

An Antigen Detection Assay for Diagnosing Filariasis

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Lymphatic filariasis, commonly known as elephantiasis, is a profoundly disfiguring disease that has a major social and economic impact. The disease is endemic in Asia, Africa, the Western Pacific and the Americas (<http://www.who.int/ctd>). It is caused by filarial nematodes, namely *Wuchereria bancrofti*, *Brugia malayi* and *Brugia timori*.¹ In Thailand, two species of the causative filarial worms have been reported. Bancroftian filariasis is endemic in a limited area in the western part of the country while cases of brugian filariasis caused by *B. malayi* are found in southern Thailand.² The infection is transmitted by both zoonotic and non-zoonotic mosquitoes, e.g. *Mansonia* spp., *Anopheles* spp. and *Culex* spp.³

Filarial infection is initially asymptomatic when microfilaremia (M) may or may not be present⁴ (<http://www.who.int/ctd>). Clinical manifestations of filariasis require repeated inoculations of the parasite's infective larvae through vec-

SUMMARY In this study we examined the diagnostic potential of monoclonal antibodies (MAB) reactive to antigens of adult *Brugia malayi*, their microfilariae and antigen of *Dirofilaria immitis*. The MAB of clone 17E10, which were of IgM isotype, reacted to the inner cuticles and internal content of both male and female worms and also to the sheath and internal content of microfilariae *in utero*. However, these MAB did not react to the sheath of blood circulating microfilariae. The MAB 17E10 produced a smear pattern between 37 to > 200 kDa in the Western blot analysis against a SDS-PAGE separated extract of *B. malayi*. The epitopes were non-protein in nature as indicated by their resistance to proteinase-K treatment. The MAB 17E10 were applied in a sandwich ELISA to detect filarial antigen in the buffy coat and plasma of patients. We tested patients with different clinical manifestations of brugian filariasis, i.e. microfilaremia (M), lymphangitis (L) and elephantiasis (E), as well as non-symptomatic inhabitants of a filariasis endemic area (NE), and compared them to samples from non-symptomatic inhabitants of disease non-endemic areas (NNE). It was found that 22 of 31 (70.9%) of M, 7 of 13 (53.8%) of L, 2 of 14 (14.2%) of E, 10 of 100 (10.0%) of NE and none (0%) of the NNE were positive for antigenaemia. The assay was also positive in 14 of 15 (93.3%) blood samples from *B. malayi* microfilaremic cats and in 7 of 7 (100%) blood samples of *Dirofilaria immitis* microfilaremic dogs. The so-developed test has a high potential for routine diagnosis of active filariasis, for epidemiological studies in both humans and reservoir animals and for monitoring treatment efficacy.

tor bites in order for the parasite to successfully mature to adult worms, which reside in the lymphatic vessels and nodes and mate. Female worms may produce thousands of microfilariae per day. Adult filarial parasites have a life span of 10 years or more in their hosts^{5,6} (<http://www.who.int/ctd>). The acute stage involves episodes of acute in-

flammation of lymphatic vessels and

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nodes (lymphangitis, L) with fever and intermittent swelling of limbs and the scrotal sac⁷ (<http://www.who.int/ctd>). The prolonged presence of maturing larvae and adult worms causes the lymphatic vessels to dilate and later thicken with cellular infiltration which results in a restriction of the lymph flow, causing an accumulation of fluid in the skin, subcutaneous tissues and body spaces, and eventually lead to swollen legs and genitalia known as elephantiasis (E). At this time, microfilariae have usually disappeared from the bloodstream of the patient.⁸ But elephantiasis is not an inevitable consequence of chronic filariasis⁹ (<http://www.who.int/ctd>).

Until recently, diagnosis of filarial infection depended on the direct demonstration of the parasite (almost always microfilariae) in the blood or skin biopsies using relatively cumbersome techniques and having to take into account the periodicity (nocturnal or diurnal) of microfilarial presentation in the blood (<http://www.who.int/ctd>). Alternative methods based on the detection of antibodies failed to distinguish between active and past infections and were insensitive due to immune complex formation.¹⁰ Moreover, antibody detection assays showed low specificity due to cross-reactivity of the ill-defined antigens used in the assays with antibodies incited by other parasites¹¹ (<http://www.filaria.org>). Circulating filarial antigen (CFA) detection should now be regarded as the "gold standard" for diagnosing acute/current filariasis. At present, two versions of antigen detection assays are available for the diagnosis of bancroftian filariasis in humans and for canine dirofilariasis. One is based on ELISA technology that yields

semi-quantitative results¹¹⁻¹³ (<http://www.who.int/ctd>) and another one is based on a simple immunochromatographic test which yields only positive or negative answers.¹³⁻¹⁴ However, no antigen detection assay has been successfully developed as yet for the routine diagnosis of brugian filariasis.

