MOLECULAR XENOMONITORING OF FILARIAL INFECTION IN MALAYSIAN MOSQUITOES UNDER THE NATIONAL PROGRAM FOR ELIMINATION OF LYMPHATIC FILARIASIS

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Abstract. The Deputy Director General of Health initiated the Malaysian National Programme for the Elimination of Lymphatic Filariasis, which aims to obtain nationwide entomological data as a criterion of confirming interruption of transmission by mosquitoes, from year 2009. Since then, nationwide entomological surveillance was conducted to eliminate LF in 2018. Molecular xenomonitoring is a promising tool for detecting pools of mosquito samples for the presence of microfilarial DNA in a mosquito population even in minute quantity. This study aimed to determine the possibility of local transmission of lymphatic filariasis by detecting the filarial DNA in field-caught mosquitoes using duplex polymerase chain reaction techniques. Mosquito samples collected from 21 endemic implementation units (red IUs) from 5 endemic states (Kedah, Pahang, Perak, Sabah, and Terengganu) and 6 non-endemic (green IUs) from 2 non-endemic states (Malacca and Selangor), were sorted and labeled. A total of 668 pools of mosquitoes were obtained from a total of 4,738 mosquitoes comprising 21 species from 6 genera. Filarial DNA was extracted from the mosquito samples, PCR amplified and electrophoresed for the specific bands of *Brugia malayi* and/or *Wuchereria bancrofti* at 322 bp and 188 bp, respectively, together with negative control of laboratory-bred *Aedes togoi*. The results confirmed that none of the mosquito samples were infected with *B. malayi* and/or *W. bancrofti*. We concluded that there was no evidence of active transmission of microfilariae by the vector population from the study areas.

Keywords: endemic implementation units, national program for the elimination of lymphatic filariasis, nationwide entomological surveillance, molecular xenomonitoring, Malaysia

INTRODUCTION

Lymphatic filariasis (LF), a 4,000-year-old disease is still a major public health problem today (Dean, 2001). Lymphatic filariasis is listed by WHO as one of the

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main diseases under the Neglected Tropical Diseases (NTD). It is caused by three species of filarial worms namely, *Wuchereria bancrofti*, *Brugia malayi* and *Brugia timori* (WHO; 1997). *Wuchereria bancrofti* accounts for 90% of cases found throughout the tropics and in some sub-tropical areas worldwide. However, *Brugia malayi* is confined to Southeast and Eastern Asia particularly in India, Indonesia, Malaysia and the Philippines. *Brugia timori* is found only in Timor and its adjacent islands (Melrose, 2002). *Brugia malayi* and *Brugia timori* account for approximately 10% or ~13 million infections of the global LF burden (WHO, 1995). Lymphatic filariasis affects an estimated 120 million people or 2.0% of the world's population in 73 countries throughout the tropics and sub-tropics of Asia, Africa, the Western Pacific, and parts of the Caribbean and South America (CDC, 2013) where 1.3 billion people (1/5 of the world's population) are at risk and more than 1/3 of these are children (WHO, 2006). Approximately 66% at risk of infection live in the WHO South-East Asia Region and 33% live in the African Region (WHO, 2014). In the United States, the infection disappeared early in the 20th century. Currently, infection with filariasis is unlikely in the USA (CDC, 2013).

B. malayi is the prevalent species in Malaysia and is often used for experimental study of LF because it can be maintained in the laboratory animals (Griffiths *et al,* 2009) unlike *W. bancrofti* which is specific to human only and has no animal model (WHO, 2013; McNulty, 2013). Although cases of lymphatic filariasis (LF) are still reported, it occurs only in very small pockets in Malaysia (WHO-CCS, 2010). However, with the increasing number of migrant workers coming into Malaysia from bancroftian endemic areas and the abundance of *Culex quinquefasciatus*, Vythilingam *et al* (2005) suggested that more thorough screening should be carried out on these workers.

One of the criteria confirming interruption of transmission by mosquito vectors is absence of filarial infection in the vectors (WHO, 1997). Thus, the need for a sensitive, rapid and species-specific diagnosis for the detection of parasites in the human and vector population is an essential parameter in the estimation of disease prevalence (Mishra *et al*, 2007) and monitoring the success of mass drug administration (MDA) and to establish end points for intervention are crucial (Fischer *et al*, 2007).

