CHAPTER III

MATERIALS AND METHODS

The design and optimization of the PCR.

1) Selection of primers.

The primers were selected from the published sequence of *C. trachomatis*, trachoma biovar, serotype B (76). The selection of these primers were performed by using the "OLIGO" program of Rychlik & Rhoads (150). The sequences and positions of these primers were shown in Fig. 3.

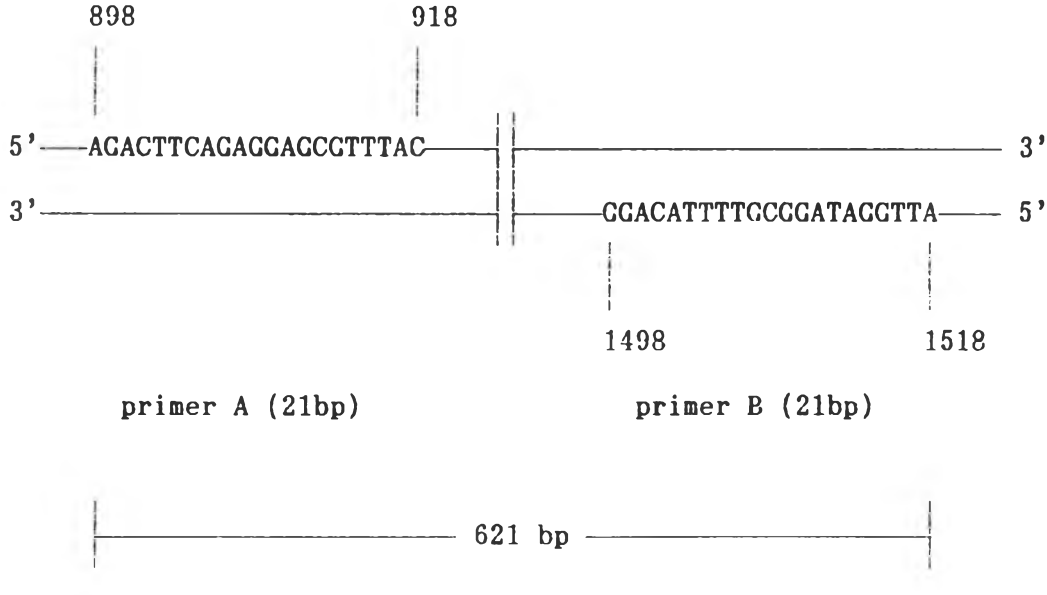

Fig 3. Positions and sequences of primers A and B. Numbers refer to the base number in the common *C. trachomatis* plasmid (76).

2) *Chlamydia trachomatis* strain.

C. trachomatis serotype L2 , in frozen culture , was provided by the Armed Forces Research Institute of Medical Science (AFRIMS).

3) Growth of *C. trachomatis*

C. trachomatis serotype L2 was grown in McCoy cell monolayer. Flasks (25-cm^2) containing monolayers of McCoy cells were inoculated with *C. trachomatis* serotype L2 and incubated for 3 hrs at 37°C. Maintanance medium (Appendix I) was added and incubated for 48-72 hrs at 35 - 37°C which resulted in chlamydial infection in at least 80-100 % of the cells.

4) Isolation of plasmid from *C. trachomatis* serotype L2

The infected cells were dislodged with glass beads and vortexed in order to lyse host cells. The cell debris was pelleted by centrifugation at 4,000 x g for 20 min. Chlamydial particles (EB and RB) in the supernatant were pelletted by centrifugation at 16,000 x g for 20 min (74), washed twice with TE buffer (Appendix III) and resuspended in 40 ul of TE buffer. Chlamydial particles were transfered with a pasteur pipette to a 1.5-ml polypropylene microcentrifuge tube conntaining 0.6 ml of lysis buffer (TE plus 4% sodium dodecyl sulfate [SDS], pH 12.4) and immediately mixed by rapid but gentle inversion of the tube. After incubation at 37°C for 20 min, the samples were brought to

pH 8.0 (± 0.5) by the addition of 30 ul of 2M Tris-HCl, pH 7.0 (APPENDIX III). The chromosomal DNA was precipitated by the addition of 0.16 ml of 5M NaCl (APPENDIX III). The tubes were chilled in an ice bath for at least 1 h and centrifuged for 5 min at 15,600 xg in a microcentrifuge (savant), and the supernatant was decanted immediately into another microcentrifuge tube. The plasmid DNA (pCHL2) was precipitated by adding 0.55 ml of cold isopropanol and incubating at -20 °C for 30 min. The precipitate was collected by centrifugation for 3 min in the microcentrifuge. The supernatant was discarded and the sediment was dried under vacuum. The sediment was resuspended in 30 ul of TE buffer (150). Ten microliters of the sample were subjected to electrophoresis in a Tris - acetate buffered agarose gel (Appendix III). The concentration of pCHL2 was estimated by comparing the DNA band intensity with those of λ /Hind III molecular size marker.

