

Rapid Detection and Identification of *Wuchereria bancrofti*, *Brugia malayi*, *B. pahangi*, and *Dirofilaria immitis* in Mosquito Vectors and Blood Samples by High Resolution Melting Real-Time PCR

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Abstract: A simple, rapid, and high-throughput method for detection and identification of *Wuchereria bancrofti*, *Brugia malayi*, *Brugia pahangi*, and *Dirofilaria immitis* in mosquito vectors and blood samples was developed using a real-time PCR combined with high-resolution melting (HRM) analysis. Amplicons of the 4 filarial species were generated from 5S rRNA and spliced leader sequences by the real-time PCR and their melting temperatures were determined by the HRM method. Melting of amplicons from *W. bancrofti*, *B. malayi*, *D. immitis*, and *B. pahangi* peaked at $81.5 \pm 0.2^\circ\text{C}$, $79.0 \pm 0.3^\circ\text{C}$, $76.8 \pm 0.1^\circ\text{C}$, and $79.9 \pm 0.1^\circ\text{C}$, respectively. This assay is relatively cheap since it does not require synthesis of hybridization probes. Its sensitivity and specificity were 100%. It is a rapid and technically simple approach, and an important tool for population surveys as well as molecular xenomonitoring of parasites in vectors.

Key words: *Wuchereria bancrofti*, *Brugia malayi*, *Dirofilaria immitis*, *Brugia pahangi*, high resolution melting real-time PCR, dog, mosquito

INTRODUCTION

Lymphatic filariasis, a mosquito-borne disease, is caused by filarial nematodes *Wuchereria bancrofti*, *Brugia malayi*, and *Brugia timori* [1]. It is a major public health problem, particularly in the tropics and subtropics, including Thailand and Myanmar [2,3]. Although it is not a major cause of mortality in humans, chronic pathology causes permanent disability and handicap. World Health Organization has categorized lymphatic filariasis as one of the 6 diseases which can be totally eliminated [4]. Several additional species of filarial nematodes,

for example, *Brugia pahangi* and *Dirofilaria immitis*, infect various animals, such as dogs, cats, tigers, otters, tree shrews, and monkeys, in endemic areas of Southeast Asia, Malaysia, the Philippines, Indonesia, Pacific islands, and Thailand [5-7] and possibly cause human zoonotic diseases [8,9].

Control of lymphatic filariasis in human populations and zoonotic filariasis in feline and canine reservoirs should result in a decrease in human cases. The availability of effective methods to monitor the presence or absence of filarial larvae in the mosquito vector and/or microfilariae in the definitive hosts is important for interruption of the transmission of infection. However, microscopic methods for detection of filarial larvae in mosquito vectors and/or of microfilariae in the blood circulation are laborious and require highly experienced personnel, and still the sensitivity is not so high. Thus, a molecular detection tool is necessary to distinguish between closely related species or subspecies. Such tools include conventional

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PCR [10] and semi-nested PCR-RFLP [11]. However, these methods require analysis by agarose gel electrophoresis which is slow, having limited throughput and a bias toward carry-over contamination as well as spurious results. Recently, quantitative real-time PCR was reported to successfully detect *W. bancrofti* [12], *B. malayi*, *B. pahangi* [13,14], *D. immitis* [15], and *Dirofilaria repens* [16]. Also recently, a real-time PCR-based high-resolution melting (HRM) analysis was reported for detection and identification of *B. malayi*, *B. pahangi*, and *D. immitis* in blood samples in a single-tube assay [17]. This is a rapid and technically simple method which does not require fluorophore probes or oligonucleotides nor agarose gel electrophoresis [18]. However, a HRM-based real-time PCR for simultaneous detection of 4 filarial species, *W. bancrofti*, *B. malayi*, *B. pahangi*, and *D. immitis* has not been developed yet.

This study was conducted to develop a single tube, HRM-based real-time PCR for differentiation and detection of *W. bancrofti*, *B. malayi*, *B. pahangi*, and *D. immitis* in mosquito vectors and blood samples. The analytical sensitivity and specificity of the method were evaluated.

