

Immunodiagnosis of *Strongyloides stercoralis* infection: a method for increasing the specificity of the indirect ELISA

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Abstract

Indirect enzyme-linked immunosorbent assay (ELISA) allows sensitive detection of serum immunoglobulin (Ig) G against a soluble extract of *Strongyloides stercoralis* infective larvae. In this study, 40/40 (100%) human strongyloidiasis sera had high levels of anti-*S. stercoralis* IgG, but 30/40 (75%) filariasis sera, and 12/40 (30%) necatoriasis sera also had higher levels than control sera from UK residents. In attempts to increase the assay specificity by absorption of cross-reactive IgG, the effectiveness of pre-incubation of sera with extracts of different parasitic nematodes was investigated. One hour of incubation with 20 µg/ml aqueous extract of *Onchocerca gutturosa* absorbed cross-reactive IgG in most filariasis and necatoriasis sera, reducing the proportion with IgG levels above the positivity threshold by more than one-half. Preliminary results suggest that absorption with extracts of other filarial nematodes is equally effective, and that some of the cross-reactive IgG is directed against phosphorylcholine. Cross-reactive IgG in most necatoriasis sera was effectively absorbed with 20 µg/ml extract of *Necator americanus*. Cross-reactive IgG was not effectively absorbed with an extract of *Ascaris lumbricoides*. Absorption of cross-reactive IgG is an effective means of increasing the specificity of the indirect ELISA, for use in the immunodiagnosis and immuno-epidemiology of *S. stercoralis* infection.

Introduction

The intestinal nematode *Strongyloides stercoralis* is frequently difficult to detect in individuals with a low intensity of infection (GROVE, 1989). This causes widespread underestimation of infection prevalences in endemic communities (PAWLOWSKI, 1989; GENTA, 1989), and increases the risk of unexpected induction of severe hyperinfection in carriers receiving immunosuppressive therapy for other conditions (IGRA-SIEGMAN *et al.*, 1981; DE VAULT *et al.*, 1990).

In attempts to increase the sensitivity of, and decrease the examination time required for, diagnosis, several immunodiagnostic methods have been developed (GENTA, 1986). The most widely tested of these is the indirect enzyme-linked immunosorbent assay (ELISA) for detection of serum immunoglobulin (Ig) G against total aqueous antigenic extracts of infective filariform (L3) larvae of *S. stercoralis* (NEVA *et al.*, 1981; SATO *et al.*, 1985; GAM *et al.*, 1987), *S. ratti* (TRIBOULEY-DURET *et al.*, 1978; CARROLL *et al.*, 1981; NEVA *et al.*, 1981), or *S. cebus* (BAILEY, 1989).

The specificity of the indirect ELISA is difficult to assess accurately, however, due to the limited sensitivity of any confirmatory parasitological test. Several investigators have referred to the possibility of false positivity in the ELISA, due to cross-reactive IgG responses induced by filarial or hookworm infections, although published information is limited. Among individuals with filariasis, for example, GAM *et al.* (1987) noted that 5 of 12, and BAILEY (1989) noted that one of 3, were seropositive in the *Strongyloides* ELISA. Although the ELISA has been used as a sensitive means of primary screening of individuals (SATO *et al.*, 1990), seropositivity in the absence of parasitological or clinical indications is considered to have insufficient positive predictive value for diagnosis (GENTA, 1986).

In this report, an assessment is made of the frequency of seropositivity in the indirect ELISA for *Strongyloides* among individuals with lymphatic filariasis and hookworm infections. A single step method of serum absorption is described which increases the specificity of detection of anti-*S. stercoralis* IgG.

Materials and Methods

Human sera

Sera were derived from 40 individuals with *S. stercoralis* infections, 20 of whom were British ex-Far East prisoners of war and had acquired their infections more than 30 years before serum sampling; the remaining 20

had presented to clinics in Britain or Dubai after an undetermined period of infection. Sera were also obtained from 40 individuals with *Wuchereria bancrofti* infections, most of whom lived in Sri Lanka and had clinical lymphatic filariasis, for whom information on intestinal helminth infections was unavailable. Sera were also provided from 40 individuals with *Necator americanus* infections from a study village in Zimbabwe where *N. americanus* was the only human helminthiasis detected (BRADLEY *et al.*, 1992). Control sera were from 20 residents of the UK.

