

Endemic Bancroftian Filariasis in Thailand: Detection by Og4C3 Antigen Capture ELISA and the Polymerase Chain Reaction

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Abstract

Lymphatic filariasis, mainly caused by *Wuchereria bancrofti* and *Brugia malayi*, has been targeted for elimination by the World Health Organization by the year 2020. To achieve this goal, highly sensitive and specific diagnostic tests are necessary for close monitoring and evaluation of the control program. We employed an ELISA to detect the Og4C3 antigen and a polymerase chain reaction-based assay for diagnosis of *W. bancrofti* infection, among the Thai-Karen population in Tak province, Thailand. We found that this endemic area had a microfilarial rate of 10 per cent, while the antigen assay could detect cases about two fold as many (23%). The repeated PCR for the detection of *Ssp I* of *W. bancrofti* was positive in 12 per cent of the population under this study. Our data emphasize the need for using highly sensitive and specific assays for assessment of the real burden of the disease.

Key word : *Wuchereria bancrofti*, *Ssp I* repeat, Og4C3 Antigen, PCR, Antigen Detection Assay

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Lymphatic filariasis has been estimated to affect more than 120 million people in endemic countries⁽¹⁾. More than 90 per cent of the disease burden is due to *Wuchereria bancrofti*. The disease has been targeted by the World Health Organization for elimination as a public health problem by the year 2020⁽²⁾. In Thailand, as assessed by using a

conventional microscopic method which has low sensitivity, the disease has been controlled to the prevalence of 0.99 per 100,000 population⁽³⁾. For bancroftian filariasis in Thailand, the active transmission areas are limited to 3 provinces (Tak, Kanjanaburi, and Mae-Hong-Son) bordering Thailand and Myanmar. To achieve the goal to eliminate the

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disease, it will be necessary for a lymphatic filariasis control program to assess the real burden of the disease. Part of the control strategies will include annual mass treatment with diethylcarbamazine and ivermectin or albendazole⁽⁴⁾. Therefore, evaluation of infection in communities is necessary before initiating mass treatment⁽⁵⁾. Close monitoring and assessment of the intervention programs require highly sensitive and specific diagnostic assays.

The routine method for diagnosis of lymphatic filariasis is to detect microfilariae in peripheral nocturnal blood by microscopic method. This time-consuming and tedious method is difficult to differentiate one filarial species from another⁽⁶⁾. Furthermore, conventional parasitological procedures fail to identify amicrofilaremic infections or individuals with very low microfilaria levels⁽⁷⁾. Assays that detect circulating *W. bancrofti* antigen can also detect nocturnal periodic infections in day blood, as well as other occult infections^(8,9). The Og4C3 monoclonal antibody has been used to diagnose microfilaremic individuals, as well as amicrofilaremic individuals, with high specificity and sensitivity⁽⁹⁻¹¹⁾.

A polymerase chain reaction (PCR)-based assay for diagnosis of *W. bancrofti* infection using species-specific deoxyribonucleic acid (DNA) probes, has been reported by several authors⁽¹²⁻¹⁴⁾. Subsequently, a very sensitive and specific PCR, based on a highly repeated DNA sequence (*Ssp* I repeat), has been developed⁽¹⁵⁾. The PCR can detect *W. bancrofti* genomic DNA in blood samples⁽¹⁶⁾, mosquitoes⁽¹⁷⁻¹⁹⁾, as well as in paraffin-embedded tissues⁽²⁰⁾, and urine samples⁽²¹⁾.

We describe here the assessment of bancroftian filariasis in an endemic area of Thailand by using the ELISA for Og4C3 antigen and a PCR-based assay to detect *W. bancrofti* DNA in blood samples collected from Thai-Karen population, living in the endemic area of Tak province, Thailand.

MATERIAL AND METHOD

Study areas and populations

Recruited for the study were 196 Thai-Karens residing in Mae Chan sub-district, Umphang district, Tak province, at the Thai-Myanmar border. The Umphang district is 60 km from Mae Sot district of Tak province. The Vector-borne Diseases Control Center is located in Mae Sot 500 km northwest of Bangkok. The study was organized in cooperation with the Filariasis Division, and the Vector-

borne Diseases Control Center 18 (Mae Sot district), Department of Communicable Disease Control, Ministry of Public Health, Thailand. As almost none of the Thai-Karens speak and read Thai or English, verbal informed consent was obtained from each individual, or parents or guardians of children recruited in the study, in the presence of two witnesses. We had a translator for Thai and Karen languages for the communication. Each individual was informed as to the purpose and scope of the study. Individuals were also educated about lymphatic filariasis and how to prevent the disease. Individuals who were microfilaremic were treated with standard treatment (diethylcarbamazine, DEC). This study was approved by the Ethics Committee of the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand.

