



## Parasitology

# The role of glycosylated epitopes in the serodiagnosis of *Strongyloides stercoralis* infection ☆☆☆★

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## ABSTRACT

Carbohydrates of pathogen antigens have been disrupted by periodate oxidation, in order to reduce nonspecific bindings and improve serodiagnosis of parasite infections. In the present study, the enzyme-linked immunosorbent assay (ELISA) was carried out with filariform larvae antigen treated, or not treated, with sodium metaperiodate. Groups of sera from patients with *Strongyloides stercoralis* infection, with other intestinal parasites and a normal control, were used. The oxidation of *Strongyloides stercoralis* glycosylated epitopes reduced the seroreactivity of sera from patients with *S. stercoralis* infection as demonstrated by ELISA, with a decrease in sera optical densities. The number of cross-reactions of IgG and IgE-ELISAs increased by 12% and 16%, respectively, after antigen treatment with metaperiodate. This was more often observed in patients infected with *Schistosoma mansoni* and hookworm. Moreover, the IgG depletion from sera tested by IgE-ELISA led to the detection of previous false-negative samples from *S. stercoralis*-infected patients.

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## 1. Introduction

*Strongyloides stercoralis* is widely distributed in tropical and subtropical regions, and it is estimated that approximately 35 million people are infected worldwide, with frequencies above 6% in Brazil (De Bona and Basso, 2008; Inês et al., 2011; Kothbary et al., 1999; Oliveira et al., 2002; Olsen et al., 2009). *S. stercoralis* infection in immunocompetent patients usually results in asymptomatic chronic disease. Nevertheless, hyperinfection and dissemination can occur in high-risk groups, such as patients undergoing glucocorticoid therapy; those with hematologic malignancy; those co-infected with human T-lymphotropic virus type I (HTLV-1) and HIV; and those suffering from chronic alcohol abuse (reviewed by Keiser and Nutman, 2004). The diagnosis of *S. stercoralis* infection usually relies on the detection of larvae in stool samples. The majority of cases involve a chronic infection with an intermittent and small larvae load in the feces, and a decrease in the sensitivity of parasitologic tests. It has been recommended that at least 4 negative results for stool examinations

are required to rule out the *Strongyloides stercoralis* infection (Dreyer et al., 1996; Liu and Weller, 1993; Roxby et al., 2009; Uparanukraw et al., 1999). Stool culture in agar plate has been shown to be a more sensitive diagnostic tool than the Baermann–Moraes technique, although it is more laborious and time consuming (Inês et al., 2011; Jongwutiwes et al., 1999; Koga et al., 1992). To bypass the limitations of parasitologic methods, an enzyme-linked immunosorbent assay (ELISA), to detect IgG anti-*S. stercoralis*, has been used. Nevertheless, the cross-reactivity with other intestinal helminths overestimates the true prevalence of *S. stercoralis* infection and represents a great limitation to serologic assays (Costa-Cruz et al., 2003; Dreyer et al., 1996; Uparanukraw et al., 1999). The demonstration of specific IgE response in human strongyloidiasis by ELISA has been used for diagnosis, since the cross-reactivities with other helminthes are lower than those reactions with specific IgG. In spite of this, IgE anti-*S. stercoralis* may not be detected by ELISA due to the presence of excessive amounts of circulating IgG which produces a competitive inhibition of IgE binding sites (Costa-Cruz et al., 2003; Leoratti, 2004). Moreover, patients under steroid therapy or coinfecting with *S. stercoralis* and HTLV may have lower circulating specific IgE (Machado et al., 2011; Porto et al., 2001).

Previous studies have shown that a specific antibody reactivity with glycoproteins of pathogen antigens was improved after periodate oxidation of their glycosylated epitopes at acid pH (Alarcón de Noya et al., 2000; Albuquerque et al., 2005; Kouguchi et al., 2011). The disruption of carbohydrates from *Schistosoma mansoni* egg soluble antigen increased the specificity of schistosomiasis immunodiagnosis

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(Alarcón de Noya et al., 2000; Noya et al., 2002) and allowed the discrimination between the chronic and acute forms of parascidiosis by using a carbohydrate-modified antigenic preparation to test the reactivity towards IgG and IgM (Ferreira et al., 2008). On the other hand, with visceral leishmaniasis, the metaperiodate treatment of *Leishmania infantum/chagasi* antigens resulted in a reduction of the seroreactivity by ELISA (Atta et al., 2004).