Indirect ELISA

The indirect ELISA for detection of serum IgG against *S. stercoralis* was based on those described by NEVA *et al.* (1981), SATO *et al.* (1985), and GAM *et al.* (1987). For antigen preparation, *S. stercoralis* filariform larvae were obtained in Jamaica by faecal culture from a patient with strongyloidiasis. Larvae were washed 5 times in phosphate-buffered saline (PBS), suspended in 0.25% sodium hypochlorite for 5 min, washed 5 times again, and suspended in PBS with protease inhibitors (5 mM EDTA, 5 mM EGTA, 5 mM NEM, 5 mM pepstatin, 1.7 mM PMSF, 0.5 mM TPCK)*. Larvae were disrupted by sonication (6 × 20 sec pulses at 8 µm, 4°C), centrifuged at 13 000 g for 30 min at 4°C, and the supernatant was used as the crude soluble antigen preparation after storage at -70°C. This antigen preparation was used at 100 µl/well and 2 µg/ml protein concentration to sensitize 96-well Linbro® plates (Flow Laboratories, UK). After removal of unbound antigen by 3 × 3 min washes, 150 µl of 2% bovine serum albumin in PBS were added to each well for 1 h. Wells were washed 3 times, and serum samples were added (100 µl at 1/200 dilution, in triplicate) and incubated at room temperature for 2 h. After washing wells 3 times, a 1/2000 dilution of horseradish peroxidase-conjugated polyvalent rabbit and anti-human IgG antibody (Dakopatts®, Dako Ltd.) was incubated (100 µl/well) for 2 h. Wells were washed 3 times, and 100 µl of 0.01% o-phenylenediamine with 0.003% H₂O₂ were added to each well. After 30 min, 25 µl of 5 N H₂SO₄ were added to each well to stop the enzyme-substrate reaction.

Indirect ELISAs were also performed identically using plates coated with 2 µg/ml of PBS-soluble extract of

*Respectively, ethylenediaminetetraacetic acid, ethylene-bis (β-aminoethyl ether) N,N,N',N'-tetraacetic acid, N-ethylmaleimide, isovaleryl-Val-Val-Sta-Ala-Sta, phenylmethylsulphonyl fluoride, and N-tosyl-L-phenylalanine chloromethyl ketone.

adult *Onchocerca gutturosa*, or PBS with 1.5% *n*-octyl glycoside (*n*OG) extracts of adult *Necator americanus*, adult *Ascaris lumbricoides*, or adult *Trichuris trichiura* (see LILLYWHITE *et al.*, 1991). *O. gutturosa* were obtained from the nuchal ligaments of naturally infected cattle slaughtered locally, while *N. americanus*, *A. lumbricoides* and *T. trichiura* were isolated from patients' stools following chemotherapy.

The threshold of positivity was taken as an optical density (O.D.) value equal to the mean value + 3 standard deviations obtained with the 20 control (UK) sera.

Pre-absorption of sera

Pre-absorption experiments were performed by mixing and incubating each serum (at 1/200 dilution) for 1 h with 20 µg/ml PBS-soluble adult *O. gutturosa* extract, 20 µg/ml PBS (with *n*OG)-soluble adult *N. americanus* extract, 50 µg/ml (with *n*OG)-soluble adult *A. lumbricoides* extract, 20 µg/ml PBS-soluble adult *Dirofilaria immitis* extract, 20 µg/ml PBS-soluble adult *Brugia pahangi* extract, or incubation buffer only (PBS with 0.5% Tween 20®). Pre-absorption with 10 mM phosphorylcholine was also investigated; because of its low solubility in PBS, this experiment, and its control, were conducted in Tris-buffered saline (pH 8.5).

