REVIEW

DNA-BASED DIAGNOSIS OF LYMPHATIC FILARIASIS

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Abstract. Lymphatic filariasis (LF) is still a major public health problem. The disease is ranked by the World Health Organization (WHO) as the second leading cause of permanent and long-term disability, and has been targeted for elimination by 2020. Effective diagnosis LF is required for treatment of infected individuals, for epidemiological assessment and for monitoring of the control program. Conventional diagnosis of LF depends on detection of microfilariae (Mf) in blood specimens, which has low sensitivity and specificity. Detection of specific circulating filarial antigens is regarded by WHO as the ‘gold standard’ for diagnosis of LF. However, the limitations of the antigen tests are cost and inconsistent availability. Although anti-filarial IgG4 antibody levels are associated with active LF infections, however, cross-reactivity with other filarial parasites is common. Not as sensitive as antigen tests, DNA-based techniques have been developed to diagnose and differentiate filarial parasites in humans, animal reservoir hosts, and mosquito vectors. These include DNA hybridization, polymerase chain reaction (PCR) amplification using specific primers (eg Ssp I repeat, pWb12 repeat, pWb-35 repeat, and LDR repeat for Wuchereria bancrofti and Hha I repeat, glutathione peroxidase gene, mitochondrial DNA for Brugia malayi), and universal primers, multiplex-PCR, PCR-restriction fragment length polymorphism (PCR-RFLP), PCR-enzyme linked immunosorbent assay (PCR-ELISA), as well as quantitative PCR. Furthermore, because bancroftian filariasis is endemic on the Thai-Myanmar border, the potential now exists for a re-emergence of bancroftian filariasis in Thailand, and random amplified polymorphic DNA (RAPD) analysis has proved effective to differentiate Thai and Myanmar strains of W. bancrofti.

INTRODUCTION

Lymphatic filariasis (LF), caused by mosquito-transmitted filarial worms Wuchereria bancrofti, Brugia malayi and Brugia timori, is the second leading cause of long-term disability and seriously affects socio-economic worldwide. The disease affects over 120 million people in 83 countries worldwide (Molyneux et al, 2003). Most of infected individuals are asymptomatic, while the common chronic clinical manifestations are lymphedema, hydrocele and elephantiasis.

In Thailand, the prevalence LF has been reduced to 0.32 per 100,000 population due
to the effective national control program (Filariasis Division, 2008). Bancroftian filariasis is endemic at the Thai-Myanmar border, while brugian filariasis is endemic in southern Thailand (Triteeraprapab et al, 2000; 2001b; Nuchprayoon et al, 2003b, 2005). The nocturnally subperiodic W. bancrofti (rural/Thai strain), found in infected Thai-Karens, employs the mosquito Ochlerotatus (Aedes) niveus group as the main vector (Gould et al, 1982; Kanjanopas, 1995). It has been reported that Myanmar migrant workers in Thailand carry W. bancrofti (urban/Myanmar strain) with prevalence of 3-8% (Triteeraprapab and Songtrus, 1999; Triteeraprapab et al, 2001b; Nuchprayoon et al, 2003a), with Culex quinquefasciatus as the main vector (Macdonald, 1991). In Thailand, Cx. quinquefasciatus is prevalent in urban areas. The Thai strain of Cx. quinquefasciatus has a potential to transmit Myanmar strain of W. bancrofti in laboratory study (Triteeraprapab et al, 2000). Together with the high prevalence of W. bancrofti infection in Myanmar migrant workers, this has prompted a concern in the public health community that a re-emergence of bancroftian filariasis in Thailand may be imminent.

Advances in treatment and diagnosis for LF had led to a paradigm shift in the 1990s that postulated that it might be feasible to eliminate LF. The Global Program to Eliminate Lymphatic Filariasis (GPELF) has targeted LF to be eliminated by 2020, by using selective diagnosis to identify endemic areas followed by repeated cycles of mass drug administration (MDA), to reduce both infection prevalence and transmission rates to levels below those required for sustained transmission (Ottesen et al, 1997; Molyneux, 2001; Ottesen, 2006). For the annual or bi-annual, single-dose, two-drug regimens being advocated [albendazole 400 mg plus diethylcarbamazine (DEC) 6 mg/kg; or albendazole 400 mg plus ivermectin 200 μg/kg], the period has been estimated to be 4-6 years, corresponding to the reproductive life span of the parasite.