In the present study, we describe, for the first time to our knowledge, an ELISA for detection of *Strongyloides stercoralis* IgG and IgE antibodies using parasite soluble antigens treated with sodium metaperiodate to remove the glycosylated molecules. Furthermore, the efficacy of previous depletion of IgG from the sera of *S. stercoralis*-infected patients for detection of IgE reactivity was evaluated.

## 2. Materials and methods

### 2.1. Patients and sera samples

The present study was carried out from November 2009 to December 2010 on individuals seen at the Clinical Analysis Laboratory of Pharmacia College, Federal University of Bahia, Brazil. Daily, around 2–3 sera samples from patients were randomly selected according to the coproparasitologic results of spontaneous sedimentation, Baermann–Moraes method, and agar plate culture methods. A total of 100 sera samples from parasitized individuals consisting of 50 from *S. stercoralis* monoinfected patients and 50 with other intestinal parasites, including hookworm (10), *Ascaris lumbricoides* (9) *Schistosoma mansoni* (10), *Trichuris trichiura* (10), *Enterobius vermicularis* (2), and *Trichostrongylus* sp. (9) were used to perform the ELISA for detection of *Strongyloides*-specific IgG and IgE. As negative controls, 34 sera samples from newborns of mothers with negative parasitologic test results and 14 from healthy adults, who were members of the laboratory staff, were used. False IgE-negative results from patients shedding *S. stercoralis* larvae in feces were evaluated in 10 IgG-depleted samples.

This study was approved by the Committee of Ethics in Research of the Gonçalo Moniz Institute, Oswaldo Cruz Foundation, and an informed consent for participation was obtained from each patient during delivery of clinical specimens.

### 2.2. *Strongyloides stercoralis* soluble antigen

*Strongyloides stercoralis* soluble antigen (SsAg) was prepared as described previously (Arakaki et al., 1990). Briefly, *S. stercoralis* filariform larvae were obtained from feces culture of hyperinfected patients. Larvae were washed 5 times in 0.15 mol/L of phosphate-buffered saline (PBS, pH 7.2) by centrifuging for 7 min at  $1.8 \times g$ . Parasites were suspended for 5 min in 0.25% sodium hypochlorite and then washed 5 times in PBS as described above. The larvae were suspended in PBS with protease inhibitors (5 mmol/L EDTA, 1 mmol/L phenyl-methylsulfonyl fluoride [Sigma], 0.05 mmol/L TPCK/TLCK, 1 µg/mL leupeptin) and sonicated in an ice bath for a duration of 9 cycles of 80 s at 40 kHz (Branson Sonifier Cell Disruptor, Branson Instruments, Danbury, CT, USA). The larvae homogenate was then centrifuged at  $11,000 \times g$  for 30 min at 4 °C. The supernatant with soluble antigen was analyzed for protein content according to Lowry et al. (1951), divided into aliquots, and stored at –70 °C until use.

### 2.3. IgG-depleted sera

IgG depletion from human serum was carried with rheumatoid factor (RF) absorbent according to the manufacturer's instructions (Siemens, Marburg, Germany). Briefly, sera samples were diluted 1:4 in PBS–0.05% Tween-20 and 1% BSA followed by a new 1:2 dilution in RF absorbent. Samples were well mixed, incubated for 18 h at 4 °C,

