



## Rapid detection of *Dirofilaria immitis* in mosquito vectors and dogs using a real-time fluorescence resonance energy transfer PCR and melting curve analysis

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### ABSTRACT

A real-time fluorescence resonance energy transfer (FRET) PCR supplemented with melting curve analysis for the rapid molecular detection of *Dirofilaria immitis* in mosquito vectors and dog blood samples was developed. This real-time FRET PCR was based on the fluorescence melting curve analysis of a hybrid between an amplicon generated from the *D. immitis* ribosomal RNA gene sequence and specific fluorophore-labeled probes. The sensitivity, specificity, accuracy, and positive and negative predictive values of this method were all 100%. Besides being highly sensitive and specific, this PCR is fast and offers a high throughput. Therefore it is a suitable and powerful tool for the diagnosis and for epidemiological surveys of canine dirofilariasis as well as for molecular xenomonitoring of *D. immitis* in mosquito vectors.

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### 1. Introduction

The heartworm *Dirofilaria immitis* is a mosquito-borne pathogenic nematode of domestic dogs and some canids (McCall et al., 2008). The worm has also been found in seals, horses, beavers, bears, nutrias and muskrats (Mar et al., 2002). Its life cycle consists of two phases, the first in mosquitoes and the second in the definitive hosts. The sexually mature worms reside in the right side of the hearts of the definitive hosts and release microfilariae into the blood circulation. After susceptible mosquitoes ingest

the larvae from a microfilaremic host, the worms develop to the infective, third stage (L3) within 10–14 days. When the infective L3 are transmitted to the definitive host by an infected mosquito during a blood meal, the parasites develop in the subcutaneous tissue, abdomen or thorax and migrate to the pulmonary arteries by day 90–120 (McCall et al., 2008). People living in heartworm endemic areas are at risk of being infected by mosquitoes (Orihel and Eberhard, 1998). Several reports have revealed occasional infections of humans (Abadie et al., 1965; Moorhouse, 1978; Goldstein and Smith, 1985; Rodríguez et al., 2002) which is why this helminthiasis could be regarded as a parasitic zoonosis.

An important goal of the elimination programme for filariasis is the interruption of the transmission of the infection. Therefore, the availability of effective methods to monitor the presence or absence of filarial larvae in the

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mosquito vector and/or microfilaria in the definitive hosts is important. Microscopical methods for the detection of filarial larvae in mosquito vectors and/or of microfilaria in the blood circulation are labour-intensive, have a low sensitivity and require highly experienced personnel. Serological tests based on enzyme-linked immunosorbent assays and immunochromatography techniques are considered highly specific, but false negative results can occur in very light infections (McCall et al., 2008).

Conventional polymerase chain reaction (c-PCR) assays using different primers have been developed to detect filarial DNA in definitive hosts and mosquito vectors (Favia et al., 1996; Mar et al., 2002; Cancrini et al., 2003; Casiraghi et al., 2006; Lee et al., 2007). The methods are sensitive and accurate in discriminating the microfilaria of the different filarial species that infect dogs. However, all of these procedures demand analysis by agarose gel electrophoresis which is slow, has a limited throughput and has a tendency to carry-over contamination as well as illusive results. Therefore this study was conducted to develop a real-time fluorescence resonance energy transfer (FRET) PCR combined with a melting curve analysis which does not need agarose gel electrophoresis for the detection of *D. immitis* DNA in mosquito vectors and infected dog blood samples.

## 2. Materials and methods

### 2.1. Mosquitoes and experimental infection

*Aedes aegypti*, *Culex pipiens quinquefasciatus*, *Mansonia uniformis*, and *Armigeres* spp. mosquitoes were collected in urban areas of Khon Kaen Province, Northeastern Thailand. Additionally *Aedes togoi* was collected from Koh Nom Sao, Chanthaburi Province, Eastern Thailand. All mosquito larvae were taken from their breeding places and reared in an insectarium. The *Ae. aegypti*, *Ae. togoi* and *Cx. quinquefasciatus* mosquitoes were artificially infected with *D. immitis* (strain from Khon Kaen Province), nocturnally subperiodic *B. malayi* (strain from Narathiwat Province, Southern Thailand) (Maleewong et al., 1987) and nocturnally periodic *W. bancrofti* (strain from Burmese immigrant, Tak Province Northwestern Thailand) (Lulitanond et al., 2004), respectively.