Thailand has launched a national control program using DEC combined with albendazole for MDA in high risk areas during 2002-2006. Besides the main strategy, health education and community cooperation have been emphasized. The GPELF includes evaluation of the disease situation, before and after the program implementation, together with close monitoring and interim assessments of the input, process and output of the program by using different diagnosis tools. A report from the Ministry of Public Health (Filariasis Division, 2008) shows that ten rounds of DEC mass administration has the potential to interrupt transmission of LF in endemic areas of Thailand, as no new case was found in children aged less than 4 years.

In the past, clinical examination (hydrocele, lymphedema or elephantiasis) and detection of Mf have been implemented for diagnosis of LF. However, the clinical examination is not a sensitive indicator of changes in infection or transmission rates following MDA.

Conventionally, laboratory diagnosis of LF depends on detection of microfilariae in night blood specimens. Although sensitivity has been increased by concentration techniques and using provocative test, traditional parasitological detection methods fail to identify microfilaremics or individuals with very low Mf levels (Chanteau et al, 1991). Moreover, this time-consuming, labor intensive, and tedious method has difficulty to differentiate one filarial species from another (Poole and Williams, 1990). The efficacy of Mf detection is further decreased by the long pre-patency and nocturnal/subnocturnal periodicity. Thus, there have been considerable efforts to develop other
diagnostic techniques, including immunooassays to detect specific circulating parasite antigens and anti-filarial antibodies, as well as molecular-biology-based assays to detect the parasite DNA.

Detection of anti-filarial IgG4 antibodies enhances the specificity of immunodiagnostic assay for LF (Ottesen et al, 1985; Lal and Ottesen, 1988; Kwan-Lim et al, 1990). The advantages of the antibody assays are the low cost of the tests, high sensitivity and availability for B. malayi. Moreover, ELISAs for anti-filarial antibody are high throughput assays and can be used with small amounts of day- or night-blood samples. However, anti-filarial IgG4 assay has limited use in cross-sectional surveys since it cannot discriminate between active infection and past exposure. Moreover, cross-reactivity to other filarial parasites is common, thereby limiting specificity of anti-filarial IgG4 assay.

Detection of circulating filarial antigen (CFA), released by adult worms of W. bancrofti into blood circulation, is regarded by WHO as the ‘gold standard’ for diagnosis of LF. Commercially available assays have 2 formats, ICT filariasis card test and Og4C3 ELISA, which are more sensitive than Mf detection and can be used with day- or night-blood samples. The antigen levels are related to the numbers of adult filarial worms in host and correlate with current active infection (Chanteau et al, 1994a). Another advantage of the antigen detection assay is that the test that detects CFA can also detect occult infection (Weil et al, 1987; Turner et al, 1993). However, limitations of the antigen tests are high cost, inconsistent availability, especially in developing countries, and not widely applicable for B. malayi. Furthermore, certain infected individuals remain antigen-positive for years after treatment, although they have no detected Mf.

Recent advances in molecular biology techniques have been employed in designing species-specific primers for PCR-based identification of parasites including diagnosis of LF both in human and animal reservoir blood, as well as in mosquitoes (Table 1). For example, PCR techniques based on the identification of repetitive DNA sequences Ssp I (for W. bancrofti) and Hha I (for B. malayi) have been developed. Other PCR-based assays, such as DNA hybridization, PCR-restriction fragment length polymorphism (PCR-RFLP), PCR-enzyme linked immunosorbent assay (PCR-ELISA), quantitative PCR and random amplified polymorphic DNA (RAPD) have also proved effective for diagnosis and epidemiological studies of LF.