Collection of specimens

Collection of blood specimens was performed as previously described^(19,22,23). Two to five ml of blood samples were collected from each individual between 8.00-12.00 p.m. Approximately 60 μ l of blood samples were smeared onto microscope slides in duplicate, air-dried and stained with Giemsa's stain. Identification of the parasite species was established. All of the microfilaria-positive specimens were the larvae of *W. bancrofti*.

Detection of specific Og4C3 antigen

The ELISA for detecting and quantifying the *W. bancrofti*-specific Og4C3 antigen was performed according to the manufacturer's instructions (Trop-Ag *W. bancrofti*, JCU Tropical Biotechnology Pty Ltd, Townsville, Queensland, Australia). The assay was performed on the polystyrene 96-well microtiter plates coated with Og4C3 monoclonal antibody that was blocked with nitrogen casein. One hundred microliters of each serum sample was boiled with 300 μ l of sample diluent (0.1 M Na₂EDTA, pH 4.0) for 5 minutes. After centrifugation, the supernatant was recovered and 50 μ l was added to each well, and kept in a humid container at room temperature for 90 minutes or at 4°C overnight. After washing with 0.01 M Phosphate buffered saline with Tween 20, pH 7.4 (PBS, 0.05% Tween 20), 50 μ l of diluted anti-*Onchocerca* antibody were added to each well and kept at room temperature for 1 hour. After another wash, 50 μ l of diluted sheep anti-rabbit immunoglobulin-horseradish peroxidase (HRP) conjugate were added to each well. The sam-

ple, hyperimmune rabbit antibody, and conjugate were each diluted with a blocking solution (PBS/T, 0.5% high nitrogen casein). Plates were kept at room temperature for one hour. After washing, 100 μ l of ABTS substrate was added to each well. The plates were kept at room temperature for 30 minutes. The reaction was stopped with 50 μ l of 2N H₂SO₄. The absorbance was read spectrophotometrically at 405 nm. The positive samples had antigen titers more than 100 units/ml.

Extraction of parasite DNA from blood samples

The DNA was extracted as previously described⁽²⁴⁾. Briefly, 100 μ l of human blood was mixed with 500 μ l of TE buffer and centrifuged. The pellet was then washed with 500 μ l of TE buffer, resuspended in 500 μ l red-cell-lysis buffer (RCLB) and incubated for 5 min to lyse red blood cells. After centrifugation, the red blood cells digestion step was repeated, then resuspended in 200 μ l of DSP buffer (20 mM Tris, pH 8.0, 50 mM KCl, 2.5 mM MgCl₂, 0.15 mg/ml Proteinase K, 0.5% Tween 20). Incubation in DSP was performed at 60°C for 3 h to lyse the microfilariae and release the DNA. The proteinase K in DSP was then inactivated by incubating the samples at 90°C for 10 min. Following brief centrifugation to pellet debris, the supernatant was kept for PCR analysis.

The polymerase chain reaction

The PCR was performed with 1 or 2 μ l of the extracted DNA as template in a final volume of 50 μ l. The reaction included 2 units of *Taq* polymerase, 400 pM each of NV1 and NV2 primers, and 200 μ M of each deoxynucleotide triphosphate in 50 mM KCl, 10 mM Tris-HCl, pH 9.0, 0.1 per cent Triton X-100, and 1.5 mM MgCl₂.

The sequences of NV1 and NV2 were 5'-CGTG ATGGCATCAAAGTAGCG- 3' and 5'-CCCTC ACTTACCATAAGACAAC- 3', respectively. The DNA was denatured at 94°C for 5 min, followed by 35 cycles of 1 min at 94°C, 1 min at 55°C, 1 min at 72°C, and a final 10 min extension at 72°C. The 188 bp PCR product (*Ssp* I repeat) was resolved and visualized on a 2 per cent ethidium bromide-stained agarose gel. The positive control DNA was prepared from adult *W. bancrofti* (kindly provided by Dr. N Raghavan).

