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Short communication

Detection of filarial parasites in domestic cats by PCR-RFLP of ITS1

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Abstract

Lymphatic filariasis has been targeted by the World Health Organization (WHO) to be eliminated by the year 2020. In addition to chemotherapy and vector control, the control of reservoir hosts is necessary for the control program to succeed. Malayan filariasis, caused by *Brugia malayi*, is endemic in the South of Thailand where domestic cats serve as the major reservoir host. However, in nature, domestic cats also carry *B. pahangi*, *Dirofilaria immitis* and *D. repens* infections and it is difficult to distinguish the different filarial species from each other just by morphology. To assess the burden of filarial parasites, we performed a study on domestic cats in an endemic area of malayan filariasis in the Prasang district, of Surat Thani, a province in Southern Thailand. Together with Giemsa staining and acid phosphatase activity studies, we performed PCR-RFLP analysis on the first internal transcribed spacer (ITS1) region of ribosomal DNA (rDNA). PCR-RFLP with *Ase* I could clearly differentiate between *B. malayi*, *B. pahangi*, *D. immitis* and *D. repens*. Out of the 52 cats studied, filarial parasites were identified in 5 (9.5%) cats, of which 4 (7.6%) were *B. pahangi* and 1 (1.9%) *D. immitis*. This PCR-RFLP technique detected two additional cats that were not detected by microscopy. The domestic cats are not an important host of *B. malayi* in this region. We could develop the PCR-RFLP assay test for differentiating filarial nematodes which can be applied to survey human, animal reservoir hosts and mosquito vectors in endemic areas.
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Keywords: Filarial parasites; PCR-RFLP; ITS1; Domestic cats; Thailand

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1. Introduction

Lymphatic filariasis, mainly caused by filarial nematodes *Wuchereria bancrofti* and *Brugia malayi*, is

still a major debilitating and disfiguring disease. It is estimated that 1.1 billion people, in more than 83 countries, are at risk of acquiring the infection, while over 120 million individuals have already been infected (Molyneux, 2003). In Thailand, bancroftian filariasis and malayan filariasis are endemic in the Thai-Myanmar and Thai-Malaysia borders, respectively (Triteeraprapab et al., 2000, 2001). At least two physiological types of *B. malayi*, i.e. the nocturnally subperiodic and diurnally subperiodic types have been identified (Filariasis Division, 2001). Lymphatic filariasis caused by subperiodic *B. malayi* is still prevalent in Narathiwat and Surat Thani Provinces (Filariasis Division, 2001; Triteeraprapab et al., 2001). This is due to favorable mosquito breeding sites (large swamp areas), and the existence of animal reservoir hosts, especially domestic cats (Chansiri et al., 2002).

Subperiodic *B. malayi*, zoonotic filariae have also been found in cats in endemic areas of Indonesia (Java, Kalimantan, and Sumatra), Malaysia (Peninsula Malaysia), the Philippines, and other Pacific islands (Lim and Mak, 1983). Control programs in endemic areas of subperiodic *B. malayi* have been reported to be complicated by continuing zoonotic transmission from reservoir hosts (Lim and Mak, 1983). Therefore, besides chemotherapy and vector control, the lymphatic filariasis control program should also pay attention to reservoir host control. Not only *B. malayi*, but also other filarial species, including *B. pahangi*, *Dirofilaria immitis* and *D. repens* can infect domestic cats (Chansiri et al., 2002). It is difficult to differentiate the filarial species by Giemsa stain under a microscope. Acid phosphatase staining could be used to differentiate filarial species (Yen and Mak, 1978), but it requires fresh samples to yield the best results. However, both Giemsa staining and acid phosphatase activity studies require a certain degree of expertise to identify and confirm the species. The PCR-linked restriction fragment length polymorphism (PCR-RFLP) technique has provided an alternative approach for the identification of filarial parasites (Chansiri et al., 2002; Nuchprayoon et al., 2003). The PCR-RFLP, of the first internal transcribed spacer (ITS1), digested with *Ase* I could differentiate *W. bancrofti*, *B. malayi*, *B. pahangi*, *D. immitis* and *D. repens* (Nuchprayoon et al., 2005). Using highly sensitive and specific PCR-RFLP, reliable epidemio-

logical assessment will be helpful for designing control strategies for the lymphatic control program.

