IMPROVED ANTIGENS FOR IgG-ELISA DIAGNOSIS OF STRONGYLOIDIASIS

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Abstract. Two preparations of antigens for the diagnosis of strongyloidiasis were prepared from an extract of the infective larvae of *Strongyloides stercoralis*: a crude antigen (CA) and a molecular weight cut-off antigen (MWCOA). Both antigens were analysed by indirect ELISA against the sera of strongyloidiasis (26 cases), other helminthiases (167) and normal controls (30). The larvae were obtained from fecal culture by a modified polyethylene tube technique after screening tests by triple simple smears per case. The larvae were extracted with distilled water and further sonicated to obtain a supernatant, the CA. A part of the CA was separated for an antigen containing molecules of lower than 30kDa by an ultrafree-MC centrifugal filter tube (PLTK): this was designed as the MWCOA. The CA gave 96.15% sensitivity and 40.12% (67/167) specificity at a cut-off value of 0.980 (5SD); false positives were produced by 19 of 20 different helminthiases. The MWCOA produced 96.15% sensitivity at cut-off value of 0.71 (4SD); the specificity of the test was 78.44% (131/167), higher than that of CA. False positives also appeared with 15 other helminthic infections. This study suggests that MWCOA is more specific than CA. A purified MWCOA will be necessary in order to reduce cross-reactivity and provide the suitable diagnosis of strongyloidiasis.

INTRODUCTION

The intestinal nematode *Strongyloides stercoralis* is prevalent in tropical and subtropical regions. It is one of the soil-transmitted nematodes whose larvae infect man by skin penetration. Two phases of the parasite's life cycle are passed in the host: the infective larvae penetrate the skin and the adult worms dwell in the host's mucosae. Strongyloidiasis has been recorded in non-tropical countries, imported by immigrants, tourists or other travellers from the tropics (Gentilini and Duffo, 1986; Piekarski, 1987) In Thailand, strongyloidiasis is found in every part of the country (Jongsuksantigul *et al*, 1992). Pitisuttithum and Juntra (1990) found that 25% (47/189) of Thai adults presented with intestinal parasitic infections; these adults resided in and around Bangkok Metropolis and strongyloidiasis accounted for 38.3% (18/47) of the infections in the survey. *Strongyloides stercoralis* can cause a broad spectrum of diseases -from chronic asymptomatic infection to a hyperinfective, often fatal, syndrome; diarrhea is another common symptom. A group at risk of infection is immunocompromised patients (Rivals *et al*, 2000). Involvement of the central nervous system may occur in patients with the acquired immunodeficiency syndrome (AIDS) (Armignacco *et al*, 1989) and also non-immunosuppressed patients (Owor and Wamukota, 1976; Lahn *et al,* 1994). Moreover, autoinfection (inordinate multiplication) of this parasite may occur in those infected and potentially produce illness and fatalities. The early negative results for strongyloidiasis that may be produced by many stool examination techniques can result in the death of patients who are given a prolonged course of corticosteroid therapy (Suvajdzic *et al*, 1999).

The low density of larvae in stool, duodenal contents and other clinical specimens creates a problem for the differential diagnosis for strongyloidiasis. Although the agar-plate method of stool culture is said to be more sensitive than the other parasitological techniques for detection of *S. stercoralis* (Arakaki *et al*, 1990; Koga *et al*, 1991; De Kaminsky, 1993), it is, on occasion, necessary to conduct immunological tests in order to diagnose strongyloidiasis. The immunodiagnosis of *S. stercoralis* infections may be performed using indirect ELISA. A crude extract of filariform larvae can give a sensitivity of 85-100% (Neva *et al*, 1981; Corrol *et al*, 1981; Bailey, 1989; Conway *et al*, 1993a). However, the specificity of the ELISA is lowered by cross-reactive antibodies (Gam *et al*, 1987; Conway *et al*, 1993a, b); in areas that are highly endemic for different kinds of parasitic infections, this results in high cross-reactivity and low specificity. Improved antigens should be developed in order to obtain high sensitivity and specificity. This

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study aimed to obtain serological data from crude and molecular weight cut-off antigens produced from the infective larvae of *Strongyloides stercoralis*; these antigens were evaluated by indirect ELISA.