In this communication, monoclonal antibodies reactive to *B. malayi* and *D. immitis* antigens were produced. They were used in a monoclonal antibody-based antigen detection assay for the diagnosis of current brugian filariasis using plasma plus buffy coat as target specimen. The assay could also detect antigens of *Dirofilaria immitis* in animal samples. The details of the experiments for the assay development form the basis of this research article.

MATERIALS AND METHODS

Preparation of filarial antigen

A *B. malayi* infected cat with a high density of microfilariae in the blood was used as source of filarial antigen. The species of microfilariae were identified by histochemical staining for acid phosphatase activity using the method established by Chalifoux *et al.*¹⁵ Four to five day old female *Aedes togoi* (Nomsao strain) mosquitoes, which are susceptible to *B. malayi* infection,¹⁶ were allowed to feed on the blood of the anaesthetized microfilaremic cat. Infective third stage *B. malayi* larvae were recovered by dissection from the mosquitoes fourteen days after they had fed on the cat's blood. The live infective larvae were injected intraperitoneally into young adult Mongolian gerbils (*Meriones unguiculatus*).

Four months thereafter, adult *B. malayi* and their microfilariae were harvested from the infected gerbils by means of peritoneal washing with normal saline solution (NSS). Adult worms were picked individually using a needle, and washed thoroughly with distilled water. The microfilariae in the NSS were recovered by filtering the peritoneal lavage through a membrane filter (8 μ m pore size) connected to a suction flask. The microfilariae retained on the filter membrane were washed alternately with NSS and distilled water in order to get rid of blood cells and other debris. The membrane filter was then placed in a petri-dish which contained NSS. The microfilariae moved out from the membrane into the NSS which was then centrifuged at 10,000 \times g at room temperature for 20 minutes. The pellets containing microfilariae were resuspended in a small volume of distilled water. Adult *B. malayi* and their microfilariae were ground separately in distilled water containing protease inhibitors (0.1 M PMSF, 0.1 M TPCK and 0.1 M EDTA). The preparations were individually subjected 10 times to sonication at 20 kHz per second (Labline Ultratip, Melrose Park, USA) at 4°C for 5 minutes and dialyzed against distilled water at 4°C overnight. The protein contents and the dry weights of the preparations were determined.¹⁷ Homogenates of adult worms and microfilariae were obtained and kept at -20°C in small aliquots until use.

Preparation of heterologous antigens

Heterologous antigens were prepared from various parasites including nematodes, cestodes, trem-

atodes and protozoa (Table 1). Homogenates of all parasites were prepared as for the adult *B. malayi* and their microfilariae.

Human specimens

Two sets of human specimens were obtained from May 1999 to August 2002 from various parts of Thailand. Oral informed consent was obtained from each subject before collecting the specimen.

The first set of specimens were plasma samples collected during May 1999 to December 2000 from 104 inhabitants of various districts of Thailand; i.e. Sugai-Padi, Yi-Ngo, Cho-Ai-Rong, Tak Bai and Sugai-Kolok of Narathiwat Province in southern Thailand, which are endemic areas of brugian filariasis. The samples were classified into four groups: group 1 consisting of 26 microfilaricemic individuals (M1); group 2 consisting of 24 individuals with lymphangitis (L1); group 3 consisting of 14 individuals with elephantiasis (E1); and group 4 consisting of 40 normal inhabitants residing in the filariasis endemic areas (NE1). Examination of stool samples of all included subjects of the filaremic areas by direct stool smear and formalin-ether concentration method revealed that most of them harboured mixed intestinal parasites, i.e., *Ascaris*, *Trichuris*, hookworms, *Entamoeba histolytica*, *E. coli*, *Giardia lamblia* and/or *Strongyloides stercoralis*. Plasma of 40 normal individuals of a filariasis non-endemic area (Bangkok) was collected as negative control (NNE1). Their stool samples at the time of the blood sample collection did not contain any intestinal parasites (group 5). Aliquots of the first set of samples were used in

the antigen detection assay either directly or after treatment with disodium ethylene diamine tetraacetic acid (EDTA) or sodium dodecyl sulphate and Triton X-100.

The second set of specimens consisted of finger prick blood samples collected into EDTA hematocrit tubes from 159 inhabitants of the same brugian filariasis endemic areas as for the first set during October 2001 to August 2002. They were also divided into four groups: group 1 consisting of 31 asymptomatic microfilaricemic individuals (M2); group 2 consisting of 13 individuals with lymphangitis (L2); group 3 consisting of 14 individuals with elephantiasis (E2); and group 4 consisting of 100 normal individuals living in the brugian endemic areas (NE2) (different subjects from NE1). Similar intestinal parasites to those of the first set were found in the stools of the second set of subjects. Blood samples were also collected from 40 normal, parasite-free individuals of a filariasis non-endemic area, i.e. Bangkok (NNE2) (different subjects from NNE1). Three hematocrit tubes (about 180 μ l) of blood samples of each subject were centrifuged and the buffy coat and about 50-60 μ l of plasma were collected together in an Eppendorf tube. Fifty microliters of distilled water, 25 μ l of 10% SDS and 25 μ l of 2% Triton X-100 solution were added to the sample tube, mixed and boiled for 20 minutes. These samples were subsequently used in the sandwich ELISA for antigen detection.

