DETECTION OF IgG ANTIBODIES OF BRUGIAN FILARIASIS WITH CRUDE MALE AND FEMALE ANTIGENS OF *DIROFILARIA IMMITIS*

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Abstract. Crude antigens from male and female *Dirofilaria immitis* were used to detect antibody to Brugian filariasis in humans by indirect ELISA. Both antigens were tested with 42 cases of Brugian filariasis, 131 cases of 20 heterologous infections and 35 healthy controls. The results - using male and female antigens - showed sensitivity of 88.1% and 88.1%, and specificities of 64.1% and 51.8%, respectively. Cross-reaction from other helminthic infections using crude male antigen gave false-positives with 48 sera from 13 heterologous diseases at the threshold value of 0.180, while the female antigen gave 63 sera from 15 diseases, at 0.309. Serum antibodies from patients with other helminthic infections - gnathostomiasis, strongyloidiasis, hookworm infections, trichinellosis, capillariasis, angiostrongyliasis, ascariasis, trichuriasis, toxocariasis, neurocysticercosis, cystic echinococcosis, taeniasis and opisthorchiasis - resulted in false-positives with both male and female antigens. One each of sparganosis and paragonimiasis heterotremus sera cross-reacted with only crude female antigen and their OD values were close to the threshold value. Although crude male antigen showed better specificity than crude female antigen, both female and male worms are sources of antigens needed for further purification. This study provides baseline data for further serodiagnosis of Brugian filariasis using dirofilaria antigen.

INTRODUCTION

Lymphatic filariasis is an important disease in tropical and sub-tropical regions of the world. Three lymphatic filariae are found in humans: *Wuchereria bancrofti*, *Brugia malayi*, and *B. timori*. In Thailand, human filariasis is caused by worms of the species *W. bancrofti* and *B. malayi*. *W. bancrofti* is found along the western border in 7 provinces: Lamphun, Mae Hong Son, Tak, Kanchanaburi, Ratchaburi, Phetchaburi, and Prachuap Khirikhan. *B. malayi* is still a problem in the southern part and spreading in people of 6 provinces: Nakhon Si Thammarat, Surat Thani, Phatthalung, Pattani, Yala, and Narathiwat provinces (Filariasis Division, 1998). Thick blood smear examination is the routine method for diagnosis of *Wuchereria* and *Brugia* in endemic areas. This diagnostic method depends on the detection of microfilariae, but requires nighttime blood collection and is unpopular with the local population. Furthermore, this method is relatively insensitive and difficult to perform accurately and consistently in field situations (Rahmah *et al*, 2004).

Serodiagnosis is the method of choice for detecting infection without disturbing patients and for studying groups in regional foci at night-time (WHO, 1998). Crude extracts, surface antigen, excretory-secretory products, purified antigens, and fractionated antigens of complex extracts of different stages of both human and animal filarids have been used to detect antibodies against human filariasis (Kaushal *et al*, 1984; Maizels *et al*, 1987; Ata *et al*, 1993; El Serougi *et al*, 2000; Rahmah *et al*, 2001). *D.immitis* is a useful filarial animal model for studies on the serodiagnosis of *W. bancrofti* infection and *D. immitis* in humans (Philipp *et at*, 1984; Harnnoi *et al*, 1996; Dekumyoy *et al*, 2000; Riyong *et al*, 2003). Adolph *et al* (1962) tested blood sera of 28 patients infected with microfilariae of *Acanthocheilonema perstans* by the hemagglutination test using *D. immitis* antigen. In this group, 26 of 27 samples (95%) gave positive reactions. Sera of 26 patients with clinical symptoms of filariasis without microfilariae in the blood were also tested in the same manner. They found that 25 of 26 sera (96%) were positive. Fujita *et al* (1970) used the extract of adult worms of *D. immitis* as antigen for hemagglutination testing against sera of humans and animals infected with various species of filariae. Normal patterns of cross-reaction were obtained from their experiments. Thus, this study evaluated crude male and female *D. immitis* adult worm antigen in detecting antibodies to Brugian filariasis by indirect-ELISA.

