CHAPTER III

MATERIALS AND METHODS

1. Materials

1.1 Population study

Blood samples were collected from infected subjects. Twenty individuals, infected with *W. bancrofti*, were collected from endemic areas in Tak province. Ten were Thai-Karens from Umphang district and the other ten were Myanmar workers from Maesot district. The filarial species from blood collected from endemic areas in Narathiwat province were identified by the staff of the Filariasis Control Center as *B. malayi*. The subjects in the group included 10 infected humans and 2 infected cats. A blood sample of *B. pahangi* was collected from an experimental cat of Parasitology Unit, Department of Pathology, Faculty of Veterinary, Chulalongkorn University. Blood samples of 10 stray dogs with *D. immitis* infection were also obtained.

1.2 Collecting specimen

Two to ten milliliters of venous blood were collected between 19.00 and 22.00 in Vacutainer^{TM} tubes containing ethylene diaminetetraacetic acid (EDTA). Individual blood samples were devided in 2 aliquots: one for microscopic examination and the other for DNA extraction. Blood samples were stored at -80°C until used.

2. Methods

2.1 Giemsa stain

A thick or thin blood film was made on a glass slide and allowed to dry overnight at room temperature. Red blood cells were lysed in water for 2-3 minutes and air-dried. The blood slide was fixed with absolute methanol for 1 minute. A dried slide was stained

with 3% Giemsa (Merck) in phosphate buffer pH 7.2 for 45 minutes, then rinsed with tap water. A blood film was examined under a light microscope.

2.2 Histochemical stain for acid phosphatase activity of microfilariae (Chalifoux & Hunt, 1971)

A thick or thin blood film was made on a glass slide and allowed to dry overnight at room temperature. Red blood cells were lysed in water for 2-3 minutes and air-dried. The blood slide was fixed with acetone at 4°C for 1 minute. Dried slide was stained for microfilarial acid phosphatase activity at 37°C for 1 hour, then rinsed with distilled water. A blood film was examined under a light microscope.

2.3 Extraction of filarial DNA from blood sample

The extraction method was modified from Steven A William's protocol (William, 1996). Two hundred and fifty microliters of blood sample were added to 1.5 mL microfuge tube and mixed with TE buffer pH 8.0 (10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0) 750 μL. The mixture was centrifuged at 12,000 rpm for 2 minutes and the supernatant was discarded. The pellet was washed with 750 μL of TE buffer at pH 8.0 and broken the pellet by vortex before centrifuged. The pellet was resuspended in 500 μL of red cell lysis buffer (RCLB; 1 M Sucrose, 10 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, 1% Triton X-100) to digest the red blood cells. The mixture was then incubated for 10 minutes at room temperature, then centrifuged and the RCLB step was repeated. After discarded the supernatant, 400 μL of warmed digestion buffer or DSP buffer (20 mM Tris-HCl pH 8.0, 50 mM KCl, 2.5 mM $MgCl_2$, 0.5% Tween 20, 150 μg/mL Proteinase K) were added to digest the pellet at 65°C for 3 hours to overnight. The enzyme activity was inactivated by incubation at 90°C for 10 minutes.

2.4 DNA purification

The digestion mixture was sequentially extracted with equal volume of Phenol: Chloroform: Isoamyl alcohol (25:24:1), mixed and centrifuged at 12,000 rpm for 5

minutes. The aqueous phase (upper phase) was transferred to a fresh tube, and sequentially extracted by 1 volume of chloroform. After centrifugation, the DNA was precipitated with 2.5 volume of ice-cold absolute ethanol, 0.1 volume of 3 M sodium acetate pH 5.2 and 2 μL of 20 mg/ml glycogen (USB, Japan) at -20°C overnight. The precipitate was centrifuged at 13,000 rpm 4°C for 30 minutes. The pellet was washed with ice-cold 70% ethanol and centrifuged again. The pellet was allowed to dry and resuspended in 10 μL of TE buffer pH 7.5 (10 mM Tris-HCl pH 7.5, 1 mM EDTA pH 8.0).

2.5 Oligonucleotide primers

Oligonucleotide primers were designed, based on reports of conserved sequences of 18S, 5.8S and 28S rRNA of filarial worm. Nevertheless, not all rRNA sequences of selected filarial worms were reported. The database of filarial rRNA sequences available online were as follows: 18S rRNA for *B. malayi* (AF036588), *W. bancrofti* (AF227234), and *D. immitis* (AF217800); ITS1-5.8S-ITS2 and a small part of 28S rRNA for only *D. immitis*; no completed 28S rRNA was reported for these filarial worms. FL1-F primer was designed from conserved region at the 3' end of filarial 18S rRNA. FL2-R primer was selected from the beginning 5' of *D. immitis* 28S rRNA, which was also conserved among reported 28S rRNA of other nematodes. Semi-nested PCR was performed to obtain higher PCR sensitivity. Two internal primers, Di5.8S 660-R and Di5.8S 558-F, were designed from *D. immitis* 5.8S rRNA region (AF217800) (figure 5).

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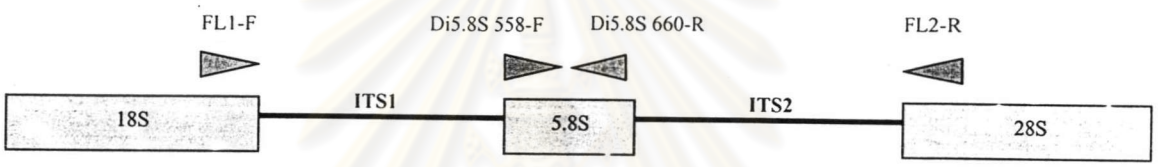

Figure 5 Schematic representation of the rDNA unit showing regions where forward and reverse primers were designed for use in PCR.