Cat samples

Blood samples were collected into EDTA hematocrit tubes from 15 microfilaricemic cats from en-

demic areas, i.e. Narathiwat Province, naturally infected with *B. malayi*. Samples were also collected from 5 other amicrofilaricemic cats from similar areas. The samples were processed like the second set of human specimens.

Dog samples

Blood samples were collected from 12 Bangkok dogs into EDTA haematocrit tubes. Seven dogs were positive for *Dirofilaria immitis* microfilariae, 5 dogs were healthy and parasite free. All samples were processed similarly to the second set of human samples.

Production of hybridomas secreting specific monoclonal antibodies (MAb) to filarial antigen

Four BALB/c mice, 6 weeks old at the beginning of the immunization, were individually immunized intraperitoneally with 100 μ g of adult *B. malayi* homogenate emulsified in complete Freund's adjuvant. Each mouse was given three booster doses containing 200 μ g of the same antigen in incomplete Freund's adjuvant at 14-day intervals. Seven days after the last booster dose, the serum antibody titers against the homologous antigen were determined by an indirect ELISA. The mouse with the highest serum antibody titer was used as the splenocyte donor during the cell hybridization for hybridoma production.¹⁸ The mouse was given an intravenous booster of 200 μ g of the same antigen. Three days after the intravenous booster, the selected mouse was bled and the serum collected as an immune serum (IS). Splenocytes of the mouse were hybridized with P3x-63-Ag8.653 non-Ig secreting myeloma cells.¹⁹ The spent culture media of all wells con-

Table 1 Parasites of which the antigens were used for checking the cross-reactivity of the monoclonal antibodies

Name of parasite	Developmental stage		Source
	Adult	Larva	
Nematodes			
1. <i>Angiostrongylus cantonensis</i>	✓	-	Experimentally infected rats ⁴⁰
2. <i>Ascaris lumbricoides</i>	✓	-	Stools of patients
3. <i>Capillaria philippinensis</i>	✓	-	Stools of infected individuals after purgation
4. <i>Dirofilaria immitis</i>	✓	-	Autopsy from dog's heart
5. Human hookworms	-	✓	<i>In vitro</i> cultured ova obtained from stools of infected children
6. <i>Gnathostoma spinigerum</i>	-	3 rd stage larvae	Livers of naturally infected eels ⁴¹
7. <i>Strongyloides stercoralis</i>	-	Filariform larvae	<i>In vitro</i> culture of rhabditiform larvae obtained from stools of infected children ⁴²
8. <i>Trichinella spiralis</i>	-	✓	Experimentally infected mice ⁴³
9. <i>Toxocara canis</i>	✓	-	Autopsy from dogs
Cestodes			
10. <i>Hymenolepis nana</i>	✓	-	Stools of infected individuals after purgation
11. <i>Taenia saginata</i>	✓	-	Stools of infected individuals after purgation
12. <i>Echinococcus granulosus</i>	-	✓	Hydatid fluid
13. <i>Cysticercus cellulosae</i>	-	✓	Autopsy from hill tribe swine
Trematodes			
Liver flukes			
14. <i>Opisthorchis viverrini</i>	✓	-	Hamsters experimentally infected with metacercariae ⁴⁴
15. <i>Fasciola gigantica</i>	✓	-	Autopsy from cow
Lung fluke			
16. <i>Paragonimus heterotremus</i>	✓	-	Cats experimentally infected with metacercariae ²⁴
Medium sized intestinal flukes			
17. <i>Echinostoma ilocanum</i>	✓	-	Stools of infected individuals after purgation
18. <i>Echinostoma malayanum</i>	✓	-	Stools of infected individuals after purgation
19. <i>Echinostoma revolutum</i>	✓	-	Stools of infected individuals after purgation
Minute intestinal flukes			
20. <i>Haplorchis taichui</i>	✓	-	Stools of infected individuals after purgation
Blood flukes			
21. <i>Schistosoma japonicum</i>	✓	-	Mice infected with cercariae obtained from infected snails ²⁸
22. <i>Schistosoma mansoni</i>	✓	-	Mice infected with cercariae obtained from infected snails ²⁸
23. <i>Schistosoma mekongi</i>	✓	-	Mice infected with cercariae obtained from infected snails ²⁸
Protozoa			
24. <i>Entamoeba histolytica</i>	✓	-	<i>In vitro</i> cultured trophozoites
25. <i>Plasmodium falciparum</i>	✓	-	Blood stage culture
26. <i>Plasmodium vivax</i>	✓	-	Blood of infected individuals
27. <i>Toxoplasma tachyzoites</i>	✓	-	Experimentally infected mice