MATERIALS AND METHODS

Larvae

Simple smear examination was used to screen for *Strongyloides stercoralis* rhabditiform larvae in patients' feces; the feces of positive cases were cultured for *S. stercoralis* filariform larvae. A modified polyethylene tube culture method (Harada and Mori, 1955) was used: this features a plastic bag (120cm wide × 180cm long without a slant-sealed buttom side). After 3-5 days, at 28-30˚C, one bottom side of the bag was cut and water was poured into a big sedimentation flask. After collection, the larvae were kept at 4˚C until being drawn into a graduated pipette and centrifuged at 12,000*g* at 4˚C for 30 minutes. The larvae were given a final wash in distilled water.

Crude antigen (CA)

The crude larval antigen was extracted with distilled water accompanied by alumina in ice and then subjected to five periods of sonication, each of one minute. The supernatant was collected by centrifugation at 12,000*g* at 4˚C for 30 minutes. The protein content was determined by the method of Bradford (1976).

Molecular weight cut-off antigen (MWCOA)

The MWCOA was prepared by the separation of crude antigen by SDS-PAGE. The SDS-treated crude antigen was analysed by immunoblot against the sera of strongyloidiasis (5 cases), other individual helminthic infections (except paragonimiasis westermani and onchocerciasis because of the need to save serum samples for ELISA) and normal controls. The MWCOA was derived from the parts of the reactive bands that reacted strongly with the antibodies to strongyloidiasis and weakly with the sera of the other helminthic infections and the normal controls. These antigens were of less than 30 kDa when compared with the low molecular weight protein standards (Fig 1). Crude antigen was then filtrated via an Ultrafree-MC centrifuge filter tube (PLTK, 30 kDa) by centrifugation at 12,000*g* at 4˚C for 10 minutes. Two repeats of the unfiltrate on the filter were washed with distilled water and recentrifuged twice. The protein content of the pooled filtrate was determined by the method of Bradford (1976).

Serum samples

Human serum samples of four groups used in the study were: those produced by the following nematode infections: strongyloidiasis (26 cases), hookworm infection (14, mainly *Necator americanus* in Thailand), ascariasis (10), trichuriasis (8), gnathostomiasis (10), trichinosis (14), capillariasis philippinensis (3), toxocariasis (7), onchocerciasis (3), *Wuchereria bancrofti* infection (10), angiostrongyliasis cantonensis (9). Sera produced by cestodes comprised neurocysticercosis (14 cases), taeniasis (10, *Taenia saginata*, cases established by the finding of segments and/or eggs; patients from endemic areas), cystic echinococcosis (10), sparganosis (3) and hymenolepiasis nana (4). Sera produced by trematodes comprised paragonimiasis heterotremus (10), paragonimiasis westermani (5), opisthorchiasis (10), schistosomiasis mansoni (6), and fascioliasis gigantica (7). The last group was 30 normal controls who were negative by simple smear and the formalin-ether flotation technique.