GPELF consists of 4 phases: phase 1 (mapping and planning), phase 2 (monitoring of MDA), phase 3 (defining endpoints of MDA), and phase 4 (post-MDA surveillance and early detection of resurgence). Different assays for filariasis are recommended for use as diagnostic tools in different phases of GPELF (Weil and Ramzy, 2007). Detection of Mf and CFA are needed as primary tools in phases 1-3, while antibody detection and molecular xenodiagnosis (detection of parasite DNA in mosquitoes) are necessary as secondary tools in phases 1-3 and as primary tools in phase 4. Detection of parasite DNA by PCR in human blood samples are recommended as secondary tool in phases 1-2 (Weil and Ramzy, 2007). This article reviews current molecular diagnosis assays and their applications in the field for control of LF in Thailand as part of GPELF.

BANCROFTIAN FILARIASIS IN THAI-KAREN POPULATION

A very sensitive and specific PCR detection method, based on a highly repeated DNA sequence (eg Ssp I repeat), has been developed (Zhong et al, 1996). This PCR


Table 1
Molecular diagnosis for lymphatic filariasis.

<table>
<thead>
<tr>
<th>Filarial species</th>
<th>Molecular technique</th>
<th>DNA target</th>
<th>Reference</th>
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<tbody>
<tr>
<td><em>Brugia malayi</em></td>
<td>DNA hybridization</td>
<td><em>Hha I</em> repeat</td>
<td>(Williams et al, 1988)</td>
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<tr>
<td></td>
<td>PCR</td>
<td><em>Hha I</em> repeat</td>
<td>(Triteeraprapab et al, 2001a)</td>
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<td></td>
<td>PCR-RFLP</td>
<td><em>Hha I</em> repeat, Glutathione peroxidase gene, ITS1</td>
<td>(Chansiri et al, 2002; Nuchprayoon et al, 2005; Thanomsup et al, 2000)</td>
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<td></td>
<td>PCR-ELISA</td>
<td><em>Hha I</em> repeat</td>
<td>(Fischer et al, 2000)</td>
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<td></td>
<td>Multiplex-PCR</td>
<td>Mitochondrial DNA</td>
<td>(Mishra et al, 2007)</td>
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<td></td>
<td>Real-time PCR</td>
<td><em>Hha I</em> repeat, Glutathione peroxidase gene</td>
<td>(Rao et al, 2006; Thanchomnang et al, 2008)</td>
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<td></td>
<td>Multiplex-PCR</td>
<td><em>Ssp I</em> repeat</td>
<td>(Mishra et al, 2007)</td>
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<td></td>
<td>Real-time PCR</td>
<td>LDR repeat</td>
<td>(Nuchprayoon et al, 2007)</td>
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<td>RAPD</td>
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<tr>
<td><em>Wuchereria bancrofti</em></td>
<td>DNA hybridization</td>
<td>pWb12 repeat, pWb-35 repeat</td>
<td>(Dissanayake et al, 1991; Siridewa et al, 1994)</td>
</tr>
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<td></td>
<td>PCR</td>
<td><em>Ssp I</em> repeat</td>
<td>(McCarthy et al, 1996; Ramzy et al, 1997; Williams et al, 2002)</td>
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<td></td>
<td>PCR-RFLP</td>
<td>ITS1</td>
<td>(Nuchprayoon et al, 2005; Thanomsup et al, 2000)</td>
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<td></td>
<td>PCR-ELISA</td>
<td><em>Ssp I</em> repeat</td>
<td>(Fischer et al, 1999)</td>
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We previously described the assessment of bancroftian filariasis in an endemic area of Thailand, by using ELISA for Og4C3 antigen and a PCR-based assay to detect *W. bancrofti* DNA (*SspI*) in blood samples collected from Thai-Karen population living in Tak Province (Nuchprayoon et al, 2001). This population had a microfilarial rate of 10%, while the antigen assay could detect 23% of the cases. PCR was positive in 12% of the population, which is less sensitive than the Og4C3 antigen assay. Although the PCR did not detect as many cases as the antigen assay, its detection of the parasite in mosquito vectors has been successful (see below) (Triteeraprapab et al, 2000). Our data emphasized that MDA as control strategy, as well as continuous monitoring, is necessary for endemic areas.

