

# A POLYMERASE CHAIN REACTION ASSAY FOR THE SURVEY OF BANCROFTIAN FILARIASIS

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**Abstract.** A polymerase chain reaction (PCR) assay based on a highly repeated DNA sequence found in *Wuchereria bancrofti* (SspI repeat) has been modified for the survey of bancroftian filariasis in expatriate workers (Myamese, Karen and Mon) from Myanmar where human filariasis is endemic. The PCR was very sensitive with the ability to detect the presence of as little as 10 pg of parasite DNA. The primers used in this PCR also showed highly specific amplification of parasite DNA without the presence of non-specific and non-target PCR products such as *Brugia malayi*, *Plasmodium falciparum* and human DNA. The primers were used to investigate filariasis in four provinces in the central and western Thailand, Samut Songkram, Ratchaburi, Nakhon Pathom and Tak during 1997-2001. Among them, Tak and Ratchaburi are the only endemic areas of bancroftian filariasis. In this field study, 1,299 human blood samples (501 from Samut Songkram, 510 from Ratchaburi, 109 from Nakhon Pathom, and 179 from Tak) were collected and screened by PCR. The result showed that 1, 2, 3, and 33 patients from Samut Songkram, Ratchaburi, Nakhon Pathom, and Tak respectively were infected with *W. bancrofti*. These numbers were corresponded to the prevalence rate of infection of 0.2, 0.4, 2.8, and 18.5%, respectively. The PCR was able to detect the third-stage infectious larvae (L3) from *Culex quinquefasciatus*, mosquito vector of the *W. bancrofti*, that was experimentally fed to infected patient. The PCR screening of each of field mosquito pools from two endemic areas, Ratchaburi and Tak, showed that no L3 of *W. bancrofti* was detected.

## INTRODUCTION

*Wuchereria bancrofti* is a mosquito-transmitted parasitic nematode that causes lymphatic filariasis in humans (Grenfell and Micheal, 1992; Devaney and Yazdanbakhsh, 2001). Lymphatic filariasis is endemic in tropical and subtropical Africa, Central and South America, Asia and Oceania (Siridewa *et al*, 1994). Traditional methods of diagnosis (blood smear and membrane filtration) are tedious, time consuming and labor-intensive; in addition, the collection, dissection, and microscopic examination of wild

mosquitos are not always reliable: the mosquitos may carry both human and animal parasites that cannot be distinguished by either biochemical or morphological methods. There is a need for a simple and effective diagnostic method that would facilitate parasite detection and enable more rapid progress to be made toward an understanding of the epidemiological and clinical course of lymphatic filariasis (Singh, 1997).

In this study, the species-specific primers for PCR amplification were derived from a repetitive DNA sequence of *W. bancrofti* (Siridewa *et al*, 1994; McCarthy *et al*, 1996; Ramzy *et al*, 1997). The specificity and sensitivity of this assay were evaluated using persons who had been naturally infected. The PCR technique was used for a survey of bancroftian filariasis in workers from Myanmar, where human filariasis is endemic. Crude dried blood samples and membrane filtered microfi-

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larvae were collected at night on account of the nocturnal periodicity of microfilaria. There are many workers from Myanmar in Thailand and they may be carriers of the disease. One local mosquito, *Culex quinquefasciatus*, is a potential vector of imported bancroftian filariasis in Thailand. The mosquitoes were allowed to feed a microfilaremic volunteer and infectious stage larvae (L3) were collected for PCR testing.

## MATERIALS AND METHODS

### Parasite collection

Blood samples from workers from Myanmar in 4 provinces of Thailand (Samut Songkram, Ratchaburi, Nakhon Pathom, and Tak) were investigated during 1997-2001. The blood samples were collected at night when parasitemia peaked. One milliliter of blood containing microfilaria was collected by intravenous acropuncture. Infected blood was filtered through a polycarbonated membrane to which the intact parasites became attached. The parasite-laden membrane was washed with 5 ml of normal saline until the red blood cells disappeared. The intact parasites were then transferred to a 15 ml tube containing 1.5% SDS in 1 ml of normal saline and 100 µg/ml proteinase K. The mixture was gently mixed and subjected to overnight agitation at 55°C before DNA extraction by conventional phenol/chloroform methods.