Indirect ELISA

On the basic of checkerboard titration, the proper concentrations of CA and MWCOA for indirect ELISA were 2.5 μg/ml and 5 μg/ml respectively; the serum dilution was 1:400; secondary antibody was 1:2,000. The diluted antigens with carbohydrate-bicarbonate buffer, pH 9.6, were individually coated in micro ELISA-wells. The unbound antigens were washed twice with PBS, pH 7.4, containing 0.05% Tween 20 (PBS-T) by microshaker, before blocking with 150 μl 0.5% BSA-0.02% NaN_3 in PBS-T and incubated in a humidified chamber for 1 hour at 37˚C. After washing twice with PBS-T, 100 μl of diluted sera (1:400) were added to the wells and kept at 37˚C for 1.5 hours. After washing five times with PBS-T, 100 μl of diluted secondary antibody (rabbit anti-human IgG) conjugated with peroxidase (1:2,000) were added to each well and incubated at 37˚C for 1.5 hours. After the final wash, 100 μl of ABTS [2, 2-azino-di(3-ethyl-benzthiazoline sulfonate)] substrate (Kirkegaard & Perry Laboratory, KPL, USA) were added and kept for 30 minutes at room temperature. The action was stopped by adding 50 μl of 1% SDS into each well and the optical density of the reactions was determined at 405 nm by an ELISA reader (Titertex Multiskan Plus, MK II).

RESULTS

The crude antigen of *Strongyloides stercoralis* infective larvae was analysed by IgG-indirect ELISA while normal serum controls determined a cut-off value at 0.980 from a mean OD of 0.44 and a 5SD (0.545). When the antigen reacted with all the serum samples, the sensitivity and the specificity of the test were

96.15% and 40.12% respectively. Antibodies of strongyloidiasis cases gave the highest OD (2.400): the lowest was 0.900 (Fig 2). For the MWCOA, the cut-off value of 0.710 was based on a mean of 0.326 plus 4SD (0.384). The highest OD of the strongyloidiasis cases was 1.62 and the lowest was 0.632. The sensitivity of the test was the same as that of the crude antigen and the specificity was 78.44% (Fig 3).

Nineteen of the 20 other helminthic diseases (167 cases) were cross-reactive with the CA; in 100 cases, the ODs were >0.980 (the cut-off value), (Table 1; Fig 2). Of the nematode infections (88 cases), fifty nine cases (67.05%) produced serum IgG recognition of the cross-reactive molecules of the CA and were false positives. Of the cestode and trematode infections, 23/41 cases (56.1%) and 18/38 cases (47.37%) respectively produced cross-reactions. In the soil-transmitted helminth (STH) group of this study, IgG against hookworm produced false positives in nine cases. Of the 10 ascariasis cases, 3 (30%) had ODs of 1.6-1.1, over the cut-off value. Three of the eight cases of trichuriasis also gave false positives. The majority of the gnathostomiasis, trichinosis, angiostrongyliasis, toxocariasis, *Wuchereria bancrofti* infections, and onchocerciasis, produced high ODs in the reactions of IgG with the crude antigen. High cross-reactivity was seen in all the three cases of onchocerciasis (100%); nearly all of the angiostrongyliasis cases (8/9; 88.84%) were positive. Of the cestode infections, only sparganosis (3/3; 100%) contained cross-reactive antibodies that bound with the crude antigen. Of all the serum samples used, that of the sparganosis cases gave the highest OD (2.240). It is accepted that a crude antigen is frequently bound with the antibodies of several diseases. Surprisingly, in the trematode group, all five cases of *Paragonimus westermani* infections gave true negatives but five of the ten cases of *P. heterotremus* infections gave false positives.

Turning to the MWCOA, fifteen of the twenty other helminthiases gave false positivities (Table 1; Fig 3). The consistent ODs under the cut-off value were observed in the paragonimiasis westermani cases. In addition, all cases of paragonimiasis heterotremus, capillariasis, taeniasis and schistosomiasis gave true negatives. Most of the serum numbers and their ODs reduced false positivity when using the MWCOA. It was found that only opisthorchiasis increased the number of false positives: one case produced an OD of 0.757. Its OD reduced from 0.789 when using the crude antigen and this value was truely negative at 0.980 of the cut-off value. However, OD of 0.757 was higher than the border line value, 0.710. Antibodies against *Trichinella spiralis* parasites could react with antigenic molecules of MWCOA: their ODs were 0.404-0.8. Only two cases persisted to be false positive and not high OD values. Two ODs (1.170-0.912) of onchocerciasis cases were separate from OD (0.518) of the other case by the cut-off value. False positive from *Wuchereria bancrofti* infection was reduced from eight cases to one case which its OD was not high from the cut-off value. Of the cestode group, all the cases of taeniasis produced ODs of between 0.33 and 0.65.