An 8-year-old mixed-breed female dog naturally infected with *D. immitis* was used as microfilariae (mf) donor (54 mf/20 µl blood). Female 3–5-day-old mosquitoes (fasted for 12 h) were allowed to feed on the *D. immitis*-infected blood by membrane feeding. Briefly, a culture flask containing warm water was covered with a Parafilm membrane and filled with infected blood. The membrane feeder was placed over a netted mosquito cup and the mosquitoes were permitted to feed on the blood for 1 h. The fed mosquitoes were reared at 27 °C. To ensure infectivity, each mosquito was dissected in normal saline solution 14 days post feeding, and the number of larval worms found in each was counted under a stereomicroscope and recorded. Only infected mosquitoes were used for the experiments. After dissection, all *D. immitis* larvae, and the insect's body were mixed and put into a 1.5-ml microcentrifuge tube, labeled, and kept at –20 °C for DNA

extraction. The range, mean ± SD and median parasite number per infected mosquito were 1–10, 5.40 ± 3.23 and 5.50 larval worms, respectively.

*W. bancrofti* infected *Cx. quinquefasciatus*, *B. malayi* infected *Ae. togoi* as well as non-infected *Ae. aegypti*, non-infected *Cx. quinquefasciatus*, non-infected *Ma. uniformis* and non-infected *Armigeres* spp. were used for DNA extraction. The eluted DNA was used as the source for specificity evaluation.

### 2.2. Source of blood specimens and control DNAs for sensitivity and specificity

A total of 71 EDTA dog blood samples were obtained from the Animal Hospital, Faculty of Veterinary Science, Chulalongkorn University and the Animal Hospital, Faculty of Veterinary Science, Khon Kaen University. The specimens were microscopically examined and the presence of parasite objects was confirmed by staining a thick blood film (20 µl blood/slide) with Giemsa (World Health Organization, 1991) and by special staining for acid phosphatase activity (Yen and Mak, 1978). Out of these 71 samples, 30 were positive for *D. immitis* (mean microfilarial density ± SD = 29.73 ± 3.85; median = 17.5; range = 1–158), 6 for *Brugia pahangi*, 2 for *Trypanosoma evansi*, 1 for *Ehrlichia canis*, 2 for *Hepatozoon canis*, 1 for *E. canis* and *H. canis*, 2 for *E. canis* and *Babesia* spp., and 1 for *Babesia* spp. Twenty-six negative blood samples from healthy dogs and one negative blood sample of a healthy cat were obtained as controls. Adult *D. immitis* were recovered from heart of infected dog. All specimens were blindly labeled and further processed by DNA extraction.

One DNA specimen extracted from *Plasmodium falciparum* infected human red blood cells was received from the frozen bank, Department of Parasitology, Faculty of Medicine, Khon Kaen University.

The study protocol was approved by the Animal Ethics Committee of Khon Kaen University, based on the Ethics of Animal Experimentation of the National Research Council of Thailand (Reference No. 0514.1.12.2/50).

### 2.3. Preparation of specimens for real-time FRET PCR

Each mosquito specimen, was put in a 1.5-ml microcentrifuge tube, homogenized with disposable polypropylene pestles (Bellco Glass, Vineland, NJ), and extracted using the Nucleospin Tissue kit (Macherey-Nagel, Duren, Germany). The DNAs were eluted in 100 µl of 5 mM Tris-HCl, pH 8.5 and stored at –20 °C until analysis. 5 µl of each DNA was used per reaction. Regarding blood samples, 50 µl were used per sample for DNA extraction and purification as above.

