III. RESEARCH DESIGN, MATERIALS AND METHODS

1. Research design

C. trachomatis positive samples were collected from the STD high risk patients attending the Venereal Disease and AIDS Control Center, Region 10, Chiang Mai province and from the Research Institute for Health Sciences, Chiang Mai University. The samples were examined for the genotypes and nucleotide sequence variation of the MOMP gene. For genotyping, the VD4-MOMP gene was amplified and then analyzed by using the restriction fragment length polymorphism (RFLP) with 4 restriction endonucleases (RE); *Alu*I, *Hind*III, *Dde*I *and Eco*RII digestion. The RFLP patterns were visualized on 6% polyacrylamide gel electrophoresis and compared with the RFLP pattern of reference serotypes shown in Table 1. In order to investigate the nucleotide sequence variation, the MOMP gene was cloned into the pGEM vector and then sequenced by the cycle sequencing technique using the ABI PRISM® Big Dye ™ Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer, Applied Biosystem, USA). The products were analyzed by the ABI 310 automated DNA sequencer (Perkin-Elmer, Applied Biosystems, USA). The nucleotide sequence of the MOMP gene from each sample was then compared with the reference strains with the aid of the computer programme, Autoassembler™ 1.4.0 (Perkin-Elmer, Applied Biosystem, USA). The steps of the whole processes are shown in Fig. 2.

