EVALUATION OF CRUDE ANTIGEN OF *DIROFILARIA IMMITIS* THIRD-STAGE LARVA FOR DETECTION OF ANTIBODY AGAINST *WUCHERERIA BANCROFTI* INFECTION BY INDIRECT ELISA

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Abstract. *Dirofilaria immitis* is an important heart worm in dogs. An immunodiagnostic test is frequently applied to use an alternative antigen from other parasites. A crude antigen from infective third stage larva (L3) of *D. immitis* was employed in detecting the antibody to Bancroftian filariasis in humans by indirect ELISA. It was shown that 25 cases of Bancroftian filariasis (76%) at a cut-off value of 0.230, were positive. Cross-reactivity was tested using available sera of other helminthic infections. These sera were 47% (23/49) positive. They comprised a major intestinal helminthic infection, 7 from 15 (46%) strongyloidiasis sera, none from 5 (0%) hookworm infection sera, 6 from 10 (60%) trichinosis sera, 2 from 10 (20%) cysticercosis sera and 8 from 9 (88%) gnathostomiasis sera. The mean OD of sera from Bancroftian filariasis patients was not significantly different from that of the other helminthic infections (p>0.05). In this study, crude antigen may be valuable for the serodiagnosis of *Wuchereria bancrofti* when subjects do not have tissue helminth infections. However, the crude antigen should be purified to obtain a better sensitivity and specificity of the test.

INTRODUCTION

Lymphatic filariasis is an important widespread disease, especially in the tropical regions of the world, such as southern and south-eastern Asia, the Pacific and eastern Africa. The disease is spreading among the poor people of the developing countries in these regions. There are three human lymphatic filariae, *ie Wuchereria bancrofti*, *Brugia malayi* and *B. timori* (WHO, 2001). In Thailand, *W. bancrofti* is spreading in 15 provinces, particularly in hilly, forested and mountainous areas at the Myanmar-Thai border (Division of Malaria, 1998). Control measures for filariasis include mass chemotherapy and vector control, *ie* mass treatment with diethylcarbamazine (DEC), DEC-medicated salt and ivermectin; and insecticide and biological control by using bacteria (*Bacillus sphaericus*) (WHO, 1999). Although these methods have been used to reduce both parasites and insect vectors, they have never overcome natural transmissions of the causative agents of filariasis. The detection of filariasis has been classically demonstrated by characteristics of microfilariae in circulating blood. Precise detection is one of the important points by which the distribution of the disease can be stopped after treatment.

Immunological methods are particularly useful for the detection of antibodies and circulating antigens in the peripheral blood of infected persons, when microfilariae are present in small numbers or totally absent from the circulation (Lim, 1992; WHO, 1992; Cross, 1993). Ottesen *et al* (1982) showed that immunological techniques could detect filariasis in asymptomatic individuals living in endemic areas. Recent applications of molecular biology and biotechnology have contributed to the development of potentially specific and sensitive assays for the diagnosis of filariasis (Lim, 1992; WHO, 1992; Cross, 1993).

Both human and animal filarial parasites are involved in producing various kinds of antigens, *eg* surface, ES and somatic, and purified antigens, such as *B. malayi* (Kaushal *et al*, 1982; Maizels *et al*, 1989), *B. pahangi* (Sutanto *et al*, 1985; Devaney, 1988), *W. bancrofti* (Maizels *et al*, 1986; Morgan *et al*, 1986), and *D. immitis* (Sawada *et al*, 1969). Somatic extracts can be produced from many stages of filarial parasites, *eg* microfilariae, infective larvae and adult worms of *B. malayi* and *B. pahangi* (Gusmao *et al*, 1981; Hamilton *et al*, 1981; Kaushal *et al*, 1984), and *Onchocerca volvulus* (Weiss *et al*, 1982). However, the use of somatic extracts frequently poses cross reactivity with antibodies from other parasitic infections. In contrast, Kaushal *et al* (1984) showed minimal reactivity between antigens derived from *Brugia* adults and non-filarial pool sera, despite the presence of strong antibody reactivity with their homologous antigens in those pools. Adolph *et al* (1962) tested the sera of 27 infected patients with

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microfilariae of *Acanthocheilonema perstans* using *D. immitis* antigen in the hemagglutination test. In this group, 26 of 27 samples (96.3%) gave positive reactions. Sera from 26 patients with clinical symptoms of filariasis, without microfilaria in the blood, were also tested in the same manner. Twenty-five of 26 sera (96.2%) were found positive.