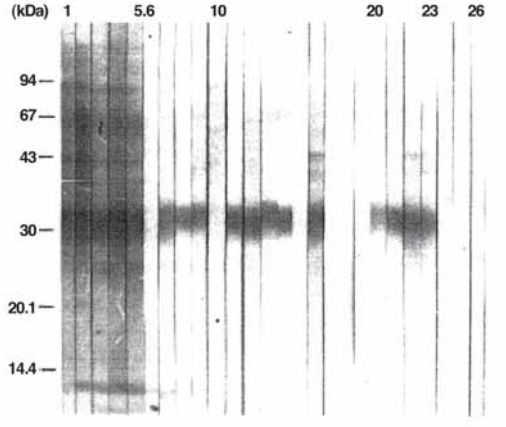

Fig 1- Immunoblot reactivity of IgG in a representative group of individual human sera with strongyloidiasis (lanes 1-5), other helminthic infections (6-23) and normal controls (24-26). Other helminthiases individually presented were; gnathostomiasis, hookworm infection, trichinosis, angiostrongyliasis, ascariasis, toxocariasis, capillariasis, trichuriasis, bancroftian filariasis, neurocysticercosis, taeniasis, hymenolepiasis nana, cystic echinococcosis, sparganosis, paragonimiasis heterotremus, opisthorchiasis, fascioliasis and schistosomiasis respectively.

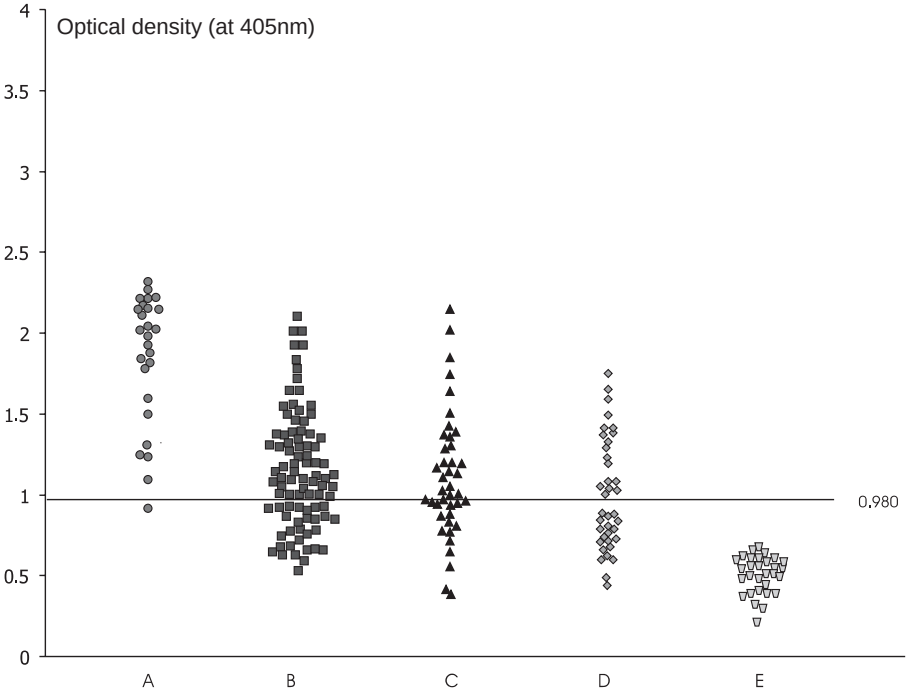

Fig 2- Distribution of absorbance values: strongyloidiasis (A), nematode infections (B), cestode infections (C), trematode infections (D), and normal controls (E). Crude antigen of *Strongyloides stercoralis* infective larvae was used; the cut-off value was 0.980.

