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

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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 amicrofilaremics 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 immunoassays 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.

Filarial species	Molecular technique	DNAtarget	Reference
Brugia malayi	DNA hybridization	*Hha* I repeat	(Williams *et al,* 1988)
	PCR	*Hha* I repeat	(Triteeraprapab *et al,* 2001a)
	PCR-RFLP	*Hha* I repeat, Glutathione peroxidase gene, ITS1	(Chansiri *et al,* 2002; Nuchprayoon *et al,* 2005; Thanomsub *et al,* 2000)
	PCR-ELISA	*Hha* I repeat	(Fischer *et al,* 2000)
	Multiplex-PCR	Mitochondrial DNA	(Mishra *et al,* 2007)
	Real-time PCR	*Hha* I repeat,Glutathione peroxidase gene	(Rao *et al,* 2006; Thanchomnang *et al,* 2008)
Wuchereria bancrofti	DNA hybridization	pWb12 repeat, pWb-35 repeat	(Dissanayake *et al,* 1991; Siridewa *et al,* 1994)
	PCR	*Ssp* I repeat	(McCarthy *et al,* 1996; Ramzy *et al,* 1997; Williams *et al,* 2002)
	PCR-RFLP	ITS1	(Nuchprayoon *et al,* 2005; Thanomsub *et al,* 2000)
	PCR-ELISA	*Ssp* I repeat	(Fischer *et al,* 1999)
	Multiplex-PCR	*Ssp* I repeat	(Mishra *et al,* 2007)
	Real-time PCR	LDR repeat	(Rao *et al,* 2006)
	RAPD		(Nuchprayoon *et al,* 2007)

method can detect *W. bancrofti* genomic DNA in blood sample (Williams *et al,* 1996), infected mosquito (Chanteau *et al,* 1994b; Nicolas *et al,* 1996; Triteeraprapab *et al,* 2000), paraffin-embedded tissue (McCarthy *et al,* 1996) and urine sample (Lucena *et al,* 1998).

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 337Myanmar 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 preva-

lent in Narathiwat Province, southern Thailand. The conventional microscopic method is insensitive and may fail to identify amicrofilaremics, 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* microfilariae 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

Table 2
RFLP analysis of filarial ITS1 PCR products digested with *Ase* I.

Filarial species	Undigested ITS1(bp)	PCR/RFLP products (bp) after *Ase* I digestion
Tested species		
Wuchereria bancrofti	482	12, 64, 100, 104, 202
Brugia malayi	504	133, 153, 218
Brugia pahangi	510	218, 292
Dirofilaria immitis	595	205, 390
Dirofilaria repens	602	602
Predicted from published sequences		
Onchocerca volvulus	513	198, 315
Mansonella ozzardi	480	20, 198, 262
Dipetalonema reconditum	446	337, 99

RFLP, Restriction fragment length polymorphism; ITS1, Internal transcribed spacer 1; PCR, Polymerase chain reaction; bp, basepairs

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.

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