MATERIALS AND METHODS

Mosquitoes and experimental infections

Culex pipiens quinquefasciatus and *Aedes aegypti* mosquitoes were collected in urban areas of Khon Kaen Province, north-eastern Thailand. Additionally, *Aedes togoi* was collected from Koh Nom Sao, Chanthaburi Province, eastern Thailand [19]. *Cx. quinquefasciatus*, *Ae. togoi*, and *Ae. aegypti* mosquitoes were artificially infected as previously described with *W. bancrofti* [12], *B. malayi* [20], and *D. immitis* [15], respectively.

Blood containing *W. bancrofti* microfilariae (density 44 microfilariae/20 μ l) was collected from a male carrier, a Burmese immigrant, at Mae Sot District, Tak Province, northwestern Thailand. Blood containing *B. malayi* microfilariae (density 160 microfilariae/20 μ l) was obtained from an experimentally infected cat. *D. immitis*-infected dog blood (density 54 microfilariae/20 μ l) came from a veterinary clinic in Khon Kaen Province.

The mosquitoes were allowed to feed on heparinized blood using an artificial membrane feeding technique [21]. Fourteen days after feeding, each mosquito was dissected in normal saline solution, and the number of larvae was counted under a dissecting microscope. After dissection, all larvae, together with the insect's body, were mixed and put into a 1.5-ml microcen-

trifuge tube. Specimens were labeled and kept at -20°C for DNA extraction.

This study was approved by the Human Ethics Committee of Khon Kaen University, based on the Ethics of Human Experimentation of the National Research Council of Thailand (Reference No. HE 42036).

Source of blood specimens and control DNAs for sensitivity and specificity

One *W. bancrofti*-infected blood sample was collected from an infected Burmese immigrant and 1 *B. malayi*-infected blood sample was collected from an experimentally infected cat (see above). A total of 14 DNA samples were extracted from EDTA blood samples of dogs obtained from the Animal Hospital of the Faculty of Veterinary Science, Chulalongkorn University, Bangkok, and from the Faculty of Veterinary Science, Khon Kaen University, Khon Kaen. Blood specimens were microscopically examined, and the presence of parasites was confirmed by staining a thick blood film (20 μ l blood/slide) with Giemsa [22] and by special staining for acid phosphatase activity for differentiation of *B. malayi* and *B. pahangi* [23,24]. Out of these 14 DNA samples, 5 were from blood samples positive for *D. immitis* (range of microfilarial density = 2-20/50 μ l), 5 for *B. pahangi* (range of microfilarial density = 1-78/50 μ l), 2 for *Hepatozoon canis* and 2 for *Babesia* spp. DNAs extracted from blood samples from healthy dogs (n = 5), a cat (n = 1), and a human (n = 1). DNAs from human red blood cells infected with *Plasmodium falciparum* (n = 1) and *P. vivax* (n = 1) were used for specificity evaluations.

For preparation of DNA specimens for real-time PCR, each mosquito specimen was put into a 1.5-ml microcentrifuge tube, homogenized with a disposable polypropylene pestle (BellCo Glass, Vineland, New Jersey, USA), and DNA extracted using a NucleoSpin Tissue Kit (Macherey-Nagel, Düren, Germany). The DNAs were eluted in 100 μ l of 5 mM Tris-HCl, pH 8.5, and stored at -20°C until use. A 5- μ l sample of each DNA was used per reaction. Fifty- μ l blood samples were used for DNA extraction and purification as described above.

HRM real-time PCR assay

LightCycler® PCR detection and analysis systems were used for amplification and quantification. Specific primers, i.e., SLX-F (5' GGAATCCCAGGTGTTGTAGACAT 3') and SLX-R (5' GGGCTGAAACATTCAATTACCTC 3') (Proligo, Helios, Singapore), were designed from *B. malayi* 5S rRNA gene and spliced

leader sequence SL1 (GenBank accession no. D87037). According to bioinformatic analysis, the primers can anneal with *W. bancrofti*, *B. malayi*, *B. pahangi*, and *D. immitis* genes, yielding amplicons of different sizes.

