CHAPTER III

MATERIALS AND METHODS

1. Study area

Pra-sang district is located in Surat-thani province, the southern part of Thailand. Two subdistricts in Pra-sang district were selected for this study. Tri-khung subdistrict consists of 13 villages, 200 houses, with the total population about 700 persons, and E-pun subdistrict consists of 12 villages, 200 houses, with the total population about 600 persons. In this area, cats are popular household pets. The agricultures in this endemic area are rubber plantations, palm oil plantations, as well as other agricultural crops. A permanent large fresh water swamp, with a various kinds of aquatic plants, grasses, and weeds, is located in Tri-khung subdistrict. This swamp is a suitable breeding site of *Mansonia* mosquitoes, a main vector of *B. malayi* (Filariasis Division, 2002).

2. Specimen collection

Five hundred to 1,000 μl venous blood of 52 domestic cats from 2 villages of Tri-khung and of E-pun subdistricts, Pra-sang district, Surat-thani province, were collected between 9.00 and 17.00. Infected blood samples from experimental cats from the Parasitology Unit, Department of Pathology, Faculty of Veterinary Science, Chulalongkorn University, were used as positive control. Non-infected cat blood samples were used as negative control. Individual blood sample was devided into 2 aliquots: one for microscopic examination and the other for molecular study.

3. Giemsa stain

Thick and thin blood films were made on glass slides and allowed to dry at room temperature. Red blood cells were lysed in water for 2-3 minutes and air-dried. The slides were 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, and then rinsed with tap water. A blood film was examined under a light microscope by 2 independent individuals.

4. Histochemical stain for acid phosphatase activity of microfilariae

Histochemical stain for acid phosphatase activity of microfilariae was performed as previously described (Chalifoux and Hunt, 1971). After lyses red blood cells on thick and thin blood films in water and air-dried. Blood slides were fixed with acetone at 4°C for 1 minute. The dried slides were stained for microfilarial acid phosphatase activity using napthol AS-TR phosphate and diazotized pararosaniline as substrate and coupling agent, respectively, then rinsed with distilled water. Each blood film was examined under a light microscope by 2 independent individuals.

5. Extraction of filarial DNA from blood samples

The extraction method was modified from previously described 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 incubated for 10 minutes at room temperature, and 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.

6. 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 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).

7. Semi-nested PCR amplification

7.1 First PCR for ITS1-5.8S-ITS2 region:

The entire ITS1-5.8S-ITS2 region was amplified by PCR using FL1-F (5'-TTCCGTAGGTGAACCTGC-3') and FL2-R (5'-ATATGCTTAAATTCAGCGGG-3') oligonucleotide primers. The hot-start PCR reaction was performed in a 50 μl reaction containing PCR buffer (10 mM Tris-HCl, pH 9.0, 1.5 mM $MgCl_2$, 50 mM KCl) (Amersham Phamacia, Freiburg, Germany); 200 μM each of dATP, dCTP, dGTP and dTTP (Promega, Wisconsin, USA); 0.625 Units *Taq* DNA polymerase (Amersham Phamacia); 5 pmol of each primer (FL1-F and FL2-R); and 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; followed by 30 cycles of temperature cycling parmeters at 94°C for 30 seconds of denaturation; 55°C for 30 seconds of annealing, and 72°C for 90 seconds of extension. The final amplification cycle included an addition of 10 minutes extension at 72°C.

7.2 Second PCR for ITS1 region:

The ITS1 region was amplified by using 1 μl of the first PCR product as a DNA template. The PCR reaction was performed in a 50 μl reaction containing PCR buffer, as described above, with 5 pmol of each primer of FL1-F and Di5.8S 660-R (5'-ACCCTCAACCAGACGTAC-3'). 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, followed by 30 cycles of 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 included an addition of 10 minutes extension at 72°C.

8. Single PCR amplification for ITS1 with ITS1-F and ITS1-R primers

The ITS1 region was amplified by using 1 μl DNA template. The PCR reaction was performed in a 50 μl reaction containing PCR buffer, as described above, with 5 pmol of each primer of ITS1-F and ITS1-R. After incubation at 94°C for 5 minutes, amplification was carried out for 35 cycles with the following temperature cycling parameters: 94°C for 1 minute of denaturation; 58°C for 1 minute of annealing; and 72°C for 1 minute of extension. The final amplification cycle included an addition of 10 minutes extension at 72°C.

9. Restriction fragment length polymorphism (RFLP)

After precipitation, the PCR product was digested with 5 units of *Ase* I 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.

10. Agarose gel electrophoresis

The analysis of DNA fragments was performed by submarine agarose gel electrophoresis (Sambrook *et al.*, 1989). 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.

11. DNA purification from gel slice

PCR product of each filarial nematode species were separated on an ethidium bromide-stained 1% TAE agarose gel. A band was excised from agarose gel with a clean, sharp razor blade and purified using QIAquick® Gel Extraction Kit (Qiagen, Chatsworth, CA). Three volumes of Buffer QG to one volume of gel were added and the tube was placed in a 50°C water bath incubator. After agarose gel was completely dissolved, one gel volume of isopropanol was added, mixed and applied to the QIAquick column. After centrifugation at 10,000 g for 1 minute, the flow-through solution was discarded. The DNA fragments was washed with Buffer PE and centrifuged for 1 minute. Buffer EB (10 mM Tris·Cl, pH 8.5) or ddH_2O was added to elute DNA and was then centrifuged for 1 minute, stored at – 20°C.