The identification of *B malayi* in domestic cats in endemic areas of Thailand is ambiguous. *Brugia* spp. has been found in domestic cats in endemic areas in Southern Thailand with a prevalence ranging from 1.59% to 3.01% (Filariasis Division, 2001). Prasang district, Surat Thani Province, Southern Thailand has been an endemic area for diurnally subperiodic *B. malayi* for 5 decades (Filariasis Division, 2003). This area was surveyed continuously. But, the survey was discontinued in 1992. To evaluate the situation, surveys have been started again in 2002. In Sin jarern, one subdistrict of Prasang district, showed *B. malayi* in 5 out of 790 (0.63%) humans and 7 out of 294 (2.4%) domestic cats (Filariasis Division, 2002). However, the prevalence in both human and domestic cats in our study area, an adjacent area to the Sin jarern subdistrict, is still unknown. The Filariasis Division needs a collaborator to survey domestic cats in this area to aid the filariasis control program. To expand the study, we surveyed domestic cats in two villages in E-pun and Tri kheung subdistricts, the area where human filariasis was detected during the last 10 years. The PCR-RFLP of ITS1 was used to assess the burden of filarial nematodes in domestic cats which was then compared to morphological studies by Giemsa and acid phosphatase stains.

2. Materials and methods

2.1. The study area and specimen collections

One milliliter of venous blood was collected from all 52 domestic cats in village no. 10 of the Tri kheung subdistrict, and village no. 10 of the E-pun subdistrict, Prasang district, Surat Thani Province, Thailand during March 2002. Infected blood samples from experimental cats from the Parasitology Unit, Department of Pathology, Faculty of Veterinary Science, Chulalongkorn University, were used as positive controls. Non-infected cat blood samples were used as negative controls. All blood samples were collected by veterinarians using a sterile technique. This study was approved by the Ethics Committee of the Faculty of Medicine at Chulalongkorn University, Bangkok, Thailand.

2.2. Morphological studies

2.2.1. Giemsa staining

Thick blood films were made from 60 μl blood on glass slides and allowed to dry at room temperature. Red blood cells were lysed in water for 2 min and air-dried. The slides were fixed with absolute methanol for 1 min. The dried slides were stained with 3% Giemsa (Merck, NJ) in phosphate buffer, pH 7.2, for 45 min, and then rinsed with tap water. Each blood film was examined under a light microscope by two independent individuals.

2.2.2. Histochemical staining

Histochemical staining for acid phosphatase activity of microfilariae was performed as previously described (Chalifoux and Hunt, 1971). Each blood film was examined under a light microscope by two independent individuals.

2.3. Extraction of filarial DNA from blood samples

The extraction method was performed as previously reported (Williams et al., 1996) with a slight modification (Nuchprayoon et al., 2003). Two hundred and fifty microliters of each blood sample was 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, 200 μl of lysis 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) was added and incubated at 65 °C for 3 h. Enzyme activity was inactivated by incubation at 90 °C for 10 min.

DNA was isolated by phenol chloroform extraction followed by ethanol precipitation. The extracted DNA was resuspended in 10 μl of TE buffer pH 7.5 (10 mM Tris–HCl pH 7.5, 1 mM EDTA pH 8.0).

2.4. Primers for filarial ITS1 amplification

Oligonucleotide forward and reverse primers were designed based on data of 18S and 5.8S rDNA conserved sequences of filarial parasites, as previously described (Nuchprayoon et al., 2005). The ITS1 forward primer (ITS1-F) was 5′-GGTGAACCTGCG-GAAGGATC-3′ and the ITS1 reverse primer (ITS1-R) was 5′-GCGAATTGCAGACGCATTGAG-3′. All oligonucleotide primers were purchased from the Bioservice Unit, NSTDA, Bangkok, Thailand.