taining growing hybrids were screened for antibodies against the homologous antigen by an indirect ELISA. Cells from antibody positive wells were cloned using the limiting dilution method. Spent media of wells which contained single clones were rechecked for antibodies to the homologous antigen and for cross-reacting antibodies against the panel of heterologous antigens prepared from other parasites listed in Table 1 by indirect ELISA. Selected cells were subjected to expanded growth in a serum-free medium. The spent culture media of individual clones (hybridomas) at their maximum growth were collected, dialyzed against distilled water and checked by the indirect ELISA for antibody titers and immunoglobulin isotypes. The antigenic specificities of antibodies were checked by Western blot analysis against the SDS-PAGE-separated homologous antigen. Localizations of the MAb specific epitopes in the worm sections were determined by immuno-alkaline phosphatase staining.^{20, 21}

Preparation of rabbit polyclonal antibodies (PAb) against filarial antigen

A rabbit, weighing about 2 kg, was immunized intramuscularly with 500 µg of adult *B. malayi* extract mixed with an equal volume of complete Freund's adjuvant. Three booster doses of the same antigen in an incomplete Freund's adjuvant were given to the rabbit at two week-intervals using the same route and dose. Two weeks after the last booster dose, the animal was tested and the antibody titer against the homologous antigen was determined. IgG were purified from the rabbit antiserum by ammonium

sulphate precipitation²² and protein A-Sepharose column chromatography (Pharmacia, Uppsala, Sweden).²³ The polyclonal anti-filarial IgG (PAb) were subsequently used as capture antibody in the sandwich ELISA for antigen detection.

The indirect ELISA

An indirect ELISA was used for checking antibody titers in the mouse/rabbit immune sera, for screening for antibodies in spent media of growing hybrid cells, for cross-reactivity checking of antibodies secreted by hybridomas and for the quantitative determination of monoclonal antibodies. It was performed as previously described²⁴ using microplates coated individually with 100 µl of antigen diluted in a carbonate-bicarbonate buffer, pH 9.6 (5 µg dry weight per ml). A result was declared positive when the read-out OD was ≥ 0.05 . One indirect ELISA unit (EU) was the smallest amount of the antibody preparation that still gave a positive indirect ELISA result.

The sandwich ELISA

The wells of polyvinyl microtiter plates (Maxi-sorb, Nunc, USA) were coated overnight at 4°C with 100 µl of rabbit polyclonal IgG to filarial antigen (PAb as capture reagent; 20 µg per ml in carbonate/bicarbonate buffer, pH 9.6). The wells were washed with PBS-T and uncoated sites were blocked with 200 µl per well of PBS-T/1%, BSA/0.2% gelatin at 37°C for 1 hour. After another washing with PBS-T, the plates were kept at -20°C in a plastic wrap until use. For use, the plates were allowed to warm up at room temperature; 100 µl of the

sample to be tested for the presence of filaria antigen was added into the PAb-coated well and incubated at 37°C for 2 hours. The plates were washed and 100 µl of MAb (the detection reagent) containing 256 EU per ml of specific antibodies to filarial antigen was added, incubated at 37°C for 2 hours and washed, after which 100 µl of rabbit anti-mouse immunoglobulin-biotin conjugate (Dakopatts, Glostrup, Denmark, 1:1,000) was added, incubated at 37°C for 1 hour, and washed. Then streptavidin-HRP conjugate (Dakopatts, Glostrup, Denmark, 1:1,000) was added. After incubating for 1 hour at 37°C, the plates were washed and freshly prepared peroxidase substrate was added. The enzyme-substrate reaction was stopped after 30 minutes of reaction by adding 50 µl of 1 N NaOH. The optical density (OD) of the content of each well was read at 492 nm against the blank (PBS or spent medium of myeloma cells) using an ELISA reader (Labsystem, Multiscan Ex, Finland). The OD of the mean (\bar{X}) plus 3 or 4 standard deviations (SD) of the normal inhabitants of the non-filariasis endemic area was used as a cut-off level between positive and negative presence of filarial antigen in the tested samples. The precision of the assay was determined by testing the same serial dilutions of adult *B. malayi* homogenate more than 8 times. The OD values were determined and the coefficient of variation (% CV) was calculated with the formula:

$$\% \text{ CV} = \frac{\text{SD}}{\bar{X} \text{ of OD}} \times 100$$

Reproducibility of the assay according to the criteria of Palmer and Cavallero was met.²⁵

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis (WB)

SDS-PAGE was carried out in a vertical slab gel apparatus (Miniprotean, Bio-Rad Laboratories, USA) according to the system of Laemmli.²⁶ A 4% acrylamide stacking gel and 12% acrylamide separating gel were used in the procedure. Western blot analysis (WB) was performed as previously described.^{27,28}