The heterologous species of filarial worms have been employed in the diagnosis of human filariasis, *W. bancrofti* and *B. malayi*. One of these models is *D. immitis*, which has been frequently used in the immunodiagnosis of human and dog dirofilariasis, but there has been little study in human filariasis. *D. immitis* is an important filarial parasite in dogs and cats, and the worm is a useful model for human filariasis. In 1996, Harnnoi and colleagues worked on serologic cross-reactions between adult somatic antigens of *D. immitis* and helminthiasis sera using the ELISA and Western blot techniques. They found that Bancroftian filariasis and cysticercosis sera did not give a positive result with ELISA. Recently, Insun (1997) also investigated the ES and surface antigen of *D. immitis* adult worms. The results indicated that both kinds of antigen gave no satisfaction in the diagnostic test of IgG-ELISA. The infective third-stage larvae of *D. immitis* may be more suitable, as fewer proteins, which still contain antigens that react *to W. bancrofti* antibodies in sufficient quantity, are expected. An obstacle to antigen preparation from human filarial parasites occurs in the development of immunodiagnosis, because of the maintenance of parasites in animal hosts (monkey) and the quality required for antigen extraction. Therefore, the main purpose of this study was to use animal filaria, infective third stage filarial larvae (L3) of *D. immitis* prepared crude antigen. The value of crude antigens could be tested by indirect ELISA with human filariasis sera infected with *W. bancrofti.*

MATERIALS AND METHODS

Aedes togoi mosquitos

The laboratory strain of *Aedes togoi* was originally obtained from Koh Nom Sao, Chantaburi Province, southeastern Thailand. This strain had been maintained in the insectarium of the Department of Parasitology, Faculty of Medicine, Chiang Mai University, since 1996. This mosquito strain was proven to be highly susceptible to *D. immitis* (Choochote *et al*, 1987).

Infecting mosquitos with *D. immitis* microfilariae

Ten milliliters of dirofilariae dog's blood were drawn by venipuncture into a heparinized syringe for each bleeding. The preferable number of microfilariae for feeding mosquitos ranges from 40-60 microfilariae per 20 μl of blood. The high density of microfilariae in the dog's blood was diluted with Hank's balance salt solution (HBSS). Three-day old adult females of *Ae. togoi* were allowed to feed on heparinized blood containing microfilariae of *D. immitis* by the artificial membrane feeding technique, as described by Chomcharn *et al* (1980). After feeding, the engorged mosquitos were reared in the insectarium at a temperature of 27°± 2°C with a relative humidity of 70-80%. The engorged mosquitos were fed with 10% sugar solution soaked into a cotton ball until ready for dissection.

Harvesting the infective larvae (L3)

After 15 days of infected-blood feeding, the mosquitos were dissected and examined for infective larvae. The head, thorax and abdomen of individual mosquitos were torn apart in separate pools of normal saline solution and examined for infective larvae under a dissecting microscope. The infective larvae recovered were pooled and stored at -70°C.

Serum collection

Blood samples were collected by venipuncture and divided into 3 groups. Group A consisted of the sera from 33 individuals with *W. bancrofti* microfilariae. Group B consisted of the sera from 40 individuals in a non-endemic area of filariasis. Group C consisted of the sera from 49 individuals who were infected with other parasitic infections. Serum samples were prepared by centrifugation of blood and storage at -70°C.

Preparation of crude antigen

The crude antigen was prepared following the method of Cox *et al* (1981), with some modifications. The infective third stage filarial larvae (L3) were suspended in a buffer [0.5 mM ethylenediaminetetra-acetic acid (EDTA), 1 mM phenylmethyl sulphonyl fluoride (PMSF), 0.01 mg/ml leupeptin-antipen], crushed by a tissue grinder and sonicated on ice. The supernatants were collected in an aliquot and stored at -70°C. Protein determination of the crude antigen was estimated by measuring absorbance at 280 nm. The level of anti-filarial antibody of the crude antigen was determined by ELISA using microtiter plates. The Student's *t*-test was used to compare OD distribution between the filariasis group and other helminth-infected groups. The sensitivity, specificity, positive and negative predictive values and the efficacy of the assay were calculated using the method of Galen (1979).

Indirect enzyme-linked immunosorbent assay (Indirect ELISA)

ELISA was performed on a microtiter plate, as described by Voller *et al* (1975), with slight modifications. The optimal concentrations of crude antigen, positive pooled serum, negative pooled serum and conjugate were predetermined by a checkerboard titration. The positive pooled serum was collected from ten sera of group A, and the negative pooled serum from ten sera of group B.