Molecular xenomonitoring is a promising tool for detecting pools of mosquito samples for the presence of microfilarial DNA in a mosquito population even in minute quantity for monitoring transmission (Williams *et al*, 2002; Fischer *et al*, 2007; Moses, 2007; Weil and Ramzy, 2007; Vasuki *et al*, 2012).

The objective of this study was to determine the possibility of local transmission of lymphatic filariasis in field-caught mosquitoes from several Implementation Units (IUs) in Malaysia using duplex polymerase chain reaction techniques.

MATERIALS AND METHODS

Study sites

Mosquito samples collected from 21 endemic Implementation Units (red IUs) from 5 endemic states (Kedah, Pahang, Perak, Sabah, and Terengganu) and 6 non-endemic (green IUs) from 2 non-endemic states (Malacca and Selangor), were sorted and labeled accordingly.

Informed consent

Informed consents were obtained from the collectors prior to the study.

Mosquito sampling

Adult mosquitoes collected from 21 endemic Implementation Units (red IUs) which had undergone 5 cycles of MDA together with 2 non-endemic areas were collected by using the bare leg catch (BLC) method from 07:00 PM to 06.00 PM and CDC light traps method to attract adult mosquitoes from 07:00 PM to 06:00 PM. Collected samples were sent by the state entomologists to Medical Entomology Unit, WHO Collaborating Centre for Vectors, Institute for Medical Research, Kuala Lumpur, Malaysia.

Negative control

Laboratory-bred *Aedes togoi* fed with 10% sucrose solution fortified with 1% vitamin B complex and maintained in the insectarium of Medical Entomology Unit at 27±2°C and 80±2% RH with 12:12 hour (L:D) photoperiod regime were used as negative control.

All adult mosquitoes received were further re-identified by using IMR Standard Taxonomy Keys and segregated according to species and location. Twenty mosquitoes of the same species were pooled in an eppendorf tube and labeled accordingly. The pooled mosquito samples were homogenized followed by a series of extraction of microfilaria DNA using Qiagen QIAamp DNA Mini Kit™ (Qiagen, Hilden, Germany). Extracted DNA were amplified by duplex Polymerase Chain Reaction (PCR) technique. The PCR assay was performed in a DNA thermal cycler using 2 sets of primers: one set specific for *B. malayi* (HhaI F 5′-GCG CAT AAA TTC ATC AGC-3′, HhaI R 5′-GCG CAA AAC TTA ATT ACA AAA GC-3′) and the other set specific for *W. bancrofti* (SspI F 5′-CGT GAT GGC ATC AAA GTA GCG-3′, SspI R 5′-CCC TCA CTT ACC ATA AGA CAAC-3′).

Table 1
Mosquito species collected from 21 red IUs from 5 endemic states (Kedah, Pahang, Perak, Sabah, and Terengganu) and 6 green IUs from 2 non-endemic states (Melaka and Selangor).

S/N	Genera	Mosquito species
1	*Aedes* sp	*Ae. albopictus*
2	*Anopheles* sp	*An. barbirostris*
		An. hycanus
		An. separatus
		An. tessellates
		An. umbrosus
3	*Armigeres* sp	*Armigeres* sp
4	*Coquilletidia* sp	*Coquilletidia* sp
5	*Culex* sp	*Cx. fuscocephala*
		Cx. gelidus
		Cx. mimulus
		Cx. pseudovishnui
		Cx. quinquefasciatus
		Cx. vishnui
		Cx. whitmorei
6	*Mansonia* sp	*Ma. annulata*
		Ma. annulifera
		Ma. bonneae
		Ma. dives
		Ma. indiana
		Ma. uniformis

The PCR products were electrophoresed in alternate lane of agarose gels for the specific bands of *B. malayi* and/or *W. bancrofti* at 322 bp and 188 bp, respectively (Vasuki *et al*, 2001, 2012). Positive and negative control of microfilariae of *W. bancrofti* and *B. malayi* were electrophoresed simultaneously with the test samples. Gel was viewed under ultra violet illuminator and the resulting bands were captured with a digital camera.