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MATERIALS AND METHODS

Parasite collection

Adult worms of *D. immitis* were collected from

the right ventricles and pulmonary arteries of naturally infected dogs killed by road accidents. The worms were washed with 0.25% sodium hypochlorite for 10 minutes on a rocking platform, followed by 1-2 times in sterile saline solution, and kept at -70˚C.

Preparation of crude antigen

Crude antigen was prepared separately from both sexes of frozen adult *D. immitis* worms according to the method described by Dekumyoy *et al* (2000). The worms were washed with distilled water and macerated with alumina paste. The suspension was sonicated by Supersonicator and centrifuged at 20,000*g* for 30 minutes, at 4˚C. The supernatant was dialyzed and concentrated using Amicon (PM 10). The protein contents were determined by Coomassie Plus Protein Assay Reagent Kit (Pierce, USA).

Serum collection

The serum samples were divided into three groups. Group A: 42 infected individuals with *B. malayi* microfilariae. Group B: 131 cases of other helminthic infections diagnosed by either parasitological or serological examinations, as follows: gnathostomiasis (10), strongyloidiasis (10), hookworm infections (10), trichinellosis (10), capillariasis (3), angiostrongyliasis (10), ascariasis (7), trichuriasis (7), toxocariasis (6), neurocysticercosis (10), cystic echinococcosis (4), taeniasis (10), sparganosis (3), hymenolepiasis nana (3), paragonimiasis heterotremus (9), opisthorchiasis (10), haplorchiasis taichui (5), giardiasis (2), amebiasis (2). Group C: 35 healthy controls that tested negative for parasites by fecal examination.

Indirect ELISA

Crude male and female antigens of *D. immitis* were diluted with 0.05 M carbonate-bicarbonate buffer at pH 9.6, to a proper microgram per milliliter. One hundred microliters of antigen dilution were added to each well of a microtiter plate (Nunc, Denmark). The plates were incubated at 37˚C for 1 hour, then overnight at 4˚C. Next, the unabsorbed antigen was removed by washing solution in a microshaker, 1 minute, 2 times. The unbound surface antigen of the wells were blocked with 150 µl of 1% BSA-PBS, pH 7.4-0.02% sodium azide, incubated for 1 hour at 37˚C, and washed as above. The primary antibody of serum samples was diluted with a washing solution containing 0.02% NaN_3-0.008% bromphenol blue and put in triplicate wells. The reaction occurred at 37˚C for 1.5 hour, followed by washing for 1 minute, 4 times. The immune complexes combined with 100 µl of a proper dilution of secondary antibody (conjugate: rabbit anti-human IgG linked with peroxidase, Dakopatt) were incubated at 37˚C for 1.5 hour and then washed 4 times, as above. The reactions were visualized with 100 µl of substrate ABTS [2,2-azino-di- (3-ethyl-benzthiazoline sulfonate, Sigma)] after a 30-minute incubation. Absorbance values were measured at 405 nm after adding 1% SDS to stop the reaction. In this study, the proper concentrations were determined by checkerboard titration of crude male and female antigens and were 4 µg/ml, with dilutions of serum samples at 1:200 and conjugate at 1:4,000 and 1:2,000, respectively.

RESULTS

According to the optical conditions of indirect-ELISA, the negative and positive discrimination thresholds were determined. The threshold value for male *D. immitis* antigen was 0.180 and the threshold value for females was 0.309 (Fig 1, 2). Thirty-seven of 42 Brugian filariasis cases gave higher OD values than the threshold values of male and female antigens. These critical values gave 88.1 and 88.1% sensitivity, while the specificities were 61.4 and 51.8%, respectively. According to the optical density values of both antigens, the three groups were compared for statistical analysis. The mean OD of sera from group A was not significantly different from that of group B ($p>0.05$), but was significantly higher than group C.