2.5. PCR-RFLP

The 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, WI); 0.625 Units *Taq* DNA polymerase (Amersham Phamacia); 200 pM of each primer (ITS1-F and ITS1-R); and 2 μl DNA template. After incubation at 94 °C for 5 min, amplification was carried out for 35 cycles with the following temperature cycling parameters: 94 °C for 30 s of denaturation; 58 °C for 30 s of annealing and 72 °C for 45 s of extension. The final amplification cycle included in addition, 10 min extension at 72 °C.

Five microliters of each PCR product of ITS1 was digested with 5 units of *Ase* I according to the manufacturer's protocols (New England Biolabs, MA). Analysis of DNA fragments was performed by submarine 1.5% agarose gel electrophoresis, stained by ethidium bromide and visualized under ultraviolet light.

3. Results

3.1. Study area and specimen collection

Tri khung subdistrict consists of 13 villages, 200 houses, with the total population about 700 persons, E-pun subdistrict consists of 12 villages, 150 houses, with the total population about 600 persons. Since the study area is cultivated land consisting of rubber and palm oil plantations, the houses are far away from each other. There were total of 42 houses in our studied area. The cat blood samples were collected from all domestic cats in the 42 houses in village no. 10 of Tri kheung subdistrict, and village no. 10 of E-pun subdistrict (Fig. 1). Prasang district consists of rubber and palm oil plantations, as well as other
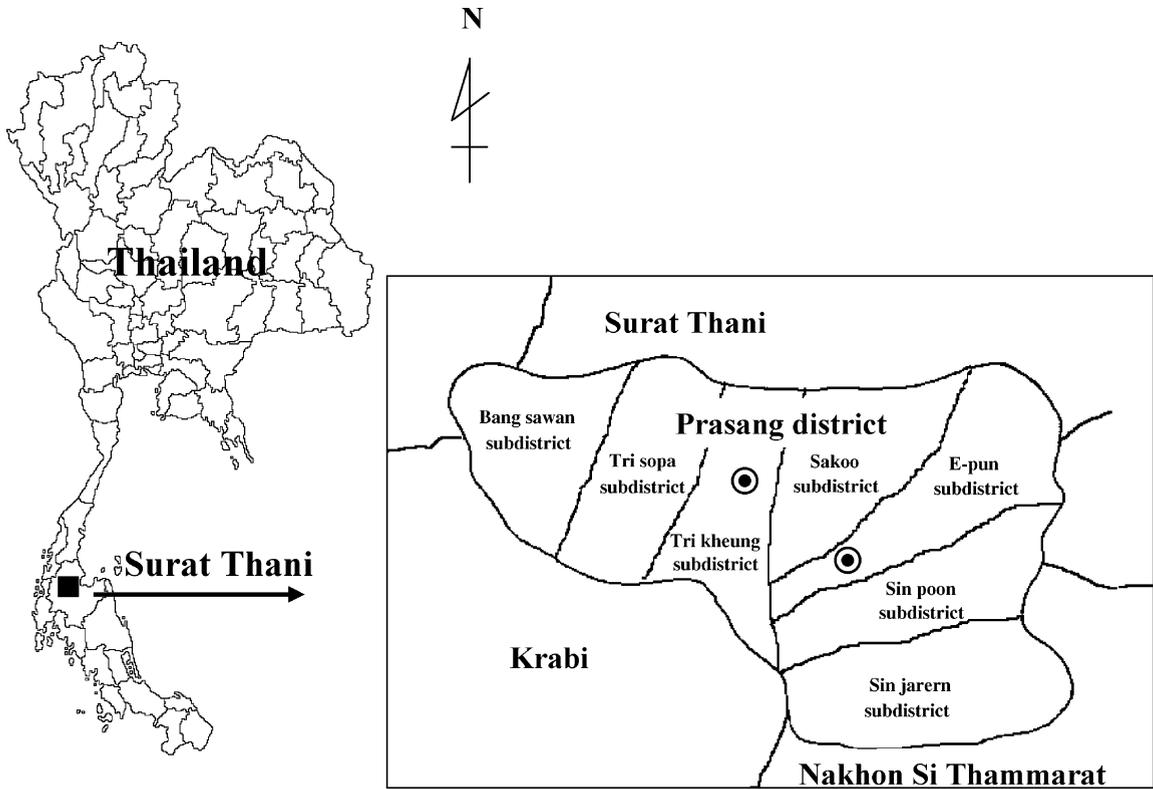
Fig. 1. Map of study area (◉), Prasang district, Surat Thani Province (■), Thailand.