5) Optimization of PCR condition for detection of *C. trachomatis*

5.1) Temperature, time, and number of cycles

The PCR is 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 52°C for 1 min (primers annealing), followed by heating to 72°C for 1 min to extend the annealed primers with the *Taq*

polymerase (primer extension). This cycle was accomplished automatically with the DNA Themal Cycler (Perkin elmer cetus). The three-step reaction was repeated about 30, 40, 45, and 50 cycles in order to determine the optimal number of cycles. After repeating a three-step PCR reaction, the sample were heated to 72°C for 10 min to complete the extension.

5.2) Setting up the PCR reaction mixture

The standard PCR is typically done in a 50 or 100 ul volume and, in addition to the sample DNA, contains 50 mM KCl, 10 mM Tris-HCl (pH 8.4 at room temp.), 1.5 mM $MgCl_2$, 0.25 uM of each primer, 200 uM of each deoxynucleotide triphosphate (dATP, dCTP, dGTP, and dTTP), and 2.5 units of *Taq* polymerase. A few drops of mineral oil are often added to seal the reaction to prevent evaporation (151). However, for those reactions in which the conditions described above do not produce the desired results, changes of the PCR reaction mixture will usually effect the outcome of the amplification. The components which effect PCR are $MgCl_2$, primers , and *Taq* polymerase. Therefore , the optimal concentration of $MgCl_2$, primers and *Taq* polymerase was determined for optimization of PCR reaction mixture. The PCR reaction mixture contains 50 mM KCl, 0.1 % Triton-x 100, 10 mM Tris-HCl (pH 8.4), 0.1 uM of each primer, 200 uM of each dNTP (dATP, dCTP, dGTP, and dTTP) and varied concentration of *Taq* polymerase (1.5, 2.0, 2.5, or 3.0 units) and $MgCl_2$ (1.5 , 2.0 , 2.5 , 3.0 , 3.5, or 4.0 mM). Double distiled water (DDW) was added to 50 ul of reaction mixture and the reaction mixture was overlayed with

50 ul of mineral oil.

In order to prevent the false-positve results due to amplicon carryover, the incorporation of dUTP instead of dTTP and uracil DNA glycosylase enzyme (UDG) were used in the PCR reaction mixture and incubated at 37°C for 10 min to allow UDG removes uracil bases from amplicon carryover. The temperature was increased to 94°C for 10 min in order to inactivate the UDG and hydrolyze the contaminants into small fragments. Two cycles of the PCR were added to the number usually employed and a final temperature was maintained at 72°C, a temperature that the UDG protein will remain inactive. The optimal concentration of dUTP was determined by varying the concentration of dUTP was 1.0, 1.2, 1.4, 1.6,1.8, and 2.0 mM dUTP,respectively.

6. Analysis of the amplified products

6.1 Agarose gel electrophoresis

Ten microliter of amplified products were electrophoresed on a 1.5 % Tris-acetate agarose gel containing 50 ug/ml ethidium bromide, in Tris-acetate buffer (Appendix IV) at 80 volts for 35 min and visualized under UV light. The positive result shows a single band of 621-bp fragment, compared with the molecular size marker (ØX174/Hae III)

6.2 Dot blot hybridization.

6.2.1) probe

A 473-bp DNA probe situated within the 621-bp fragment was labeled with biotin by amplifying *C. trachomatis* plasmid DNA (pCHL2) with the nested primers. The primers were also selected from the published sequence of *C. trachomatis*, trachoma biovar, serotype B by using "OLIGO"program. The sequence and position of these primers were shown in Fig 4.