*Brugia malayi* was collected from patient at night when parasitemia peaked. DNA was isolated and purified according to the detail above. *Plasmodium falciparum* strain K1 was gifted from Prof Peerapan Tan-areeya, Department of Microbiology, Faculty of Sciences, Mahidol University, Bangkok, Thailand. One microliter of packed intact parasites was suspended in an equal volume of normal saline 800 µl 10% SDS in the presence of 100 µg/ml proteinase K. The mixture was gently mixed and subjected to overnight agitation at 55°C before DNA extraction by conventional phe-

nol/chloroform methods. Similarly, 10 ml of human blood was collected from a volunteer. Buffy coat was taken, washed twice with 5 ml normal saline and centrifuged at 3,000 rpm for 10 minutes. The pellet was suspended in 1 ml normal saline, 400 µl 10% SDS and 100 µg/ml proteinase K. The reaction was incubated at 55°C for 12-14 hours. DNA was subsequently extracted by conventional phenol/chloroform methods.

### Blood collection and extraction of parasite DNA for multiplex PCR analysis

Sixty microliters of infected blood were collected by finger-prick from each of the Myanmar workers at the time of peak microfilaria. The age of workers ranged from 15 to 55 years; most had migrated from Rangoon, Moulemein, Mataban, Ye, and Kawkaeik Provinces. Some were from the Karen and Mon minority groups. The whole blood specimens were manipulated according to the instruction supplied by Bio-Rad for its Intragene® Genomic DNA Isolation Kit. The mixture of purified human and parasites DNAs were achieved and proceeded to PCR. The samples, containing parasite DNA, could be stored at 4°C until tested.

### Mosquitos

Hundred mosquitoes, *Culex quinquefasciatus*, were allowed to feed on a microfilaremic volunteer. The survived mosquitoes were dissected after 10 days of incubation at 28°C under 85% humidity. The infectious stage larvae (L3) were collected for PCR testing.

Approximately one thousand of field mosquitoes were collected from each of two endemic areas, Ratchaburi and Tak. Each of field samples was smashed in sterile 5ml of normal saline and the solution was transferred to a new test tube. The L3 were collected by centrifugation at 5,000 rpm for 10 minutes and supernatant was discarded. Pellet was suspended in 20 µl of 0.002% SDS, heated at 95°C for 10 minutes and proceeded to PCR amplification.

## PCR

The oligonucleotide primers, WbF and Wb2, which were specific for a repetitive region of *W. bancrofti* were used for amplification. The forward primer, WbF, was synthesized from 5' to 3' using the nucleotide sequences of CACCGGTATCGAGATTAATT. Similarly, the reverse primer, Wb2, was generated by using the nucleotide sequences of 5'TGGATGTATG TCAAAAAGCA3'. *Brugia malayi*, *P. falciparum* and human purified DNA were used as the negative control.

The PCR amplification was performed in 25 µl in the presence of 1x PCR buffer, 0.1 mM dNTP, 1.5 mM MgCl<sub>2</sub>, 1 mM of each oligonucleotide primer and 1 unit of *Taq* polymerase. The PCR mixtures were heated at 95°C for 4 minutes prior to monitoring the PCR cycle. One cycle of PCR was consisted of denaturation at 95°C for 60 seconds, annealing at 50°C for 60 seconds and polymerization at 72°C for 90 seconds. The cycle was proceeded for additional 29 cycles in Thermal Cycler (MJ research). Ten microliter of PCR products of approximately 400 and 450 bp in size were analyzed by using 1.5% agarose gel electrophoresis, ethidium bromide staining and visualized under UV transluminator.

