III. RESEARCH DESIGN, MATERIAL AND METHODS

1. Research design

The positive *C. trachomatis* samples collected from the STD high risk women attending the Venereal Disease and AIDS Control Center, region 10, Chiang Mai were investigated for the genotypes and nucleotide sequence polymorphism of the VD4-MOMP gene. DNA was extracted by phenol-chloroform extraction and used as a template for PCR. The VD4-MOMP gene was the target for the amplification. For genotyping, the PCR products were analyzed by using the restriction fragment length polymorphism (RFLP) with 4 restriction endonucleases (RE); *Alu*I, *Hind*III, *Dde*I and *EcoR*II digestion. The RFLP pattern of 4 RE, were visualized on 2% agarose gel electrophoresis and compared with the RFLP pattern of reference serotypes in Table 2. In order to ensure that the genotyping obtained from RFLP was correct and to characterize the nucleotide sequence polymorphism of the VD4-MOMP gene, the PCR products were purified and subject to the cycle sequencing using the ABI PRISM™ dye terminator cycle sequencing ready reaction kit (Perkin-Elmer, Applied Biosystems, USA). The sequencing products were purified prior to being injected into the ABI 310 automated DNA sequencer (Perkin-Elmer, Applied Biosystems, USA) and analyzed by the computer programmes; ABI 310 data collection version 3.10 and ABI 310 DNA sequencing version 2.2. Finally, the VD4-MOMP gene sequencing data from each sample was compared with the nucleotide sequence of the reference strain, obtained from the GenBank database by using the computer programme DNASIS version 2.1. The diagram below shows the steps of the whole processes in ordering.

