DIFFERENTIATION OF *BRUGIA MALAYI* AND *BRUGIA PAHANGI* BY PCR-RFLP OF ITS1 AND ITS2

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Abstract. Lymphatic filariasis has been targeted by the World Health Organization for elimination by the year 2020. Malayan filariasis, caused by *Brugia malayi*, is endemic in southern Thailand where domestic cats serve as a major reservoir host. However, in nature, domestic cats also carry *B. pahangi* infection. In addition to chemotherapy and vector control, control in reservoir hosts is necessary to achieve the elimination of the disease. Therefore, differentiation between *B. malayi* and *B. pahangi* in the cat reservoir will help the lymphatic control program to monitor and evaluate the real disease situation. It is difficult to differentiate these two *Brugia* species by microscopic examination. The technique is also time-consuming and requires expertise. We employed the polymerase chain reaction-linked restriction fragment length polymorphism (PCR-RFLP) technique of internal transcribed spacer regions, ITS1 and ITS2, of ribosomal DNA (rDNA) to differentiate *B. malayi* from *B. pahangi* species. Among the restriction enzymes tested, only the PCR product of ITS1 digested with *Ase* I could differentiate *B. malayi* from *B. pahangi*. This PCR-RFLP technique will be useful for lymphatic filariasis control programs for monitoring and evaluating animal reservoirs.

INTRODUCTION

Lymphatic filariasis, mainly caused by the filarial nematodes *Wuchereria bancrofti* and *Brugia malayi*, is a debilitating and disfiguring disease. It is estimated that 1.1 billion people, 20% of the world's population, in more than 80 countries, are at risk of acquiring the infection, while over 120 million have already been infected (WHO, 2000). Although Bancroftian filariasis has been controlled to a low level, 0.99 cases/100,000 population (Filariasis Division, 1999), Malayan filariasis is still endemic in southern Thailand. Lymphatic filariasis, caused by *B. malayi*, nocturnal subperiodic type, is prevalent mainly in Narathiwat Province. This is due to the many suitable mosquito breeding sites, large swamp areas, and the existence of animal reservoir-hosts. In Thailand, *Mansonia* sp are the main mosquito vectors of *B. malayi* (Filariasis Division, 1998). Domestic cats are important reservoir hosts for *B. malayi* (Palmieri *et al*, 1985; Phantana *et al*, 1987; Kanjanopas *et al*, 2001; Chansiri *et al*, 2002). Therefore, besides chemotherapy and vector control, the successful elimination of lymphatic filariasis should include the control of reservoir hosts. Not only *B. malayi* can infect domestic cats, but also another filarial species, such as *B. pahangi* (Nithiuthai and Chungpivat, 1992; Chungpivat and Sucharit, 1993). Co-infections of both *Brugia* species in domestic cats is not uncommon in Thailand. This raises the possibility of misdiagnosis due to the difficulty in differentiating both species by the conventional microscopic method. Misdiagnosis would jeopardize the lymphatic filariasis control program.

In endemic areas, the routine method for identifying microfilaria species is microscopic examination, based on the delineation of particular morphological features using Giemsa stain, and geographic location, that is, where the specimens have come from. Nevertheless, using this technique, it is difficult to discriminate clearly between closely related species such as *B. malayi* and *B. pahangi*. Histochemical staining, to detect acid phosphatase activity, could overcome the problem (Yen and Mak, 1978; Chungpivat *et al*, 1990; Nithiuthai and Chungpivat, 1992; Chungpivat and Sucharit, 1993). However, this technique needs fresh samples to yield the best results. Furthermore, both staining methods require expertise to identify and confirm the species. Molecular analysis has been introduced as a new tool to distinguish parasite species. Ribosomal DNA (rDNA) has been a valuable tool to discriminate closely related species among several eukaryotic organisms, including nematodes. The application of internal transcribed spacers (ITS) to identify the organism has received the most attention by nematologists during the past decade (Gasser *et al*, 1994; 1996; Zhu *et al*,

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1998; Almeyda-Artigas *et al*, 2000; Conole *et al*, 2001). Studies of PCR-linked restriction fragment length polymorphism (PCR-RFLP) profiles of the nematodes' ITS regions have provided data on nematode diversity, as well as the critical taxonomic character useful for species comparison and identification (Gasser *et al*, 1994, 1996). We report here the results of PCR-RFLP of ITS1 and ITS2 to differentiate *B. malayi* from *B. pahangi*.