agricultural crops. There are also two permanent large fresh water swamps with a various kinds of aquatic plants, grasses and weeds. The swamps are a suitable breeding site for *Mansonia* spp., the common vector of *B. malayi* (Filariasis Division, 2001). The domestic cat serve as an important household pet in this area.

In total, 52 cats were included in this study, 25 (48%) were male and 27 (52%) were female. The mean age was 4 years.

3.2. Species identification by PCR-RFLP of ITS1 digested with Ase I

PCR-RFLP analysis of ITS1 PCR products with *Ase* I allowed unequivocal delineation of samples representing the four species (Fig. 2). Digestion with *Ase* I produced three fragments of 218, 153 and 133 bp for *B. malayi*, while two fragments of 292 and 218 bp were detected for *B. pahangi*. Two fragments of 380 and 215 bp were detected for *D. immitis*, while an undigested band of 602 bp was detected for *D. repens*.
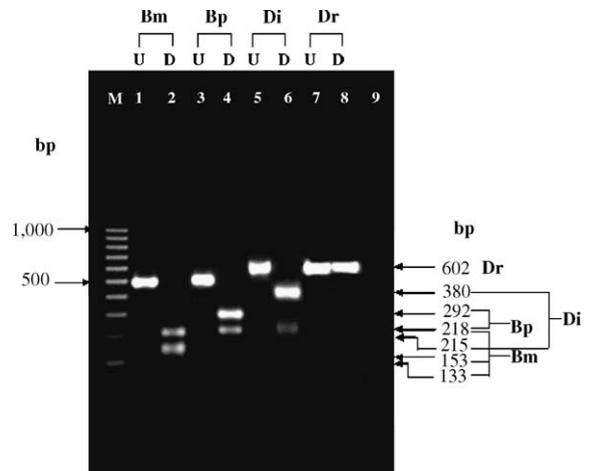

Fig. 2. PCR-RFLP analysis of filarial ITS1 digested with *Ase* I. Lane M, 100 bp ladder; lane 1, undigested (U) *B. malayi* (Bm); lane 2, digested (D) *B. malayi*; lane 3, undigested *B. pahangi* (Bp); lane 4, digested *B. pahangi*; lane 5, undigested *D. immitis* (Di); lane 6, digested *D. immitis*; lane 7, undigested *D. repens* (Dr); lane 8, digested *D. repens*; lane 9, non-infected cat blood (negative control).

Table 1
Detection of filarial infections in domestic cats by both microscopic methods and PCR-RFLP, in two villages of the Prasang district, Surat Thani Province, Thailand

Techniques	No. examined	No. of positive (%)				
		B. malayi	*B. pahangi*	*D. immitis*	*D. repens*	Total
Microscopy[a]	52	0	2 (3.8)	1 (1.9)	0	3 (5.7)
PCR-RFLP[b]	52	0	4 (7.6)	1 (1.9)	0	5 (9.5)

[a] Giemsa staining and acid phosphatase activity staining showed similar results.
[b] PCR-RFLP: PCR-RFLP of ITS1 digested with *Ase* I.