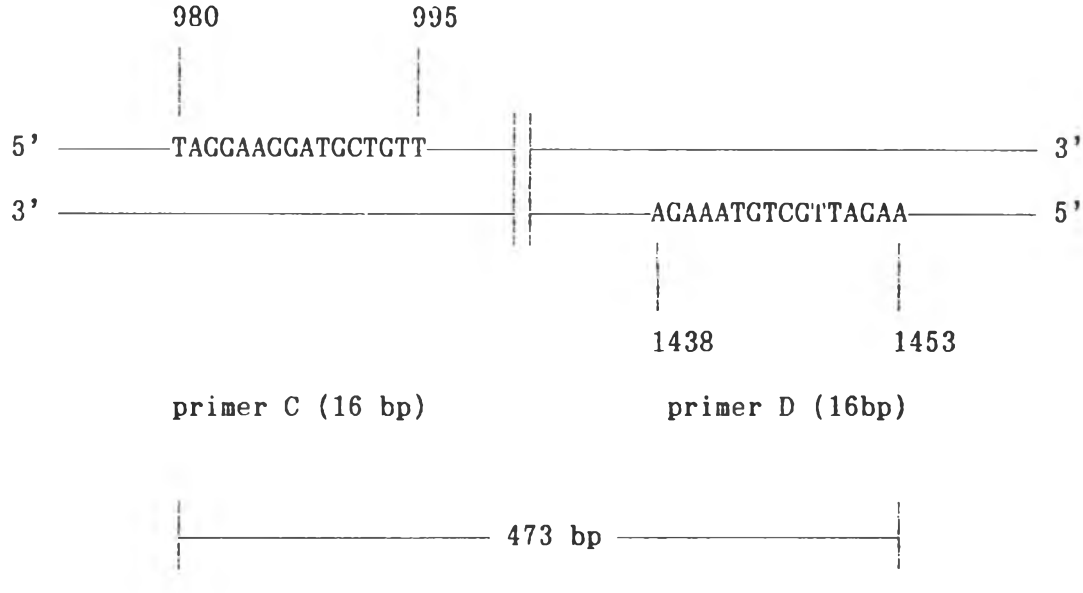

Fig.4 : Position and sequences of primer C and primer D. Numbers refer to the base number in the common *C. trachomatis* plasmid (76).

The PCR reaction mixture used for amplifying the biotinylated probes consists of 50 mM KCl, 0.1 % Triton-X 100, 10 mM Tris-HCl pH 8.0, 1.5 mM $MgCl_2$, 0.1 uM of each primer, 200 uM or each deoxynucleotide triphosphate (dTTP,dGTP,dCTP), 160 mM of dATP, 40 mM of biotin-14-dATP (Gibco-BRL) and 1 unit of *Taq* polymerase (Promega), and 4 ng of pCHL2. The DDW was added until to 100 ul of reaction mixture. The reaction mixture was overlayed with 50 ul of mineral oil. The PCR was run at 94°C for 1 min (DNA denaturation), 45°C for 1 min (primer annealing), 72°C for 1 min (primer extension), and complete extension at 72°C for 10 min.

6.2.2) Generation of hybridization dot blot

The whatman 3 mm paper was cut fit to dot blotter manifold and a nylon membrane (PhotoGene™) was cut to a desired surface area. The whatman 3 m paper and the nylon membrane were placed in distilled water for 5 min and then placed in 2 x SSC (Appendix V) for 10 min. The whatman 3 mm paper was placed on the manifold and the nylon membrane was placed on the whatman 3 mm paper. Ten microliters of each PCR products were boiled for 10 min, chilled the sample on ice immediately. With a dot blotter apparatus, the samples were applied on to the nylon membrane and washed with 100 ul of 10 x SSC (Appendix V). The samples were suctioned until dry and the dot blotter apparatus was disassembled. The whole nylon membrane was rinsed with 2 x SSC two times , air dried and baked at 80°C for at least 1 hr in vacuum oven in order to fix nucleic acid to the membrane.

6.2.3) Setting up hybridization

The nylon membrane was placed in a plastic hybridization bag and prehybridized in 250 ul prehybridization solution (Appendix V) per cm^2 of membrane surface area for 15 min at 42°C. The probe was dissolved in 50 ul 2 x hybridization buffer(Appendix XI) per cm^2 and an equal volumn of formamide was added. The probe was denatured by boiling for 10 min and chilled on ice immediately. [amount of probe (ng) = area of membrane in cm^2 x 50 ng probe per ml x o.1 ml per cm^2 membrane]. The prehybridization solution was removed from the hybridization bag and the denatured probe solution was added, the bag was incubated at 42°C for 2 hrs while agitating gently. The membrane was washed twice with 2 ml per cm^2 of 5 x SSC, 0.5 % (W/V) SDS (APPENDIX V) at 64°C for 5 min each wash. The membrane was washed once with 2 ml per cm^2 0.1 x SSC, 1 % (w/v) SDS (APPENDIX V) at 65°C for 30 min. The membrane was washed once with 2 ml per cm^2 of 2 x SSC (APPENDIX V) for 5 min at room temperature.