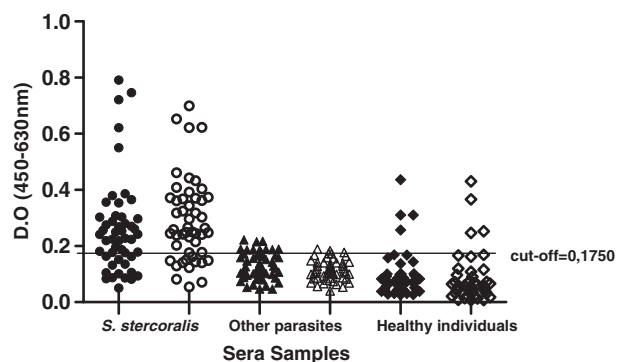
and then centrifuged for 5 min at  $1.8 \times g$ . The supernatant was used to perform the immunoassay to detect specific IgE.

### 2.4. Enzyme-linked immunosorbent assay for IgG and IgE detection

Indirect ELISA for specific IgG and IgE was conducted as previously described by Van Doorn et al. (2007) with some modifications. Briefly, the microtiter plates (Corning Costar polystyrene EIA/RIA plates, Corning) were coated with 10 µg/mL of *S. stercoralis* antigen in 0.06 mol/L carbonate–bicarbonate buffer (pH 9.6), incubated overnight at 4 °C, and washed 3 times with PBS containing 0.05% Tween-20 (PBS-T). To test the role of glycosylated epitopes, the SsAg was treated with sodium metaperiodate (MSsAg) according to Woodward et al. (1985). Soon thereafter, SsAg-coated plates, as described above, were washed with 50 mmol/L sodium acetate buffer (pH 4.5) and then glycosylated epitopes were oxidized by the addition of 100 µL of 10 mmol/L sodium metaperiodate in 50 mmol/L sodium acetate buffer (pH 4.5). Plates were then incubated for 1 h at room temperature in the absence of light, washed 3 times with PBS (pH 7.2), and incubated with 100 µL of 50 mmol/L sodium borohydride in PBS for 30 min at room temperature, followed by rinsing with 50 mmol/L sodium acetate buffer (pH 4.5). MSsAg- and SsAg-coated plates were blocked with 100 µL PBS-T plus 5% w/v skim milk (PBS-T-Milk) or with PBS-T plus 1% w/v bovine serum albumin (PBS-T-BSA) for 1 h at 37 °C for *S. stercoralis* IgG and IgE detection by ELISA, respectively. After blocking, wells were washed as described previously.

Assays for specific IgG antibodies were performed with sera samples diluted at 1:100. Plates coated with MSsAg or SsAg were incubated in duplicate with 100 µL of sera per well for 1 h at 37 °C. Thereafter, 100 µL of conjugated anti-human IgG linked to horseradish peroxidase (Sigma-Aldrich, St. Louis, MO, USA) diluted at 1:4000 was added and incubated in the same conditions. The reaction was revealed by the addition of 100 µL of substrate (100 µL of 0.051 mol/L citrate–phosphate buffer [pH 5.0] containing 0.0037 mol/L *p*-phenylenediamine and 0.04% hydrogen peroxide 30 volumes) and incubated for a period of 20 min in the absence of light, followed by the addition of 20 µL of 8N sulfuric acid to stop the reaction. The absorbance (Abs) was measured at 450–630 nm with a microplate reader (Awareness Technology, USA).

Assays for specific IgE antibodies were carried out using whole or IgG-depleted sera as discussed in Section 2.3. To each well coated with MSsAg or SsAg, 100 µL of 1:8 dilution of sera samples was added (depleted or undepleted of IgG) and incubated for 1 h at room temperature. The wells were washed 3 times with PBS-T, and 100 µL of biotinylated goat anti-human IgE (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA) diluted 1:2000 was added. After incubation for 1 h at room temperature, the wells were washed as described



**Fig. 1.** Indirect ELISA to detect IgG anti-*S. stercoralis* using *S. stercoralis* soluble antigens treated with sodium metaperiodate (black symbols) and without treatment (hollow symbols). Sera samples from infected patients with *S. stercoralis* ( $n = 50$ ) or with other intestinal parasites ( $n = 50$ ) and from healthy individuals ( $n = 48$ ) were tested. The cut-off was calculated by the ROC curve.