Results

S. stercoralis indirect ELISA without serum pre-absorption

The O.D. values (means of triplicate tests at 1/200 serum dilution) from an indirect ELISA for detection of IgG against *S. stercoralis* filariform larval extract are shown in Fig. 1. All 40 (100%) strongyloidiasis sera, 30/40 (75%) lymphatic filariasis sera, and 12/40 (30%) necatoriasis sera were designated positive.

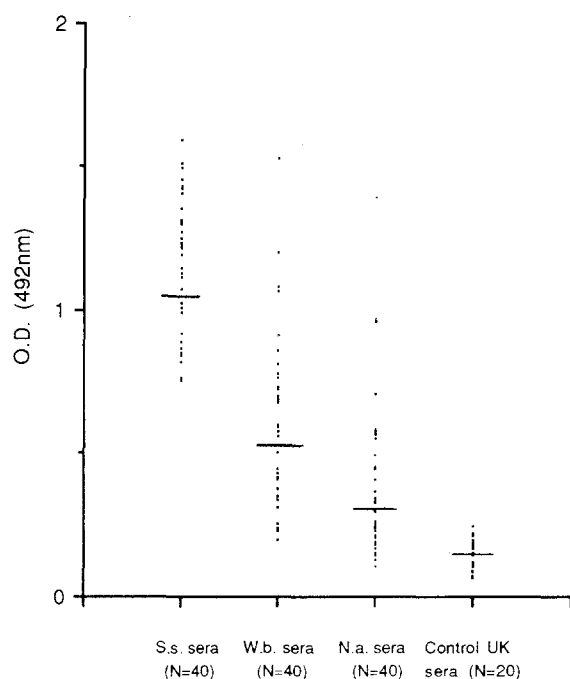


Fig. 1. Serum IgG levels against *Strongyloides stercoralis* larval extract measured by indirect ELISA (means of triplicate optical density [O.D.] values), in individuals with strongyloidiasis (S.s. sera, *n*=40), lymphatic filariasis (W.b. sera, *n*=40), necatoriasis (N.a. sera, *n*=40), and among UK residents (control, *n*=20). Horizontal bars indicate the mean value in each group of sera.

Correlations between serum IgG levels against extracts of *S. stercoralis* and other parasitic nematodes

It was considered that extracts of other parasitic nematodes might be used to pre-absorb sera to reduce the levels of cross-reactive IgG detected in the *S. stercoralis* indirect ELISA. To select potentially useful extracts, all sera

tested against the *S. stercoralis* extract were also tested in indirect ELISAs against extracts of *N. americanus*, *O. gutturosa*, *T. trichiura* and *A. lumbricoides* (using 2 µg/ml protein concentration for sensitizing wells). Table 1 shows Spearman rank order correlations between levels

Table 1. Spearman rank order correlations of optical density measurement of serum IgG against different antigenic extracts*

	Strongyloidiasis sera (<i>n</i> =40)	Filariasis sera (<i>n</i> =40)	Necatoriasis sera (<i>n</i> =40)
<i>S. stercoralis</i> vs.			
<i>N. americanus</i>	0.521**	0.526**	0.779***
<i>O. gutturosa</i>	0.662***	0.505**	0.757***
<i>T. trichiura</i>	0.359	0.177	0.574**
<i>A. lumbricoides</i>	0.629***	0.400	0.740***

*Significance of correlations is indicated thus: ***P*<0.001, ****P*<0.0001.

of IgG to *S. stercoralis* and the other nematode extracts, calculated independently for each of the groups of sera. Most of the correlations were statistically significant. Correlations between IgG levels to *S. stercoralis* and to *N. americanus* or *O. gutturosa* extracts were highly significant within all groups of sera. Correlations between IgG levels to *S. stercoralis* and *A. lumbricoides* extracts had a higher level of significance than correlations between levels to *S. stercoralis* and *T. trichiura* extracts. Therefore, it was decided to examine the effects on the specificity of the *S. stercoralis* indirect ELISA of pre-absorbing sera against extracts of *O. gutturosa*, *N. americanus* and *A. lumbricoides*.