RESULTS

Hybridoma production

Twelve hybridoma clones were established. The MAb secreted by these clones could be classified into four different groups according to their reactivity to the homologous and heterologous antigens tested by the indirect ELISA. Group 1 reacted not only to the homologous antigen, but also to *D. immitis* antigen; however, it did not react to any other non-filarial heterologous antigen listed in Table 1. Group 2 gave reactions to *B. malayi*, *A. lumbricoides* and *D. immitis*. Group 3 reacted to *B. malayi*, *A. lumbricoides*, *D. immitis* and *E. malayanum*, while those of group 4 reacted to *B. malayi*, *A. cantonensis*, *A. lumbricoides*, *D. immitis*, *E. malayanum*, *E. revolutum*, *G. spinigerum* and *T. spiralis*. A representative hybridoma of group 1, i.e. clone 17E10, was selected for subsequent use in the sandwich ELISA for the detection of filarial antigen. This hybridoma clone 17E10 secreted IgM-kappa isotypes of immunoglobulin. At a stationary phase of cell growth, the spent culture medium always had a titer

of 1:256 when tested by indirect ELISA against the homologous antigen, implying cell stability. The Western blot pattern of the MAb 17E10 against the SDS-PAGE-separated *B. malayi* homogenate is shown in lane B of Fig. 1. The MAb reacted to adult *B. malayi* antigen in a smear pattern from approximately 37 kDa to > 200 kDa. A similar Western blot pattern was seen when the SDS-PAGE separated-microfilarial homogenate was used (data not shown). The smear Western blot pattern was reproduced using the proteinase-K-treated antigens, implying the non-protein nature of the MAb 17E10 epitope.

staining revealed that the MAb 17E10 reacted to the internal cuticle and various internal organs of both female and male worms (Fig. 2A, C and E) as well as to the sheath and internal content of microfilariae *in utero* (Fig. 2G). However, the MAb did not react to the sheath of blood circulating microfilariae (Fig. 2A). Overall anatomical locations in the worm of the specific epitopes targeted by the MAb 17E10 are shown in Fig. 2.

Antigen detection

The sandwich ELISA was performed on serially diluted adult *B. malayi* homogenates and it was found that the lowest amount of the antigen that the sandwich ELISA

Immunoalkaline-phosphatase

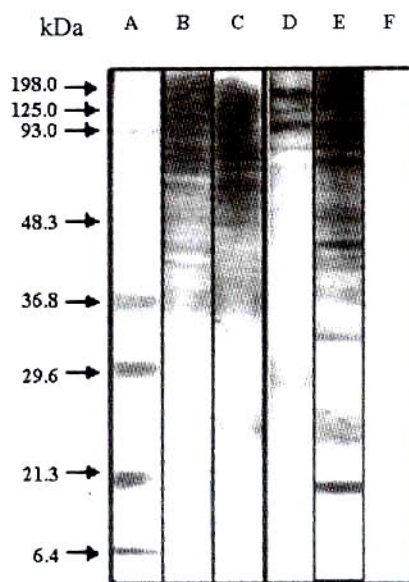


Fig. 1 Western blot analysis (WB) showing patterns of immune mouse serum (IS) and MAb of hybridoma clones against SDS-PAGE separated crude extract of adult *B. malayi*; lane A, molecular weight markers (kDa); lane B, MAbs of group 1 hybridoma clone; lane C, MAbs of group 2 hybridoma clone; lane D, MAbs of group 3 hybridoma clone; lane E, immune mouse serum (IS); lane F, culture supernatant of mouse myeloma cells (negative control).

could detect was 7.8 ng. For the untreated plasma samples of the 40 individuals of the NNE1 group, the mean (X) optical density (OD) was 0.026 and the standard deviation (SD) was 0.0065. $X + 4$ SD, which was 0.052, was used as cut-off limit between antigen positive and antigen negative status. None of the NNE1 samples was positive, which indicates a high specificity of the assay. The overall results of the antigen detection in the first set of specimens are shown in Table 2.

From the first set of specimens, it seemed that treatment of samples with SDS + Triton X-100 prior to antigen testing yielded the highest sensitivity of antigen detection by this sandwich ELISA. Thus, similar treatment was applied for all human samples of the second set which all consisted of plasma plus buffy coat (P + B), and also for the cat and dog samples, prior to subjecting them to the sandwich ELISA.

For the second set of specimens, the X of the OD was 0.028 and the SD was 0.008 for NNE2 samples. The cut-off limit between positive and negative filaria antigenemia was set at $X + 3$ SD which was 0.052. The overall results of the antigen detection of the second set of specimens are shown in Table 3. Among the 7 of 13 cases of L2 which were antigen negative, it was later revealed that three patients had received diethyl carbamazine (DEC) treatment prior to specimen collection.