**Table 1**

ELISA for detection of IgG and IgE antibodies using *S. stercoralis* soluble antigen treated with sodium metaperiodate (MSsAg) and without treatment (SsAg).

Cross-reactions with other intestinal parasitic infections					
<sup>a</sup> Infections	No. of sera tested	IgG-ELISA		IgE-ELISA	
		MSsAg	SsAg	MSsAg	SsAg
		No. (%) of positive samples		No. (%) of positive samples	
<i>Schistosoma mansoni</i>	10	5 (50%)	2 (20%)	7 (70%)	5 (50%)
Hookworm	10	3 (30%)	2 (20%)	6 (60%)	4 (40%)
<i>Trichuris trichiura</i>	10	1 (10%)	1 (10%)	6 (60%)	2 (20%)
<i>Trichostrongylus</i> sp.	9	1 (11.1%)	0 (0%)	1 (11.1%)	1 (11.1%)
<i>Ascaris lumbricoides</i>	9	1 (11.1%)	0 (0%)	3 (33.3%)	3 (33.3%)
<i>Enterobius vermicularis</i>	2	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Total	50	11 (22%)	5 (10%)	23 (46%)	15 (30%)

<sup>a</sup> Infected patients with only 1 parasite specie.

previously. The reaction was amplified by adding 100  $\mu$ L of streptavidin–peroxidase conjugate (Kirkegaard & Perry Laboratories) diluted at 1:500, followed by a 30-min incubation at room temperature. Plates were washed, and the reactions were revealed by adding the enzyme substrate 2,2'-azino-bis-3 ethyl-benzothiazoline sulfonic acid (ABTS®, 0.01 mol/L) (Kirkegaard & Perry Laboratories) and incubated for a period of 30 min in the absence of light. The absorbance was read at 405 nm.

### 2.5. Data analysis

Statistical analysis was performed using the software GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA, USA). The cut-off value, sensitivity, and specificity were established by receiver operating characteristic (ROC) curve analysis using, as negative control, 48 sample sera from healthy individuals and 50 from patients with other intestinal parasites. The comparison among the sera groups was carried out using analysis of variance test followed by Dunn's test. The different mean optical densities obtained by ELISA using antigen treated with sodium metaperiodate and without treatment were analyzed by paired *t* test. Differences were considered as statistically significant when  $P < 0.05$ .

## 3. Results

### 3.1. Specific IgG and IgE detection by ELISA

The assays performed to detect specific IgG from sera of *S. stercoralis* infected patients using MSsAg and SsAg demonstrated

reactivity in 36 (72%) and 38 (76%) out of 50 cases, respectively (Fig. 1). The results showed no significant differences between assays ( $P > 0.05$ ), although the IgG-ELISA mean optical densities of sera using MSsAg were lower ( $0.26 \pm 0.17$ ) than when SsAg was used ( $0.29 \pm 0.15$ ) ( $P < 0.05$ ). IgG-ELISA cross-reactivity was found in 11 (22%), using MSsAg, and in 5 (10%), using SsAg, out of 50 sera from patients infected with other intestinal parasites (Table 1). Sera from *Schistosoma mansoni* and from hookworm-infected patients showed a higher number of cross-reactions (Table 1). The specificities of IgG-ELISA using MSsAg and SsAg were 83.7% and 92.9%, respectively ( $P < 0.05$ ) (Fig. 3).