RESULTS

A total of 668 pools of mosquitoes were obtained from a total of 4,738 mos-

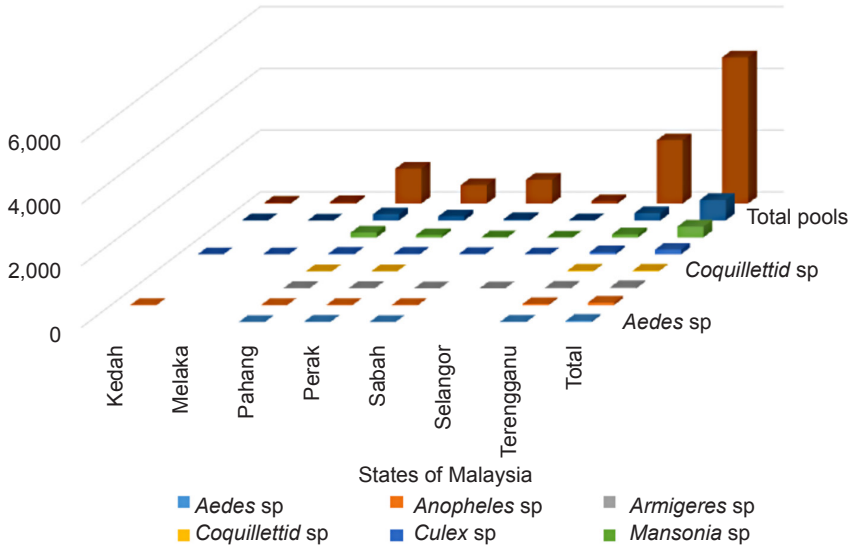

Fig 1–Mosquito samples collected from 21 red Implementation Units from 5 endemic states (Kedah, Pahang, Perak, Sabah, and Terengganu) and 6 green Implementation Units from 2 non-endemic states (Malacca and Selangor).

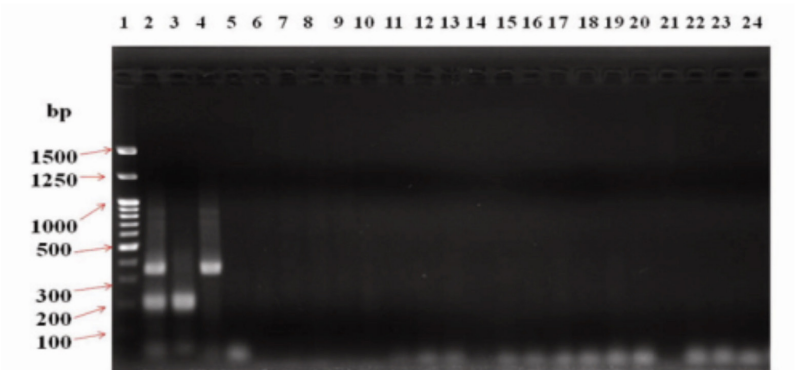

Fig 2–Gel documentation of PCR products to detect microfilariae of *B. malayi* and *W. bancrofti* in mosquito samples collected from 21 red Implementation Units from 5 endemic states (Kedah, Pahang, Perak, Sabah, and Terengganu) and 6 green Implementation Units from 2 non-endemic states (Malacca and Selangor). Lane 1, DNA size markers; lane 2, positive control of *B. malayi* (322 bp) and *W. bancrofti* (188 bp); lane 3, positive control for *W. bancrofti* (188 bp); lane 4, positive control for *B. malayi* (322 bp); lane 5, negative control; lanes 6-24, mosquitoes.

quitoes (Fig 1), comprising 21 species from 6 genera of mosquito samples collected from 21 red IUs from 5 endemic states of Kedah, Pahang, Perak, Sabah, and Terengganu and 6 non endemic (green) IUs from 2 non-endemic states of Malacca and Selangor (Table 1). The PCR results obtained confirmed that none of the mosquito samples was infected with *B. malayi* and/or *W. bancrofti* (Fig 2).

DISCUSSION

The WHO Resolution N° WHA 50.29 was adopted for global elimination of lymphatic filariasis as a public health problem by 2020 during the 50th World Health Assembly in 1997 (WHO, 1997; WHO, 2010). Elimination is defined by microfilariae rate of <1% or antigenemia <1/1,000 after 5 cycles of Mass Drug Administration (MDA).

Routine filariasis control program in Malaysia have been on going under the National Filariasis Control Programme (NFCP) since 1956. The control program successfully

Table 2
Malaysia's strategy in the National Programme for Elimination of Lymphatic Filariasis (NPELF) 2003-2017.