Data analysis

Data were recorded and analyzed by using Microsoft Excel 6.0 program. The unpaired *t*-test was used to test statistically the significant difference.

RESULTS

Characteristics of the study population

Among the 196 Thai-Karens living in Mae Chan, 114 (58%) were males and 82 (42%) females. The mean age was 28 \pm 13 (1-69) years. The majority (53%) of the subjects were in the age group >15-30 years (Table 1). There was a total of 20 microfilaremic cases accounting for the prevalence of 10 per cent. Most microfilaremic individuals were in the age group >15-30 years (17%) and >30-45 years (17%). Women had a slightly higher microfilarial infection rate than men (11% compared to 10%, respectively).

Prevalence of lymphatic filariasis assessed by using Og4C3 antigenemia

The ELISA for the specific circulating antigen (Og4C3) to assess the situation of bancroftian filariasis in this population identified 44 anti-

Table 1. Microfilaremia and Og4C3 antigenemia in Thai-Karens stratified by age and sex.

Age-group (years)	Male			Female			Total					
	Total	Mf+	Ag+	Total	Mf+	Ag+	Total	%	Mf+	%	Ag+	%
≤ 15	17	1	2	4	0	1	21	11	1	5	3	14
> 15-30	56	4	11	49	4	8	105	53	8	8	19	18
> 30-45	27	5	9	20	3	6	47	24	8	17	15	32
> 45-60	12	1	3	8	2	4	20	10	3	15	7	35
> 60	2	0	0	1	0	0	3	2	0	0	0	0
Total (%)	114	11	25	82	9	19	196	100	20	10	44	23

Note: Mf+ and Ag+ indicate microfilaremic and antigenemic patients, respectively.

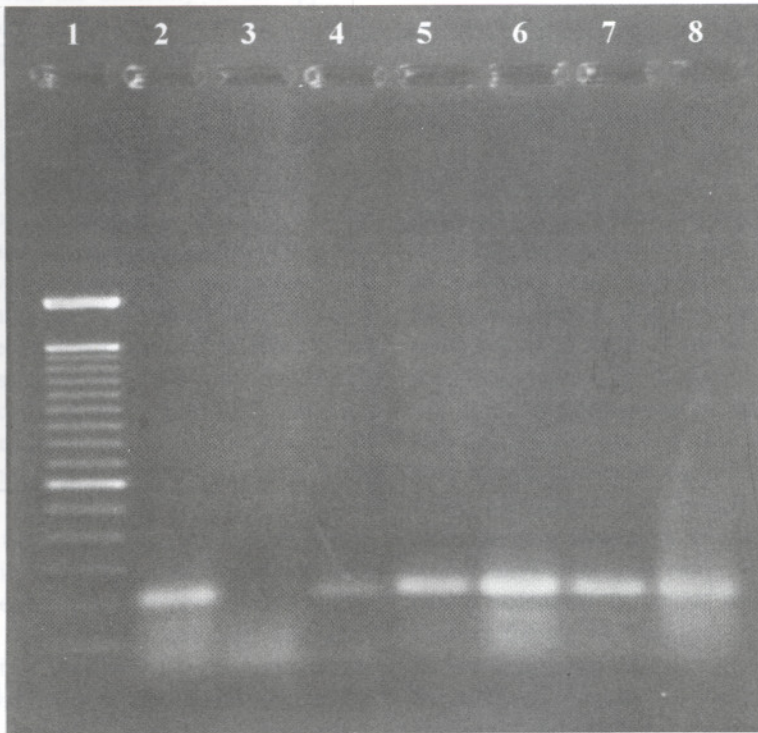


Fig. 1. Detection of 188-bp *Ssp I* repeat by a PCR assay on a 2% ethidium bromide-stained agarose gel. Lane 1: 100-bp marker, lane 2: positive control using *W. bancrofti* genomic DNA, lane 3: negative control using blood sample from non-infected healthy volunteers, lanes 5-8: five representative samples positive for *Ssp I* repeat by the PCR.

genic cases in total (Table 1). All of the microfilaremic cases were positive for Og4C3 antigenemia. The antigen detection assay was positive for 24 individuals in the amicrofilaremic group. The number accounted for a 23 per cent prevalence of antigenemia (Table 1). The antigen test could detect more positive cases than the conventional microscopic method. The majority of the antigenic cases were in the age group > 45 - 60 years (35%). Those who were in the age group < 15 years, > 15-30 years, > 30-45 years and > 60 years had antigenemic rates of 14 per cent, 18 per cent, 32 per cent and 0 per cent, respectively.

