ORIGINAL ARTICLE

Comparison of *Brugia-Elisa* and Thick Blood Smear Examination in a Prevalence Study of Brugian Filariasis in Setiu, Terengganu, Malaysia

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Summary

A total of 1134 finger-pricked blood samples were collected from residents of Setiu, Terengganu. A drop of blood was used to make thick blood smear and about four drops were used for obtaining serum. The smears were stained and examined by the State Vector Control Unit in Kuala Terengganu, while the serum samples were tested for specific IgG4 antibodies to a novel recombinant antigen using *Brugia-Elisa*. Prevalence of filariasis in these areas were found to be 0.26% (3/1134) using thick blood smear examination and 2.47% (28/1134) using *Brugia-Elisa*, thus demonstrating the greater sensitivity of the latter test. In addtion, *Brugia-Elisa* showed a high level of specificity (97.8%, 1106/1131) when compared to thick blood smear examination.

Key Words: *Brugia malayi*, Prevalence, Setiu, Thick blood smear, *Brugia-Elisa*, Recombinant antigen

Introduction

Brugian filariasis accounts for about 10% of the 120 million people infected with lymphatic filariasis worldwide. This disease, caused by *Brugia malayi*, is mainly a rural infection transmitted by *Mansonia, Anopheles* and *Aedes spp.* mosquitoes. It is endemic in parts of India, China and several South East Asian countries[1]. In Malaysia, endemic areas are mainly found in Sabah, Sarawak, Pahang, Terengganu and Kelantan. The periodic strain of *B. malayi* is principally a human parasite, whereas its subperiodic form is capable of zoonotic transmission[2]. Both strains coexist in Malaysia and more than 80% of the peak microfilarial counts were detected between 2000 and 2400 hours[3]. In 1999, Terengganu, an east coast state of Peninsular Malaysia recorded an incidence rate 0.23% microfilaria positive cases as compared to the national average of 0.41%[4]. Filariasis control program in the district of Setiu, Terengganu rely on selective chemotheraphy and vector control. However, the annual systematic surveillance by night thick blood smear examination was unable to prevent the annual reemergence of new microfilaraemic cases. A more sensitive diagnostic tool is thus required to ensure detection and

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treatment of all infected persons in order to eliminate this infection as a public health problem. This study is aimed at comparing the sensitivity of thick blood smear and a recombinant antigen-based immunoassay *(Brugia-Elisa)* in the detection of *B. malayi* infection in several areas in Setiu.

Materials and Methods

Study population

A total of 1134 finger-pricked blood samples were collected from five villages and two secondary schools in the district of Setiu, Terengganu. The five villages were chosen based on their past filariasis records, whereas the two residential co-educational secondary schools consisted of 13 to 17 year old students mostly from these five villages. Informed consents were obtained from all subjects before the night sampling, which was performed between 2030 - 2300 hours, in conjunction with a routine screening exercise conducted by the Setiu Vector-Borne Disease Control team. A drop of blood (~ 60µl) was used to make thick blood smears (TBS) and about 4 drops were placed in a tube for serum separation. The blood samples were Giemsa stained and examined by personnels from the State Vector Control Unit in Kuala Terengganu, while the 'blind' serum samples were tested in the Department of Microbiology and Parasitology, School of Medical Sciences, Universiti Sains Malaysia for specific IgG4 antibodies to a novel recombinant antigen using *Brugia-Elisa*. The results of the thick blood smear examination were only revealed after the completion of the immunoassay.