**BANCROFTIAN FILARIASIS IN MYANMAR MIGRANTS OF THAILAND**

Recently, there has been an influx of more than one million Myanmar migrants into urban areas of Thailand. These Myanmar migrants are often infected with *W. bancrofti*, nocturnal periodic (urban) type, which has *Cx. quinquefasciatus* as the main mosquito vector. A microfilarial rate of 4.4% in 654 Myanmar migrants working in Mae Sot, Tak Province was detected (Triteeraprapab and...
Songtrus, 1999). Another study showed Mf observed in 8% of 371 Myanmar migrants, while 10% of the subjects were positive with Og4C3 antigen test (Nuchprayoon et al, 2001). An estimation of prevalence based on the demonstration of anti-filarial IgG4 in sera was a remarkable 42%. A study in 2003 showed that the Og4C3 ELISA could detect 19.1% of bancroftian filariasis while the ICT test detected 12.7% in 337 Myanmar workers in Tak Province (Nuchprayoon et al, 2003a). Therefore, close monitoring and control of LF in Myanmar migrants are of public health importance.

TRANSMISSION OF NOCTURNAL PERIODIC (MYANMAR) STRAIN OF W. BANCROFTI BY THAI CX. QUINQUEFASCIATUS

It is possible that an urban cycle of transmission of bancroftian filariasis could become established in Thailand as Myanmar migrants are infected with the nocturnal periodic (urban) type W. bancrofti for which Cx. quinquefasciatus serves as the main vector. Feeding experiments demonstrated that Thai Cx. quinquefasciatus is permissive for the development of Myanmar W. bancrofti to the infective third-stage larvae, thus establishing the potential for establishing an urban cycle of transmission in Thailand (Triteeraprapab et al, 2000). PCR amplification for the Ssp I repeat was used to identify W. bancrofti-infective mosquitoes that was capable of detecting a single infective-stage larvae in a pool of 100 mosquitoes.

RANDOM AMPLIFIED POLYMORPHIC DNA (RAPD) FOR DIFFERENTIATION BETWEEN THAI AND MYANMAR STRAINS OF W. BANCROFTI

Traditionally, identification of W. bancrofti strains depends on morphological and morphometric studies. The Myanmar strain of W. bancrofti has been shown to be distinct from the Thai W. bancrofti strain based on its size and the number of nuclei between cephalic space and nerve ring (Jitpakdi et al, 1999). Our study also showed that Thai and Myanmar strains of W. bancrofti are different in body length, cephalic space length and cephalic space width (Nuchprayoon et al, 2007). However, this technique is time-consuming, laborious, and consequently not suitable for large-scale application. DNA polymorphism assay based on random amplified polymorphic DNA (RAPD) analysis has been useful for analyzing the inter- and intra-specific genetic variations and phylogenetic relationship.

Since the high prevalence of bancroftian filariasis in Myanmar migrant workers could place risk of re-emerging in Thai people, we developed a RAPD analysis that proved to be an easy, reproducible and rapid diagnostic method to distinguish between Thai and Myanmar W. bancrofti strains (Nuchprayoon et al, 2007). Each strain of W. bancrofti was shown to be genetically distinct; however, to a certain extent, they shared some similar migrated DNA bands.

A study of the epidemiological aspects related to prevalence of W. bancrofti, both Thai and the Myanmar strains, will help the filariasis control program to design strategies to control the appropriate human and mosquito populations in endemic areas. In addition to early detection and prompt treatment of infected cases, verification of W. bancrofti strains in the mosquito populations by RAPD analysis can be used as a tool to monitor and evaluate GPELF.