Normal cat samples, i.e. P + B, gave a sandwich ELISA X at 0.024 and a SD at 0.019. The cut-off used for differentiating positive and negative antigenemia in the microfilaric cats was set at $X + 3$

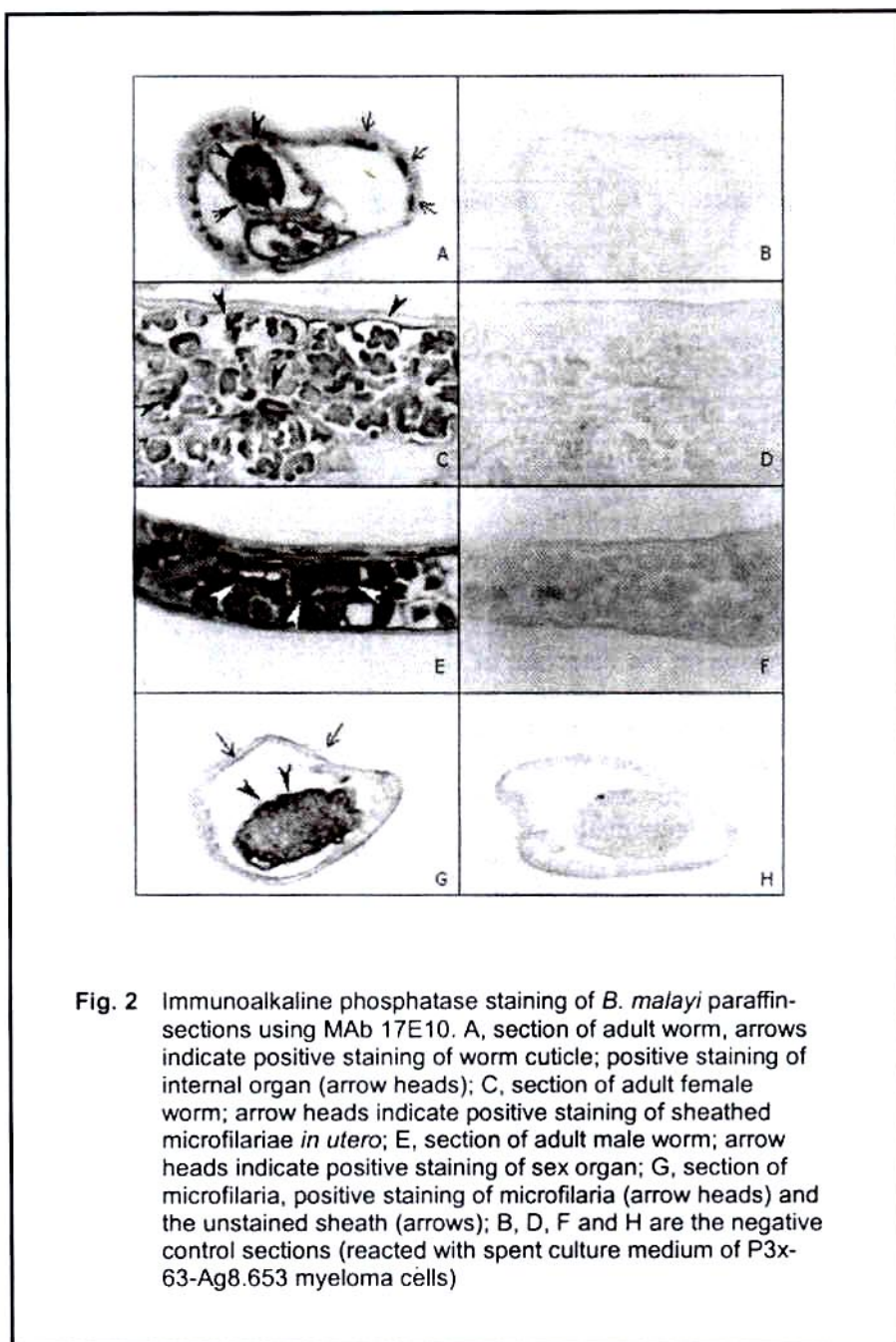


Fig. 2 Immunalkaline phosphatase staining of *B. malayi* paraffin-sections using MAb 17E10. A, section of adult worm, arrows indicate positive staining of worm cuticle; positive staining of internal organ (arrow heads); C, section of adult female worm; arrow heads indicate positive staining of sheathed microfilariae *in utero*; E, section of adult male worm; arrow heads indicate positive staining of sex organ; G, section of microfilaria, positive staining of microfilaria (arrow heads) and the unstained sheath (arrows); B, D, F and H are the negative control sections (reacted with spent culture medium of P3x-63-Ag8.653 myeloma cells)

SD which was 0.081. It was found that 14 of 15 (93.3%) of the microfilaric cats gave a positive antigen test.