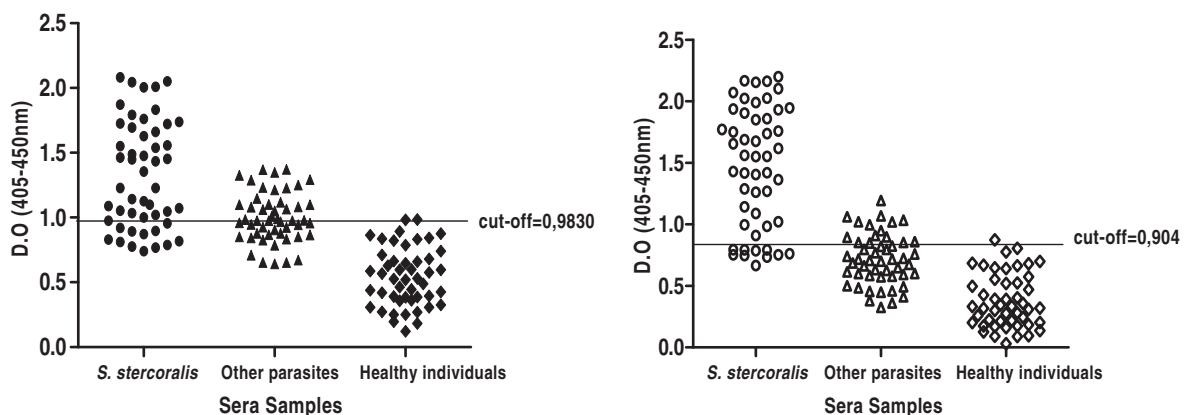
The ELISAs for specific IgE from sera of *S. stercoralis*-infected patients using MSsAg and SsAg showed seroreactivity in 37 (74%) and 40 (80%) of 50 samples, respectively, without significant differences between assays ( $P > 0.05$ ) (Fig. 2). The presence of cross-reactivity with MSsAg was 46% (23/50) and 30% (15/50) with SsAg (Fig. 2). IgE assays also produced a higher number of cross-reactions with sera from *Schistosoma mansoni* and hookworm-infected patients (Table 1). The specificity of IgE-ELISA was significantly different using MSsAg (75.5%) compared to the assay with SsAg (90.8%) ( $P < 0.05$ ) (Fig. 3).

To test the IgG competition for IgE *Strongyloides* antigen binding sites, the IgE reactivity was assessed in 10 IgE false-negative sera from parasitologically confirmed *S. stercoralis*-infected patients, after the IgG absorption. IgG-depleted sera were accompanied by a significant increase in seroreactivity of these sera as shown in Fig. 4 ( $P < 0.05$ ).

## 4. Discussion

Many parasitologic techniques have been evaluated to detect *S. stercoralis* infection. However, *S. stercoralis* is one of the most difficult intestinal parasites to diagnose due to the low parasite load and irregular larvae output in the majority of subclinical infections (Hirata et al., 2007; Inês et al., 2011). Immunoassays to measure antibodies have been applied to diagnose infectious diseases, including those caused by parasites, because of their high detection power and ease of implementation (Schaffel et al., 2001; Van Doorn et al., 2007). Unfortunately, the presence of cross-reactions, especially in regions with high prevalence of intestinal parasites, is very frequent. Moreover, the constant exposure of patients to *S. stercoralis* and maintenance of a low-parasite-load infection, not always detected by parasitologic methods, may also affect the sensitivity and specificity of immunoassays.

The development of immunologic methods using modified or purified parasite antigens could improve the sensitivity and specificity of serodiagnostic methods for *S. stercoralis* infection. In fact, parasite nematodes express many unusual complex carbohydrates at the cell surface in the form of glycoconjugates and glycol phospholipids. The



**Fig. 2.** Indirect ELISA to detect IgE anti-*S. stercoralis* using *S. stercoralis* soluble antigens treated with sodium metaperiodate (black symbols) and without treatment (hollow symbols). Sera samples from infected patients with *S. stercoralis* ( $n = 50$ ) or with other intestinal parasites ( $n = 50$ ) and from healthy individuals ( $n = 48$ ) were tested. The cut-off was calculated by the ROC curve.

