REAL-TIME PCR WITH HIGH-RESOLUTION MELTING ANALYSIS FOR DIAGNOSIS OF LYMPHATIC FILARIASIS

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ABSTRACT

Lymphatic filariasis (LF), or elephantiasis, is caused by the filarial nematodes Wuchereria bancrofti, Brugia malayi, and B. timori. Over a billion people are at risk of contracting the disease, and about 40 million people suffer from severe disfigurement and disability as a result. Diagnosis is an essential element in the management of the disease, both at the individual patient care level and at the level of disease control in endemic populations. The success of LF elimination programs has lowered the levels of microfilariae in blood samples, which in turn has reduced the sensitivity of the conventional microfilariae detection method. In this study, we used real-time PCR with high-resolution melting analysis (HRM) to detect and identify B. malayi and W. bancrofti in human blood samples. The assay can be performed using a single pair of primers targeting the mitochondrial partial 12S rRNA genes of these filarial parasites. Amplification and detection steps can be conducted in the same reaction, and no post-PCR processing is required. After assessing 30 blood samples, the HRM assay exhibited a specific melting temperature for each species, as well as high sensitivity and specificity compared with the microfilaria-detection method. Thus, the assay could be helpful for the diagnosis of LF.

Keywords: Brugia malayi, Wuchereria bancrofti, real-time PCR, high-resolution melting, lymphatic filariasis

INTRODUCTION

Lymphatic filariasis (LF), or elephantiasis, is caused by three species of thread-like filarial nematodes: Wuchereria bancrofti, Brugia malayi, and Brugia timori. Of these, 90% of all infections result from W. bancrofti, with 10% attributed to Brugia malayi and Brugia timori (Ravindran et al., 2003). The disease is transmitted by mosquito vectors; when an infected mosquito bites, the infective-stage larvae (L3) migrate into the lymphatic system; on reaching sexual maturity after 6 to 12 months, the adult female worms release millions of microfilariae into the bloodstream (Garcia, 2007). The life cycle is completed when these microfilariae are ingested by new mosquito vectors (Semnani and Nutman, 2004).

The WHO estimates that over a billion people in more than 80 countries are at risk of contracting LF and over 120 million people have already been infected with the disease, with about 40 million people suffering from severe disfigurement and disability (WHO, 1992).

Although it is easy to recognize classical obstructive filariasis, many infected individuals do not show signs or symptoms. Laboratory confirmation of the infection is necessary for the management of the disease, both at the level of individual patient care and for disease control in endemic populations. Thick blood smear staining is the conventional method for demonstrating and identifying microfilariae

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circulating in blood. This method confers diagnostic specificity, but low sensitivity in people with minimal microfilarial counts. The method is also unable to diagnose those who are not microfilaremic (Weil et al., 1997).

In 1997, the World Health Organization (WHO) established a global programme to eliminate lymphatic filariasis (GPELF), which aims to interrupt the transmission of LF through mass drug administration. Mass drug treatment reduced the prevalence of LF infection to levels at which it is assumed transmission can no longer be sustained (Molyneux et al., 2002). In an address to the 7th GAELF mapping the worldwide distribution of LF, Engels indicated that 538.6 million people were treated in 2010-2011 (Engels et al., 2012). The WHO databank of PCT reported that, as of 2011, 95.9% of the endemic populations were treated with diethylcarbamazine and/or albendazole (WHO, 2013).

The success of GPELF has decreased the prevalence of LF and lowered levels of microfilariae in blood samples, which in turn has reduced the sensitivity of the microfilaria-detection method. Furthermore, in places outside endemic areas, filarial infections are sporadically diagnosed in refugees and immigrant workers. Long-term residents in endemic areas, such as the armed services, students, and volunteers, are also intermittently diagnosed with the disease. Diagnosis of filarial infections among these individuals requires both strong clinical suspicion and expert training in infrequently practiced parasitological methods (Fink et al., 2011). Due to these limitations, DNA-based methods may be more efficient for the diagnosis of LF infection.

In the present study, we applied a real-time PCR with high-resolution melting analysis (HRM) targeting the mitochondrial partial 12S rRNA gene, which is highly conserved and contains genus- and species-specific sequence variations to detect and identify B. malayi and W. bancrofti in human blood samples.

MATERIALS AND METHODS

Ethical approval

The study protocol was approved by the Human Research Protection Unit of the Faculty of Medicine Siriraj Hospital, Mahidol University, based on the Ethics of Human Experimentation of the National Research Council of Thailand (Certificate of Approval number Si285/2013).

Source of study

Positive and negative controls

Microfilariae of Brugia malayi and Wuchereria bancrofti were used as positive controls in the present study. Blood samples obtained from healthy human subjects served as negative controls.

