**Rapid Detection of *Brugia malayi* in Mosquito Vectors Using a Real-time Fluorescence Resonance Energy Transfer PCR and Melting Curve Analysis**

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Abstract. We developed real-time fluorescence resonance energy transfer (FRET) polymerase chain reaction (PCR) combined with melting curve analysis for detection of *Brugia malayi* DNA in blood-fed mosquitoes. Real-time FRET PCR is based on a fluorescence melting curve analysis of hybrid formed between amplicons generated from a family of repeated DNA element, 153-bp *Hha*I repeated sequence, specific to genus *Brugia* and specific fluorophore-labeled probes. The *B. malayi*-infected mosquitoes were differentiated from *Wuchereria bancrofti*-infected and uninfected mosquitoes and from genomic DNA of *Dirofilaria immitis*– and *Plasmodium falciparum*-infected human red blood cells and human leukocytes by their melting temperature. Sensitivity and specificity were both 100%. Melting curve analysis produces a rapid, accurate, and sensitive alternative for specific detection of *B. malayi* in mosquitoes, allows high throughput, and can be performed on small samples. This method has the potential for endemic area mapping or monitoring effect of brugian filariasis mass treatment programs.

**INTRODUCTION**

Lymphatic filariasis, a mosquito-borne disease, is a major public health problem, particularly in the tropics and subtropics. It is caused by the nematodes *Wuchereria bancrofti*, *Brugia malayi*, and *Brugia timori*.1,2 Symptoms include acute fever and chill and chronic lymphedema and hydroceles. Bru- gian filariasis caused by *B. malayi* and *B. timori* affects ~13 million people, mainly in India and Southeast Asia.3 A principal goal of the Global Program to Eliminate Lymphatic Filaria (GPELF) is interruption of the transmission of infection.2 Hence, the availability of efficient and effective diagnostic tools to monitor the presence or absence of filarial larvae in the mosquito vector is particularly important.4 Entomologic methods for the detection of filarial larvae in mos- quito vectors are based on the dissection of mosquitoes. How- ever, these methods are laborious, tedious, and time consum- ing and carry a low sensitivity and a need for specially trained microscopists.

Many conventional polymerase chain reaction (c-PCR) assays have been developed to detect filarial DNA in human blood and mosquito vectors.5-6 All of these procedures require agarose gel electrophoresis for the analysis. However, determination by gel electrophoresis is slow, has a limited throughput, and is prone to carry-over contamination and subjective results. Recently, effective real-time PCR has greatly improved molecular detection and differential diagnosis of microorganisms belonging to the same genus and has increasingly replaced c-PCR. Effective real-time PCR is not only accurate, sensitive, fast, and can quantify specific DNA in biologic samples,7 but it also differentiates species or strains of several medically pathogenic microorganisms by melting curve analysis.5,8-10 Moreover, this method provides a high-throughput means because it does not need agarose gel electrophoresis for analysis of the amplicons and has a broad dynamic range.11 The method has great potential for epide- miologic studies and for monitoring elimination programs of infectious agents. Recently, *W. bancrofti* and *Brugia* spp. DNA have been shown in infected blood and in infected mos- quito vectors by either a Taqman probe or an Eclipse minor groove binding probe based on real-time PCR.12,13 In contrast to the reports by Rao and others5,9,12 and Fischer and others,13 another assay format using real-time fluorescence resonance energy transfer (FRET) PCR combined with a melting curve analysis has been reported by our group for *W. bancrofti* DNA detection in mosquito vectors.10 This format can be used for a differential detection of *W. bancrofti* DNA from DNA of *Dirofilaria immitis* and *B. malayi* and from human and mosquito vectors. However, *B. malayi* DNA detection based on this technique is still not feasible. Here, we have used specific primers amplifying the repetitive sequence, *Hha*I repeat, of *B. malayi* (GenBank accession no. M12691)15 and developed a genus specific and sensitive real-time FRET PCR using a LightCycler-based PCR system (Roche Applied Science, Mannheim, Germany) for the differential detection of larval stages of *B. malayi* in mosquitoes. Two individually fluorophore-labeled specific hybridization probes were used, and melting point profiles were compared with the effects gained from other control DNA.