Regarding crude female antigen and the threshold value 0.309, 51.8% specificity presented false positive with 63 sera from 15 heterologous diseases: gnathostomiasis (4/10 cases), strongyloidiasis (9/10), hookworm infections (4/10), trichinellosis (10/10), capillariasis (3/3), angiostrongyliasis (6/10), ascariasis (4/7), trichuriasis (3/7), toxocariasis (2/6), neurocysticercosis (6/10), cystic echinococcosis (4/4), taeniasis (5/10), sparganosis (1/3), paragonimiasis heterotremus (1/9), opisthorchiasis (1/10) and healthy controls (17/35 cases). Crude male antigen showed 61.4% specificity of the test at the threshold value of 0.180 and cross-reactivity occurred with 48 cases of 13 diseases: gnathostomiasis (4/10 cases), strongyloidiasis (10/10), hookworm infections (1/10), trichinellosis (9/10), capillariasis (3/3), angiostrongyliasis (6/10), ascariasis (3/7), trichuriasis (1/7), toxocariasis (2/6), neurocysticercosis (2/10), cystic echinococcosis (4/4), taeniasis (2/10), opisthorchiasis (1/10) and healthy controls (15/35 cases). Sparganosis and paragonimiasis heterotremus, which cross-reacted with the crude female antigen, became true negative with crude male antigen (Table 2). All nematode serum antibodies cross-reacted with both female and male crude antigens. However, only one or two cases of some diseases showed cross-binding and low ODs-

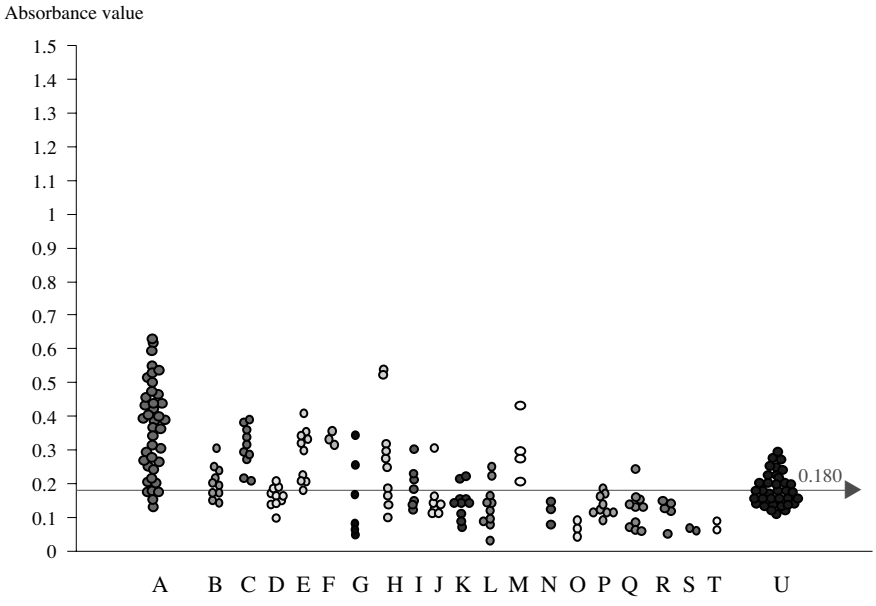

Fig 1- Distribution of ELISA values using crude male *D. immitis* antigen against filariasis sera, other helminthic infections and healthy controls at threshold value 0.180. The columns indicate as follows; A = Brugian filariasis, B = gnathostomiasis, C = strongyloidiasis, D = hookworm infections, E = trichinellosis, F = capillariasis, G = toxocariasis, H = angiostrongyliasis, I = ascariasis, J = trichuriasis, K = taeniasis, L = neurocysticercosis, M = cystic echinococcosis, N = sparganosis, O = hymenolepiasis, P = paragonimiasis heterotremus, Q = opisthorchiasis, R = haplorchiasis taichui, S = giardiasis, T = amebiasis, U = healthy controls.