### 2.4. Real-time FRET PCR assay

The LightCycler PCR detection and analysis systems (LightCycler 2.0, Roche Applied Science, Mannheim, Germany) were used for amplification and quantification. The reaction was performed in glass capillaries. Specific primers, *i.e.* DI-F (5' ATG ATG ATT GCT CAA TTA AGT AGA C 3') and DI-R (5' GAT AAT CTG ATC GAT ATT GAC CCT

3') (Proligo, Singapore), were designed to bind to the *D. immitis* ribosomal RNA gene sequence (GenBank accession number AF217800). This target sequence was previously used to design highly sensitive and specific primers for the detection of *D. immitis* DNA by c-PCR (Mar et al., 2002).

For amplification detection, the LightCycler FastStart DNA Master HybProbe Kit (Roche Applied Science) was used as recommended by the manufacturer. Briefly, a pair of adjacent oligoprobes was hybridized with the *D. immitis* ribosomal RNA gene. One probe had been labeled at the 5' end with the LightCycler Red 640 fluorophore (5' Red 640-GCT CGT GGA TCG ATG AAG AAC GCA GCT-Phosphate 3') (DILC640 probe) and the other had been labeled at the 3' end with 530 fluorescein (5' ATT TTT CAA TAA CTC TAA GCG GGG GAT CAC C-Flou 530 3') (DIFL530 probe) (Tib Molbiol, Berlin, Germany). Probe and primers were designed by the LC probe design software (Roche Applied Science). A schematic diagram of the hybridization analysis of the primers and probes is shown in Fig. 1. When the probes hybridized to the same DNA strand internal to the PCR primers, the probes came in close proximity and produced a FRET (Intapan et al., 2009).

The PCR mixture contained LightCycler Faststart DNA Master HybProbe (Roche Applied Science), 2 mM MgCl<sub>2</sub>, 0.5 μM DI-F primer, 0.5 μM DI-R primer, 0.4 μM DILC640 probe and 0.2 μM DIFL530 probe. The total reaction volume was 20 μl. The samples were run through 45 cycles of repeated denaturation (10 s at 95 °C), annealing (15 s at 45 °C), and extension (15 s at 72 °C). The temperature transition rate was 20 °C/s. After amplification, a melting curve was produced by heating the product at 20 °C/s to 95 °C, cooling it to 40 °C, keeping it at 40 °C for 30 s and then slowly heating it at 0.1 °C/s to 75 °C. The fluorescence intensity change was measured throughout the slow heating phase.

In order to determine the specificity of the oligonucleotide hybridization based on the FRET technique, DNA extracted from samples other than *D. immitis*-infected mosquitoes and infected dog samples were analyzed separately. Each run contained at least one negative control consisting of 5 μl distilled water.

For improved visualization of the melting temperatures ( $T_m$ ), melting peaks were derived as previously described (Thanchomnang et al., 2008). Melting curves were used to determine the specific PCR products, which were confirmed

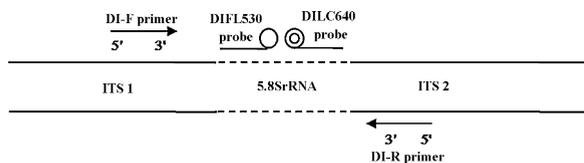


Fig. 1. Schematic illustration of the PCR primers (DI-F and DI-R primers), anchor, and detection probes hybridized with the *D. immitis* ribosomal RNA gene (AF217800). The probe DIFL530 was labeled with 530 fluorescein at the 3' end and served as anchor probe for the sensor DILC640 probe. The sensor probe was labeled with LightCycler Red 640 fluorophore (LC red 640) at the 5' end. Single circle, 530 fluorescein; double circle, LC red 640. The left and right lines indicate the internal transcribed spacer region 1 (ITS1) and internal transcribed spacer region 2 (ITS2), respectively whereas the middle dashed line indicates the 5.8S ribosomal RNA gene.

by conventional gel electrophoresis. The cycle number (Cn) indicating the target sequence copy number was presented as the number of PCR cycles needed for the fluorescence signal of the amplicons to exceed the detected threshold value. The correlation between the worm loads and the Cn was analyzed by Pearson Correlation Test.