MATERIALS AND METHODS

Specimen collection

In co-operation with the Filariasis Division, Department of Communicable Diseases Control, Ministry of Public Health, Thailand, collection of human blood specimens was performed as previously described (Triteeraprapab and Songtrus, 1999; Triteeraprapab *et al*, 2000; 2001; Nuchprayoon *et al*, 2001). Two milliliters of venous blood from domestic cats infected with *B. malayi* or *B. pahangi* were obtained from experimental cats at the Parasitology Unit, Department of Pathology, Faculty of Veterinary Science, Chulalongkorn University. *Dirofilaria immitis* microfilariae were obtained from infected stray dogs' blood. Blood from healthy volunteers, non-infected domestic cats and dogs were used as the negative control. This study was approved by the Ethics Committee of the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand.

All filarial parasites were identified and the species confirmed by Giemsa staining, and special staining for acid phosphatase activity (Yen and Mak, 1978; Chungpivat *et al*, 1990; Nithiuthai and Chungpivat, 1992; Chungpivat and Sucharit, 1993).

DNA extraction

The extraction method was modified from a previously reported protocol (William *et al*, 1996). Two hundred and fifty microliters of each blood sample were added to a 1.5 ml microfuge tube and mixed with 750 µl of TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0). The mixture was centrifuged and the supernatant was discarded. The pellet was washed with 750 µl of TE buffer, pH 8.0 and 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) twice. After centrifugation, the supernatant was discarded, 400 µl of 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 and incubated at 65˚C for 3 hours. Enzyme activity was inactivated by incubation at 90˚C for 10 minutes.

Primers design

The forward primer for the ITS1 (FL1-F) was designed from the conserved sequence of filarial parasites reported in the Genbank database (Fig 1). The reverse primer for the ITS1 (Di5.8S 660-R) and the forward primer for the ITS2 (Di5.8S 558-F) were designed from the reported 5.8S rDNA sequence of *D. immitis* (AF217800). The reverse primer for ITS2 (FL2-R) was designed from the 28S rDNA sequence of *D. immitis* (AF217800). All oligonucleotide primers were purchased from the Bioservice Unit, NSTDA, Bangkok.

Polymerase chain reactions

In order to obtain sufficient DNA for RFLP study, semi-nested PCR for ITS1 and ITS2 were performed.

First PCR ITS1-5.8S-ITS2 region. The entire ITS1-5.8S-ITS2 region (Fig 1) was amplified by PCR using FL1-F and FL2-R oligonucleotide primers. The hot-start PCR reaction was performed in a 25 µl reaction containing PCR buffer (10 mM Tris-HCl, pH 9.0, 1.5 mM $MgCl_2$, 50 mM KCl) (Amersham Pharmacia, Freiburg, Germany); 200 µM each of dATP, dCTP, dGTP and dTTP (Promega, Wisconsin, USA); 0.625 units *Taq* DNA polymerase (Amersham Pharmacia); 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 parameters 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 additional 10 minutes' extension at 72˚C.

Second PCR for ITS1 region. The ITS1 region was amplified by using 1 µl of PCR product from the first PCR 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. 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 additional 10 minutes extension at 72˚C.

Second PCR for ITS2 region. The ITS2 region

was amplified by using 1 µl of PCR product from the first PCR as a DNA template. The PCR reaction was performed in a 50 µl reaction containing PCR buffer with 5 pmol of each primer, Di5.8S 558-F and FL2-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 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 additional 10 minutes' extension at 72˚C.

Restriction fragment length polymorphism (RFLP)

After precipitation of each PCR product and resuspension in 10 µl sterile distilled water, 1 µl of PCR product was digested with 5 units of each restriction endonuclease, according to the manufacturer's protocols (New England Biolabs, Massachusetts, USA). The digestion was incubated at 37˚C for 3 hours. The following enzymes were evaluated; *Ase* I, *Acc* I, *Hinf* I and *Rsa* I. Analysis of DNA fragments was performed by 2-2.5% submarine agarose gel electrophoresis.