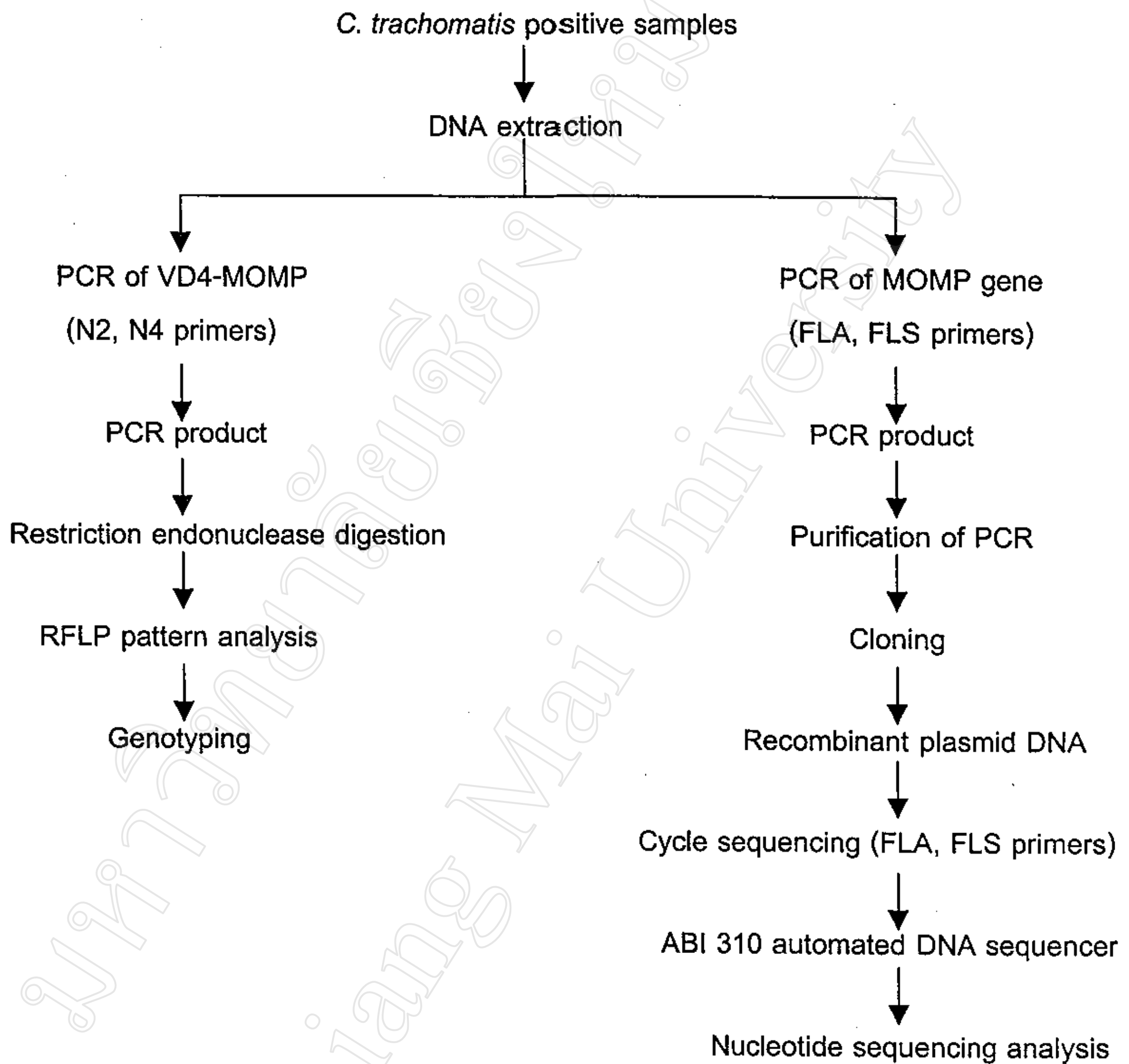

Figure 2. The schematic diagram presenting the research design of this study

2. Materials and methods

2.1 Clinical samples

Endocervical and urethral swabs were obtained from female sex workers and male patients attending the Venereal Disease and AIDS Control Center, Region 10, Chiang Mai province during January to August 1999. Endocervical swabs were routinely tested for the presence of *C. trachomatis* DNA by using the nucleic acid hybridization test (Gen-Probe PACE 2 system, USA) at the Center, with the results confirmed by PCR in this study. The urethral swabs were tested by the PCR. After testing, 23 infected samples were collected and used for this study. The other 11 samples were *C. trachomatis* isolation positive obtained from the Research Institute for Health Sciences, Chiang Mai University. Among those isolates, 6 were from female sex workers, 3 from female ex-sex workers and 2 from male drug users. All of them resided in Chiang Mai, Lumphun, Lumpang, Phayoa and Chiang Rai provinces. The isolates were collected during January 1994 to January 1999.

2.2 Laboratory studies

2.2.1 DNA extraction

2.2.1.1 Phenol/chloroform method

The samples from the Gen-Probe test were in the Gen-Probe lysis buffer. For those samples, DNA was extracted by using the phenol/chloroform method. The samples were thawed and 200 μl of DNA lysate was transferred to a 1.5 ml microcentrifuge tube. An equal volume of phenol/chloroform solution was added into the lysate and centrifuged at 10,000 rpm for 10 minutes. After collecting the supernatant in an aqueous phase, DNA was precipitated with a 0.5 volume of 7.5 M sodium acetate and a 2.0 volume of absolute ethanol. The mixture was stored at -70°C for 10 minutes, centrifuged at 10,000 rpm for 30 minutes, and then the supernatant

was discarded. The pellet was rinsed with 70% ethanol and centrifuged at 10,000 rpm for 10 minutes. The air-dried pellet was resuspended in 30 µl of sterile distilled water.

2.2.1.2 High Pure PCR Template Preparation Kit

The cervical and urine positive samples were extracted for DNA by using the High Pure PCR Template Preparation Kit (Boehringer Mannheim, Germany). The process was performed as recommended by the manufacture. In brief, 500 µl of the sample was spun down, then the pellet was resuspended in 200 µl of PBS. The 200 µl of binding buffer and 40 µl of protinase K were added into the suspension. The nucleic acid was adsorped into the High Pure filter tube. Then, the proteins and other cellular contents were washed out by passing the washing buffer through the filter tube. Finally, the DNA was eluted by 50 µl of distilled water and stored at -20°C for further analysis.

2.2.2 Primers

The oligonucleotide primers were synthesized as the sequences showed in the WHO Chlamydial PCR Workshop from June 2 to 5, 1992. All primers derived from the conserved sequence within the MOMP gene. They were common to all *C. trachomatis* serotypes. The amplified product of the MOMP DNA, using FLS and FLA primers, was approximately 1,200 bp . The VD4 region of the MOMP gene was amplified by using the Nest 2 (N2) and Nest 4 (N4) primers, which generated the product of approximately 350 bp. The nucleotide sequence and location of those primers in the MOMP gene are shown in Figure 3.