The P + B of the five non-microfilaric dogs gave ODs of 0.302, 0.049, 0.062, 0.068 and 0.038 in the sandwich ELISA for antigen detection. The cut-off limit

was set at $X + 3$ SD (0.068) to differentiate positive and negative antigenemia using the MAb-based sandwich ELISA; it was found that all of the *D. immitis* microfilaric samples (100%) were positive.

DISCUSSION

Although antigen detection

Table 2 Results of antigen detection in the first set of specimens

Clinical status	Antigen detection test results								
	Untreated plasma			EDTA treated plasma			SDS + Triton X-100 treated plasma		
	No. test	No. +ve	(%)	No. test	No. +ve	(%)	No. test	No. +ve	(%)
Microfilaraemia (M1)	17	1	(5.8)	10	0	(0)	23	9	(39.1)
Lymphangitis (L1)	22	5	(22.7)	8	0	(0)	17	8	(47)
Elephantiasis (E1)	14	2	(14.2)	3	0	(0)	9	1	(11.1)
Endemic normal (NE1)	40	6	(15)	40	0	(0)	40	5	(12.5)

assays are commercially available for bancroftian filariasis in humans and dirofilariasis in dogs, no such assay is available for the routine diagnosis of brugian filariasis. Most research reports on the detection of circulating *B. malayi* antigen in human sera showed unsatisfactory sensitivity and specificity or the assay was evaluated using serum pools rather than individual sera.²⁹⁻³¹

In this study, attempts have been made to develop a specific serodiagnosis, i.e. a monoclonal antibody based- sandwich ELISA for current brugian filariasis by detecting antigenemia in individual subjects with different stages of filariasis, as well as in normal counterparts of disease endemic areas. The same assay was also used for detection of filarial antigenemia in infected animals.

In this study, hybridomas secreting monoclonal antibodies to filarial antigen were produced from a mouse immunized with *B. malayi* antigen. The MAb secreted by the clone 17E10 were selected for further use in a sandwich ELISA for detecting filarial antigen in human and animal blood samples. These MAb reacted only to *B. malayi* and *D. immitis* and not to any non-filarial parasites. Unfortunately other filarial worms, e.g. *W. bancrofti*, *On-*

Table 3 Results of antigen detection in the second set of specimens

Clinical status	Antigen detection test results (SDS + Triton X-100 treated plasma + buffy coat)		
	No. Test	No. +ve	% +ve
Microfilaraemia (M2)	31	22	70.9
Lymphangitis (L2)	13	7	53.8
Elephantiasis (E2)	14	2	14.2
Endemic normal (NE2)	100	10	10

chocera, *B. timori*, *Loa loa*, were not available for cross-reactivity testing of the MAb.

The MAb 17E10 produced a smear pattern in the Western blot analysis against SDS-PAGE separated-homogenate of adult *B. malayi* (lane B, Fig. 1). A similar pattern was obtained when the microfilarial extract was used (data not shown) indicating that the MAb specific epitope is not a stage-specific antigen. The epitope is non-protein in nature as indicated by its proteinase-K resistant nature. The smear Western blot pattern of the MAb 17E10 against SDS-PAGE separated-filarial antigen tends to indicate a highly repetitive polymeric structure, with large numbers of identical antigenic determinants. The strength of the signal obtained

through the binding of monoclonal antibodies as detection reagent in an antigen detection assay will be much greater with such antigen (containing highly repetitive epitopes) than when there is only a single antigenic determinant per molecule.²²

It has been known that a multitude of antigens of parasites including filaria are public antigens, i.e. they are shared not only by different developmental stages but also by members of different species.³² The antigens associated with internal organs performing basic functions essential to the survival of the worms are likely to be shared by many parasite stages and species.³³ Results of immunalkaline phosphatase staining confirmed that MAb 17E10 reacted to

a common epitope of adult *B. malayi* and microfilariae and also to *D. immitis* homogenate. Similar findings have been reported previously by Wenger *et al.*³⁴ and Gonzaga and Santos.³⁵

A major problem in detecting circulating parasite antigens in the host serum or plasma is the host's antibody response to such antigenic molecules, thereby reducing their availability for antigen detection. Ideally, the best target molecules would be those that are non-immunogenic in humans but immunogenic in other species, e.g. rabbits, mice, such that specific antibodies could be produced in these animals and used as an antigen capture/detection tool for human infection. In filariasis, circulating immune complexes have been demonstrated, both in experimental animal models and in humans^{29, 36-38} and several methods have been used to identify/detect the antigenic moieties in the so-formed circulating immune complexes. Lunde *et al.*³⁸ used a two-step method to treat the circulating immune complexes in serum samples of patients infected with *W. bancrofti*. The serum sample was mixed with EDTA and 12% polyethylene glycol 6,000 (PEG); after centrifugation, the pellet was washed in a 2.4% PEG solution and the antigen-antibody complexes were dissociated with a glycine-HCl solution, before being used in an ELISA for antigen detection. In other studies, samples of *W. bancrofti* infected-subjects were treated with disodium EDTA, and heated to precipitate the complexes prior to antigen testing. In this study, the buffy coat and the plasma of the studied subjects and animals were used as source of the antigenic target for our polyclonal-monoclonal