For amplification detection, a LightCycler 480 High Resolution Melting Master Kit (Roche Applied Science, Mannheim, Germany) was used as recommended by the manufacturer. The PCR mixture contained 1×LightCycler 480 HRM Master Mix, which included HRM dye, a DNA-binding dye which can be used at high concentrations without inhibiting PCR reactions, 2.2 mM MgCl₂, 0.8 μM SLX-F primer, and 0.8 μM SLX-R primer. The total reaction volume was 20 μl. The PCR cycling for HRM curve acquisition was run under the following conditions: 1 cycle pre-incubation 10 min at 95°C; 45 cycles of 95°C for 10 sec, 55°C for 8 sec, and 72°C for 15 sec. After amplification, the reaction product was melted by raising the temperature from 60°C to 90°C, with an increment of 0.1°C/sec, to obtain information on melting profiles. Products were then cooled to 40°C for 30 sec. All samples were tested in duplicate in 96-well plates.

In order to determine the specificity of the HRM real-time PCR assay, DNAs extracted from samples (see above) were analyzed separately. Each run contained at least 1 negative control (consisting of 5 μl distilled water) and 1 positive control (plasmid in water).

Melting curve analysis to determine melting temperature (T_m) of the specific PCR product, of which identification was confirmed by conventional gel electrophoresis, was performed using LightCycler 480 gene scanning software (version 1.5) (Roche Applied Science). The cycle number indicating the target sequence copy number was presented as the number of PCR cycles needed for the change in fluorescence signal of the amplicons to exceed the detected threshold value.

Positive control plasmids

A positive control plasmid was constructed for each worm species by cloning a PCR product amplified by the SLX-F and SLX-R primers into the pGEM-T Easy plasmid (Promega, Madison, Wisconsin, USA), according to the manufacturer's instructions. Each plasmid was propagated in *Escherichia coli* JM109. Each inserted gene was sequenced in both directions; the resulting sequences were identical with the gene sequences from which the PCR primers were designed.

RESULTS

Standardization of HRM real-time PCR

The sensitivity of HRM real-time PCR was determined using 10-fold serial dilutions of *W. bancrofti*-positive control plasmid DNA (9 to 9 × 10⁷ copies), *B. malayi*-positive control plasmid DNA (2 to 2 × 10⁷ copies), *D. immitis*-positive control plasmid DNA (1 to 10⁷ copies) and *B. pahangi*-positive control plasmid DNA (4 to 4 × 10⁷ copies) in water. At a cut-off at 40 PCR cycles, the limit of detection for *W. bancrofti* was ≤ 9 copies of positive control (Fig. 1A), for *B. malayi* the limit was ≤ 2 copies of positive control (Fig. 1B), for *D. immitis* the limit was 10 copies of positive control (Fig. 1C), and for *B. pahangi* the limit was ≤ 4 copies of positive control (Fig. 1D). No fluorescence was detected when testing purified DNA from non-infected mosquitoes (*Cx. quinquefasciatus* and *Ae. aegypti*), human, dog, and cat blood samples, dog blood samples infected with *H. canis* and *Babesia* spp. or *P. falciparum*, and *P. vivax*-infected human red blood cells.

HRM real-time PCR for detection of filarial nematodes in mosquitoes and blood samples

HRM real-time PCR was applied to detect *W. bancrofti*, *B. malayi*, and *D. immitis* in infected mosquitoes and *W. bancrofti*, *B. malayi*, *D. immitis*, and *B. pahangi* in blood samples (Table 1). DNAs from uninfected dog blood samples and blood samples infected with other pathogens were not amplified by this primer set. The results of melting curve analyses are given in Fig. 2 and Table 1. Furthermore, the HRM real-time PCR could detect as little as a single L3 in mosquitoes infected with *W. bancrofti*, *B. malayi*, or *D. immitis*, or a single microfilaria of *W. bancrofti*, *B. malayi*, *B. pahangi*, and *D. immitis* per 50-μl blood sample. The sizes of the amplified products are 227 bp for *W. bancrofti*, 199 bp for *B. malayi*, 201 bp for *B. pahangi*, and 161 bp for *D. immitis*. The sensitivity and specificity were both 100%.

