used in place of the sample as a negative control.

7. Interpretation

A specimen was considered as positive if there was an agreement of the result of at least two different methods for detection, i.e. MIF test and PCR for one or both genes, IFA and PCR for one or both genes or MIF and IFA.

8. Determination of the sensitivity and specificity of PCR

To calculate sensitivity and specificity of the test, the following formulars can be used.

$$\text{Sensitivity} = \frac{\text{true positive}}{\text{true positive} + \text{false negative}} \times 100$$

$$\text{Specificity} = \frac{\text{true negative}}{\text{false positive} + \text{true negative}} \times 100$$