An aliquot (5 µl) of each field sample DNA, derived from the procedures described above, was added to the PCR reactions. The PCR was carried out in a 25 µl reaction volume as described previously. The PCR products were analyzed by electrophoresis on a 2.0% agarose

gel and visualized under ultraviolet light.

## RESULTS

PCR amplification of *W. bancrofti* microfilarial DNA using species-specific primers for *Ssp I* repetitive region produced two bands of 400 and 450 bp PCR fragments (Fig 1). The PCR was able to detect the presence of as little as 10 pg of parasite DNA. The primers used in this PCR also showed highly specific

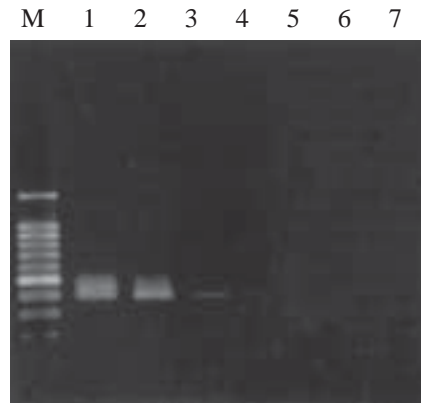


Fig 1—Sensitivity of primers WbF and Wb2 for detection of *W. bancrofti* using single PCR was manipulated at the DNA concentration of 1 ng (lane 1), 0.1 ng (lane 2), 10 pg (lane 3) and 1 pg (lane 4). The specificity of the two primers was tested against *B. malayi* (lane 5), *P. falciparum* (lane 6) and human DNA (lane 7).

Table 1

The number of *W. bancrofti* infected patients and the prevalence rate of infection of investigated Myanmar workers using PCR technique during 1997-2000.

Provinces	No. of investigated	No. of <i>W. bancrofti</i> positive	Prevalence rate of infection (%)
Samut Songkram	501	1	0.2
Ratchaburi	510	2	0.4
Nakhon Pathom	109	3	2.8
Tak	179	33	18.5

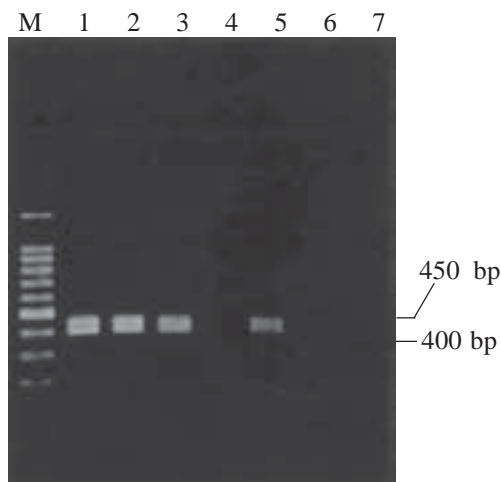


Fig 2—PCR profile of *W. bancrofti* L3 infective larva stage isolated from experimented blood-fed *Cx. quinquefasciatus* using WbF and Wb2 as specific primers. Microfilarial DNAs used for PCR amplification were indicated as followed; Lanes 1-3 and 5 represent DNA of L3 obtained from experimented blood-fed, *Cx. quinquefasciatus*, lane 4 represents DNA of *Cx. quinquefasciatus*, lanes 6 and 7 represent field mosquito samples from Ratchaburi and Tak pools. Lane M represents 100 bp ladder.

amplification of parasite DNA without the presence of non-specific and non-target PCR products such as *B. malayi* and *P. falciparum* and human DNA. The PCR method was used to analyze the field blood specimens obtained from 1,299 Myanmar workers of four provinces. The data revealed that 1 out of 501 from Samut Songkram, 2 out of 510 from Ratchaburi, 3 out of 109 from Nakhon Pathom and 33 out of 179 from Tak were *W. bancrofti* positive (Table 1). The prevalence rate of infection were 0.2, 0.4, 2.8 and 18.5%, respectively. Fig 2 demonstrated the PCR amplification of *W. bancrofti* L3 isolated from four experimentally fed mosquitos that survived after 10 days using the same primers. The data revealed that all four *Cx. quinquefasciatus* showed the positive PCR amplification while no positive PCR products were observed upon detection of field mosquito pools from Ratchaburi and Tak.