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2.6 Semi-nested PCR amplification

First round amplification:

The entire ITS1 and ITS2 regions were amplified by PCR using conserved oligonucleotide primers, FL1-F and FL2-R. The PCR reaction was performed in a 25 μL reaction containing 1x PCR buffer (10 mM Tris-HCl pH 9.0, 1.5 mM $MgCl_2$, 50 mM KCl) (Amersham Phamacia); 200 μM each of dATP, dCTP, dGTP and dTTP (Promega); 0.625 Units *Taq* DNA polymerase (Amersham Phamacia); 5 pmol of each primers FL1-F (5'- TTCCGTAGGTGAACCTGC-3') and FL2-R (5'- ATATGCTTAAATTCAGCGGG-3'); 1 μL DNA template. After incubation at 94°C for 5 minutes, amplification was carried out for 5 cycles with the following temperature cycling parameters: 94°C for 30 seconds of denaturation; 58°C for 30 seconds of annealing; and 72°C for 90 seconds of extension; and followed by 30 cycles of temperature cycling parmeters at 94°C for 3 seconds of denaturation; 55°C for 30 seconds of an annealing and 72°C for 90 sec of extension. The final amplification cycle at 72°C for 10 minutes was included.

Second round amplification:

For ITS1 region:

ITS1 region was amplified with 1 μL of PCR product from the first PCR step as DNA template. The PCR reaction was performed in a 50 μL reaction containing 1x PCR buffer (10 mM Tris-HCl pH 9.0, 1.5 mM $MgCl_2$, 50 mM KCl) (Amersham Phamacia); 200 μM each of dATP, dCTP, dGTP and dTTP (Promega); 0.625 Units *Taq* DNA polymerase (Amersham Phamacia); 10 pmol of each primers FL1-F (5'-TTCCGTAGGTGAACCTGC-3") and Di5.8S 660-R (5'- ACCCTCAACCAGACGTAC-3'). After incubation at 94°C for 5 minutes, amplification was carried out for 5 cycles with the following temperature cycling parameters: 94°C for 30 seconds of denaturation; 58°C for 30 seconds of annealing; and 72°C for 45 seconds of extension, and followed by 30 cycles of temperature cycling parameters: 94°C for 30 seconds of denaturation, 55°C

for 30 seconds of an annealing; and 72°C for 45 seconds of extension. The final amplification cycle at 72°C for 10 minutes was included.

For ITS2 region:

ITS2 region was amplified with 1 μL of PCR product from the first PCR step as DNA template. The PCR reaction was performed in a 50 μL reaction containing 1x PCR buffer (10 mM Tris-HCl pH 9.0, 1.5 mM $MgCl_2$, 50 mM KCl) (Amersham Phamacia); 200 μM each of dATP, dCTP, dGTP and dTTP (Promega); 0.625 Units *Taq* DNA polymerase (Amersham Phamacia); 10 pmol of each primers Di5.8S 558-F (5'-TAGCTGCGATAAATAGTGC-3') and FL2-R (5'- ATATGCTTAAATTCAGCGGG-3'). After incubation at 94°C for 5 minutes, amplification was carried out for 35 cycles with the following temperature cycling parameters: 94°C for 30 seconds of denaturation; 55°C for 30 seconds of annealing; and 72°C for 45 seconds of extension. The final amplification cycle at 72°C for 10 minutes was included.

2.7 PCR product precipitation

The PCR product was precipitated with 2.5 volume of ice-cold absolute ethanol, 0.1 volume of 3M sodium acetate pH 5.2 and 2 μL of 20 mg/ml glycogen (USB, Japan) at -20°C, overnight. The precipitate was centrifuged at 13,000 rpm 4°C for 30 minutes. The pellet was washed with ice-cold 70% ethanol and centrifuged again. The pellet was allowed to dry and resuspended in 10 μL of sterile distilled water.

2.8 Restriction Fragment Length Polymorphism (RFLP)

After precipitation as stated above, the PCR product (1-3 μL) was digested with 5 units (0.5 μL) of each restriction endonuclease according to the manufacturer protocols (New England Biolabs), 1x reaction buffer (provided) and sterile distilled water added to a final volume of 15 μL. The digestion was incubated at 37°C for 3 hours to overnight. The following enzymes were evaluated: *Ase* I, *Acc* I, *Hinf* I and *Rsa* I. These

endonucleases were selected based on *D. immitis* sequence (AF217800) after performed restriction mapping analysis using online software (http://www.webcutter.com)

2.9 Agarose Gel Electrophoresis

The analysis of DNA fragments was performed by submarine agarose gel electrophoresis. Two to 2.5% agarose (USB, Spain) were prepared by dissolving the gel powder in 1x TAE (40 mM Tris-acetate, 1 mM EDTA pH 8.0). The mixture was heated until it was completely dissolved. Agarose gel was allowed to cool down to around 50°C before being poured into the mold. Ten microliters of PCR product was mixed with 1 μL 6x loading dye (0.25% bromphenol blue, 40% (w/v) sucrose in water) and the mixture was loaded into the slots of the gel. Electrophoresis was performed at 80 volts for 70-90 minutes, and then stained with 0.5 μg/ml ethidium bromide. The DNA bands were visualized under ultraviolet light at 302 nm.

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