<u>Primer FLS (forward 30-mer)</u>

5' CTCTTGAAATCGGTATTAGTATTTGCCGCT 3'

<u>Primer FLA (backward 30-mer)</u>

5' TTAGAAGCGGAATTGTGCATTTACGTGAGC 3'

<u>Primer Nest 2 (N2) (forward 21-mer)</u>

5' CATGAGTGGCAAGCAAGTTTA 3'

<u>Primer Nest 4 (N4) (backward 20-mer)</u>

5' GCTTGATCGATGAGAGAGCA 3'

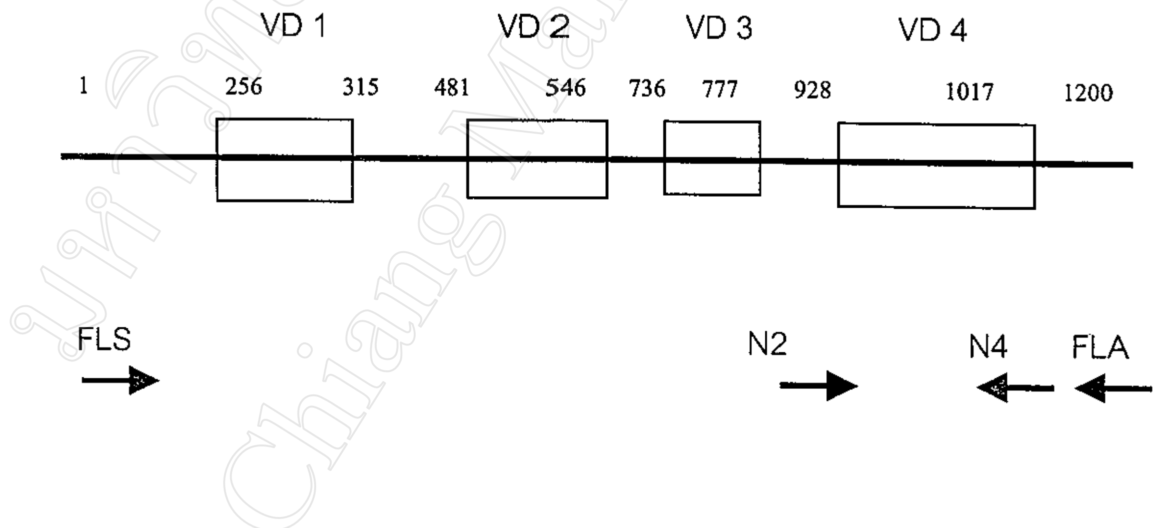

Figure 3. The sequences of FLS, FLA, N2 and N4 oligonucleotide primers and their positions on the MOMP gene of *C. trachomatis*.

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2.2.3 Polymerase chain reaction (PCR)

2.2.3.1 PCR of the MOMP gene

PCR was performed with a total volume of 50 µl. The final reaction mixture contained 0.2 mM of each deoxynucleotide triphosphate (dATP, dTTP, dGTP and dCTP); PCR buffer (50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCL, pH 8.8); 50 pmol of each FLS and FLA primer; and 1.25 U of *Tag* DNA polymerase . The initial PCR reaction contained 10 µl of DNA template. The *C. trachomatis* L2 DNA and sterile distilled water were used as a positive and negative control, respectively.

The reaction was amplified for 30 cycles in a thermocycler (GeneAmp 2,400, Perkin-Elmer, Cetus, USA.). Each cycle consisted of denaturation at 94°C for 60 seconds, annealing at 60°C for 60 seconds and extension at 72°C for 90 seconds. After the last cycle, the extension step was extended for another 7 minutes to ensure its completion.

2.2.3.2 PCR of the VD4-MOMP gene

PCR reaction for the amplification of the VD4 sequence was prepared similarly to that of the MOMP gene, except N2 and N4 primers were used instead of FLS and FLA primers.