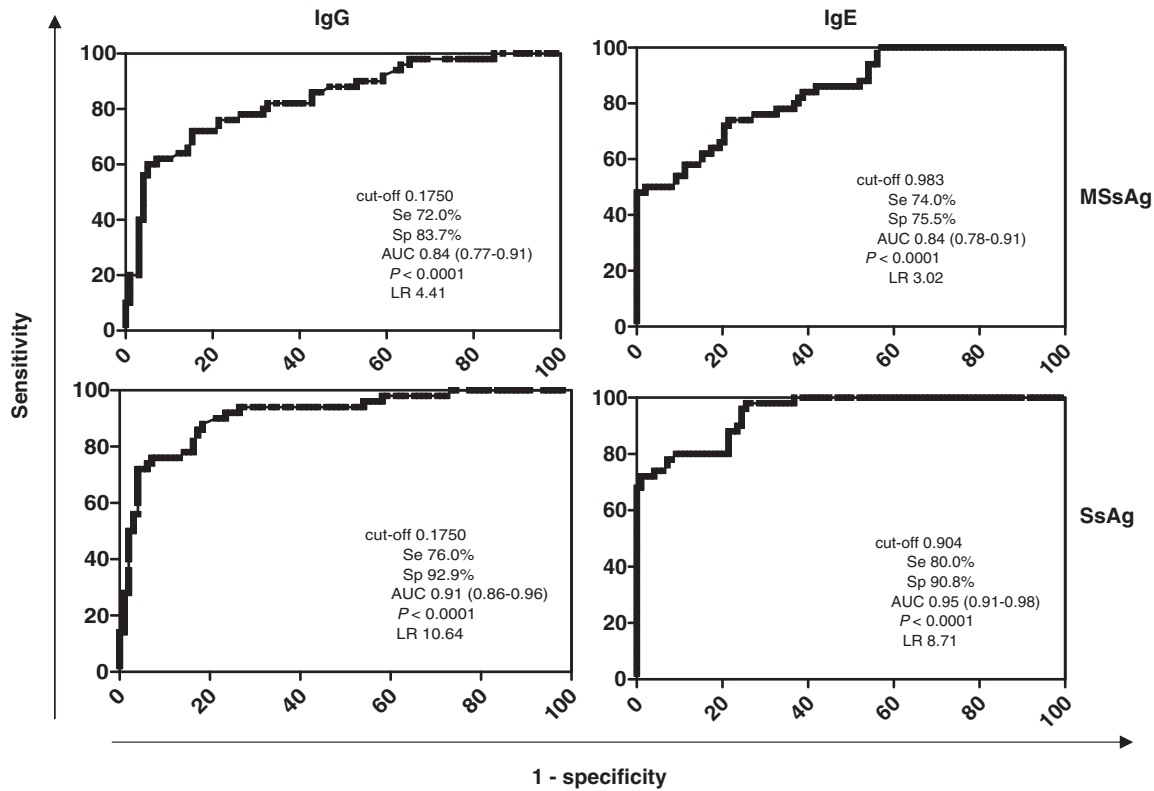


Fig. 3. ROC curve indicating the best point of the reaction (cut-off), sensitivity (Se), specificity (Sp), area under the ROC curve (AUC), and likelihood ratio (LR) for *Strongyloides stercoralis* soluble antigen treated with sodium metaperiodate (MSsAg) and without sodium metaperiodate treatment (SsAg) in sera samples for IgG and IgE detection.

cuticle surface of several nematode species is covered by a carbohydrate-rich glycocalyx that can be secreted and excreted as glycoconjugate antigens (Gonzaga, 2011; Maruyama et al., 2000). In several cases, such molecules have been shown to be involved in parasite survival, infection, and specific recognition by cells (Maruyama et al., 2000; Obregón-Henao et al., 2001; Schallig and Van Leeuwen, 1996).

The role of glycoprotein epitopes in increased antigenicity has been demonstrated in immunoassays to detect parasite antibodies (Alarcón de Noya et al., 2000; Kouguchi et al., 2011). Mild periodate oxidation destroys carbohydrates without altering protein and lipid epitopes and has been used to evaluate the carbohydrate role of antigenic glycoproteins in seroreactivity (Feizi and Childs, 1987). As shown here, for the first time, antibody reactivity to *S. stercoralis* was increased in the presence of glycosylated epitopes, thereby the sensitivity of ELISA with antigen treatment with sodium metaperiodate decreased from 76% to 72% for IgG and from 80% to 74% for IgE.