DISCUSSION

The HRM real-time PCR assay has been used for simultaneous discrimination between *B. malayi* and *B. pahangi* in cat reservoirs [25] and recently, to distinguish between *B. malayi*, *B. pahangi*, and *D. immitis* microfilariae in blood samples by different T_m [17]. *W. bancrofti* is still a major debilitating and disfiguring disease. It is considered that many tropical countries

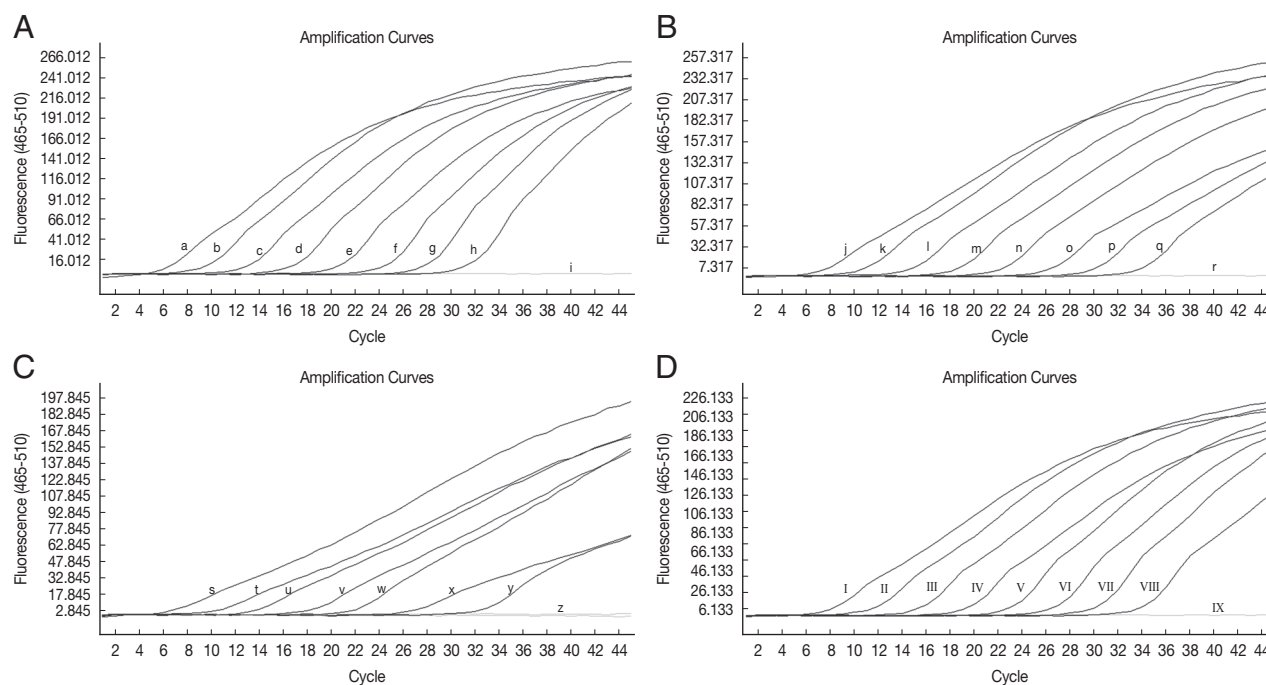


Fig. 1. Amplification plot of fluorescence (y-axis) vs cycle numbers (x-axis) showing the analytical sensitivity of HRM real-time PCR for detecting DNA of *W. bancrofti* (A), *B. malayi* (B), *D. immitis* (C), and *B. pahangi* (D) in control plasmids. (a-h) 10-fold dilutions of *W. bancrofti* plasmid from 9×10^7 to 9 copies/reaction (i) distilled water. (j-q) 10-fold dilutions of *B. malayi* plasmid from 2×10^7 to 2 copies/reaction (r) distilled water. (s-y) 10-fold dilutions of *D. immitis* plasmid from 10^7 to 10 copies/reaction (z) *D. immitis* plasmid 1 copy/reaction and distilled water. (I- VIII) 10-fold dilutions of *B. pahangi* plasmid from 4×10^7 to 4 copies/reaction (IX) distilled water.