**MATERIALS AND METHODS**

**Mosquitoes.** *Aedes togoi* mosquitoes, a mosquito species from Koh Nom Sao, Chanthaburi Province, Eastern Thai- land,16 were artificially infected with *B. malayi*. The mosquito larvae were taken from their breeding places and reared in the insectarium.

**Blood sources for mosquitoes.** Blood infected with microfi- lariae of nocturnally subperiodic *B. malayi* was obtained from an infected cat. The worms were originally taken from a 20- year-old woman in Bang Paw District, Narathiwat Province, Southern Thailand, and used to experimentally infect domestic cats and are now kept in the Department of Parasitology, Faculty of Medicine, Chiang Mai University. The mainte- nance and care of animal experiments in this study complies with the current Thai laws.

**Infection of mosquitoes.** The *B. malayi*-infected mosqui- toes were obtained as previously described.17 Briefly, blood was drawn from the femoral vein of the infected cat, and
heparin was added at a concentration of 10 units/mL blood. Next, 3- to 5-day-old female Aedes togoi mosquitoes (fasted for 12 hr) were allowed to feed on the heparinized blood using an artificial membrane feeding technique as described previously.\(^1\) To ensure infectivity, the mosquitoes were dissected in normal saline solution 14 days after feeding, and the number of larvae was counted under a dissecting microscope. Only infected mosquitoes were used for the experiments, and the number of parasites was recorded. After dissection, all B. malayi larvae and the body of the individual infected mosquito were mixed and placed in a 1.5-mL microcentrifuge tube, labeled, and kept at \(-20^\circ\text{C}\) for DNA extraction. The range and mean \(\pm\) SD of the number of parasites per infected mosquito were 1–42 and 7.06 \(\pm\) 10.35 larvae, respectively.

**Source of DNA for specificity evaluation.** Adult worms of *D. immitis* from infected dogs (from Khon Kaen Province), uninfected *Ae. togoi*, uninfected *Culex pipiens quinquefasciatus*, and *Plasmodium falciparum*-infected human red blood cells and human leukocytes were separately extracted and purified using the Nucleospin Tissue kit (Macherey-Nagel, Duren, Germany) according to the manufacturer’s recommendations. The DNA was resuspended in 100 \(\mu\)L of 5 mmol/L Tris-HCl, pH 8.5, and used for specificity evaluation. The *Cx. quinquefasciatus* mosquitoes infected with the nocturnally periodic *W. bancrofti* (from a Burmese immigrant, Tak Province, northwestern Thailand), obtained as previously described,\(^1\) were also used for DNA extraction as described above. The range and mean \(\pm\) SD of the parasite load per infected mosquito were 1–18 and 5.36 \(\pm\) 5.16 parasites, respectively.

**Preparation of specimens for real-time FRET PCR.** Each specimen, infected or uninfected, was put in a 1.5-mL microcentrifuge tube and homogenized with disposable polypropylene pestles (Bellco Glass, Vineland, NJ), followed by extraction using the Nucleospin Tissue kit (Macherey-Nagel). The DNA was eluted in 100 \(\mu\)L of 5 mmol/L Tris-HCl, pH 8.5, of which 5 \(\mu\)L were used in the real-time PCR reaction.