Phase	Year	Activity	Endemic states identified (Red)	Non-endemic states identified (Green)
1	2002-2003	Nationwide endemicity mapping of Implementation Units (red IUs)	Johore Kedah Kelantan Pahang Perak Terengganu Sabah Sarawak	Labuan Melaka Negeri Sembilan Pulau Pinang Perlis Selangor Kuala Lumpur
2	2004-2008	MDA to all red IUs covering 80% population excluding pregnant and lactating mother, children below 2 years old and patient with chronic illness	MDA 5 cycles per year for 5 successive years	
3	2009-2011	Post MDA surveillance on human and vector population initiated by Dr Lokman Hakim, Deputy Director General of Health (Public Health) in 2009	Human population surveillance Entomological surveillance data to determine the possibility of local transmission of LF in all field-caught mosquitoes	
	2011	WHO implemented Global Vector Surveillance and revised strategy of global elimination programme	Target elimination for Malaysia in 2018	
		Transmission Assessment Survey (TAS)	TAS conducted in 6 endemic states of LF in Peninsular Malaysia (Kedah, Terengganu, Kelantan, Perak, Pahang and Johor)	
	2012-2013	2 cycles of additional MDA	Sabah, Sarawak and 4 red IUs in Pahang	
	2014-2017	TAS & Post MDA surveillance 1 & 2 and vector surveillance	All 8 endemic states	
4	2018	Elimination	Elimination is defined by microfilariae rate of <1% or antigenemia <1/1,000 population in all red IUs	

Table 3
Eight endemic states with 116 Implementation Units (red IUs) conducting entomological surveillance from 2007 to 2014.

Endemic states conducting entomological surveillance	Mapping of red IUs	Red IUs conducting entomological surveillance (*n*)	% of red IUs conducting entomological surveillance (%)
Johore	6	0	0
Kedah	2	2	100
Kelantan	3	0	0
Pahang	28	15	54
Perak	12	11	92
Terengganu	19	12	63
Sabah	17	13	76
Sarawak	29	9	14
Total	116	62	53%

brought down the microfilaraemia rate from >50% in 1955-1956 to <3% in 2003 (WHO-WPRO, 2009).

In Malaysia, preparation for establishing the National Programme for the Elimination of Lymphatic Filariasis started in 2001 with the target of achieving LF elimination status by 2018. A series of constructive measures were carried out including mapping of endemicity of Implementation Units (red IUs) with sentinel sites of chosen and fixed locality which were heavily burdened with mf in 2002-2003. There are 8 states that have been identified endemic, where cases of brugian filariasis are still being reported; these include Johor, Kedah, Kelantan, Pahang, Perak, Sabah, Sarawak and Terengganu with a total of 116 Implementation Units (red IUs) (Table 3) with 1.1 million population at risk. MDA of diethylcarbamazine and albendazole was conducted for 5 years in all red IUs in 2004-2008 (Table 2) with successful coverage of 84.3%-95.1% in Peninsular Malaysia.

Following this, from the year 2009, Datuk Dr Lokman Hakim, Deputy Director General of Health (Public Health) initiated the entomological surveillance program of the Malaysian National Programme for the Elimination of Lymphatic Filariasis which aimed to obtain nationwide entomological data as a criterion of confirming interruption of transmission by mosquitoes until 2018 (Ministry of Health, Malaysia, 2012). The surveillance (2011-2018) comprised 2 additional cycles of MDA in Sabah, Sarawak, and Pahang, followed by Transmission Assessment Survey (TAS) conducted from 2011-2017 by using antibody based rapid test kit, involving 67 IUs/EU in 24 districts from 6 states of Peninsular Malaysia with a total population of 584,891 (Table 3) and post MDA surveillance 1 and 2, with the main goal of obtaining verification of elimination in 2018 (Ministry of Health, Malaysia, 2012).

Our results confirmed that no active local transmission of filarial worms was detected in the mosquito population from the areas studied. Hence, we concluded that there was no evidence of active transmission of microfilariae in the vector

population after completion of three and five rounds of MDA in 2006 and 2008, respectively (WHO, 1997; WHO-CCS, 2010).

Malaysia had achieved early deduction of the frequency of positive mosquito pools from the red IUs with only 3 rounds of MDA in 2006 as compared to the Egyptian study of Farid *et al* (2007), which took 5 rounds of MDA to significantly reduce the frequency of positive mosquito pools in the study areas.

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