PCR-BASED ASSAY FOR HHA I REPEAT OF B. MALAYI IN HUMANS IN NARATHIWAT PROVINCE

LF caused by B. malayi is highly prev-
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The conventional microscopic method is insensitive and may fail to identify microfilarems, or individuals with very low Mf levels, while the antigen assays are not widely available. A PCR-based assay to detect specific Hha I repeat of B. malayi has been developed to identify infected cases with high sensitivity and specificity (Rahmah et al, 1998; Fischer et al, 2000; Triteeraprapab et al, 2001a). Although the PCR-based assay is mainly useful to detect filarial third-stage larvae in mosquito vectors (Chanteau et al, 1994b; Nicolas et al, 1996; Vythilingam et al, 1998; Triteeraprapab et al, 2000), preliminary results suggested that the PCR could be also used to diagnose active cases who are microfilaremic (Triteeraprapab et al, 2001a).

The PCR assay of Hha I repeat could detect as little as 10 fg of B. malayi genomic DNA (Triteeraprapab et al, 2001a). As no other better tests are available, the PCR-based assay to detect specific Hha I repeat of B. malayi could be useful in field studies for GPELF.

PCR-RFLP FOR DIFFERENTIATION OF FILARIAL SPECIES

The conventional Giemsa stain to detect Mf is difficult to discriminate clearly between closely related species in human and animal reservoirs in Thailand, including W. bancrofti, B. malayi and B. pahangi or Dirofilaria immitis, D. repens, and Dipetalonema reconditum. Although histochemical staining to detect acid phosphatase activity could overcome this problem (Huynh et al, 2001), it requires fresh samples in order to yield optimal results. Furthermore, the staining method requires expertise to identify and confirm the species, as well as being time consuming and labor intensive (Nuchprayoon et al, 2001).

A previous study showed that PCR can detect Brugia malayi microfilarems in domestic cats (Chansiri et al, 2002). However, in nature, domestic cats also carry B. pahangi, Dirofilaria immitis and D. repens infections. We have reported an assay system that employs a single-step PCR followed by RFLP analysis, which discriminates between filariae at the species level (Nuchprayoon et al, 2005, 2006). The first internal transcribed spacer (ITS1) along with the flanking 18S and 5.8S rDNA were isolated and cloned from W. bancrofti, B. malayi, and B. pahangi. Sequence analysis identified conserved sites in the 18S and 5.8S rDNA sequence that could be used as universal priming sites to generate ITS1 distinctive PCR products that are useful to distinguish filariae at the genus level. Addition of Ase I digestion of the ITS1 PCR product generated a fragment profile that allowed differentiation at the species level for W. bancrofti, B. malayi, B. pahangi, D. immitis, and D. repens (Nuchprayoon et al, 2005). Based on analysis of sequence data, the predicted patterns of Ase I digestion of the ITS sequences from O. volvulus, M. ozzardi, and D. reconditum yielded different patterns diagnostic for these filarial parasites as well (Table 2). Therefore, the PCR-RFLP of ITS1 rDNA will be useful to diagnose and differentiate filarial parasites in human, animal reservoir hosts, and mosquito vectors in endemic areas.

CONCLUSION

DNA-based diagnosis is not as sensitive as antigen tests for diagnosis of lymphatic filarial parasites, especially W. bancrofti. However, it is useful to differentiate among filarial parasite species in humans, animal reservoirs, and mosquito vectors. A single-step PCR followed by RFLP analysis can distinguish almost all filarial parasites, of public health problem, at the species level. Furthermore, RAPD analysis can differentiate Thai and Myanmar strains of W. bancrofti. Therefore, DNA-based techniques are very
useful for diagnosis of filarial infections, especially in co-endemic areas, and in endemic areas where antigen tests are not available. Furthermore, DNA-based assays are essential tools for monitoring the GPELF.

ACKNOWLEDGEMENTS

I would like to thank Professor Yong Poovorawan and Dr Saravudh Suvannadabba for their advice and suggestion. I am thankful to the faculty and staff at Lymphatic Filariasis Research Unit, Department of Parasitology, and Chulalongkorn Medical Research Center, Faculty of Medicine, Chulalongkorn University, for their kind assistance. I thank all the health personnel from the Vector Borne Disease Control Center 9.3 (Tak Province), Department of Disease Control, Ministry of Public Health, Thailand for assistance in the field. I also thank Ms Alisa Junpee and Ms Vivornpun Sanprasert for manuscript preparation.

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