### 2.5. *Dirofilaria immitis*-positive control plasmids

A positive control plasmid was constructed by cloning a PCR product of the *D. immitis* ribosomal DNA gene (Mar et al., 2002) into the pCR4-TOPO vector (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. The PCR products were obtained by c-PCR using the primers DI-F and DI-R. The plasmid was propagated in *Escherichia coli* and purified by using the Wizard<sup>®</sup> plus SV Minipreps kit (Promega Corporation, Madison, WI). The nucleotide sequence of the inserted gene was sequenced in both directions and revealed to be identical to the *D. immitis* ribosomal RNA gene (GenBank accession number AF217800). The size of the plasmid was 4250 bp (including the 294-bp ribosomal RNA gene sequence).

## 3. Results

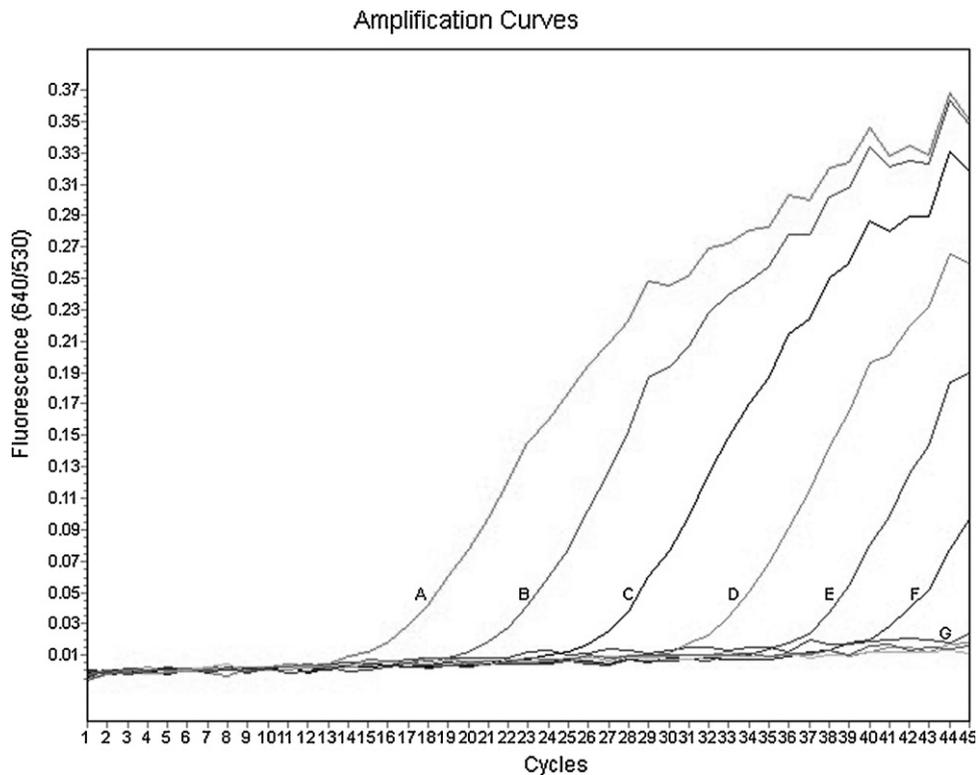
### 3.1. Standardization of the real-time PCR

The sensitivity of real-time FRET PCR was determined using 5 μl of serial dilutions (4–4 × 10<sup>8</sup> copies) of *D. immitis*-positive control plasmid in water. The detection limit of the ribosomal RNA gene target DNA sequence was 4 × 10<sup>4</sup> copies of positive control plasmid (Fig. 2) or as little as 5 × 10<sup>-3</sup> ng *D. immitis* genomic DNA (figure not shown) when considering 35 cycles as the cut-off detection limit.