Out of the 52 studied cats, filarial parasites were identified in 5 (9.5%), of which 4 (7.6%) were *B. pahangi* and 1 (1.9%) *D. immitis* (Table 1).

3.3. Species identification by Giemsa staining and acid phosphatase activity staining

Of the 52 cats examined, 3 (5.7%) had microfilariae: 2 (3.8%) with *B. pahangi* and 1 (1.9%) with *D. immitis*

as shown in Table 1. The levels of microfilaremia in 2 cats infected with *B pahangi* were 34 and 32 mf/20 μl and in a cat infected with *D. immitis* was 47 mf/20 μl. No *B. malayi* and *D. repens* were identified.

For *B. pahangi*, Giemsa staining showed the clear sheathed microfilaria and 2 terminal nuclei (TN) (Fig. 3A). Acid phosphatase staining showed heavy and diffused acid phosphatase activity along the entire body (Fig. 3B).
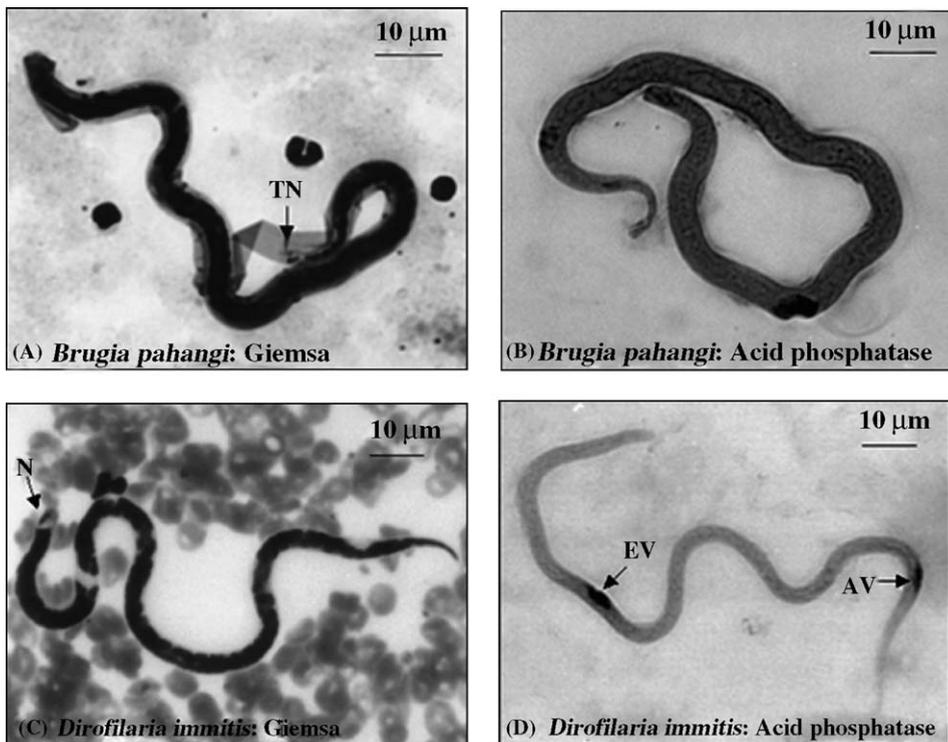

Fig. 3. Microscopic examination of *B. pahangi* and *D. immitis* microfilariae from cats. (A) Giemsa stain of *B. pahangi* showing the sheathed microfilaria and 2 terminal nuclei (TN). (B) An acid phosphatase stain of *B. pahangi* showing acid phosphatase activity along the entire body. (C) A Giemsa stain of *D. immitis* showing the unsheathed microfilaria and one nucleus (N) in the cephalic space. (D) An acid phosphatase stain of *D. immitis* showing acid phosphatase activity at the excretory vesicle (EV) and the anal vesicle (AV).