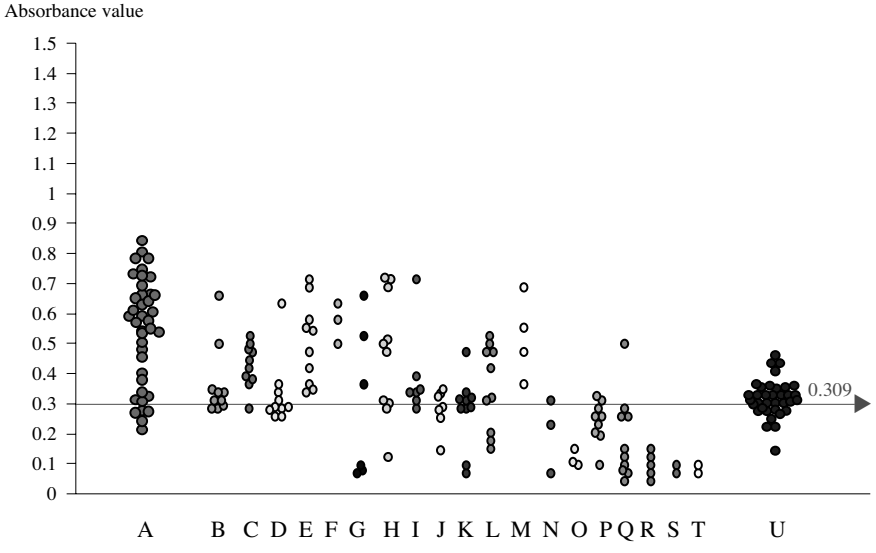

Fig 2- Distribution of ELISA values using crude female *D. immitis* antigen against filariasis sera, other helminthic infection and healthy controls at threshold value 0.309. The columns indicate as follows; A = Brugian filariasis, B = gnathostomiasis, C = strongyloidiasis, D = hookworm infections, E = trichinellosis, F = capillariasis, G = toxocariasis, H = angiostrongyliasis, I = ascariasis, J = trichuriasis, K = taeniasis, L = neurocysticercosis, M = cystic echinococcosis, N = sparganosis, O = hymenolepiasis, P = paragonimiasis heterotremus, Q = opisthorchiasis, R = haplorchiasis taichui, S = giardiasis, T = amebiasis, U = healthy controls.

ELISA close to an individual threshold values (Table 1). Sparganosis, hymenolepiasis nana, paragonimiasis heterotremus, haplorchiasis taichui, and opisthorchiasis antibodies against helminth diseases gave lower ODs than the threshold values, although 1/10 cases of opisthorchiasis gave slightly higher ODs than 0.180 when using crude male antigen. ODs-ELISA using crude female antigen gave similar results to those diseases. Neither antigen was reactive to antibodies of protozoans in this study.

Table 1
True positive and false positive results following their indirect-ELISA threshold values using crude male and female *D. immitis* antigens.

Groups tested	No. of sera tested	No. positive (%)	
		Male	Female
		Threshold value	
		0.180	0.309
True positivity			
Brugian filariasis	42	37 (88.1)	37 (88.1)
False positivity			
Other helminthic infections	131	48 (36.6)	63 (48)
Healthy controls	35	15 (42)	17 (48)

Table 2
Comparison of false positive (FP) between crude male and female *D. immitis* antigens using serum antibodies of heterologous sera and healthy controls at their threshold values, 0.180 and 0.309, respectively.