S. stercoralis indirect ELISA after serum absorption

Sera were tested in the *S. stercoralis* indirect ELISA after 1 h pre-incubation with 20 µg/ml *O. gutturosa* extract,

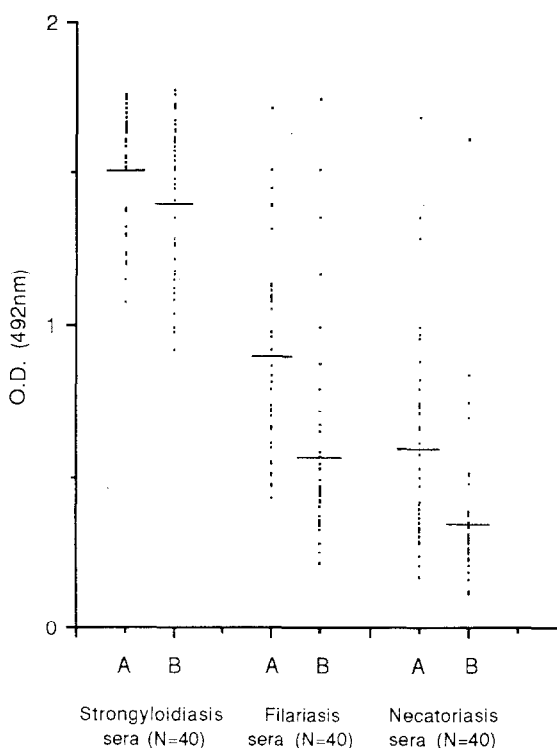


Fig. 2. Effects of pre-absorption of serum with 20 µg/ml *Onchocerca gutturosa* extract on IgG levels detected in the *Strongyloides stercoralis* indirect ELISA, among strongyloidiasis sera (*n*=40), lymphatic filariasis sera (*n*=40), and necatoriasis sera (*n*=40). Columns A show the control values after incubation in buffer alone, and columns B show the values after incubation with the *O. gutturosa* extract. O.D.=optical density; horizontal bars indicate the mean value in each group of sera.

20 µg/ml *N. americanus* extract, 50 µg/ml *A. lumbricoides* extract, or with incubation buffer alone. These protein concentrations had previously been determined to give the maximum pre-absorption effect among a limited number of sera (data not shown).

To test whether extracts of other filarial nematodes could be usefully employed in serum pre-absorption, as alternatives to *O. gutturosa*, the effects of pre-absorption of a limited number of sera with 20 µg/ml of adult *Dirofilaria immitis* or *Brugia pahangi* were studied. All 3 ex-

Table 2. The effects of pre-absorption of sera with different extracts on the proportion of sera positive^a in the indirect ELISA for anti-*S. stercoralis* IgG

	No.	Proportion positive after pre-absorption with			
		Buffer only	<i>O. gutturosa</i> (20 µg/ml)	<i>N. americanus</i> (20 µg/ml)	<i>A. lumbricoides</i> (50 µg/ml)
Strongyloidiasis sera	40	40/40 (100%)	40/40 (100%)	40/40 (100%)	40/40 (100%)
Filariasis sera	40	30/40 (75%)	14/40 (35%)	26/40 (65%)	31/40 (77.5%)
Necatoriasis sera	40	16/40 (40%)	6/40 (15%)	4/40 (10%)	10/40 (25%)

^aPositive sera were those with optical densities greater than the mean value + 3 standard deviations obtained with 20 control sera in the UK.

Pre-absorption with the *O. gutturosa* extract significantly lowered the detectable IgG against *S. stercoralis* among the 40 filariasis sera (mean O.D. reduced from 0.901 to 0.570) and the 40 necatoriasis sera (mean O.D. reduced from 0.590 to 0.348), but had only minimal effect on the 40 strongyloidiasis sera (mean O.D. reduced from 1.524 to 1.423) (Fig. 2). The proportions of positive sera among the filariasis and necatoriasis sera were reduced to less than one-half of those observed with the unabsorbed control, while all the strongyloidiasis sera remained positive (Table 2).