RESULTS

Oligonucleotide primers were designed based on the reported conserved sequences of 18S, 5.8S and 28S

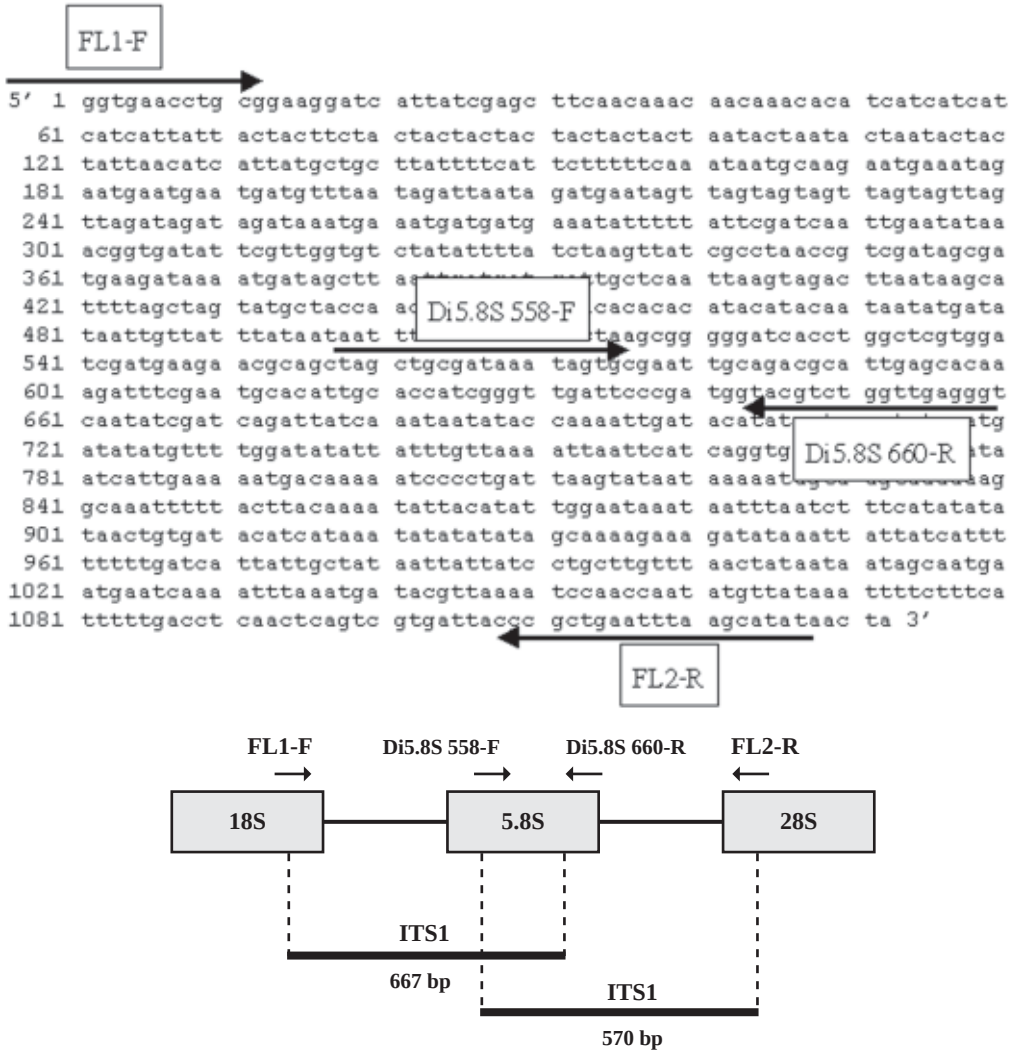

Fig 1- Forward and reverse primers for ITS1 and ITS2. The FL1-F primer is conserved in filarial parasites. All other primers were designed from the *D. immitis* 5.8S and 28S rDNA sequence (AF217800). FL1-F and Di5.8S 660-R are primers for the amplification of ITS1; Di 5.8S 558-F and FL2-R primers are for ITS2.

rDNA of filarial worms (*B. malayi*, *W. bancrofti* and *D. immitis*). Nevertheless, not all rDNA sequences of the selected filarial worms were completely reported. The available filarial rDNA sequences from the online database (Genbank) were as follows: 18S rDNA from *B. malayi* (AF036588), *W. bancrofti* (AF227234), and *D. immitis* (AF217800); ITS1, 5.8S and ITS2 sequence from *D. immitis* (AF217800); partial 28S rDNA sequence from *D. immitis* (AF217800) and *B. malayi* (AF499130). The FL1-F primer was designed from the conserved region at the 3´ end of *B. malayi*, *W. bancrofti* and *D. immitis* 18S rDNA (Fig 1). The FL2-R primer was selected from the beginning 5´ end of *D. immitis* 28S rDNA, which was also conserved among the reported 28S rDNA from other nematodes. After the first round of PCR, very faint DNA bands of ITS1-5.8S-ITS2 were observed. Subsequently, semi-nested PCR was performed to obtain a high yield of PCR products. Two internal primers, Di5.8S 660-R and Di5.8S 558-F, were designed from the *D. immitis* 5.8S rDNA region (AF217800) in order to amplify the ITS1 and ITS2 regions, respectively.