Briefly, to find the optimal concentration of surface antigens, microtiter plate wells were filled with 100 µl of coating buffer containing protein concentrations of 2.5, 5 and 10 µg/ml. The plate was kept in a humidified chamber, then incubated at 4°C overnight before washing 3 times with PBS containing 0.05% Tween 20 (PBS-Tween). After that, each well was filled with 100 µl of pooled filariasis positive sera and negative sera that had been previously diluted at a ratio of 1:50 with PBS-T (duplicated in each serum dilution). Blank wells received PBS-T, instead of diluted serum. The plate was incubated at 37°C for 1 hour, and then washed 5 times. One hundred microliters of peroxidase conjugate anti-human IgG, diluted at a ratio of 1:2,500 in PBS-T, were added to each well, with the exception of the blank wells, and the plate was incubated for 1 hour at 37°C. After washing 3 times with PBS-Tween, 100 µl of freshly prepared substrate solution containing O-phenylenediamine dihydrochloride was added to each well and the enzyme-substrate reaction was allowed to occur in the dark at room temperature. After 30 minutes' incubation, the reaction was stopped by adding 50 µl of 8 N H_2SO_4. The optical density measurement of each well was made against the blank well at 492 nm by using a microplate spectrophotometer. After checkerboard titration, an optimal concentration of antigen (5 µg/ml), dilution ratios of serum at 1:50, and of conjugate at 1:2,500, were used for further tests with filariasis cases, various other helminth-infected and normal healthy control sera. The cut-off level was determined and ELISA sensitivity, specificity, and positive and negative predictive values were calculated.

RESULTS

The total crude antigens collected from 1,743 of all *D. immitis* (L3) comprised 212 µg of proteins or 0.12 µg/worm. The crude antigens from *D. immitis* (L3) were used in the indirect ELISA for detecting antibody levels to *W. bancrofti*. An optimal concentration of crude antigens was 5 µg/ml at a conjugated dilution ratio of 1:2,500, with both positive and negative pooled sera at a dilution ratio of 1:50.

A total of 33 serum specimens of Bancroftian filariasis were tested for a specific antibody. Sera taken from 40 apparently healthy adults and 49 other helminth infections were also run for control and cross-

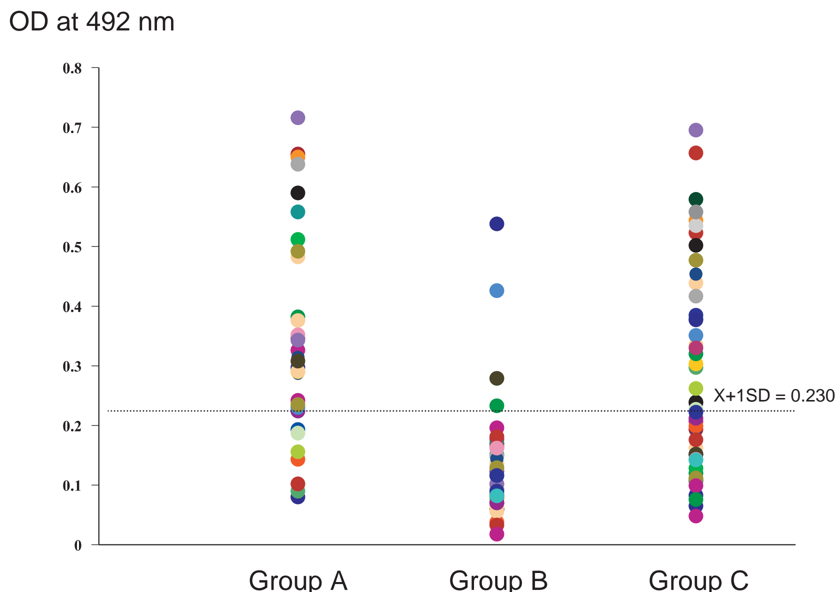

Fig 1- Distribution of ELISA values of filariasis sera, healthy controls and other helminth infections. Group A = 33 patients with Bancroftian filariasis; Group B = 40 normal healthy controls; Group C = 49 patients with other helminthic infections.

reactivity, respectively. It was found that 25 cases of group A (76%), 4 of group B (10%) and 23 of group C (47%) were positive (Fig 1). According to the optimal conditions above, the OD of sera from the three groups were compared for statistical significance. The mean OD of sera from group A was not significantly different from that of group C (p>0.05), but was significantly higher than that of group B. The mean, SD and OD ranges of the sera from 33 Bancroftian filariasis patients were 0.363, 0.187 and 0.070-0.800, respectively. The mean+1SD of 0.230 was determined from the OD group of normal healthy adults and used as the threshold value to calculate the positivity of the serum samples (Table 1). The sensitivity, specificity, positive and negative predictive values of the test were 76%, 70%, 49% and 88.5%, respectively. Regarding the cross-reaction with helminthiasis sera, the present study found that 7 of 15 (46%) strongyloidiasis sera cases, none of 5 (0%) hookworm infection cases, 6 of 10 (60%) trichinosis cases, 2 of 10 (20%) cysticercosis sera cases (20%) and 8 of 9 (88%) gnathostomiasis sera cases, were positive (Table 2).