Table 1. HRM real-time PCR showing cycle number (Cn) and melting temperatures (Tm) for the detection of *Wuchereria bancrofti*, *Brugia malayi*, *Dirofilaria immitis*, and *Brugia pahangi* in infected mosquitoes and blood samples

Samples (n)	HRM results					
	Cn			Tm (°C)		
	Range	Mean \pm SD	Median	Range	Mean \pm SD	Median
<i>W. bancrofti</i> infected ^a (6)	23.2-36.3	29.0 \pm 4.9	27.2	81.2-81.7	81.5 \pm 0.2	81.6
<i>B. malayi</i> infected ^b (6)	19.1-35.2	28.0 \pm 6.2	25.7	78.1-79.3	79.0 \pm 0.3	79.1
<i>D. immitis</i> infected ^c (10)	21.0-25.7	22.7 \pm 2.1	22	76.6-76.9	76.8 \pm 0.1	76.8
<i>B. pahangi</i> infected ^d (5)	19.1-25.5	22.7 \pm 2.4	23.1	79.8-80.0	79.9 \pm 0.1	79.9

^a5 *W. bancrofti*-infected *Cx. quinquefasciatus* and 1 infected human sample.

^b5 *B. malayi*-infected *Ae. togoi* and 1 infected cat blood sample.

^c5 *D. immitis*-infected *Ae. aegypti* and 5 infected dog blood samples.

^d5 *B. pahangi*-infected dog blood samples.

are at risk of acquiring the infection [26]. In Thailand, bancroftian filariasis is endemic in the Thai-Myanmar border regions [3]. This prompted us to develop the HRM real-time PCR assay for differential detection of 4 filarial species; *W. bancrofti*, *B. malayi*, *D. immitis*, and *B. pahangi* in mosquito vectors and blood samples. The HRM assay could detect the 5S rRNA gene and spliced leader sequences by using a single primer pair that can amplify products from all 4 species. The assay could distinguish each species according to its melting temperature

based on different GC content and the length of the amplicon [27]. No PCR products were amplified from a wide range of control DNAs from other parasites or from intermediate and definitive hosts, indicating high specificity.

On a cost-per-sample basis, this method is cheaper than using multiplex real-time PCR methods that require synthesis of specific labeled probes. The assay can be performed on a large number of samples at the same time, with only very small sample volumes required.

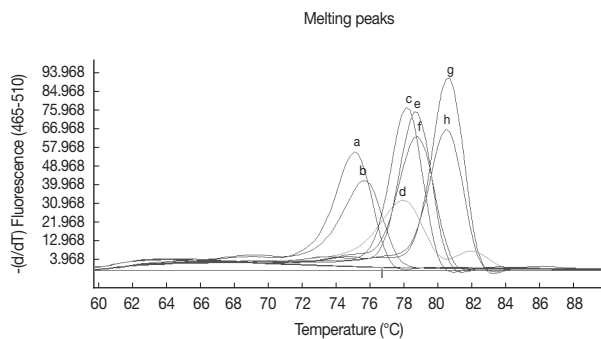


Fig. 2. Representative melting curve analyses of the amplification products of *W. bancrofti*, *B. malayi*, *D. immitis*, and *B. pahangi* 5S rRNA gene and spliced leader sequence. The figure shows the melting curves of *D. immitis* plasmid DNA (10^6 copies) (a), *D. immitis*-infected blood sample (b), *B. malayi* plasmid DNA (2×10^4 copies) (c), *B. malayi*-infected blood sample (d), *B. pahangi* plasmid (4×10^4 copies) (e), *B. pahangi*-infected blood sample (f), *W. bancrofti* plasmid (9×10^4 copies) (g), *W. bancrofti*-infected blood sample (h), non-infected *Ae. aegypti* and *Cx. quinquefasciatus*, *P. falciparum*-infected and *P. vivax*-infected human red blood cells, non-infected cat, human, and dog blood samples, *Babesia* spp., and *H. canis*-infected dog blood DNA, respectively, and distilled water (all i).