**Real-time FRET PCR assay.** The LightCycler PCR and detection system (Roche Applied Science, Mannheim, Germany) was used for amplification and quantification. PCR was performed in glass capillaries. A specific primer pair, BM-F (5’-TCATTAGACAAAGGATTGGTTC-3’) and BM-R (5’-TTAAGACTAAAATGACAACACA-3’) (Tib Molbiol, Berlin, Germany), was designed to bind to the *Hha* repetitive sequence of the *B. malayi* genome as described previously (GenBank accession no. M12691).\(^1\) The reasons of using this target sequence were 1) the sequence is present in high copy number in the *B. malayi* genome (\(>7,000\) copies per haploid genome) and 2) several c-PCR tests for the detection of *Brugia* DNA have used this sequence as a target, and no sequence variation has been shown in different strains of *B. malayi* and *B. timori*.\(^2\) For amplification detection, the LightCycler FastStart DNA Master HybProbe Kit was used as recommended by the manufacturer. Briefly, a pair of adjacent oligoprobes was hybridized to an internal genus-specific repetitive sequence of *B. malayi*. One probe was labeled at the 5’ end with the LightCycler Red 705 fluorophore (5’-Red-705-TGGTACACGTAGTGCTGTGCTGTA-Phosphate-3’; BMLC705 probe), and the other was labeled at the 3’ end with 530 fluoroscein (5’-AAATTAATTTGACTATGGTATCGGA-Flou 530-3’; BMFL530 probe; Tib Molbiol). Probes and primers were designed by using the LC probe design software (Roche Applied Science). Schematic diagram of the hybridization analyzes used in the assay is shown in Figure 1.
gen, Carlsbad, CA), according to the manufacturer’s instructions. The PCR products were obtained by c-PCR using primers BM-F (5’-TCATTAGCAAGGATATTGGTTC-3’/H11032) and BM-R (5’-TTTAACTATAAAATGACAACACA-3’/H11032) (Tib Molbiol). The plasmid was propagated in Escherichia coli, and the nucleotide sequence of the inserted gene was sequenced in both directions. The nucleotide sequence of the cloned HhaI repeat revealed an identical structure to the B. malayi genome (GenBank accession no. M12691). The size of the plasmid was 4,109 bp (including the 153-bp HhaI sequence).

RESULTS

Standardization of the real-time FRET PCR. Five microliters of serial dilutions (4 to 4×10^{10} copies) of B. malayi–positive control plasmid in water was tested to assess the sensitivity of hybridization real-time PCR. When using cycle numbers of 35 as the cut-off limit of detection, the reliable detection limit of the HhaI repeat target DNA sequence was ∼4×10^2 copies of positive control plasmid (Figure 2). No fluorescence signal was obtained when purified DNA from W. bancrofti–infected Cx. quinquefasciatus, D. immitis, uninfected Ae. togoi and Cx. quinquefasciatus, P. falciparum–infected human red blood cells, or human leukocytes were tested.

To determine the capability of detection by real-time FRET PCR, each pool of 10, 30, 60, and 100 uninfected Ae. togoi adult mosquitoes inoculated with one infected mosquito that harbored one B. malayi larva were used. These samples were used for DNA extraction and examined the real-time FRET PCR assay. This method could detect filarial DNA of as little as one B. malayi larva infected in one mosquito inoculated in 100 uninfected Ae. togoi (data not shown).

Real-time FRET PCR to detect B. malayi in mosquitoes. We used the Brugia-specific DNA sequence Hhal repeat to detect B. malayi in infected mosquitoes using the real-time FRET PCR assay combined with a melting curve analysis of the PCR product. A total of 30 B. malayi–infected Ae. togoi, 30 uninfected Ae. togoi, and 30 W. bancrofti–infected Cx. quinquefasciatus were separately determined. The real-time PCR could detect as little as one larva in a single mosquito. The melting curve analysis is shown in Figure 3. When using B. malayi–specific primers and probes, the mean ± SD of Tm values of B. malayi was 57.31 ± 0.07 (N = 30), whereas neither Tm value was shown in W. bancrofti–infected and unin-
fected mosquito groups or other control DNAs, respectively. A total of 30 B. malayi–infected Ae. togoi were positive by melting curve analysis, whereas all 30 uninfected Ae. togoi and 30 W. bancrofti–infected C. quinquefasciatus were negative by melting curve analysis. The sensitivity and specificity were both 100%.

All B. malayi–infected mosquitoes and positive control plasmids were amplified by real-time PCR (Figure 4, Lanes 2–7) and showed the prominent 153-bp product, whereas genomic DNA from uninfected Ae. togoi (Figure 4, Lane 8), W. bancrofti–infected (Figure 4, Lanes 9–12) and uninfected (Figure 4, Lane 13), Cx. quinquefasciatus, and P. falciparum–infected human red blood cells (Figure 4, Lane 15) and human leukocytes (Figure 4, Lane 16) were amplified the faint bands. An ~300-bp amplification product was seen with D. immitis DNA (Figure 4, Lane 14). A significant correlation between cycle number and parasite load in mosquito controls is shown in Figure 5 ($R = -0.582; P < 0.01$). These results showed that real-time PCR can provide data on parasite densities in vectors.