DISCUSSION

In this study, 33 patients with *W. bancrofti* infections, 40 normal healthy controls and 49 patients with other helminthic infections were tested against the crude antigen of *D. immitis* (L3) using the indirect ELISA test. The result revealed that sensitivity and specificity, at the cut-off level 0.230, were 76% and 70%, respectively. Twenty-five of 33 cases (76%) of Bancroftian filariasis, 4 of 40 (10%) normal healthy controls and 23 of 49 (47%) cases of other helminthic infections, gave positive reactions. Cross-reaction from other helminthic infections, *ie* 7 of 15 (46%) strongyloidiasis cases, 6 of 10 (60%) trichinosis cases, 2 of 10 (20%) cysticercosis cases, and 8 of 9 (88%) gnathostomiasis cases, showed OD above the cut-off values. These findings were more or less similar to those of Harnnoi *et al* (1996), who tested the somatic antigen of adult *D. immitis* with sera from helminth infections. They found that a high percentage of sera from trichinosis, gnathostomiasis, angiostrongyliasis, opisthorchiasis and paragonimiasis patients cross-reacted with somatic antigens of *D. immitis, ie* 78.3%, 35%, 85%, 27.7% and 26.7%, respectively. Insun (1997) also investigated the ES and surface antigens produced from *D. immitis* adult worms and could not obtain good sensitivity and specificity with IgG-ELISA. In that study, the use of ES and surface antigens of *D. immitis* adult worms produced sensitivities of 80% and 63%, and specificities of 62% and 61% respectively. The sera from patients with other helminthic infections: gnathostomiasis, strongyloidiasis, trichinosis, angiostrongyliasis, ascariasis, echinococcosis, taeniasis, sparganosis and opisthorchiasis, gave cross-reactions with both ES and surface antigens and showed OD above the cut-off values.

The crude antigen of *D. immitis* (L3) in this study gave satisfactory results in the diagnostic test of Bancroftian filariasis sera using indirect ELISA. However, this antigen may need to be purified in order

Table 1
Positivity of sera from 3 groups of patients at cut-off value 0.230 (mean+1SD).

Groups tested	No. of sera tested	No. positive (%)
A. Bancroftian filariasis	33	25 (76)
B. Healthy controls	40	4 (10)
C. Other helminthic infections	49	23 (47)

Table 2
Positivity of helminth sera infection groups at cut-off value 0.23 (mean+1SD).

Other helminth group	No. of sera tested	No. positive (%)
Strongyloidiasis	15	7 (46)
Hookworms	5	0 (0)
Trichinosis	10	6 (60)
Cysticercosis	10	2 (20)
Gnathostomiasis	9	8 (88)

to eliminate cross-reaction with other helminthic infections. Theodore and Kaliraj (1990) prepared the crude surface antigen of the adult bovine filaria *S. digitata* by EDTA extraction, and purified it by affinity chromotography using Sepharose-bound human filaria antibody obtained from chronic human filarial sera. The purified and crude antigens were used in the ELISA test for the detection of serum antibodies in filariasis caused by *W. bancrofti*. In summary, the results of this study indicated that the crude antigen of infective third stage larva of *D. immitis* is potentially useful for the immunodiagnosis of Bancroftian filariasis using indirect ELISA.

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REFERENCES

Adolph PE, Kagan IG, McQuay RM. Diagnosis and treatment of *Acanthochilonema perstans* filariasis. *Am J Trop Med Hyg* 1962;11:76-88.

Chomcharn Y, Surathin K, Bunnag D, Sucharit S, Harinasuta T. Effects of a single dose of primaquine on a Thai strain of *Plasmodium falciparum*. *Southeast Asian J Trop Med Public Health* 1980;11:408-12.

Choochote W, Keha P, Sukhavat K, Khamboonruang C, Sukontason K. *Aedes* (Finlaya) *togoi* Theobald 1907, Chanthaburi strain, a laboratory vector in studies of filariasis in Thailand. *Southeast Asian J Trop Med Public Health* 1987;18:259-60.

Cox GN, Kusch M, Edgar RS. Cuticle of *Caenorhabditis elegans*: its isolation and partial characterization. *J Cell Biol* 1981;90:7-17.