Polymerase chain reaction (PCR) for diagnosis of lymphatic filariasis

The molecular biology technique using PCR to detect genetic material, DNA, of infectious

organisms has been used widely. For lymphatic filariasis, the DNA region named *Ssp I* repeat was used in the polymerase chain reaction (PCR) for the detection of the filarial parasite, *Wuchereria bancrofti*. The PCR result showed the 188-bp *Ssp I* repeat as shown in Fig. 1. All blood samples from microfilaremic patients were positive when tested by the PCR. In addition, four more subjects who had been earlier classified in the amicrofilaremic group were found positive for the *Ssp I* repeat. Therefore, the PCR was positive in 12 per cent of the study population.

High Og4C3 antigen levels among microfilaremic individuals

To study the correlation between the Og4C3 antigen level and microfilaremic status, we analysed the data and it was tested with unpaired *t*-test. The

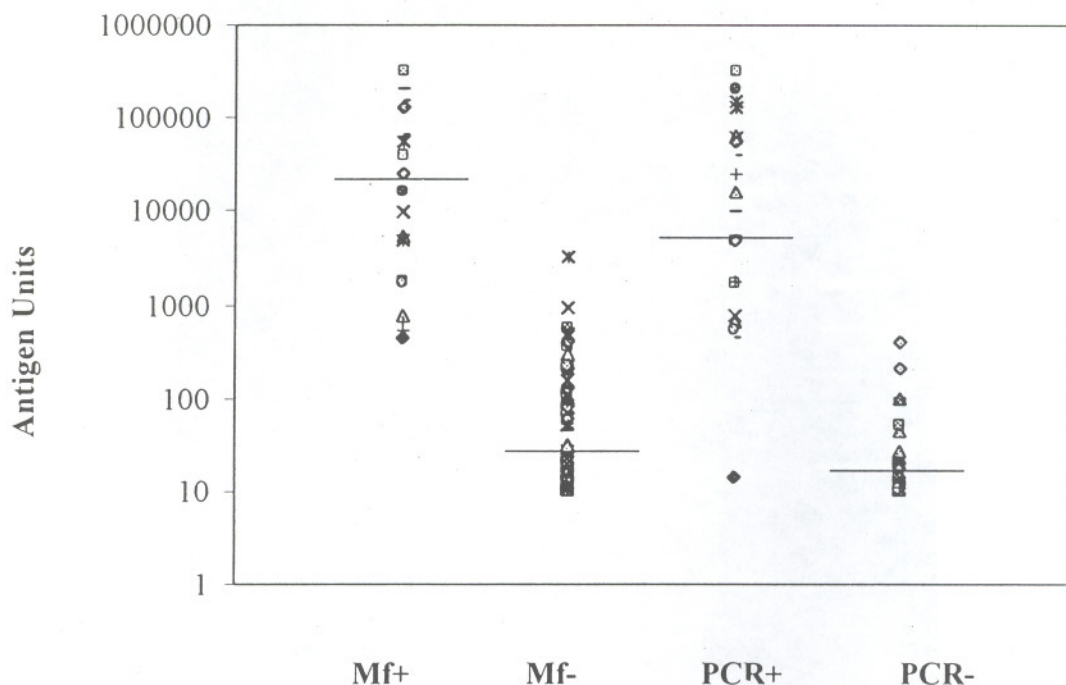


Fig. 2. The antigen units classified by microfilaremic status and detection of *Ssp I* repeat. Mf+: microfilaremic group, Mf-: amicrofilaremic group, PCR+: *Ssp I*-positive group, PCR- : *Ssp I*-negative group.

range of the antigen level was 10 - 3,156 units in the amicrofilaremics, and 439- 321,907 units in the microfilaremics (Fig. 2). The level of Og4C3 antigen among microfilaremics (GM = 11,507 units) was higher than those in amicrofilaremics (GM = 23 units) with statistic significance (unpaired *t*-test, $p = 3.9 \times 10^{-11}$).