Study samples

Microfilaria-positive blood samples were obtained from 10 patients infected with B. malayi and 5 patients infected with W. bancrofti, and were used as study samples. Fifteen blood samples obtained from malaria-infected and healthy persons were also used to study the specificity of the assay.

Extraction of DNA from whole blood

200µL of blood from capillary tubes were used for DNA extraction. A High Pure PCR Template Preparation Kit (Roche Germany) was used to extract DNA, while DNA concentration was measured by Nanodrop (Thermo Fisher Scientific) according to the manufacturer’s instructions. The DNA was used for the HRM real-time PCR.

Primers

Previously designed primers targeting the mitochondrial partial 12S rRNA gene, which is highly conserved and contains genus-and species-specific sequence variations, were used in this study (Wongkmachai et al., 2013). It was found that the primer sequence also recognized a 111-bp region of the mitochondrial partial
12S rRNA gene of W. bancrofti. A BLAST search (www.ncbi.nlm.nih.gov/BLAST/) was performed to check the specificity of the primers to their respective DNA targets. Figure 1 shows the primers that are based on alignments of the mitochondrial partial 12s rRNA genes of B. malayi (GenBank accession number AJ544843; positions 177-287) and W. bancrofti (GenBank accession numbers NC_016186.1; positions 7619-7729).

**Sensitivity and reproducibility of the real-time PCR with HRM analysis**

The detection limit of the assay was established. The lowest levels of B. malayi and W. bancrofti were determined in 10-fold serial dilutions of genomic DNA. To verify the reproducibility of the assay, DNA from each species was tested 5 times within the same run (intra-assay variation) and on 3 different runs on different days (inter-assay variation). Mean, standard deviation (SD), and the coefficient of variance (%CV) were calculated.

**Real-time PCR with HRM analysis for diagnosis of lymphatic filarial infection in blood samples**

A real-time PCR with HRM analysis was performed in a single run on a LightCycler LC480 instrument (Roche Diagnostics, Germany). A 10µL reactive mixture was generated, which consisted of (a) 3µL of DNA, (b) 0.25pmol of the forward and reward primers, and (c) 5µL of the LightCycler 480 High-Resolution Melting Master mixture containing Reso Light dye and 3mM MgCl₂ in PCR grade water. DNA extracted from normal blood samples was used as the negative control. Positive controls for B. malayi and W. bancrofti were performed in each run. The reaction conditions included an activation step at 95°C for 5min, followed by a 40-step amplification of 10s at 95°C, 10s at 58°C, and 10s at 72°C. Subsequently, the products were heated to 95.8°C for 1 min and then cooled to 40°C for 1 min. HRM was performed from 65°C to 95°C, rising at 1°C/s with 25 acquisitions per degree. The final cooling step was 40°C for 10s. Melting curves were generated, normalized, temperature-shifted, and converted to difference plots by LightCycler 480 gene-scanning software (Roche Applied Science, Germany).

For the study’s blood samples, the DNA from 15 microfilaremic human blood samples as well as 15 human blood samples obtained from patients infected with malaria (P. vivax and P. falciparum), and healthy subjects, were amplified and the resulting amplicons were analyzed.

**RESULTS**

Compared with thick blood-smear staining, the sensitivity, specificity, positive predictive value (PPV), and negative predictive value...
(NPV) were all 100%.

For intra-assay variation, Tms of B. malayi and W. bancrofti revealed the mean±SD of 76.26±0.01 and 77.35±0.03°C with %CV at 0.02% and 0.18% respectively. The inter-assay variation, Tms of B. malayi and W. bancrofti showed the mean±SD at 76.23±0.01 and 77.28±0.19°C, with %CV at 0.19% and 0.21%, respectively.

Figure 1 presents alignments of the mitochondrial partial 12s rRNA genes of B. malayi (GenBank accession number AJ544843; positions 177-287) and W. bancrofti (GenBank accession numbers NC_016186.1). Figure 2 shows representative melting peaks (Tms), normalized difference curves, and the normalized and temperature-shifted difference plot of the amplified product of the study.

**Fig. 2** Melting peaks (a, b), normalized difference curves (c, d), and the normalized and temperature-shifted difference plot (e, f) of the amplified product of W. bancrofti and B. malayi, obtained by LightCycler® 480 gene-scanning software; 7-13 represent study blood samples and control samples (Bm, B. malayi and Wb, W. bancrofti).
samples, obtained by LightCycler® 480 gene-scanning software.

The Tm samples identified as B. malayi showed mean + SD as 76.32 ± 0.06, range as 76.24-76.41, and median as 76.34. The Tm of the samples identified as W. bancrofti indicated the mean ± SD as 77.17 ± 0.41, range as 76.73-77.57, and median as 77.37. Table 1 shows the results of the real-time PCR with HRM analysis of all study samples.