PCR-RFLP patterns of ITS1 and ITS2

The 667 bp band of *D. immitis* ITS1 was amplified as expected (Fig 2A). However, both *B. malayi* and *B. pahangi* ITS1 had smaller ITS1 sizes of about 590 bp. In contrast, the ITS2 from *D. immitis* was 570 bp, and for *B. malayi* and *B. pahangi* ITS2 about 650 bp (Fig 2B). Both *B. malayi* from humans and domestic cats showed the same size of PCR products and similar digested patterns for ITS1 and ITS2 (Fig 2A, B). There were 2 bands of about 290 bp and 300 bp from the *Ase* I digested *B. pahangi* ITS1 (Fig 2A), while about 140 bp, 160 bp and 290 bp bands were from the *B. malayi* digested ITS1. PCR-RFLP of ITS1 with *Acc* I and with *Hinf* I did not differentiate *B. malayi* from *B. pahangi*. Similary, the PCR-RFLP of ITS2 with *Rsa* I and with *Ase* I did not discriminate either *Brugia* species.

PCR-RFLP of *D. immitis* ITS1 by *Ase* I showed the 455 bp and 212 bp predicted products. All other digested ITS1 and ITS2 showed the predicted PCR-RFLP patterns. We demonstrated that the semi-nested PCR-RFLP of ITS1 with *Ase* I could differentiate *B. malayi*, *B. pahangi* and *D. immitis*.

DISCUSSION

The conventional method used for the detection and species differentiation of lymphatic filarial parasites is the identification of microfilariae by Giemsa stain. Although this method is not expensive, it requires skill and experience to differentiate among closely-related species. Histochemical stain of acid phosphatase activity is another useful technique for species identification. It clearly discriminates different filarial nematode species (Yen and Mak, 1978; Chungpivat *et al*, 1990; Nithiuthai and Chungpivat, 1992; Chungpivat and Sucharit, 1993). However, the enzymatic activity is rather sensitive to light and requires fresh specimens. As a result, PCR-RFLP of the ITS regions may be an alternative method to differentiate closely-related species, which are indistinguishable by morphology (Gasser *et al*, 1998). Furthermore, PCR-RFLP could be used to identify parasites in developmental stages (Gasser and Chilton, 1995; Newton *et al*, 1998; Almeyda-Artigas *et al*, 2000) in different geographic distributions (Gasser and Chilton, 1995; Ramachandran *et al*, 1997).

The ITS1 and ITS2 regions of rDNA are useful for investigating some variations among closely-related species (Gasser *et al*, 1996; Ramachandran *et al*, 1997). The ITS regions are flanked by conserved rDNA genes, and the sequences can be used to design primers to amplify the intervening regions by PCR. Moreover, the presence of multiple copies provides a large number of target sequences for PCR in most organisms (Long and Dawid, 1980). As a result, the ITS regions of filarial nematodes are suitable targets for amplification and detection, even in mildly infected individuals. Although the first round PCR was inadequate to provide a detectable amount of PCR products, a subsequent semi-nested PCR has been utilized to increase the sensitivity for the detection of the ITS1 and ITS2 regions. Since the filarial DNA was extracted from whole blood specimens of infected individuals, host genomic DNA might possibly interfere with the PCR reaction. However, the bases at the 3´ end of both internal primers designed for semi-nested PCR of ITS1 and ITS2 were not complementary to human rDNA (U13369). Therefore, no ITS1 or ITS2 products were amplified from the semi-nested PCR of the non-infected human blood samples (data not shown). There was no reported sequence of ITS1 and ITS2 from domestic cats and dogs. Nevertheless, no PCR products of ITS1 and ITS2 were amplified from the non-infected blood samples of domestic cats and dogs (data not shown).

The PCR-RFLP profiles of domestic cat *B. malayi* and human *B. malayi* ITS1 and ITS2 were similar for all restriction endonucleases used in our study. Our results supported the suggestion that domestic cats play an important role as animal reservoirs of *B. malayi*, as reported previously by studies of microfilarial morphology, periodicity, PCR-RFLP of *Hha* I repeat