Pre-absorption with *N. americanus* extract significantly reduced the positive proportion of necatoriasis sera, and slightly reduced the proportion among the filariasis sera, whereas pre-absorption with *A. lumbricoides* had little effect on either group (Table 2).

tracts of adult filariae had similar effects on the level of cross-reactive IgG (Fig. 3a). Pre-absorption with phosphorylcholine, a likely target of some cross-reactive antibody responses to filarial nematodes and *S. stercoralis* (see LAL & OTTESEN, 1989), reduced the anti-*S. stercoralis* IgG levels detected in some of the filariasis and necatoriasis sera, but did not have as marked an effect as the crude filarial extracts (Fig. 3b). This suggests that phosphorylcholine was only one of a number of cross reactive components in crude extracts of these nematodes.

Discussion

Pre-absorption of sera with particular nematode extracts increased the specificity of anti-*S. stercoralis* IgG detection by indirect ELISA. Without serum absorption, cross-reactive IgG was detected in many sera from indi-

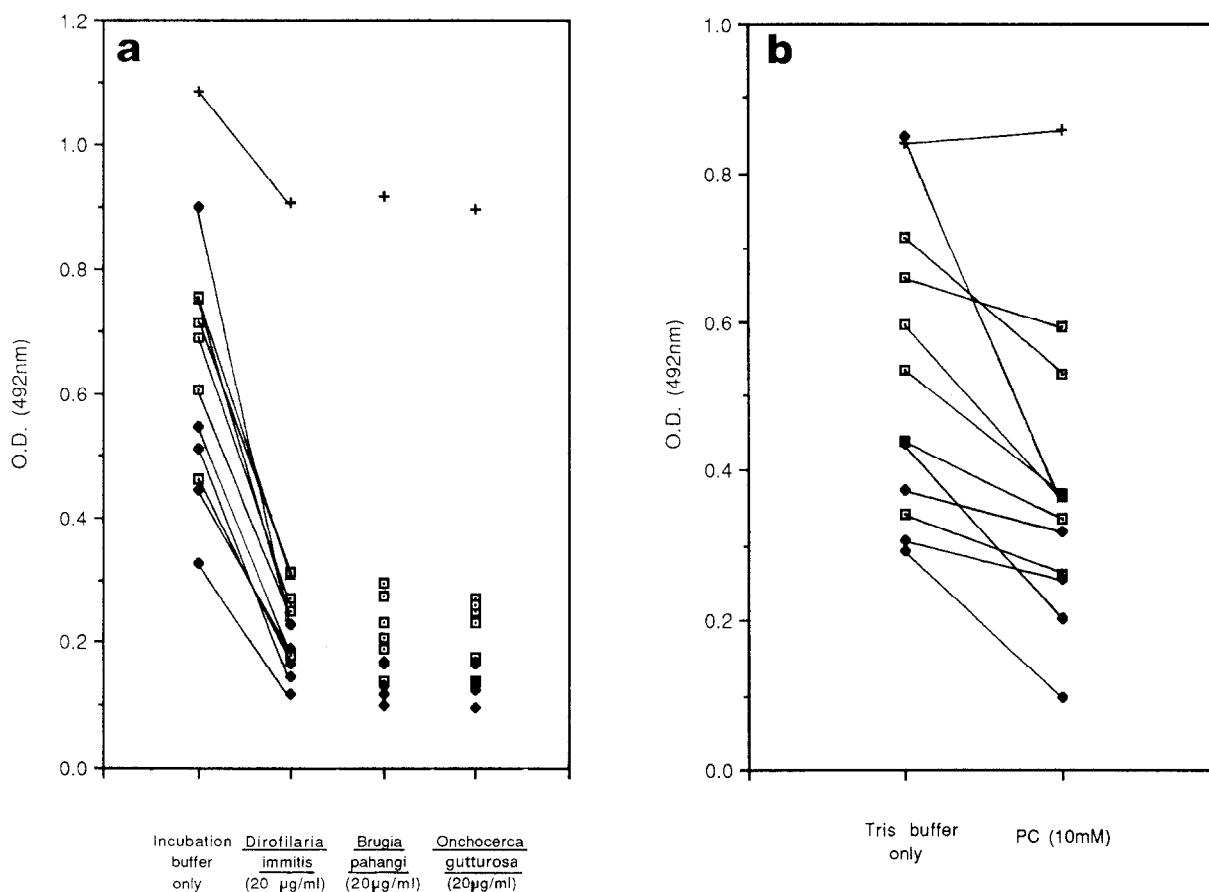


Fig. 3. (a) The effect of 1 hour pre-incubation with (a) 20 µg/ml extracts of different filarial nematodes, and (b) 10 mM phosphorylcholine (PC), on IgG levels detected in the *Strongyloides stercoralis* ELISA. In (b), the sera were diluted in Tris buffered saline which allowed PC to remain in solution. O.D.=optical density; □, filariasis sera; ◆, necatoriasis sera; +, strongyloidiasis serum.

viduals with filarial or hookworm infections. However, in most of these sera, the cross-reactive IgG was effectively absorbed by 1 h incubation with PBS-soluble extract of adult *O. gutturosa*. Incubation with adult *N. americanus* extract was effective in absorbing cross-reactive IgG in most necatoriasis sera, but not in most filariasis sera. Incubation with adult *A. lumbricoides* extract was ineffective in absorbing cross-reactive IgG in almost all sera tested. Tests on a limited number of sera suggest that absorption of cross-reactive serum IgG may be achieved as successfully by incubation with extracts of the filarial nematodes *D. immitis* and *B. pahangi* as it is with the extract of *O. gutturosa*.

Even after absorption with *O. gutturosa* extract, 14/40 (35%) of filariasis sera and 6/40 (15%) of necatoriasis sera had detectable anti-*S. stercoralis* IgG levels higher than the threshold of seropositivity. Some of these sera had substantially reduced anti-*S. stercoralis* IgG activity after absorption, although the ELISA O.D. values did not decline beneath the seropositivity threshold. The seropositivity threshold could be arbitrarily raised to increase discrimination between strongyloidiasis sera and other sera. This option is not necessarily advisable, because, although in this study all strongyloidiasis sera were highly positive in the assay, in other studies a minority of strongyloidiasis sera has been shown to contain low levels of anti-*S. stercoralis* IgG (GENTA, 1986; GAM *et al.