In conclusion, a rapid, sensitive, and specific HRM real-time PCR for the detection of 4 filarial nematodes in mosquito vectors and blood samples is presented. This procedure is a suitable tool not only for diagnosis and epidemiological surveys of filariasis and dirofilariasis in final hosts but also for molecular xenomonitoring of *W. bancrofti*, *B. malayi*, *B. pahangi*, and *D. immitis* in mosquito vectors. As *D. repens* has also been reported as a causative agent of human filariasis in Thailand [28], we intend to seek material of this species to extend the experimental work reported here.

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REFERENCES

- Weil GJ, Ramzy RM. Diagnostic tools for filariasis elimination programs. *Trends Parasitol* 2007; 23: 78-82.
- World Health Organization. Lymphatic filariasis: the disease and its control. Fifth report of the WHO Expert Committee on Filariasis. *Tech Rep Ser* 1992; 821: 1-71.
- Triteeraprapab S, Nuchprayoon I, Porksakorn C, Poovorawan Y, Scott AL. High prevalence of *Wuchereria bancrofti* infection among Myanmar migrants in Thailand. *Ann Trop Med Parasitol* 2001; 95: 535-538.
- Center for Disease Control and Prevention. Recommendation of International Task Force for Disease Eradication. *Morbidity and Mortality Weekly Report. Recommendation and Reports* 1993; 42: 1-38.
- Orihel TC. Morphology of the larval stages of *Dirofilaria immitis* in the dog. *J Parasitol* 1961; 47: 251-262.
- Denham DA, McGreeny PB. Brugian filariasis: epidemiological and experimental studies. *Adv Parasitol* 1977; 15: 243-309.
- Lim KC, Mak JW. Human behaviour and zoonotic disease in Malaysia. In Cross JH, Croll N eds, *Human Ecology and Infectious Disease*. London, UK. Academic Press. 1983, p 49-72.
- Palmieri JR, Ratiwayanto S, Masbar S, Tirtokusumo S, Rusch J, Marwoto HA. Evidence of possible natural infections of man with *Brugia pahangi* in South Kalimantan (Borneo), Indonesia. *Trop Geogr Med* 1985; 37: 239-244.
- Simón F, Siles-Lucas M, Morchón R, González-Miguel J, Mellado I, Carretón E, Montoya-Alonso JA. Human and animal dirofilariasis: the emergence of a zoonotic mosaic. *Clin Microbiol Rev* 2012; 25: 507-544.
- Latrofa MS, Weigl S, Dantas-Torres F, Annoscia G, Traversa D, Brianti E, Otranto D. A multiplex PCR for the simultaneous detection of species of filarioids infesting dogs. *Acta Trop* 2012; 122: 150-154.
- Nuchprayoon S, Sangprakarn S, Junpee A, Nithiuthai S, Chungpivat S, Poovorawan Y. Differentiation of *Brugia malayi* and *Brugia pahangi* by PCR-RFLP of ITS-1 and ITS-2. *Southeast Asian J Trop Med Public Health* 2003; 34: 67-73.
- Lulitanond V, Intapan PM, Pipitgool V, Choochote W, Maleewong W. Rapid detection of *Wuchereria bancrofti* in mosquitoes by LightCycler polymerase chain reaction and melting curve analysis. *Parasitol Res* 2004; 94: 337-341.
- Thanchomnang T, Intapan PM, Lulitanond V, Choochote W, Manjai A, Prasongdee TK, Maleewong W. Rapid detection of *Brugia malayi* in mosquito vectors using a real-time fluorescence resonance energy transfer PCR and melting curve analysis. *Am J Trop Med Hyg* 2008; 78: 509-513.
- Thanchomnang T, Intapan PM, Chungpivat S, Lulitanond V, Maleewong W. Differential detection of *Brugia malayi* and *Brugia pahangi* by real-time fluorescence resonance energy transfer PCR and its evaluation for diagnosis of *B. pahangi*-infected dogs. *Parasitol Res* 2010; 106: 621-625.
- Thanchomnang T, Intapan PM, Lulitanond V, Sangmaneedet S,