For analysis of the PCR results, we found that those who were positive for *Ssp I* repeat also had higher antigen levels (14 - 321,907 units; GM = 8,359 units) than those who were negative for the *Ssp I* repeat (10 - 389 units; GM = 13 units) (unpaired *t*-test, $p = 3.5 \times 10^{-10}$).

DISCUSSION

The data from the Filariasis Division, Department of Communicable Disease Control, Ministry of Public Health show that Thailand has controlled lymphatic filariasis to a prevalence rate of 0.99 per 100,000(3). Our study, however, showed

a much higher microfilarial rate in the endemic area of Tak province. The microfilarial rate (10%) in a Thai-Karen population living in Mae Chan sub-district, Umphang district was much higher. Therefore, this endemic area is considered a high-risk area with active transmission(25). The continuous monitoring and annual mass treatment as control strategies are necessary for the area. Advanced diagnostic techniques were found useful as indicators for assessment the real prevalence of the disease. The data also indicated that the specific Og4C3 antigen assay was a good indicator of an active infection. Although the PCR did not detect as many cases as the antigen assay, its detection of the parasite in mosquito vectors has been proved successful(19).

The PCR did not detect the *W. bancrofti*-infected cases as high as the antigen detection assay. Therefore, the PCR for detection of *Ssp I* repeat is less sensitive than the Og4C3 antigen

assay. Together with the difficulty in the application of this technique in the endemic areas, the PCR has obviously less applicability for the field study than the antigen detection assay. Another advantage of the antigen detection assay is that it directly correlated with the current active infection⁽²⁶⁾.

The high prevalence of bancroftian filariasis assessed by the detection of microfilariae, Og4C3 antigen and *Ssp* I repeat found among this population deserves considerable attention from the public health community in Thailand. The collected data demonstrated the real burden of bancroftian filariasis as assessed by the Og4C3 antigen assay, which is an indicator of active infection. The high prevalence of the disease in the endemic area invites closer monitoring and control strategies for the disease.

Part of the reason for such a high prevalence of lymphatic filariasis is that this remote area is difficult to access by health personnel. Furthermore, the local villagers are not well educated; being unaware of its danger and prone to infection. Poverty is another major hindrance preventing this population from access to health care services.

To eliminate the disease successfully, health personnel need to have access to the remote areas as well as to provide health education.

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โรคเท้าช้างในแหล่งโรคชุกชุมของประเทศไทย: การวินิจฉัยโดยวิธีอีไลซา เพื่อหา Og4C3 และปฏิกิริยาลูกโซ่โพลีเมอร์เรส

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โรคเท้าช้างมีสาเหตุหลักมาจากพยาธิโรคเท้าช้าง *Wuchereria bancrofti* และ *Brugia malayi* ซึ่งมีการแพร่ระบาดขององค์การอนามัยโลกมีนโยบายที่จะกำจัดให้หมดไปภายในปี พ.ศ. 2563 ในการที่จะบรรลุเป้าหมายนี้มีความจำเป็นที่จะต้องอาศัยการวินิจฉัยที่มีความไวและความจำเพาะสูงในการติดตามและประเมินโครงการควบคุมโรค ผู้วิจัยได้ใช้วิธีอีไลซาในการตรวจแอนติเจน Og4C3 และใช้ปฏิกิริยาลูกโซ่โพลีเมอร์เรสในการวินิจฉัยโรคติดต่อพยาธิโรคเท้าช้าง *W. bancrofti* ในประชากรที่อาศัยอยู่ในพื้นที่ที่มีโรคชุกชุมเขตจังหวัดตากของประเทศไทย พบว่ามีอัตราการตรวจพบเชื้อไมโครฟิลาเรีย 10% ในขณะที่การตรวจแอนติเจนสามารถตรวจการติดเชื้อได้เพิ่มขึ้นประมาณ 2 เท่า (23%) และวิธีปฏิกิริยาลูกโซ่โพลีเมอร์เรสตรวจส่วน Ssp I repeat พบได้ 12% ข้อมูลดังกล่าวเน้นให้เห็นความจำเป็นที่จะต้องใช่วิธีวินิจฉัยที่มีความไวและความจำเพาะสูงในการประเมินสถานการณ์โดยตามความเป็นจริง

คำสำคัญ : วูเชอเรีย แบนครีออฟไต, Ssp I repeat, แอนติเจน Og4C3, ปฏิกิริยาลูกโซ่โพลีเมอร์เรส, การตรวจแอนติเจน

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