## DISCUSSION

PCR diagnosis was used for screening of *W. bancrofti* in Myanmar workers in Thailand. The majority of these Myanmar workers migrated from the endemic areas of filariasis in their countries, who legally held the work permission in Thailand, would be treated by using drug diethylcarbamazine (DEC) due to the policy of Ministry of Public Health of Thailand. However, a number of the illegal and unrecorded expatriate workers that are the crucial carriers of the diseases. The numbers of DEC-treated Myanmar workers are mainly distributed in the small and medium industrial areas of Thailand. Samut Songkram and Nakhon Pathom are the two provinces of the non-endemic area of central Thailand which are approximately 70 km far from the capital city, Bangkok. The investigation of human carriers in these areas during 1997-2001 revealed that the filariasis control was effective in Samut Songkram with the prevalence rate of infection at 0.2% while this value was higher (2.8%) when the Myanmar workers at Nakhon Pathom were investigated. However, these levels were ineffective for the natural transmission by *Cx. quinquefasciatus*. The repeat dose of DEC for complete eradication of the bancroftian filariasis in Samut Songkram and Nakhon Pathom should be accomplished.

Ratchaburi and Tak, the endemic border provinces in the western of Thailand, possessed a number of both legal and illegal expatriate workers due to the abundance of small and medium industries. Since the two is closed to Myanmar with the long border line so that it is difficult to achieve the eradication in this area. From this investigation at Tak Province, we found that 18.5% of infection rate was observed. Furthermore, the plenty of *Cx. quinquefasciatus* in this area makes it suitable for transmission of the bancroftian filariasis. However, the distribution of the disease in Thai residences of Tak area has not yet been reported. In contrast, the infection rate of the disease obtained from Ratchaburi Province was rather low. This may be due to the workers were treated with DEC

at the time of their immigration. In conclusion, the ability of PCR diagnosis of bancroftian filariasis from human and mosquitos is likely to be of significantly rapid and reliable assistance for investigation of the large number of specimens as well as for the control and eradication programs of filariasis.

#### ACKNOWLEDGEMENTS

This research, ID 00-1-HEL/MAL-10-004, was granted by T-2, National Science and Technology Development Agency (NSTDA), Thailand. Our deeply thank to Dr Nopporn Sarataphan for his contribution in collection of *W. bancrofti* L3 infective larva stage.

#### REFERENCES

- Devaney E, Yazdanbakhsh M. Prospects and challenges in lymphatic filariasis. *Parasitol Immunol* 2001; 23: 323-5.
- Grenfell BT, Micheal E. Infection and disease in lymphatic filariasis: an epidemiological approach. *Parasitology* 1992; 104: S 81-90.
- McCarthy JS, Zhong M, Gopinath R, Ottesen EA, Williams SA, Nutman TB. Evaluation of a polymerase chain reaction-based assay for diagnosis of *Wuchereria bancrofti* infection. *J Infect Dis* 1996; 173: 1510-4.
- Ramzy RM, Farid HA, Kamal IH, *et al.* A polymerase chain reaction-based assay for detection of *Wuchereria bancrofti* in human blood and *Culex pipiens*. *Trans R Soc Trop Med Hyg* 1997; 91: 156-60.
- Singh B. Molecular methods for diagnosis and epidemiological studies of parasite infections. *Int J Parasitol* 1997; 27: 1135-45.
- Siridewa K, Siridewa K, Karunanayake EH, *et al.* Cloning and characterization of a repetitive DNA sequence specific for *Wuchereria bancrofti*. *Am J Trop Med Hyg* 1994; 51: 495-500.