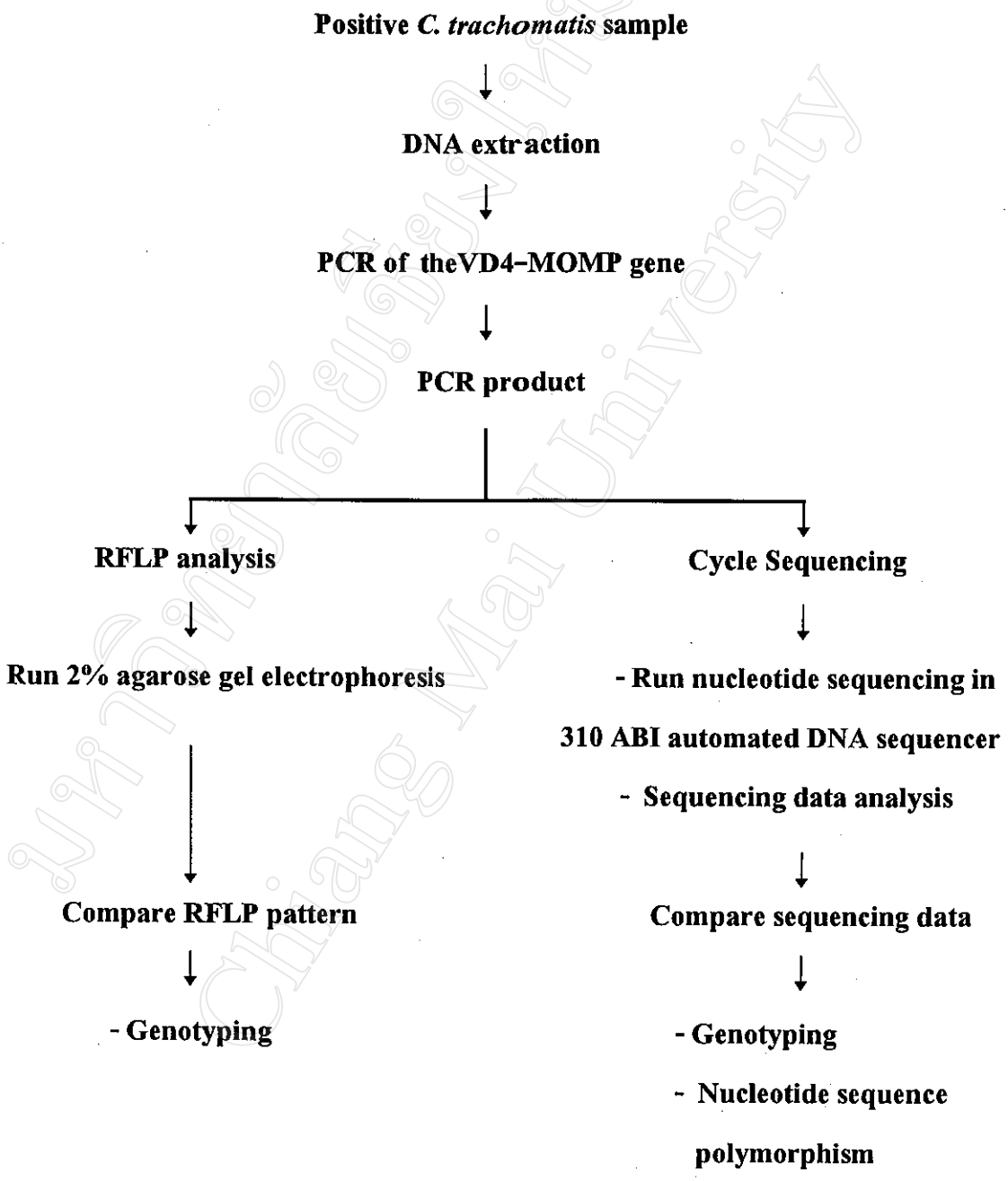

Figure 2. The schematic diagram presenting the research design in this study.

2. Materials and methods

2.1 Clinical samples

During May to July 1996, 377 local commercial female sex workers were enrolled for the screening of *C. trachomatis* infection by the Venereal Disease and AIDS Control Center, Region 10, Chiang Mai. All of them were asymptomatic at the time of examination. Endocervical swabs were collected and tested for the presence of *C. trachomatis* DNA by using the nucleic acid hybridization test (Gen-Probe PACE 2 system, USA). After testing, 52 samples indicated infection of *C. trachomatis*. Forty of these positive samples were have chosen randomly for this study. The other 10 were *C. trachomatis* isolates obtained from the Research Institute for Health Sciences, Chiang Mai University. They were isolated from endocervical swabs collected from those commercial female sex workers attending the Venereal Disease and AIDS Control Center, Region 10, Chiang Mai during January to April 1995.

2.2 Laboratory studies

2.2.1 DNA extraction

The DNA was extracted and purified by using the phenol/chloroform method. The sample was thawed and 200 µl of DNA lysate 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 0.5 volume of 7.5 M sodium acetate and 2.0 volume of absolute ethanol. The mixture was stored at -70°C for 30 minutes, centrifuged at 10,000 rpm for 30 minutes, and then discarded with the supernatant. The pellet was rinsed with 70% ethanol and centrifuged at 10,000 rpm for 10 minutes. The air-dried pellet was resuspended in 25 µl of sterile distilled water.

2.2.2 Primers

The oligonucleotide primers were synthesized as shown in the sequences at the chlamydial workshop (England, 1992). The primers derived from the conserved sequence within the MOMP region, which could amplify all *C. trachomatis* serotypes. Part of theVD4-MOMP gene was amplified using primer Nest 2 (N2) and Nest 4 (N4), which generated the approximate 350 bp amplified product. The nucleotide sequence of the primers and the location of those in the MOMP gene are shown in Figure 3.