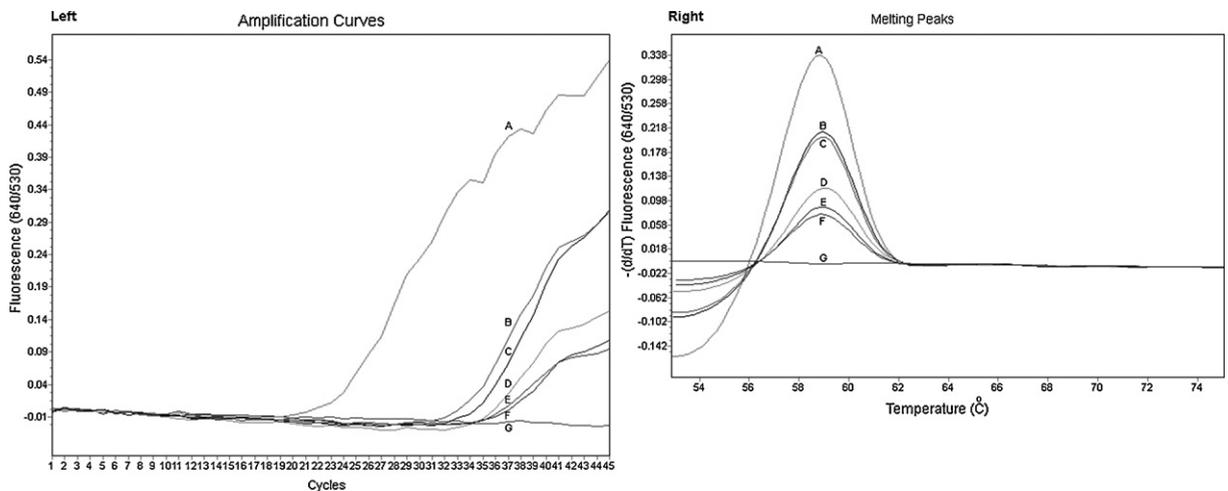
No fluorescence signal was obtained when purified DNA from *W. bancrofti*-infected *Cx. quinquefasciatus*, *B. malayi*-infected *Ae. togoi*, non-infected mosquitoes (*Ae. aegypti*, *Cx. quinquefasciatus*, *Ma. uniformis*, and *Armigeres* spp.), non-infected dog and cat blood samples, dog blood samples with infections other than *D. immitis*, or *P. falciparum*-infected human red blood cells were tested. The capability of detection was determined with each pool of 1, 5, 10, 15 and 20 uninfected *Ae. aegypti* adult mosquitoes inoculated with one infected mosquito that nourished one *D. immitis* L3. After DNA extraction and examination of the real-time FRET PCR assay, this method could detect *D. immitis* DNA from as little as one *D. immitis* larva infected in one mosquito vector spiked in 20 uninfected *Ae. aegypti* (Fig. 3).