**DISCUSSION**

Molecular xenomonitoring in mosquito vectors is another diagnostic choice for the GPELF. It can be used for detecting lymphatic filariasis-endemic areas and selecting regions for inclusion in the program. Although the classic method for the detection of filarial parasites in mosquitoes, dissection, is an inexpensive method, it requires technicians well trained in the identification of larvae in dissected mosquitoes. This classic method of dissection becomes increasingly inefficient as the prevalence of infection in the vector population decreases, as well as impractical for regions with low vector infection rates after mass drug administrations. c-PCR has previously been suggested as a promising tool for monitoring the work progress of eliminating lymphatic filariasis. Real-time PCR provides additional value to these procedures because it is highly specific and sensitive, allows a high throughput, and can be done on very small samples. In this study, we developed a real-time FRET PCR combined with a melting curve analysis to detect B. malayi DNA in infected mosquitoes. Two primers were used to produce the genus-specific amplicon, which was subsequently shown by its combined melting peak profile with two hybridization fluorophore-labeled probes. The method can detect as little as one filarial larva harbored in one infected mosquito inoculated in one pool of 100 uninfected mosquitoes. The result showed high sensitivity of method and possibly can be useful to apply for detection in field samples. In addition, this report described the first use of real-time FRET PCR for detection of B. malayi in mosquito vectors.

This assay differentiated B. malayi DNA in infected vectors from DNA of D. immitis and W. bancrofti. However, faint bands were shown with other control DNA (Figure 4, Lanes 8–13, 15, and 16) and an ~300-bp amplification product was seen with D. immitis DNA (Figure 4, Lane 14). This does not cause a problem because no specific fluorescence signal was shown during melting peak analysis. The above described technique showed a 100% sensitivity and specificity, and merging it with a melting curve analysis offered a rapid and reliable procedure for differentially identifying lymphatic filariasis. Moreover, the real-time FRET PCR used for amplifying the repetitive sequence, HhaI repeat, of B. malayi (GenBank accession no. M12691) showed a correlation between the cycle numbers and the parasite numbers in the mosquitoes (Figure 5). This information suggests a quantitative result; however, results should be interpreted carefully because PCR can not differentiate between parasite stages, and the amount of DNA may vary between stages. Nevertheless, our assay represents another useful choice for the detection of Brugia DNA.

Recently, Fischer and others showed the persistence of B. malayi DNA in vector and non-vector mosquitoes by Taqman probe or an Eclipse minor groove binding probe based on real-time PCR. This does not pose a problem if xenomonitoring is done selectively in vectors only. For epidemiologic studies and eradication programs, confirmation assays detecting Brugia DNA in the blood of the population at risk and transmission monitoring of vectors need to be done. Al-

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**Figure 4.** Ethidium bromide staining patterns of the PCR products on a 1.5% agarose gel. Arrow indicates 153-bp B. malayi–specific band. Lane 1, negative control containing no DNA; Lane 2, PCR products obtained from positive control plasmid; Lanes 3–7, PCR products obtained from B. malayi–infected mosquitoes; Lane 8, PCR products from uninfected Ae. togoi; Lanes 9–12, PCR products from W. bancrofti–infected mosquitoes; Lane 13, PCR product from uninfected Cx. pipiens quinquefasciatus; Lane 14, PCR product from D. immitis; Lane 15, PCR product from P. falciparum–infected human red blood cells; Lane 16, PCR product from human leukocyte genomic DNA; Lane M, DNA size markers (1 kb plus DNA ladder from Invitrogen).

**Figure 5.** The number of larvae (y-axis) plotted against the cycle numbers (x-axis). Negative correlations were observed between the number of larvae and cycle numbers (Spearman rank correlation test, $R = -0.582; P < 0.01$).
through *Brugia* DNA detection is only an indirect measure of transmission, it does provide information on rates of lymphatic filarial transmission or the potential of transmission.

In summary, this study shows that real-time FRET PCR and melting curve analysis are sensitive and specific methods for *Brugia* DNA detection. The assay can be used to screen mosquito vectors for mapping areas of endemicity or human blood specimens for diagnosis. The method should be helpful for evaluating changes in transmission after mass drug administration and should be an important diagnostic tool for the GPELF.

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