Disease (No.)	ELISA					
	Crude male (0.180)			Crude female (0.309)		
	FP	%FP	ODs	FP	%FP	ODs
Gnathostomiasis (10)	4	40	0.209-0.333	4	40	0.347-0.635
Strongyloidiasis (10)	10	100	0.184-0.359	9	90	0.381-0.537
Hookworm infections (10)	1	10	0.182	4	40	0.327-0.624
Trichinellosis (10)	9	90	0.184-0.400	10	100	0.326-0.709
Capillariasis (3)	3	100	0.299-0.340	3	100	0.532-0.628
Angiostrongyliasis (10)	6	60	0.236-0.542	6	60	0.463-0.709
Ascariasis (7)	3	42.86	0.183-0.293	4	57.14	0.312-0.648
Trichuriasis (7)	1	14.28	0.311	3	42.86	0.209-0.333
Toxocariasis (7)	2	33.3	0.225-0.389	2	33.3	0.472-0.652
Neurocysticercosis (10)	2	20	0.226-0.264	6	60	0.314-0.539
Cystic echinococcosis (4)	4	100	0.189-0.422	4	100	0.373-0.696
Taeniasis (10)	2	20	0.209-0.219	5	50	0.317-0.444
Sparganosis (3)	-	-	-	1	33.33	0.323
Hymenolepiasis nana (3)	-	-	-	-	-	-
Paragonimiasis heterotremus (9)	-	-	-	1	11.1	0.310
Opisthorchiasis (10)	1	10	0.237	1	10	0.507
Haplorchiasis taichui (5)	-	-	-	-	-	-
Giardiasis (2)	-	-	-	-	-	-
Amebiasis (2)	-	-	-	-	-	-
Total number	**48**			**63**		
Healthy control	15	42.86	0.183-0.283	17	48.57	0.314-0.427

DISCUSSION

In this study, antibodies of 42 patients with *B. malayi* were determined with 88.1% sensitivity of ELISA following different threshold values when using individual antigens of *D. immitis* worm extracts. The ODs-range of true positives using female crude antigen was higher than male crude antigen. It may be that several complicated molecules of the female structure itself can react with antibodies of Malayan filariasis. This evidence supports the higher cross-reaction of heterologous serum samples in our study. Female crude antigen gave 51.8% specificity, while male crude antigen was higher at 61.4%. Crude antigens can be produced from many stages of filarial parasites, *eg*, infective stage larvae, microfilariae and adult worms such as *B. malayi* and *B. pahangi* (Hamilton *et al*, 1981; Kaushal *et al*, 1982), *Onchocerca volvulus* (Weiss *et al*, 1982), *D. immitis* (Sawada *et al,* 1969) and *W. bancrofti* (Maizels *et al,* 1986; Morgan *et al,* 1986). However, the use of crude antigens frequently involves cross-reactivity with antibodies from other helminthic infections. Riyong *et al* (2003) detected antibody to Bancroftian filariasis in humans using crude antigen from infective third-stage larvae (L3) of *D. immitis,* by indirect ELISA. The result revealed that the sensitivity and specificity at the cut-off level of 0.230 were 76 and 70%, respectively. The sera from patients with other helminthic infections – gnathostomiasis, strongyloidiasis, hookworm infections, trichinellosis and cysticercosis – also displayed cross-reactions with crude antigen. This result was similar to our study, which found cross-reaction in both crude male and female antigens. Thus, crude antigen should be suitable for baseline serodiagnostic data but needs to be purified to eliminate cross-reaction with other helminthic infections. Theodore and Kaliraj (1990) prepared the crude surface antigens of the adult bovine filarial parasite *Setaria digitata* by EDTA extraction, purified by affinity chromatography using sepharose-bound human filarial antibodies obtained from chronic human filarial sera. The purified and crude antigens were used in ELISA to detect serum antibodies in filariasis due to *W. bancrofti*. Alli *et al* (2000) fractionated *B. malayi* microfilarial excretory-secretory (mf ES) and phosphate-buffer-saline-soluble (mf S) antigens by fast protein liquid chromatography (FPLC) on a Superdex 200 HR 10/30 gel filtration column. The active antigen fractions were identified and explored in comparison with whole mf ES and mf S antigens to detect filarial IgG antibodies in different groups: those with microfilaremia, acute, chronic and occult filarial cases *of W. bancrofti* infection, endemic cases and non-endemic cases. They concluded that the pooled FPLC purified *B. malayi* mf antigens (a fraction of mf ES and two fractions of mf S antigens) with higher specificity are preferable to whole mf ES and mf S antigens to detect active filarial infection in patients with microfilaremia or evidence of filariasis.

In our study, crude male antigen showed better specificity than crude female antigen. However, both antigens need to be further purified. This study provides baseline data for further immunodiagnosis of Brugian filariasis.

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