### 3.2. Real-time FRET PCR for the detection of *D. immitis* in infected mosquitoes and dogs

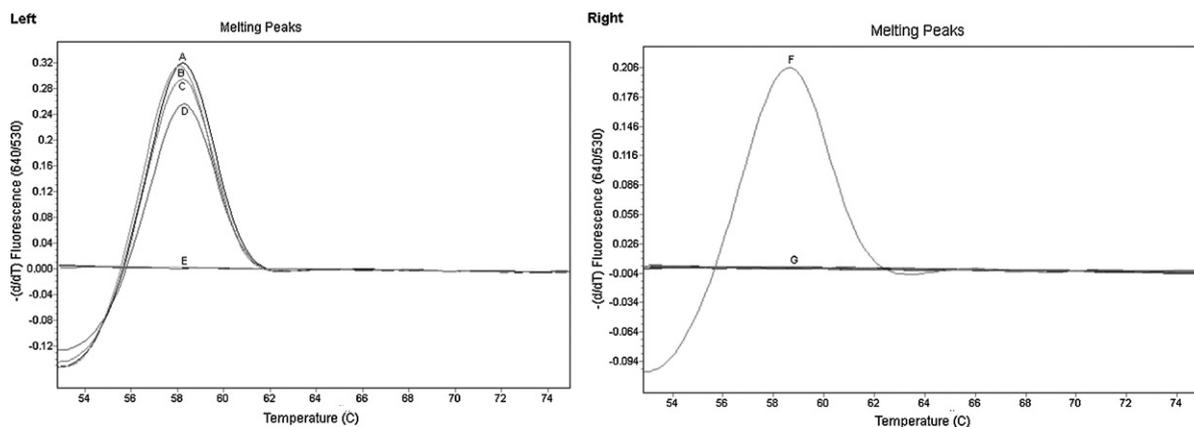
The method combined with a melting curve analysis of the amplicon products was applied to detect *D. immitis* DNA in infected mosquitoes and dogs. A total of 30 *D. immitis*-infected and 30 non-infected *Ae. aegypti* as well as 30 *D. immitis*-infected and 26 non-infected dog samples and 15 dog samples infected with other biological agents were separately analyzed. The melting curve analyses are shown in Fig. 4. When using *D. immitis*-specific primers and probes, the mean ± SD, range and the median of the  $T_m$



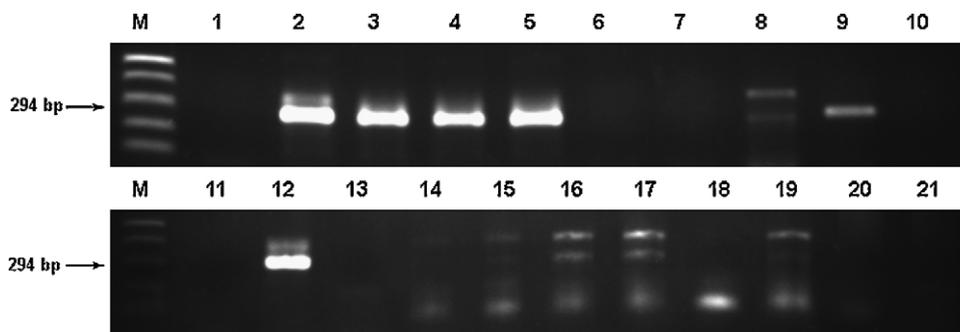
**Fig. 2.** Amplification plot of fluorescence (y-axis) vs cycle numbers (x-axis) showing the analytical sensitivity of the real-time FRET PCR for detecting *D. immitis* plasmid DNA. (A) *D. immitis*  $4 \times 10^8$  copies/reaction; (B) *D. immitis* plasmid  $4 \times 10^7$  copies/reaction; (C) *D. immitis* plasmid  $4 \times 10^6$  copies/reaction; (D) *D. immitis* plasmid  $4 \times 10^5$  copies/reaction; (E) *D. immitis* plasmid  $4 \times 10^4$  copies/reaction; (F) *D. immitis* plasmid  $4 \times 10^3$  copies/reaction; (G) *D. immitis* plasmid  $4 \times 10^2$ ,  $4 \times 10^1$  copies/reaction, and distilled water, respectively.



**Fig. 3.** Amplification plot of fluorescence (y-axis) vs cycle numbers (x-axis) showing the analytical sensitivity of the real-time FRET PCR for detecting one *D. immitis* larva harbored in one infected mosquito inoculated in pools of 1, 5, 10, 15 and 20 non-infected mosquitoes (left figure) and the representative melting curve analysis of two fluorophore-labeled probes hybridized to the amplification products of the *D. immitis* ribosomal RNA gene. The melting temperature ( $T_m$ ) of the double-stranded fragment is visualized by plotting the negative derivative of the change in fluorescence divided by the change in temperature in relation to the temperature  $[-(d/dT) \text{ Fluorescence (640/530)}]$ . The turning point of this converted melting curve results in a peak and permits identification of the fragment specific  $T_m$  (right figure). (A) *D. immitis* positive control plasmid ( $4 \times 10^7$  copies/reaction); (B)–(F) are the results of DNA extracted from one *D. immitis* larva harbored in one infected mosquito inoculated in 1, 5, 10, 15 and 20 of non-infected mosquitoes, respectively; (G) negative control containing no DNA.