*, 1987; GROVE, 1989), and raising the threshold of seropositivity could slightly decrease the sensitivity of the assay. A small number of filariasis and necatoriasis sera had high anti-*S. stercoralis* IgG levels which were not markedly diminished after absorption with *O. gutturosa* extract, as was noted for all the strongyloidiasis sera. The possibility of these individuals harbouring an undetected, *S. stercoralis* infection cannot be excluded. No information was available concerning intestinal parasites in the individuals with filariasis, and any low intensity *S. stercoralis* infections might have escaped detection in the individuals with necatoriasis. Although not used in the present study, a recently described agar plate culture method may increase the sensitivity of parasitological diagnosis of low intensity infections (ARAKAKI *et al.*, 1990; KOGA *et al.*, 1991).

In studies in communities where filarial and hookworm infections are endemic, it is probable that cross-reactive IgG induced by these infections is the main cause of false positivity in the *Strongyloides* indirect ELISA. Interestingly, among British ex-Far East prisoners of war, over 10% of whom had acquired *S. stercoralis* infections during internment more than 30 years previously and were still infected, BAILEY (1989) reported an ELISA specificity of 99%, much higher than that noted in studies on endemic populations. Other helminth infections were absent from the ex-Far East prisoners of war group, and it is therefore likely that cross-reactive IgG responses were at a minimum, which probably accounts for the high specificity observed. In addition, it is improbable that persistent IgG from previous *S. stercoralis* infections is a major cause of 'false positivity' as, 6 months after successful albendazole therapy, a large proportion of patients became seronegative (J. W. Bailey *et al.*, unpublished results).

In this study, serum absorption with an extract of *O. gutturosa* has been shown to reduce the proportions of positive results in the *Strongyloides* indirect ELISA among individuals with *W. bancrofti* or *N. americanus* infections by more than one-half. Application of this method should increase the positive predictive value of the assay for immunodiagnosis, and the ease of interpretation of ELISA results in future immuno-epidemiological studies on *S. stercoralis* in populations where filarial and hookworm infections are endemic.

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