Primer Nest 2 (N2) (forward 21-mer)

5' **CATGAGTGGCAAGCAAGTTTA** 3'

Primer Nest 4 (N4) (backward 20-mer)

5' **GCTTGATCGATGAGAGAGCA** 3'

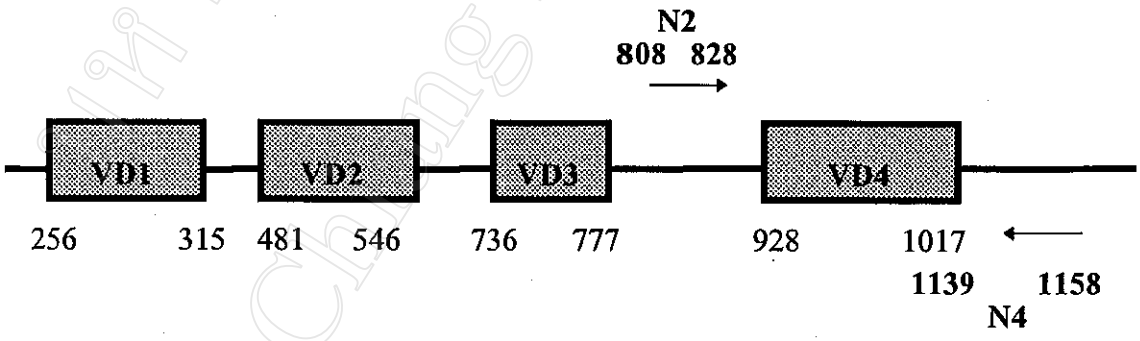

Figure 3. The sequences of oligonucleotide primers Nest 2 and Nest 4 and their position on the MOMP gene of *C. trachomatis*.

2.2.3 Polymerase chain reaction (PCR)

PCR was performed with a total volume of 50 μl. The final reaction mixture contained 0.2 mM of each deoxynucleoside triphosphate (dATP, dTTP, dGTP and dCTP); PCR buffer (50 mM KCl, 1.5 mM $MgCl_2$, 10mM Tris-HCl, pH 8.8); 50 pmol of each primer Nest 2 and Nest 4; and 1.25 U of *Tag* DNA polymerase (Perkin-Elmer, Cetus, USA). The initial PCR reaction contained 5 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 performed by using a thermocycler (GeneAmp 2400, Perkin-Elmer, Cetus, USA) that started with a 5 minute denaturation step at 95°C followed by 40 cycles of amplification. Each cycle consisted 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 to ensure complete extension.

2.2.4 Detection of the PCR product

Ten μl of the amplified product was analysed on 1% agarose gel electrophoresis in a TAE buffer at 100 volts for 45 minutes. It was then stained with ethidium bromide for 15 minutes, destained with distilled water for 15 minutes and visualized under a long wavelength ultraviolet light using the ultraviolet transilluminator. The approximate 350 bp DNA banding 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 amplified DNA was performed with 4 restriction endonucleases (RE), *Alu*I, *Hind*III, *Dde*I and *Eco*RII (GIBCO-BRL, Gaintherburg, USA). Ten μl of amplified PCR product was digested with 10 U of each RE in 20 μl of reaction volume and incubated at 37°C for 2 hours. The digested products were analyzed on 2% agarose 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 1 Kb DNA Ladder marker (GIBCO-BRL, Gainthersburg, USA). The serotypes were identified by comparison to the RFLP pattern table (Table 2.) constructed from the nucleotide sequences of the 18 reference serotypes.

2.2.6 Cycle sequencing technique

The nucleotide sequence of the *C. trachomatis* VD4-MOMP gene was determined by using the ABI PRISM dye terminator cycle sequencing ready reaction kit (Perkin-Elmer, Applied Biosystems, USA). This ready reaction kit combined the unique properties of AmpliTag DNA polymerase, FS enzyme, for dye terminator sequencing with the convenience of the ready reaction format. In this kit, the same formulation of reagents was ready for use and included in a single tube. The reagents were suitable for performing fluorescence-based cycle sequencing reactions on PCR products. In addition, this reaction was protected from exposure to light in order to prevent the excitation of the fluorescent dye tagged on the products. The purification and quantitation of the PCR product for the DNA template in the cycle sequencing reaction are recommended by the manufacture (Perkin-Elmer, Applied Biosystems, USA)(43).