**Fig. 4.** Representative melting curve analyses of two fluorophore-labeled probes hybridized to the amplification products of *D. immitis* ribosomal RNA gene. The left figure shows the melting peaks of *D. immitis* infected mosquitoes (A), *D. immitis* infected dog blood (B), a positive control plasmid (C), *D. immitis* genomic DNA (D), non-infected *Ae. aegypti*, non-infected *Cx. quinquefasciatus*, *W. bancrofti*-infected and *B. malayi*-infected mosquitoes DNA and the negative control containing no DNA (E). The right figure shows the melting peaks of a positive control plasmid (F) and *P. falciparum*-infected human red blood cells, non-infected cat and dog blood samples, *B. pahangi*-, *Babesia* spp.-, *H. canis*-, *E. canis*-, and *T. evansi*-infected dog blood DNA, respectively, as well as non-infected *Armigeres* spp. and non-infected *Ma. uniformis* (G) DNA.



**Fig. 5.** Ethidium bromide staining patterns of the PCR products on a 1% agarose gel. The arrows indicate the 294 bp *D. immitis* specific bands. Negative control containing no DNA (lanes 1 and 11); the PCR products obtained from the positive control plasmid (lanes 2 and 12); *D. immitis* genomic DNA (lane 3); *D. immitis*-infected mosquitoes (lane 4); *D. immitis*-infected dog sample (lane 5); non-infected *Ae. aegypti* (lane 6); non-infected *Cx. quinquefasciatus* (lane 7); non-infected dog blood (lane 8); *W. bancrofti*-infected (lane 9) and *B. malayi*-infected mosquitoes (lane 10); *P. falciparum*-infected human red blood cells (lane 11); *B. pahangi* infected dog blood (lane 12); non-infected cat blood (lane 13); *Babesia* spp.-infected (lane 14); *H. canis*-infected (lane 15); *E. canis*-infected (lane 16); *T. evansi*-infected dog blood samples (lane 17); non-infected *Armigeres* spp. (lane 18) and non-infected *Ma. uniformis* (lane 19); lane M, DNA size markers (1 kb plus DNA ladder from Invitrogen).

values of the *D. immitis*-infected mosquitoes were  $58.40 \pm 0.11$ ,  $58.30$ – $58.58$  and  $58.35$ , respectively and of the *D. immitis*-infected dogs were  $58.52 \pm 0.13$ ,  $58.47$ – $58.79$  and  $58.53$ , respectively. A total of 30 *D. immitis*-infected *Ae. aegypti* (Cn range 24.90–31.0; mean  $\pm$  SD =  $27.90 \pm 2.34$ ; median = 27.39) and 30 *D. immitis*-infected dog samples (Cn range 23.37–34.40; mean  $\pm$  SD =  $26.60 \pm 3.45$ ; median = 27.30) were positive by real-time FRET PCR and melting curve analysis, whereas all of the specificity control DNA was negative. The sensitivity, specificity, accuracy and positive and negative predictive values were all 100%.