Brugia-Elisa

The preparation of ***BmR1*** recombinant antigen and the protocol for *Brugia-Elisa* was performed as described by *Rahmah et al* (2000)[5]. Briefly, the recombinant bacteria culture was induced with 1mM IPTG for 3h at 30°C. The bacterial pellet was lysed and the soluble portion was purified by affinity chromatography using Ni-NTA column (Qiagen, Germany). Protein-containing fractions were pooled and its concentration estimated using Bio-Rad reagent, aliquoted and frozen at -20°C until use. Each well of the microtiter plate was coated with 20µg/ml protein in $NaHCO_3$ buffer (pH 9.6) and kept at 4°C, overnight in a humid container. After a washing step with 0.05% Tween-20 in phosphate buffered saline (PBST), each well was blocked with 0.5% bovine serum albumin (BSA) in PBS for 0.5h at 37°C. Next serum samples were added at 1:45 dilution (in duplicate wells) and incubated at 37°C for 2h. After washing off the excess sera, mouse monoclonal anti-human IgG4-HRP (CLB, Netherlands) was added at 1:4500 dilution (in PBS) and incubated for 0.5h. Following a washing step, ABTS substrate (2,2'-Azino-di-[3-ethylbenzthiazoline sulfonate]; Roche Boehringer Mannheim, Germany) was added and the results were read at 410 nm with an ELISA spectrophotometer (Dynatech, USA). A PBS control, positive and negative controls were included in each plate and the optical density (O.D.) readings were blanked with the PBS control. O.D. readings of ≥0.300 (mean O.D. of negative samples + 3 S.D.) were considered as being positive.

Three months later venous night blood samples were obtained from 19 of the 28 consenting individuals who were positive by *Brugia-Elisa* but negative by the TBS examinations. The nine remaining individuals either refused participation or had left the area. Each sample was then subjected to membrane filtration and PCR-ELISA.

Membrane filtration examination

One ml fresh blood sample in ethylenediaminetetraaceticacid (EDTA) solution was passed through a 5µm, 25mm diameter polycarbonate membrane filter (Whatman, England), followed by a 20ml wash with PBS, pH 7.2, and a 10ml distilled water. Excess water was expelled by forcing 3 syringe-volumes of air. The filter was placed on a slide and air-dried

thoroughly at room temperature. Four filters were prepared from the blood sample of each individual. Filters were stained for an hour using 3% (v/v) Giemsa in PBS, pH7.0 and then examined under a microscope.

PCR-ELISA

PCR-ELISA was conducted as previously described by *Rahmah et al* (1998)[6]. Briefly, blood DNA was extracted using the High Pure™ PCR template preparation kit (Roche Boehringer Mannheim, Germany). Template DNA was amplified with the same pair of primer sequences and PCR amplification programme as described by *Lizotte et al.* (1994)[7]. Each PCR run included the following controls: DNA elution buffer, DNA from microfilaraemic blood (positive control), and the DNA from normal blood (negative control). A 25-mer *B. malayi* DNA probe (5"-ACGTGAATTGTACCAGTGCTGG TCG-3') labelled with biotin at the 5' end, was used for the species-specific detection of the PCR products. Each streptavidin-coated well (Roche Boehringer Mannheim, Germany) was incubated with 45µl of either one of the following: PCR product, DNA elution buffer, positive control DNA or negative control DNA. Duplicate wells were used for each sample. After 5 x 3 mins wash with 0.05% Tween 20 in Tris-buffered saline (TBST), anti-digoxigenin-peroxidase (Roche Boehringer Mannheim) diluted to 1:800 in 3% skim milk was added. Washing steps were repeated, followed by 30 mins incubation with ABTS substrate. Results were then read at 410nm using an ELISA spectrophotometer (Dynatech, USA). The cut-off O.D. value used was 0.480 (mean O.D. of negative samples + 4 standard deviation).

Results

Out of 1134 pricked blood samples collected, three individuals (0.26%, 3/1134) from Jelapang were found to be microfilareamic by TBS examination and all three were also positive by *Brugia-Elisa*. In addition *Brugia-Elisa* detected an additional 25 actively infected individuals, thus recording an infection prevalence of 2.47% (28/1134). Of the 19 venous blood samples resampled based on the positive *Brugia-Elisa* results, two (10.5%) were found to be positive by both the membrane filtration and PCR ELISA techniques.

Jelapang recorded the highest percentage of positive cases: 8.4% for *Brugia-Elisa* and 1.3% by Giemsa-stained TBSE. This is followed by Kg. Kasar (4.3% & 0%), SMK Sungai Tong (1.9% & 0%) and SMA Setiu (0.3% & 0%). None of the samples was positive with both assays in Chalok Barat and Kg Beris Tok Ku (Table I). As shown in Table II, using TBS examination as the gold

Table I
Comparison of *Brugia-Elisa* and Thick Blood Smear Examination in the Detection of *B. Malayi* Infection