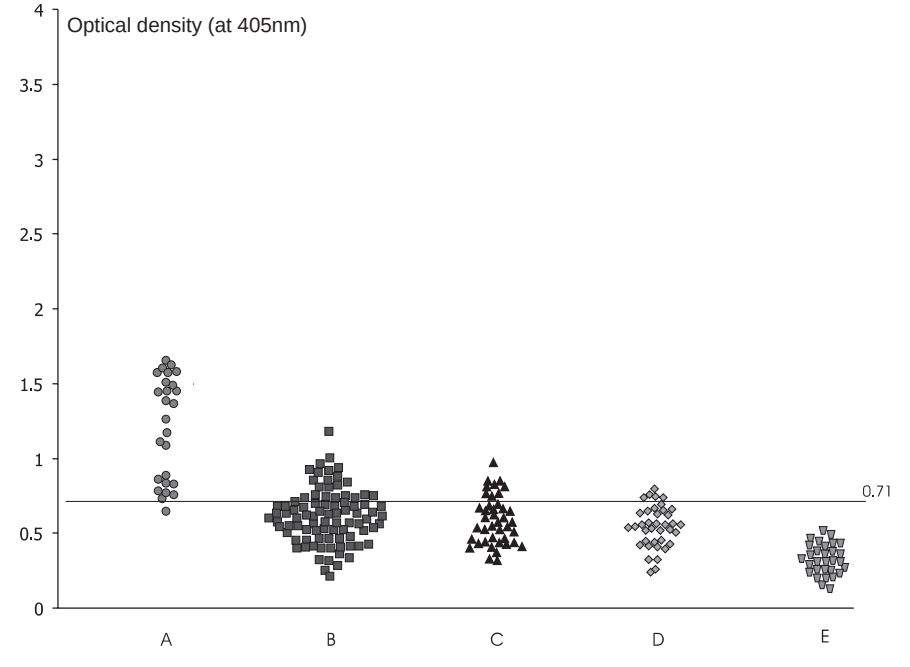

Fig 3- Distribution of absorbance values: strongyloidiasis (A), nematode infections (B), cestode infections (C), trematode infections (D), and normal controls (E). A molecular weight cut-off antigen of *Strongyloides stercoralis* infective larvae was used; the cut-off value was 0.710.

Table 1
Optical densities of false positives produced by other helminthic infections by crude antigen and molecular weight cut-off antigen.

Diseases	Number of sera	Crude antigen, cut-off value (0.980)	No. of false positive	MWCOA, cut-off value (0.710)	No. of false positive
Gnathostomiasis	10	1.870 – 1.030	8	0.933 – 0.739	6
Hookworm infection	14	2.000 – 1.000	9	0.945 – 0.730	2
Trichinosis	14	2.100 – 1.012	12	0.800 – 0.740	2
Angiostrongyliasis	9	2.000 – 1.100	8	0.909 – 0.756	5
Ascariasis	10	1.600 – 1.10	3	0.999 – 0.733	2
Toxocariasis	7	2.100 – 1.000	4	0.757	1
Capillariasis	3	1.000	1	–	0
Trichuriasis	8	1.600 – 1.400	3	0.712	1
Filariasis	10	1.991 – 1.090	8	0.806	1
Onchocerciasis	3	2.200 – 1.100	3	1.170 – 0.912	2
Neurocysticercosis	14	1.900 – 1.032	9	0.972 – 0.764	4
Taeniasis	10	2.100 – 1.200	7	–	0
Hymenolepiasis nana	4	1.790	1	0.809 – 0.800	2
Cystic echinococcosis	10	1.439 – 0.990	3	0.840 – 0.801	2
Sparganosis	3	2.240 – 1.000	3	0.748	1
Paragonimiasis heterotremus	10	1.700 – 1.080	5	–	0
Paragonimiasis westermani	5	–	0	–	0
Opisthorchiasis	10	1.800 – 1.400	3	0.770 – 0.744	4
Fascioliasis	7	1.660 – 1.030	7	0.800	1
Schistosomiasis	6	1.400 –1.020	3	–	0
Total	**167**		**100**		**36**