The amplification cycle composed of denaturation at 94°C for 45 seconds, annealing at 55°C for 45 seconds and extension at 72°C for 45 seconds. After the last cycle, the extension step was extended for another 7 minutes.

2.2.4 Detection of the PCR product

The amplified product was analyzed on 1% agarose gel electrophoresis in a TAE buffer at 100 volts for 45 minutes. It was then stained with ethidium bromide and visualized under a long wavelength ultraviolet light using the ultraviolet transilluminator. The PCR product of the MOMP gene was approximately 1,200 bp,

while the PCR product of the VD4-MOMP gene was approximately 350 bp. The PCR product size was demonstrated as comparable with a 1 Kb DNA Ladder marker (GIBCO-BRL, Gaintherburg, USA).

2.2.5 Restriction fragment length polymorphism (RFLP)

The RFLP analysis of the VD4-MOMP gene was performed with four restriction endonucleases (RE), *Alu*I, *Hind*III, *Dde*I and *Eco*RII (GIBCO-BRL, Gaintherburg, USA). Ten microlitres of VD4-MOMP amplified product was digested with 5 U of each RE in 20 µl of reaction volume and incubated at 37°C for 4 hours. The digested products were analyzed on 6% polyacrylamide gel electrophoresis. The DNA fragments were visualized under a long wavelength ultraviolet light using the ultraviolet transilluminator, and measured for the fragment size as a reference to the 50 bp DNA Ladder marker (GIBCO-BRL, Gaintherburg, USA). The genotypes were identified by their comparison with the RFLP pattern table (Table 1.).

2.2.6 MOMP gene cloning

In order to keep the *C. trachomatis* MOMP DNA for repeated analysis or further investigations, the PCR product of the MOMP gene from each isolate was cloned as follows.

2.2.6.1. Purification of the PCR product

The amplified product of the MOMP gene was purified by using the Agarose Gel DNA Extraction Kit (Boehringer Mannheim, Germany). The procedure of purification was performed, as recommended in the manufacturer's instruction manual. Briefly, the amplified DNA fragment was separated on 0.8% agarose gel (Ultrapure BRL, USA), then the DNA band was cut by using a sterile blade under ultraviolet light. The gel slice was warm in the gel solubilization buffer containing the silica matrix at 60 ^{O}C for 10 minutes. The reaction tube was centrifuged at 14,000 rpm

for 30 seconds and the supernatant was discarded . The matrix containing DNA was resuspended in 500 μl of nucleic acid binding buffer and spun down, and the supernatant was then removed, as before. The pellet was washed twice in washing buffer and dried at room temparature. Thirty microlitres of distillled water was added to elute the DNA.

2.2.6.2 Ligation of purified MOMP DNA

The purified MOMP DNA was cloned by using the pGEM® -T Easy Vector (Promega,USA.). The ligation of plasmid and MOMP DNA was performed according to the protocol recommended by the manufacture. In brief, 50-100 ng of the purified DNA from 2.2.6.1 was added to the ligation reaction that contained the ligation buffer, 50 ng of the pGEM ®-T Easy Vector and 1 Weiss units of T4 DNA ligase. The reaction was incubated at 4°C overnight. After ligation, the recombinant plasmid was transformed into *E. coli* .

2.2.6.3 Transformation of recombinant plasmid DNA

The recombinant plasmid was transformed into *E.coli* competent cells (DH5-α strain). The transformation was carried out by adding 5 μl of recombinant plasmids into 200 ul of competent cells in a screw cap glass tube and put on ice for 60 minutes. The cells were heat shocked for 2 minutes in a water bath at exactly 42 ^{O}C. Then the tubes were returned immediately to the ice bath for 5 minutes. One point five millitres of LB broth were added into the reaction tube and incubated for 1.5 hours at 37 ^{O}C, while slowly shaking (~150 rpm). One hundred microlitres of transformation culture were plated onto amplicillin plates. The plates were incubated overnight at 37 ^{O}C. The colonies of bacteria were picked up and examined for recombinant plasmid by using a PCR assay.