For *D. imimitis*, Giemsa staining showed unsheathed microfilaria and one nucleus (N) in the cephalic space (Fig. 3C). Acid phosphatase staining showed phosphatase activity at the excretory vesicle (EV) and the anal vesicle (AV), while the rest of the body remained unstained (Fig. 3D).

4. Discussion

Prasang district, Surat Thani Province, Southern Thailand is an endemic area of diurnally subperiodic *B. malayi* parasites (Shutidamrong and Phantana, 1986). In 2002, a survey in Sin jarern subdistrict, a subdistrict in the Prasang district, identified *B. malayi* in both human and domestic cats (Filariasis Division, 2002). Infected individuals in Sin jarern subdistrict have been treated annually with a single dose of 400 mg albendazole and 6 mg/kg diethylcarbamazine citrate (DEC) as recommended by the WHO (1998). All domestic cats that were *Brugia* spp. positive were also treated using a single dose of 100 μg/kg ivermectin by subcutaneous injection.

For this study, we surveyed the domestic cats in two villages, E-pun and Tri kheung. Fifty-two blood samples were examined using PCR-RFLP of ITS1 digested with *Ase* I. The PCR-RFLP could detect all three microfilariae positive cats and could also detect *B. pahangi* in two more blood samples, increasing the prevalence from 5.7 to 9.5% (Table 1). The species identification by the PCR-RFLP technique provided a result concordant with the microscopic techniques. However, no *B. malayi* were identified. The result is consistent with the data found by the Filariasis Division survey in 2003 that there was no filarial nematodes detected in humans in the studied areas (Filariasis Division, 2003). Therefore, the finding that no *B. malayi* was identified in domestic cats in the studied area could imply the effectiveness of the filariasis control program.

Accurate identification of filarial nematode species is necessary for the control of zoonotic transmission. It is difficult to differentiate among the filarial parasites, particularly the diagnostic stage, microfilaria. Although the Giemsa and the acid phosphatase stained blood films are useful to differentiate filarial species, this technique is insensitive, time-consuming, and labor intensive. Furthermore, staining methods require expertise to identify and confirm the species. The molecular techniques such as DNA hybridization (Williams et al., 1988) and PCR of repeated sequences (Lizotte et al., 1994) have been used in the diagnosis of *Brugia* infections and have provided the opportunity for improved diagnosis. However, the limitation of these techniques are identification of a single species at a time.

We could adjust the PCR reaction to detect as little as 1 pg of parasite DNA (data not shown), which allows for the presence of only a single microfilaria in 250 μl blood sample to be detected. Our assay has higher sensitivity in the case of amicrofilaremic than Knott's assay which has a sensitivity 1 mf/ml (Melrose et al., 2000). Furthermore, the PCR-RFLP of ITS1 rDNA could detect and differentiate *D. immitis*, the worldwide distributed agent of canine cardiovascular dirofilariasis which causes pulmonary dirofilariasis in man, as well as *D. repens* which causes subconjunctiva and subcutaneous nodules in man (Boreham, 1988). Thus, PCR-RFLP technique will be useful to monitor and evaluate *Brugia* spp. and *Dirofilaria* spp. in animal reservoir hosts. The PCR-RFLP technique represents an important alternative for specific diagnosis of filarial nematodes in cats and it can be applied to diagnose and differentiate pathogenic filarial species in human, and mosquito vectors in endemic areas.

References

Boreham, P.F.L., 1988. Dirofilariasis in man. In: Boreham, P.F.L., Atwell, R.B. (Eds.), Dirofilariasis. CRC Press, Florida, pp. 217–226.

Chalifoux, L., Hunt, R.D., 1971. Histochemical differentiation of *Dirofilaria immitis* and *Dipetalonema reconditum*. J. Am. Vet. Med. Assoc. 158, 601–605.

Chansiri, K., Tejangkura, T., Kwaosak, P., Sarataphan, N., Phantana, S., Sukhumsirichart, W., 2002. PCR based method for identification of zoonostic *Brugia malayi* microfilariae in domestic cats. Mol. Cell. Probes. 16, 129–135.