DISCUSSION

Ideal diagnostic antigens produce tests of high sensitivity and specificity. Cross-reactivity with the antibodies of other diseases is common when using a crude antigen or antigens produced by faulty techniques. Our baseline data of strongyloidiasis by indirect ELISA was investigated using a crude antigen of *Strongyloides stercoralis* infective larvae. It was found that this crude antigen was highly cross-reactive with nineteen other helminthic infections. The results suggested high sensitivity (96.15%) but poor specificity (40.12%). The false positives (one hundred cases) were due to several cross-reactive molecules of the crude antigen that were able to react with the various disease-specific antibodies (except those of paragonimiasis westermani).

A properly prepared molecular weight cut-off antigen could improve testing by eliminating the cross-reactive molecules of the CA. The lower 30 kDa antigen was less reactive to IgG-antibodies from other parasitic infections and reacted with anti-*Strongyloides* antibodies. Although the MWCOA gave higher specificity (78.44%) than the crude antigen (40.12%), cross-reactive antibodies from fifteen diseases were still bound. It was observed that case numbers of each disease also reduced false positives. Only opisthorchiasis increased one case of false positives when comparing serum numbers using the CA. Its OD reduced from 0.789 using the CA to 0.757 using the MWCOA. However, OD of 0.757 was higher than the border line value, 0.710. It may be explained that IgG-antibodies of this case mostly reacted with the cross-reactive molecules of lower than 30 kDa. The

specificity of the test was increased by the reduction of some of the cross-reactive molecules of the crude antigen.

When comparing the crude antigen from filariform larvae of *Strongyloides stercoralis*, Sato *et al* (1985) showed an 88% sensitivity for ELISA although the crude antigen was cross-reactive with antibodies from schistosomiasis japonicum, paragonimiasis westermani and trichinosis; the antigen did not react with antibodies from schistosomiasis mansoni, fascioliasis, paragonimiasis miyasakii. Genta (1988) used an extract of *S. stercoralis* infective larvae and reacted it with the antibodies of hookworm infection, filariasis (bancroftian filariasis, onchocerciasis, *Loa loa* infection), ascariasis, trichuriasis, schistosomiasis mansoni and intestinal protozoan infections. The sensitivity of ELISA was 88%; specificity was high (99%); cross-reactivity was evident with *Loa loa* infection and ascariasis. Conway *et al* (1993a) reduced cross-reactivity in serum samples: antibodies to other individual helminths were pre-absorbed by crude antigen of *S. stercoralis* infective larvae; the sensitivity was 100% but antibodies of filariasis (30/40 cases) and necatoriasis (16/40 cases) cross-reacted with the antigen. In the same year, antibody absorption by *Onchocerca guttucerca* antigen was attempted: it gave 91% sensitivity in both ELISA and immunoblot. The cross-reactive antibodies are not absolutely eliminated because the serum samples of ascariasis, trichuriasis, onchocerciasis and filariasis continued to react with the antigen (Conway *et al*, 1993b). In the present study, 80% of bancroftian filariasis were positives by crude extract of infective larvae; 10% of filariasis sera produced false positives with the MWCOA.

It was found that the molecular weight cut-off antigen (MWCOA) was more specific than the crude antigen: this may have been due to the reduction of cross-reactive molecules of over 30 kDa by the microporous membrane. However, the MWCOA requires further purification in order to produce yet higher sensitivity and specificity. Further cross-reactivity testing, using more parasitic infections is indicated.

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