2.2.6.4 Plasmid purification

The transformed bacteria were cultured in LB broth at 37^0C for 24 hours. The plasmid was purified by using the High Pure Plasmid Isolation Kit (Boehringer Mannheim) according to the procedure recommended by the manufacture's instruction. Briefly, 1.5 ml of transformed bacteria were spun down. Then the pellet was resuspended and mixed in 250 µl each of suspension buffer and lysis buffer, and incubated for 5 minutes at room temparature. After that, 350 µl of binding buffer was added, mixed and centrifuged. The supernatant was collected and filtered through the High Pure filter tube, and the protein and other cellular contents were washed out by passing the washing buffer through the same filter tube. Then the plasmids were eluted by 60 µl of warm distilled water (60^0C).

2.2.7 Cycle sequencing technique

The nucleotide sequence of the *C.trachomatis* MOMP gene was determined by using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer, Applied Biosystem, USA). The kit combined all essential reagents, the dye terminators, deoxynucleotide triphosphates, AmpliTag DNA Polymerase, FS , thermally stable pyrophosphatase, magnesium chloride and buffer, into a single tube called a Ready Reaction Premix.

2.2.7.1 Quantitation of the purified plasmid DNA

The plasmid DNA concentration was determined by using the spectrophotometry. The DNA was diluted in distilled water at a dilution of 1:50 and the optical density (OD) measured for nucleic acid and protein at wavelength 260 and 280 nm using the UV spectrophotometer (Shimadz model 1101, Japan). The purity of the DNA sample was determined by the OD 260/280 ratio. Only a ratio of the above

1.8 was used for the sequencing experiment. The concentration of DNA was calculated by using the following equation:

Quantity of DNA (ng/μl) = OD at 260 nm x dilution factor x 50

2.2.7.2 Dye terminator cycle sequencing

Approximately 500 ng of purified template was mixed with 8.0 μl of dye terminator premix containing A-dye T, C-dye T, G-dye T, T-dye T, dNTPs, Tris-HCL pH 9, $MgCl_2$, AmpliTag DNA Polymerase, FS with thermally stable pyrophosphatase. The sequencing primer (FLS or FLA) was added in a concentration of 3.2 pmol and adjusted to the total volume of 20 μl with sterile distilled water. The mixture was mixed and placed in the GeneAmp 2,400 thermocycler (Perkin-Elmer, Cetus, USA). The reaction ran for 25 cycles. Each cycle was performed in 3 steps: denaturation at 96 ^{O}C for 10 seconds, primer annealing at 50 ^{O}C for 5 seconds and extension at 60 ^{O}C for 4 minutes.

2.2.7.3 Purification of sequencing products

The unincorporated nucleotide was removed from the reaction mixture by using ethanol precipitation. Two microliters of 3M sodium acetate pH 4.6 and 50 μl of 95% ethanol were added and placed on ice for 10 minutes, then centrifuged at 10,000 rpm at 4 ^{O}C for 30 minutes. The supernatant was discarded and the pellet was washed once with 70% ethanol. After the ethanol was removed, the pellet was dried at 37 ^{O}C for 5 minutes and kept at -20 ^{O}C until sequence analysis.

2.2.7.4 ABI 310 automated sequencer analysis

The pellet of purified sequencing product was resuspended in 25 μl of Template Suppression Reagent: TSR (Perkin-Elmer, Applied Biosystem,USA). The

tube was mixed and centrifuged briefly before heating at 95 ^{O}C for 2 minutes and then placed on ice.

The fluorescence emitted from the dye-labelled DNA fragments was detected by passing it through the capillary electrophoresis using POP6 sequencing gel (Perkin-Elmer, Applied Biosystem, USA). The capillary electrophoresis was performed at 12.2 kV, at 50 ^{O}C for 120 minutes. The withdrawn fluorescence was collected and transferred into digital signals, then analyzed automatically by the computer programs, ABI 310 data collection version 3.10 and ABI 310 DNA sequencing version 2.2 (Perkin-Elmer, Applied Biosystem, USA). The nucleotide sequencing data was shown as an electrophoregram. Finally, the multiple nucleotide sequence alignments between the samples and reference serotypes were obtained by using the computer program, Autoassembler ™ 1.4.0 (Perkin-Elmer Applied Biosystem USA).