Filariasis Division, 2001. Annual Report, Filariasis Division, CDC Department, Ministry of Public Health, Bangkok, Thailand (in Thai).

Filariasis Division, 2002. Annual Report, Filariasis Division, CDC Department, Ministry of Public Health, Bangkok, Thailand (in Thai).

Filariasis Division, 2003. Annual Report, Filariasis Division, CDC Department, Ministry of Public Health, Bangkok, Thailand (in Thai).

Lim, B.L., Mak, J.W., 1983. Human behaviour and zoonotic disease in Malaysia. In: Cross, J.H., Croll, N.A. (Eds.), Human Ecology and Infectious Disease. Academic Press, London, pp. 49–72.

Lizotte, M.R., Supali, T., Partono, F., Williams, S.A., 1994. A polymerase chain reaction assay for the detection of *Brugia malayi* in blood. Am. J. Trop. Med. Hyg. 51, 314–321.

Melrose, W.D., Turner, P.F., Pisters, P., Turner, B., 2000. An improved Knott's concentration test for the detection of microfilariae. Trans. R. Soc. Trop. Med. Hyg. 94, 176.

Molyneux, D.H., Bradley, M., Hoerauf, A., Kyelem, D., Taylor, M.J., 2003. Mass drug treatment for lymphatic filariasis and onchocerciasis. Trends Parasitol. 19, 516–522.

Nuchprayoon, S., Sangprakarn, S., Junpee, A., Nithiuthai, S., Chungpivat, S., Poovorawan, Y., 2003. Differentiation of *Brugia malayi* and *Brugia pahangi* by PCR-RFLP of ITS-1 and ITS-2. Asian Pac. J. Allergy Immunol. 34, 67–73.

Nuchprayoon, S., Junpee, A., Poovorawan, Y., Scott, A.L., 2005. Detection and differentiation of filarial parasites by universal primers PCR and RFLP analysis. Am. J. Trop. Med. Hyg. 73, 895–900.

Shutidamrong, C., Phantana, S., 1986. Periodicity of microfilaria in Thailand. Com. Dis. J. 12, 227–237.

Triteeraprapab, S., Kanjanopas, K., Suwannadabba, S., Sangprakarn, S., Poovorawan, Y., Scott, A.L., 2000. Transmission of the nocturnal periodic strain of *Wuchereria bancrofti* by *Culex quinquefasciatus*: establishing the potential for urban filariasis in Thailand. Epidemiol. Infect. 125, 207–212.

Triteeraprapab, S., Karnjanopas, K., Porksakorn, C., Sai-ngam, A., Yentakan, S., Loymak, S., 2001. Lymphatic filariasis caused by *Brugia malayi* in an endemic area of Narathiwat province, Southern of Thailand. J. Med. Assoc. Thai. 84, S182–S188.

Williams, S.A., Nicolas, L., Lizotte-Waniewski, M., Plichart, C., Luquiaud, P., Nguyen, L.N., Moulia-Pelat, J.P., 1996. A polymerase chain reaction assay for the detection of *Wuchereria bancrofti* in blood samples from French Polynesia. Trans. R. Soc. Trop. Med. Hyg. 90, 384–387.

Williams, S.A., DeSimone, S.M., McReynolds, L.A., 1988. Species-specific oligonucleotide probes for the identification of human filarial parasites. Mol. Biochem. Parasitol. Mar. 28, 163–169.

World Health Organization, 1998. Epidemiologic approaches to lymphatic filariasis elimination: initial assessment, monitoring and certification. WHO, Atlanta, GA, USA, *WHO/FIL*/99.195, 1–35.

Yen, P.K.F., Mak, J.W., 1978. Histochemical differentiation of *Brugia, Wuchereria, Dirofilaria* and *Breinlia* microfilariae. Ann. Trop. Med. Parasitol. 72, 157–162.