Village/school	Sample Size	Microfilaria Positive Individuals	*Brugia-Elisa* Positive Individuals
Kg. Beris Tok Ku	96	0	0
SM(A) Setiu	353	0	1
Chalok Barat 2	125	0	0
SMK Sungai Tong	213	0	4
Jelapang	237	3	20
Kg. Kasar	70	0	3
Permaisuri	40	0	0
TOTAL	1134	3	28

Table II
Sensitivity and Specificity of *Brugia-Elisa* for the Diagnosis of Brugian Filariasis

	Giemsa Stained Thick Blood Smear Examination		
Brugia-Elisa	**Positive**	**Negative**	**Total**
Positive	3	25	28
Negative	0	1106	1106
	3	1131	1134

The sensitivity and specificity of Brugia Elisa is 100% and 97.8% respectively.

standard, the sensitivity and specificity of *Brugia -Elisa* were found to be 100% (3/3) and 97.8% (1109/1134) respectively.

Discussions

In this study, the five villagers and two schools were selected by the Setiu health office based on their previous filariasis records. Thus the surveys were performed based on purposive and convenient sampling in conjunction with the routine screening exercises conducted by the Setiu Vector-Borne Disease Control team. As such, the results are not intended to reflect the normal prevalence of brugian filariasis in Setiu.

In this 'blind' study, *Brugia-Elisa* was able to detect 25 more cases of *B. malayi* infection as compared to Giemsa-stained TBS examination, which represents an increase of about 9 times (28 cases) more positive cases as compared to the traditional method (3 cases). From the 19 venous blood samples positive by *Brugia-Elisa* but negative by TBS, PCR-ELISA was able to detect circulating *B. malayi* DNA in two of the individuals. These two samples were also found to be positive by membrane filtration; they were from a 28 year old woman with a total of two microfilaria per 4ml blood and a 56 year old man with a total microfilaria count of 58 per 4ml blood. Since TBS uses ~60µl blood, these represents ≤ I mf/slide, thus the parasite could easily be missed by the microscopist. Although the membrane filtration and PCR-ELISA are more sensitive techniques than TBS, both assays failed to detect 17 other individuals who were positive by *Brugia-Elisa*. These individuals were probably having cryptic infections whereby infected individuals are amicrofilaraemic and thus unlikely to be detected by methods that rely on the presence of circulating microfilaria. Since these individuals are likely to harbour live adult worms in their lymphatics, they were or will become microfilaraemic at some stage and thus may contribute to the transmission of the infection. This could thus explain the perpetual existence of brugian filariasis in Setiu despite the annual screening and selective chemotherapy. Treatment and follow-up of these amicrofilaraemic infected people are being planned in the near future. We would expect a decrease in the anti-filarial IgG4 titres post-treatment, which would provide further evidence that these amicrofilaraemic individuals were indeed infected.

Although membrane filtration is more sensitive than TBS examination, the collection of venous blood requires expertise, is time consuming; frightens the villagers, especially small children and is impractical for mass screening. PCR-based assays require technical skill, are more expensive and probably unsuitable for mass screening in undeveloped/underdeveloped areas. *Brugia-Elisa* thus provides a good alternative tool for mass screening to detect active brugian filariasis. This assay, which is based on anti-filarial IgG4 antibody detection of a specific recombinant antigen has demonstrated specificity and

sensitivity rates of 96% - 100% using sera bank from five institutions from three countries[6]. Various other published reports have also shown anti-filarial IgG4 is a good indicator of active filarial infection and that the IgG4 antibody levels decline post-treatment[8,9,10,11,12,13].

In this field-based evaluation, *Brugia-Elisa* demonstrated sensitivity and specificity rates of 100% and 97.8% respectively. This assay does not require night blood sampling, employs commonly-used diagnostic laboratory equipment, do not require high technical skill and many samples (up to 93 samples/plate) could be analysed in a single day. Thus Brugia-Elisa would potentially be very useful to be used in the screening of villagers in endemic areas.

Conclusion

Brugia-Elisa was able to detect nine times more *B. malayi* infected individuals as compared to thick blood smear examination in a field study of 1134 individuals in Setiu, Terengganu. Therefore, in comparison to the thick blood smear examination, *Brugia-Elisa* is a much more sensitive test that will enable more accurate determination of the prevalence of *B. malayi* infection.

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