2.2.6.1 Purification of PCR products

The amplified DNA fragments were separated on a 0.6% agarose gel (Ultrapure BRL,USA) and stained with ethedium bromide. The DNA band was cut out by using a steriled blade under ultraviolet light, and then extracted and purified by means of the Glass MAX® Spin Cartridge System (Gibco-BRL, Canada), as recommended by the manufacture (Perkin-Elmer, Applied Biosystems, USA). In brief, the gel slice was heated in the binding solution (6 M NaI) at 50°C for 7 minutes until it was fully dissolved. The solution was applied to a Glass MAX® spin cartridge and passed though a silica-based membrane by centrifugation at 10,000 rpm for 1 minute at room temperature. DNA was bound in the presence of NaI to a silica-based membrane. Residual impurities and NaI were then removed from DNA by passing a 400 μl of wash buffer through the membrane. The purified DNA was eluted in 40 μl of sterile distilled water.

2.2.6.2 Quantitation of the purified PCR products.

Approximately 100-180 ng of purified DNA were used for the cycle sequencing reaction as recommended by the manufacture (Perkin-Elmer, Applied Biosystem, USA). The concentration of DNA was determined by the optical density (OD) at 260 nm using the spectrophotometer (Shimadzu model 1101, Japan). The DNA was diluted with distilled water at a dilution ratio of 1:50 and measured at 260 nm and 280 nm. The purity of DNA samples was determined by the OD260/OD280 ratio. Only a ratio of above 1.8 was used for the sequencing experiment. The concentration of DNA was calculated by using the following equation:

$$\text{Quantity of DNA (ng/μl)} = \text{OD at 260 nm} \times \text{dilution factor} \times 50\ \text{ng/μl}$$

2.2.6.3 Dye terminator cycle sequencing

The nucleotide sequence target was amplified using the PCR technique with *Tag* DNA polymerase. The PCR product was purified and measured, then used as the template for sequence analysis using an ABI PRISM™ dye terminator cycle sequencing ready reaction kit (Perkin-Elmer, Applied Biosystems, USA). Approximately 100-180 ng of purified template was mixed with 8.0 μl of terminator premix containing A-dye T, C-dye T, G-dye T, T-dye T, dNTPs, Tris-HCl (pH9.0), $MgCl_2$, thermal stable pyrophosphatase and AmpliTag DNA polymerase, FS. The sequencing primer (Nest 2 or Nest 4) was added in a concentration of 3.2 pmol and adjusted to the total volume of 20 μl by using sterile distilled water. The mixture was mixed and placed in a programmable heat block of the GeneAmp 2400 thermocycler (Perkin-Elmer, Cetus, USA). The reaction ran for 25 cycles. Each cycle was performed in 3 steps, denaturation at 96°C for 10 seconds, primer annealing at 50°C for 5 seconds and extension at 60°C for 4 minutes.

2.2.6.4 Purification of sequencing products

In the sequencing product purification, the unincorporated nucleotide was removed from the reaction mixture by using the 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°C for 30 minutes. The supernatant was then removed and air dried for 30 minutes at room temperature. The pellet was kept in store at -20°C.

2.2.6.5 ABI 310 automated sequencer

The pellet of purified sequencing product was resuspended in 25 μl of Template Suppression Reagent: TSR (Perkin-Elmer, Applied Biosystems, USA). The tube was mixed and centrifuged briefly before heating at 95°C for 2 minutes and placed on ice. It was then put into the ABI 310 automated DNA sequencer (Perkin-Elmer, Applied Biosystems, USA).

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 Biosystems, USA). The capillary electrophoresis was performed at 12.2 KVs, at 50°C for 120 minutes. The withdrawn fluorescence was collected and transferred into digital signals, then analyzed automatically by the computer programmes ABI 310 data collection version 3.10 and ABI 310 DNA sequencing version 2.2 (Perkin-Elmer, Applied Biosystems, USA). The data showed the nucleotide sequences with the electrophoregram. Finally, the multiple nucleotide sequence alignments between Chiang Mai's isolates and reference serotypes were obtained by using the computer programme DNASIS version 2.1.