Cross J. Recent advances in epidemiological field techniques in filariasis. *Southeast Asian J Trop Med Public Health* 1993;24(suppl 2):40-4.

Devaney E. The biochemical and immunochemical characterisation of the 30 kilodalton surface antigen of *Brugia pahangi*. *Mol Biochem Parasitol* 1988;27: 83-92.

Division of Malaria, Department of Communicable Disease Control, Ministry of Public Health. Annual Report. 1998.

Galen RS. The predictive value of laboratory testing. *Orthop Clin North Am* 1979;10:287-97.

Gusmao RD, Stanley AM, Ottesen EA. *Brugia pahangi*: immunologic evaluation of the differential susceptibility to filarial infection in inbred Lewis rats. *Exp Parasitol* 1981;52:147-51.

Hamilton RG, Hussain R, Ottesen EA, Adkinson NF Jr. The quantitation of parasite-specific human IgG and IgE in sera: evaluation of solid-phase RIA and ELISA methodology. *J Immunol Methods* 1981;44:101-14.

Harnnoi T, Morakote N, Uparanukraw P. Reactivity of sera from helminthiosis patients with saline extract of *D. immitis*. *Chiang Mai Bull* 1996;35:97-102.

Insun D. Evaluation of excretory-secretory and partial surface antigens of *D. immitis* against antibody of *W. bancrofti* infection by indirect ELISA and IgG, IgG4-immunoblot. Bangkok: Mahidol University. 1997. MSc Thesis.

Kaushal NA, Hussain R, Nash TF, Ottesen EA. Identification and characterization of excretory-secretory products of *Brugia malayi*, adult filarial parasites. *J Immunol* 1982;129:338-43.

Kaushal NA, Hussain R, Ottesen EA. Excretory-secretory and somatic antigens in the diagnosis of human filariasis. *Clin Exp Immunol* 1984;56:567-76.

Lim PK. Recent advances in diagnostic techniques in filariasis. *Southeast Asian J Trop Med Public Heath* 1992;24(suppl 2):45-50.

Maizels R, Burke J, Sutanto I, Purnomo, Partono F. Secreted and surface antigens from larval stages of *Wuchereria bancrofti*, the major human lymphatic filarial parasite. *Mol Biochem Parasitol* 1986;19:27-34.

Maizels RM, Gregory WF, Kwan-Lim GE, Selkirk ME. Filarial surface antigens: the major 29 kilodalton glycoprotein and a novel 17-200 kilodalton complex from adult *Brugia malayi* parasites. *Mol Biochem Parasitol* 1989;32:213-27.

Morgan TM, Sutanto I, Purnomo, Sukartono, Partono F, Maizels RM. Antigenic characterization of adult *Wuchereria bancrofti* filarial nematodes. *Parasitology* 1986;93:559-69.

Ottesen EA, Weller PF, Lunde MN, Hussain R. Endemic filariasis on a Pacific island. II. Immunologic aspects: immunoglobulin, comple-

ment and specific antifilarial IgG, IgM and IgE antibodies. *Am J Trop Med Hyg* 1982;31:953-61.

Sawada T, Sato K, Sato S. Studies on the skin test antigen FST for immunodiagnosis of filariasis. 1. Electrophoretic analysis and fractionation of antigen FST. *Jpn J Exp Med* 1969;39:427-33.

Sutanto I, Maizels RM, Denham DA. Surface antigens of a filarial nematode: analysis of adult *Brugia pahangi* surface components and their use in monoclonal antibody production. *Mol Biochem Parasitol* 1985;15:203-14.

Theodore JG, Kaliraj P. Isolation, purification and characterization of surface antigen of the bovine parasite *Setaria digitata* for the immunodiagnosis of bancroftian filariasis. *J Helminthol* 1990;64: 105-14.

Voller A, Draper C, Bidwell DE, Bartlett A. Microplate enzyme-linked immunosorbent assay for Chagas' disease. *Lancet* 1975;1:426-8.

Weiss N, Hussain R, Ottesen EA. IgE antibodies are more species-specific than IgG antibodies in human onchocerciasis and lymphatic filariasis. *Immunology* 1982;45:129-37.

WHO. Epidemiologic approaches to lymphatic filariasis elimination: initial assessment, monitoring and certification. World Health Organization, Atlanta, Georgia, USA. *WHO/FIL/99.195.* 1999:1-35.

WHO Expert Committee on Filariasis. Lymphatic filariasis : the disease and its control. Fifth report of the WHO Expert Committee on Filaiasis. *WHO Tech Rep Ser* 1992;821:1-71.

WHO. Regional strategic plan for elimination of lymphatic filariasis. New Delhi: World Health Organization, 2001:1-30.