**DISCUSSION**

LF diagnosis usually depends on clinical features, history of exposure in endemic areas, and laboratory findings. Diagnostic tests performed in the laboratory include microfilarial detection in peripheral blood and serological detection (Mondal, 2012).

The detection of microfilariae in night blood samples by thick blood smear staining procedure, Knott’s concentration procedure, or membrane filtration technique through polycarbonate filters, have been used for a long time. Nevertheless, these traditional parasitological methods have low sensitivity and are unable to identify the disease in non-microfilaremic individuals (Chanteau et al., 1991). The current success of the global program to eliminate LF has decreased microfilarial levels, in turn decreasing the sensitivity of microfilarial detection. Moreover, the migration of workers from LF-endemic areas is increasing (Fink et al., 2011), potentially carrying the parasites to non-endemic areas (Poole and Williams, 1990; Yen and Mak, 1982).

Recently, the nucleic acid detection technique by polymerase chain reaction (PCR) procedure has gained popularity. PCR can demonstrate the presence of parasitic DNA and is now the most sensitive technique in use (Lizotte et al., 1994; Bockarie et al., 2000; Kluber et al., 2001; Fischer et al., 2002; Goodman et al., 2003; Helmy et al., 2004; Rao et al., 2006). Both conventional PCR and real-time PCR assays have been developed for the detection and identification of many filarial species. In LF, the PCR-based assay offers a direct indication of active infection (Rahmah et al., 2010). These assays have shown great promise for high-level sensitivity and specificity (Fink et al., 2011).

The present study used a real-time PCR with HRM analysis to detect and identify two LF infections in blood samples: W. bancrofti and B. malayi. This PCR assay has been found to have equal or higher sensitivity compared with conventional parasitological methods. This assay also demonstrated high specificity, since no false positives were detected in any blood sample obtained from malaria-infected

<table>
<thead>
<tr>
<th>Blood sample</th>
<th>Number</th>
<th>Amplification curve</th>
<th>Tm range (°C)</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. malayi mf positive</td>
<td>10</td>
<td>Positive</td>
<td>76.32 – 76.41</td>
<td>B. malayi</td>
</tr>
<tr>
<td>W. bancrofti mf positive</td>
<td>5</td>
<td>Positive</td>
<td>76.73 – 77.57</td>
<td>W. bancrofti</td>
</tr>
<tr>
<td>Malaria positive</td>
<td>5</td>
<td>Negative</td>
<td>-</td>
<td>Negative</td>
</tr>
<tr>
<td>Normal</td>
<td>10</td>
<td>Negative</td>
<td>-</td>
<td>Negative</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>30</td>
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</tbody>
</table>

Mf = microfilariae, Tm = melting temperature
Compared with conventional PCR, a real-time PCR with HRM has distinct advantages; the amplification and detection steps can be conducted in the same reaction tube or well, and no post-PCR processing is required (Dobrowolski et al., 2009; Nettuwakul et al., 2010).

A real-time fluorescence resonance energy transfer (FRET) PCR combined with a melting curve analysis has been developed for differentiating B. malayi and B. pahangi DNA in blood samples. The FRET PCR used one set of primers and fluorophore-labeled hybridization probes (Thanchomnang et al., 2010). The difference from FRET is that no probe is required in a real-time PCR with HRM analysis. The master mix used in the HRM analysis contains a DNA-binding dye that stains target sequences and results in sharp melting signals that are analyzed using the gene scanning software in the HRM instrument (Dobrowolski et al., 2009). This means that both parasites can be detected and identified using a single pair of primers.

Compared with conventional parasitological methods, the PCR assay had equal or a higher sensitivity. Using a molecular technique reveals a higher prevalence of filarial infections compared with the levels detected from a thick blood smear (Albrechtova et al., 2011; Wongkamchai et al., 2014).

In conclusion, the sensitivity and specificity of this assay make it a potentially useful tool for the efficient diagnosis of LF in blood samples. The method also uses a smaller amount of blood, which could be collected from a finger prick. Since no refrigeration is required, samples may be transported by mail from endemic areas to a laboratory where the PCR assay may be performed. Furthermore, the assay may be utilized for the diagnosis of LF outside disease-endemic areas, where the equipment and practical skills required for the PCR procedure are available. The PCR does not require specialized knowledge of parasite morphology or classification, and can be run by laboratory personnel with generalized training (Fink et al., 2011). In addition to the sensitivity and specificity factors, cost-effectiveness is another essential component of any diagnostic technique. The HRM real-time PCR procedure is cost-effective, since it requires no expensive probe, and a large number of samples can be identified in a single run.

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