12. Ligation of PCR products into plasmid vector

The ligation of gel purified ITS1 was carried out in a 10 μl reaction mixture containing pGem®-T (Promega, Madison, WI, USA) vector and ITS1 DNA in the molar ratio 1:3, 2 units of T_4 DNA ligase and 1X buffer (provided). The pGem®-T vector is approximate 3.0 kb and supplied at 50 ng/μl. The amount of the DNA insert was calculated from the following equation:

$$\frac{\text{ng of vector x size (kb) of insert}}{\text{size (kb) of vector}} \text{ X insert : vector molar ratio = ng of insert}$$

The ligation reaction was carried out at 16°C for 16-18 hours and the ligation products were used to transform *Escherichia coli* competent cells prepared by $CaCl_2$ method.

13. Preparation of *E. coli* competent cells by $CaCl_2$ method

A single colony of *E. coli* was inoculated into 3 ml of LB broth and incubated at 37°C with 200 rpm shaking for 16-20 hours. The overnight culture was diluted 1:100 into 100 ml of new LB broth and incubated at 37 °C until an OD_{600} of 0.4-0.5. The cell culture was chilled on ice for 10 minute prior to pipetting the 10 ml aliquots of the culture into 15 ml sterile polypropylene tubes. The cells was pelleted by centrifugation at 4,000 rpm for 10 minute at 4°C, the pellet was suspended in 5 ml of ice-cold 0.1 M $MgCl_2$. After centrifugation at 4,000 rpm for 10 minute at 4°C, the pellet was suspended in 5 ml of ice-cold 0.1 M $CaCl_2$ and left on ice for 30 minutes to

establish competency. After centrifugation at 4,000 rpm for 10 minutes at 4°C, the pellet was resuspended in 750 µl of 15% (v/v) glycerol and 0.1 M $CaCl_2$. The cells were kept in 200 µl aliquots at –80°C until required.

14. Transformation of *E. coli* competent cells

One hundred microlitters of JM109 *E. coli* competent cells were mixed with 2 µl of ligation products and immediately placed on ice for 30 minutes. The cells were subjected to heat-shock at 42°C for 45 seconds and placed on ice for an additional 3 minutes. The transformed cells were mixed with 900 µl of LB broth and incubated at 37°C for 1 hour with shaking at 200 rpm. Finally, 100 µl of the transformed culture was spread on an LB agar plate containing 50 µg/ml ampicillin, 40 µl of stock solution of X-gal (20 mg/ml in dimethylformamide) and 8 µl of a solution of isopropylthio-β-D-galactoside (IPTG) (100 mg/ml) and incubated at 37°C overnight. After transformation, the white colonies containing ITS1 DNA were selected and extracted by alkaline lysis method.

15. Plasmid DNA extraction by alkaline lysis method

A single white colony of bacteria was inoculated in 3 ml LB broth and incubated at 37°C with 200 rpm shaking for 16-20 hr. The cells were harvested in 1.5 ml microcentrifuge tubes by centrifugation at 5,000 rpm for 3 minutes and then resuspended in 100 µl of solution I (50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl, pH 8.0) with 20 µg/ml RNase A. To the cell suspension, 200 µl of freshly prepared solution II (1% SDS, 0.2 N NaOH) was added and incubated on ice for 5 minutes. Then, 150 µl of solution III (3 M potassium acetate, 11.5% glacial acetic acid) was

added to the mixture and incubated on ice for 5 minutes. The mixture was pelletted by centrifugation at 10,000 rpm for 10 minutes. The supernatant was moved to a new tube. The plasmid DNA was recovered from the supernatant by adding 7/10 volume of isopropanol and standing in room temperature for 10 minutes. The content was centrifuged at 12,000 rpm for 10 minutes. The pellet was washed with 500 µl of 70% ethanol, centrifuged at 12,000 rpm for 5 minutes and dried at room temperature. The DNA pellet was resuspended in 30 µl of TE buffer pH 7.5 (10 mM Tris-HCl, pH 7.5; 1 mM EDTA) or sterile water. Then the plasmid DNA were digested with *Eco*R I and separated on agarose gel again to check for successful cloning.

16. Digestion of restriction endonucleases and analysis

About 500 ng of plasmid DNA digested with 5 units of *Eco*R I according to manufacturer's protocols (New England Biolabs), 1X reaction buffer (provided), and sterile distilled water added to a final volume of 20 µl. The digestion was incubated at 37°C for 3 hours. After digestion, agarose gel electrophoresis was performed and analyzed to screen for plasmids, which contain ITS1 DNA.

17. DNA Sequencing

Positive colonies were sequenced using ABI Prism™ Dye Terminator Cycle Sequencing Ready Reaction kit with Amplitaq® DNA Polymerase FS Version 2 (Applied Biosystems, Foster city, CA). The PCR reaction was carried out in a 10 µl reaction containing 4 µl of terminator ready reaction mix, 200 nM of primer and 500 ng DNA template. After incubation at 95°C for 5 minutes, amplification was carried out for 25 cycles with the following temperature cycling parameters; 95°C for 10

seconds of denaturation, 50°C for 5 seconds of annealing and 60°C for 4 minutes of extension. The DNA was then precipitated by the addition of 1/10 volume of 3 M sodium acetate and 2 volume of absolute ethanol and incubated at -20°C overnight. After centrifugation at 12,000 rpm for 10 minutes, the pellet was washed with 500 µl of 70% ethanol, centrifuged at 12,000 rpm for 5 minutes and air dried. The DNA pellet was resuspended in 10 µl Template Suppression Reagent (Perkin) and sequencing was preceded using an automated sequencer (Applied Biosystems, Foster city, CA) according to manufacturer's protocol. To confirm the sequence at least 3 clones of each PCR product were sequenced in both directions using M13 forward and reverse primers.

18. Sequences analysis

Alignments of the ITS1 sequences were made using CLUSTAL X multiple alignment program (EMBL).