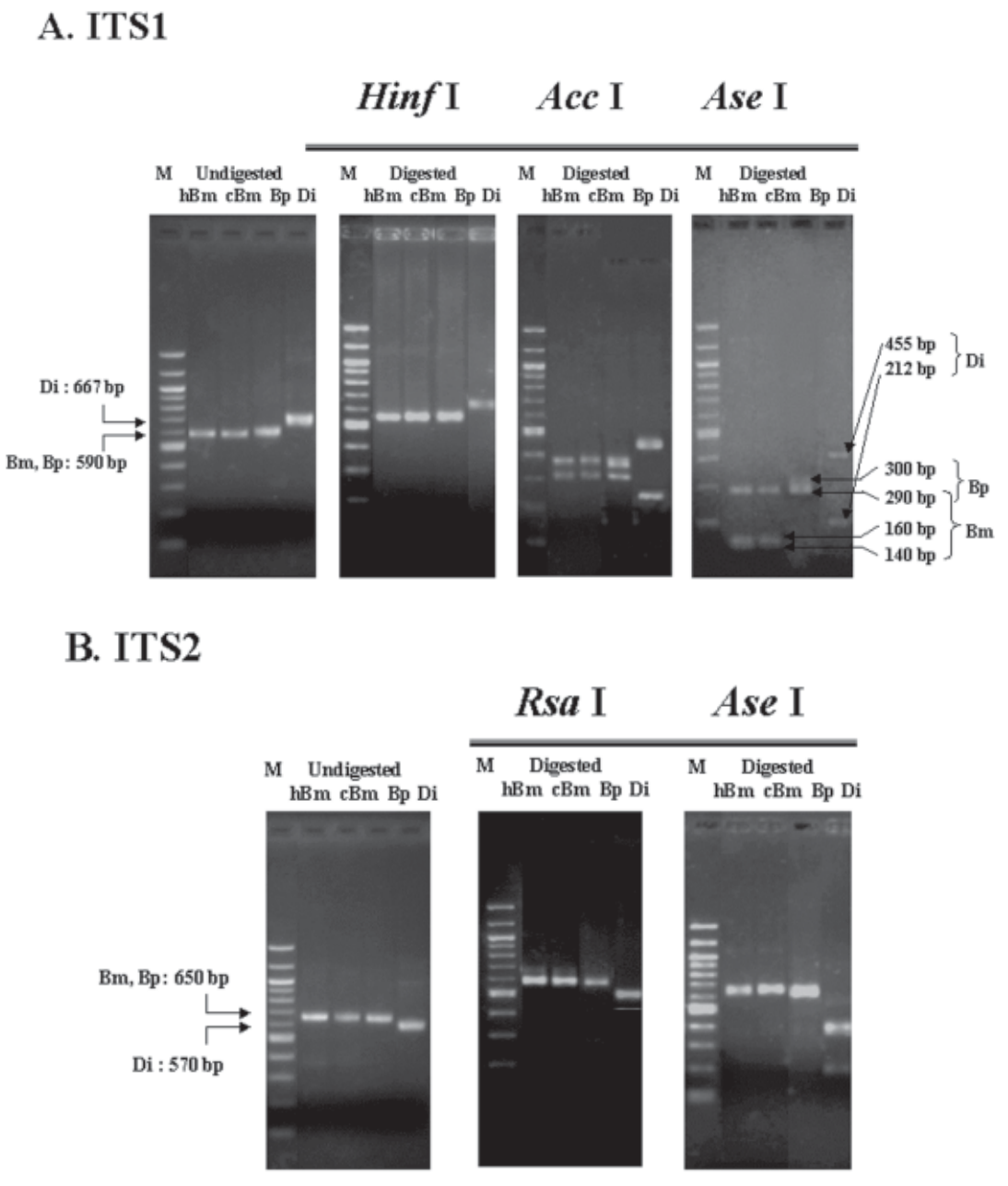

Fig 2- PCR-RFLP of *B. malayi* and *B. pahangi* ITS1 (A) and ITS2 (B). M: 100 bp ladder molecular weight marker**;** hBm: human *B. malayi*; cBm: cat *B. malayi*; Bp: *B. pahangi*; Di: *D. immitis*; *Ase* I, *Acc* I, *Hinf* I and *Rsa* I are restriction enzymes used for the digestion of ITS1 and ITS2 PCR products.

genes and PCR of Trans-spliced Leader Exon I (SLX) (Chansiri *et al*, 2002).

In summary, we demonstrated that the PCR-RFLP of ITS1, digested with *Ase* I, could differentiate *B. malayi*, *B. pahangi* and *D. immitis*. Recently, it has been shown that domestic cats in Thailand can carry not only *B. pahangi* and *B. malayi*, but also *D. immitis* (Chansiri *et al*, 2002). Further study, for the field application of the PCR-RFLP method to monitor and evaluate infections in the reservoir hosts, would help the on-going lymphatic filariasis control programs.

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