6.2.4) Detection of hybridization

The detection of hybridization was performed by using PhotoGene™ nucleic acid detection system. The hybridized membrane was washed in 1 ml per cm^2 of TBS-Tween 20 (Appendix V) for 1 min. Then, the non specifec protein binding sites on the

membrane were blocked by incubating the membrane in 0.75 ml/cm^2 of blocking solution (Appendix V) at 65^o C for 1 hr in hybridization bag. The tube of streptavidin-alkaline phosphatase (SA-AP) conjugate was microcentrifuged at 10,000 g for 5 min at room temperature. With a sterile pipet tip, carefully remove 7 ul SA-AP from the supernatant solution for each 100 cm^2 of membrane area. Avoid pipetting any pelletted material. Dilute the supernatant SA-AP 1:1000 in TBS-Tween 20. The membrane was incubated with SA-AP solution for 10 min at room temperature by gently agitating the membrane during incubation. Make certain that the membrane was completely covered by the SA-AP solution, and that they do not stick to one another. The membrane was washed twice with TBS-Tween 20 at room temperature for 15 min. Prepare the final wash buffer by diluting the 10x final wash buffer 1:10 with distilled water. The membrane was washed with 1 ml/cm^2 1x final wash buffer for 60 min at room temperature, agitating gently. The membrane was placed in a PhotoGene™ development folder provided with the system. Pipet 0.01 ml/cm^2 of detection reagent over the membranes. Immediately cover the membrane with the development folder and roll a 10 ml pipet over the plastic sheet to spread the detection reagent and to remove the air bubbles over the membrane. The edges of the development folders may be sealed with tape. Store the membrane in the dark at 23^{o}C to 25^{o}C for 10 min and detect the light emission by exposure the membrane to X-ray film. Light emission from the PhotoGene™ system results from two reactions : the dephosphorylation of the PPD (4-methoxy-4-(3-phosphatephenyl) spiro [1,2-deoxetane-3,2'- adamantane]) and the decay of the

dephosphorylated dioxetane.

7. Determination of PCR sensitivity

The sensitivity of the PCR with the primer A and B was determined by adding decreasing amounts of pCHL2 to the reaction. The starting amounts of pCHL2 was 2.0×10^{-10} g per ul. The DNA was diluted ten-fold serially to 2.0×10^{-18} g per ul. One microliter of each dilution was tested by the PCR. The results were analysed by agarose gel electrophoresis and dot blot hybridization.

8. Evaluation of the PCR efficiency for detection of *C. trachomatis* from clinical specimens

8.1 Clinical specimens

To evaluate the detection of *C. trachomatis* by using the PCR, the endocevical specimens were obtained from 100 woman attending a clinic for sexually transmitted diseases at Bangrak Hospital. The ENT swab (Medical Wire and Equipment Co.,Ltd., Corsham, England) was rotated in the endocervical canal and immediately placed into 1.5 ml transport medium (AppendixII) in the ice box. They were stored at -70°C until tested.

8.2 Specimens for the PCR

The specimens were thawed and 200 ul of the specimens were pelleted by centrifugation at 10,000 x g in microcentrifuge for 30 min. The supernatant was removed, the pellet was resuspended in 20 ul of 0.5% nonidet P-40 (Boehringer Mannheim GmbH),0.5% tween20 (Sigma),and 100 ug/ml of proteinase K (Amresco). The suspension was incubated at 60°C for 1 hr and boiled for 10 min (152). Five microliter of the treated specimens were tested by the PCR.

8.3 Positive and negative control for the PCR

Part of each sample was spiked with 20 pg of pCHL2 as a control to detect the presence of amplification inhibitor in each samples. Five microliters of the specimens were substituted by DDW as negative control for the PCR. A positive control containing 20 pg of pCHL2 and a negative control containing no DNA were included in each run.

8.4 Specimens for cell culture

The specimens were thawed rapidly in a water-bath at 37°C and agitated vigorously with 4-5 particles of sterile glass beads using vortex mixer about 1 min. If the specimens contained blood , centrifuge them for 5 min at 200 x g. The supernatant was ready for inoculation.

8.5 Isolation of *C. trachomatis* by cell culture

McCoy cells, 1.5 x 10^{-5} cells, were grown on the coverslip vial (13 mm in diameter) at 37°C for 24 hrs in order to form McCoy cells monolayer. Pour off the cell culture medium (Growth medium : Appendix I) and inoculate with 0.5 ml of clinical specimen, centrifuge 3,000 x g at room temperature for 1 hr. The supernatant was removed, add 1 ml of maintanance medium and incubate at 37°C for 48-72 hrs. The infected McCoy cells were stained with iodine solution (Appendix I), examined and counted the inclusion under light microscope.