antibody based-antigen detection assay. The buffy coat was used because independent of whether the antigens were free in the blood or already had formed immune complexes they had to be engulfed by blood phagocytes and processed, either for further presentation to T lymphocytes in order to induce the host immune response or for being broken down to the smallest units, e.g. amino acids, sugars, etc. which might be re-used or discarded. Moreover spinning of the anticoagulated blood should also concentrate the intact microfilariae, if there were any, to the buffy coat area. Plasma was included in order not to miss any of the circulating antigen, which had escaped phagocytosis. For antigen detection, blood collected in three hematocrit tubes was used. SDS and Triton X-100 were added to lyse the leucocyte cytoplasmic membrane, the endosomal/lysosomal membrane as well as the sheath of the microfilariae thus releasing the respective intracellular contents including the target antigens. It was found that the addition of the SDS, Triton X-100 and distilled water not only prevented sample coagulation but also eliminated the background endogenous enzymes that would otherwise interfere with the immunoassay interpretation.

The sandwich ELISA performed on SDS and Triton X-100 treated samples yielded the highest sensitivity when compared with the assay performed on untreated or EDTA-treated samples. The sensitivity of the ELISA for the microfilaremic group (M2) was 70.9% while for samples of patients with lymphangitis (L2) and elephantiasis (E2) was 53.8 and 14.2%, respectively. Weil *et al.*³⁹ also found sam-

ples of microfilaremic and amicrofilaremic patients with clinical bancroftian filariasis, i.e. lymphangitis, and elephantiasis, which were negative by an antigen test. Several reasons may be given for these findings which were also observed in this study about brugian filariasis. The negative results in some of the cases may be first due to the patients no longer harboring the adult parasites (most probable in elephantiasis cases, but also possible in lymphangitis and microfilaremics).¹¹ Second, these results may be due to the pathway of clearance of the parasite antigens from the blood. It is known that besides the clearance of the circulating antigen or immune complexes by phagocytosis, other mechanisms also deprive the antigen from the plasma and phagocytes. Much of the complement component (C3b, C4b) coated antigens could be efficiently bound by red blood cells via the CR1 (CD 35), which is a complement derived opsonin receptor found abundantly on the erythrocyte membranes. As the blood circulates, the complement coated antigens would subsequently be destroyed in the liver and spleen. It is also possible that the negative antigen results of the microfilaremic samples (~ 30%) in this study were due to the inadequate amount of blood samples used for preparing the test samples. Three hematocrit tubes might not contain detectable levels of antigen for the sandwich ELISA in some cases (detection limit is 7.8 ng of adult worm homogenate); thus, larger volumes of blood might be needed. Unfortunately, owing to the limited amount of blood originally obtained and the subsequent mass treatment of the microfilaremic cases in the endemic areas launched by the Ministry of Public

Health, Thailand, it was not possible to retest the blood samples of the antigen negative, microfilaria positive cases by the sandwich ELISA. Filarial antigen was detected in patients with lymphangitis and elephantiasis in whom microfilariae could not be detected by microscopic examination and in 10% of normal inhabitants of the filaria endemic areas. These findings agreed with the work of Weil *et al.*³⁹ who performed an antigen detection assay of bancroftian filariasis and found the antigen in samples of amicrofilaremic patients with clinical manifestations.

The sandwich ELISA, using MAb 17E10 as detection reagent, could also detect antigens of *D. immitis* in animal samples. Ideally the sandwich ELISA developed in this study should be tested on anticoagulated blood samples of patients or animals infected with other filarial parasites in order to see whether the test is positive in those cases as well. Unfortunately, such samples were not available. Thus, it is too early to speculate whether the assay could be used for pan diagnosis of filarial infection. Nevertheless, it offers an alternative to the current standard diagnostic procedures for filariasis, especially brugian filariasis, for which no antigen test is presently available. The target antigen of the sandwich ELISA is heat stable and can be obtained from the subject at any time of the day, which is an advantage over the detection of circulating microfilariae for which only nocturnal blood samples can be used. The test has a high sensitivity and specificity; it is rapid and inexpensive. Several samples can be tested at the same time without much increase in the overall test time. All of these character-

istics make this sandwich ELISA attractive as an alternative routine diagnostic test for clinical use as well as for mass screening of samples in an epidemiological survey of filariasis. Moreover, the test may be used for evaluation of the treatment efficacy as it is reasonable to speculate that a positive antigen result should be reversed after successful chemotherapy.

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