- Chungpivat S, Taweethavonsawat P, Choochote W, Maleewong W. Rapid detection of *Dirofilaria immitis* in mosquito vectors and dogs using a real-time fluorescence resonance energy transfer PCR and melting curve analysis. *Vet Parasitol* 2010; 168: 255-260.
16. Duscher G, Feiler A, Wille-Piazzai W, Bakonyi T, Leschnik M, Miterpáková M. Detection of *Dirofilaria* in Austrian dogs. *Berl Munch Tierarztl Wochenschr* 2009; 122: 199-203 (in German).
 17. Wongkamchai S, Monkong N, Mahannol P, Taweethavonsawat P, Loymak S, Foongladda S. Rapid detection and identification of *Brugia malayi*, *B. pahangi*, and *Dirofilaria immitis* by high-resolution melting assay. *Vector Borne Zoonotic Dis* 2013; 13: 31-36.
 18. Wittwer CT, Reed GH, Gundry CN, Vandersteen JG, Pryor RJ. High-resolution genotyping by amplicon melting analysis using LCGreen. *Clin Chem* 2003; 49: 853-860.
 19. Choochote W, Keha P, Sukhavat K, Khamboonruang C, Sukontason K. *Aedes (Finlaya) togoi* Theobald 1907, Chanthaburi strain, a laboratory vector in studies of filariasis in Thailand. *Southeast Asian J Trop Med Public Health* 1987; 18: 259-260.
 20. Maleewong W, Choochote W, Sukhavat K, Khamboonruang C, Arunyanart C. Scanning electron microscopic study of third-stage larva of *Wuchereria bancrofti* and *Brugia malayi* in Thailand. *Southeast Asian J Trop Med Public Health* 1987; 18: 261-264.
 21. Choochote W, Leemingsawat S, Sucharit S, Kunnaphuti J, Wongkamchai S, Sukontason KL, Jitpakdi A. *Aedes albopictus*, a potential vector of nocturnally subperiodic *Wuchereria bancrofti* and dengue type 2 virus. *Southeast Asian J Trop Med Public Health* 2001; 32: 621-624.
 22. World Health Organization. Basic laboratory methods in medical parasitology. Geneva, Switzerland. World Health Organization. 1991, p 39-61.
 23. Yen PK, Mak JW. Histochemical differentiation of *Brugia*, *Wuchereria*, *Dirofilaria* and *Breinfilaria* microfilariae. *Ann Trop Med Parasitol* 1978; 72:157-162.
 24. Chungpivat S, Taweethavonsawat P. The differentiation of microfilariae in dogs and cats using Giemsa's staining and the detection of acid phosphatase activity. *J Thai Vet Pract* 2008; 20: 47-55.
 25. Areekit S, Kanjanavas P, Pakpitchareon A, Khawsak P, Khuchareontaworn S, Sriyaphai T, Chansiri K. High resolution melting real-time PCR for rapid discrimination between *Brugia malayi* and *Brugia pahangi*. *J Med Assoc Thai* 2009; 92(suppl 3): S24-S28.
 26. World Health Organization. Global programme to eliminate lymphatic filariasis. *Wkly Epidemiol Rec* 2007; 82: 361-380.
 27. Ririe KM, Rasmussen RP, Wittwer CT. Product differentiation by analysis of DNA melting curves during the polymerase chain reaction. *Anal Biochem* 1997; 245: 154-160.
 28. Jariya P, Sucharit S. *Dirofilaria repens* from the eyelid of a woman in Thailand. *Am J Trop Med Hyg* 1983; 32: 1456-1457.