For validation of this method, the DNAs from the *D. immitis*-infected mosquitoes, the *D. immitis*-infected dogs, the *D. immitis* genomic DNA, and positive control plasmids were amplified by real-time FRET PCR (Fig. 5, lanes 2–5), to verify the presence of the prominent 294-bp product, whereas genomic DNA from other specificity control DNAs were not amplified. However, when the genomic DNAs from non-infected dog samples (Fig. 5, lane 8), *W. bancrofti*-infected *Cx. quinquefasciatus* (Fig. 5, lanes 9), dog blood

infected with *B. pahangi* (Fig. 5, lane 14), *Babesia* spp. (Fig. 5, lane 16), *H. canis* (Fig. 5, lane 17), *E. canis* (Fig. 5, lane 18), *T. evansi* (Fig. 5, lane 19) and non-infected cat blood (Fig. 5, lane 15) were amplified the faint bands but no specific fluorescence signal was detected during melting peak analysis. Significant correlations between the Cn and the microfilarial load in *D. immitis*-infected dog bloods ( $R = -0.553$ ;  $P < 0.05$ ) and between the Cn and the larval load in infected mosquitoes ( $R = -0.591$ ;  $P < 0.05$ ) were revealed. These results show that real-time FRET PCR can present information on larval densities in canine blood and in vectors.

#### 4. Discussion

With regards to animal health, the canine and feline heartworm disease caused by adult *D. immitis* worms is severe and potentially fatal. Laboratory diagnosis of the disease can be done using blood tests that detect circulating microfilariae or adult antigen, however other

diagnostic tests are needed to investigate the severity of the disease and treatment options (McCall et al., 2008). Molecular methods to detect filarial DNA are sensitive and accurate tools for the differential diagnosis and are helpful in cases of morphological abnormalities of the microfilariae. Moreover, molecular procedures are useful for the survey of filarial infections in vectors (Lee et al., 2007) and have potential for mapping endemic areas of diseases. These methods can be used for surveillance based control of arthropods and for the management of such vector-borne diseases.

Recently, quantitative real-time PCR was reported for detection of *D. repens* in Austrian dogs (Duscher et al., 2009). The real-time FRET PCR, a variation of the real-time PCR, is being used effectively for the rapid molecular detection of *W. bancrofti* and/or *B. malayi* DNAs in mosquito vectors (Lulitanond et al., 2004; Thanchomnang et al., 2008; Intapan et al., 2009). Here, we reported for the first time a real-time PCR based on two pairs of specific primers and individually labelled hybridization probes merged with melting curve analysis for the detection of *D. immitis* infection in mosquito vectors and dogs. As little as one single *D. immitis* L3 infection of one mosquito mixed in 20 uninfected *Ae. aegypti* and one microfilaria in 50 µl of dog blood sample could be detected. These results present a high sensitivity and suggest that this method can be applied for the field detection of *D. immitis* in dogs as well as for molecular xenomonitoring of mosquito vectors, even in regions with a low intensity level of infection. Nevertheless, this method gave negative results with 3 dog blood samples that antigen positive-negative microfilaria for *D. immitis* (data not shown). The method also had 100% specificity. No fluorescence was documented when a large range of control DNA from other parasites and from intermediate and definitive hosts was investigated. However, we need further investigation to test against *D. repens* DNA for make sure the specificity of procedure.

The real-time FRET PCR provides an alternative to the available classic methods and more modern molecular or immunologic procedures for *D. immitis* detection in vectors and dogs. Although detection of *D. immitis* DNA is an indirect method of filarial detection, it does supply data on the *D. immitis* epidemiology. The entire procedure is rapid and provides a high throughput since there is no need for presenting the amplicons with agarose gel electrophoresis. A large number of samples can be examined at the same time and only very small sample volumes are needed for analysis. The method also abolishes the requirement for the microscopic examinations by experienced personnel.

In conclusion, a rapid, specific, and sensitive real-time FRET PCR for the detection of heartworm infection in mosquito vectors and in animal blood is presented here. This method is a suitable and powerful tool not only for the diagnosis and for epidemiological surveys of canine dirofilariasis but also for molecular xenomonitoring of *D. immitis* in mosquito vectors. As survey results of heartworm disease are not promptly available in Asian countries (McCall et al., 2008), the method presented in this study could potentially support surveys on prevalence and distribution of *D. immitis* in this area.

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