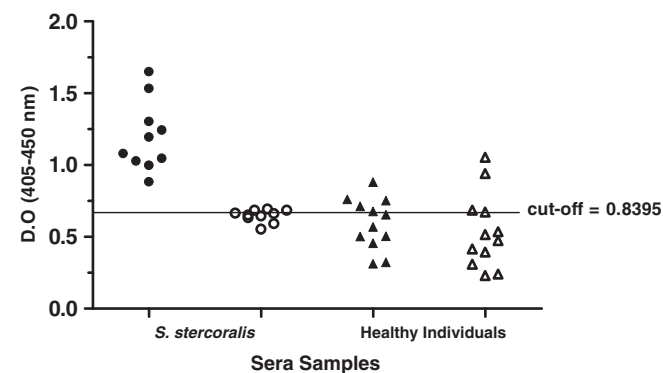


Fig. 4. Indirect ELISA to detect IgE anti-*S. stercoralis* using IgG-depleted (black symbols) and undepleted sera (hollow symbols). The cut-off was calculated by the ROC curve.

The mean optical densities of positive sera for *S. stercoralis* also decreased significantly in IgG immunoassays when the carbohydrate molecules were disrupted. These findings suggest a reduction of specific immunoreactivity when glycosylated epitopes are removed from parasite antigens, as previously demonstrated in immunoassays with sera from patients infected with *Hemonchus contortus* (Schallig and Van Leeuwen, 1996; Vervelde et al., 2003), *Taenia solium* (Obregón-Henao et al., 2001), and *Leishmania* (Atta et al., 2004).

Glycoprotein antigens are also associated with cross-reactions in immunodiagnostic tests, as observed previously in human sera from patients infected with *Schistosoma mansoni* (Alarcón de Noya et al., 2000), *Leishmania* sp. (Gomes-Silva et al., 2008), and *Echinococcus granulosus* (Kanwar and Vinayak, 1993; Younis et al., 2008). On the other hand, in the present study, the depletion of glycosylated epitopes from *S. stercoralis* antigens increased the cross-reactions with other parasites from 10% to 22% in IgG and from 30% to 46% in IgE ELISAs, thereby the specificity of both IgG and IgE assays decreased after carbohydrate depletion. These results are in agreement with those published by Albuquerque et al. (2005) who reported higher cross-reactivity by ELISA with *Paracoccidioides brasiliensis* antigen in the absence of glycosylated epitopes. The high level of cross-reactions after depletion of glycosylated epitopes may be associated with exposure of nonspecific antigenic binding sites hidden by carbohydrates. These results may also suggest that lectin-binding specific sites are present in *S. stercoralis* soluble antigens.

Circulating specific IgE may be a marker to distinguish current parasite infection from a past infection. In addition, IgE-ELISA has shown higher specificity than IgG-ELISA, presenting few cross-reactions with other intestinal parasites (Conway et al., 1993; Costa-Cruz et al., 2003). In this work, the conventional IgE-ELISA showed sensitivity and specificity of 80% and 90.8%, respectively. This is in agreement with other studies of the detection of IgE anti-*S. stercoralis* (Conway et al., 1993; Neva et al., 1981; Schaffel et al., 2001; Uparanukraw et al., 1999). However, there was no reduction in

cross-reactions in IgE-ELISA compared to IgG-ELISA, which may be explained by the constant exposition of the patients to *S. stercoralis* infection and by the maintenance of a low parasite load undetectable by parasitologic methods, but with detectable IgE specific antibodies. The absorption of serum samples with other helminth antigens has also been used to reduce the cross-reactivity in serologic tests (Conway et al., 1993). Nevertheless, difficulties in obtaining antigens from various parasite species, often requiring experimental models, make this approach unfeasible for immunodiagnosis. The depletion of IgG may increase the sensitivity of diagnostic tests, avoiding competition for the same IgE-antigen binding site (Rihet et al., 1992) as demonstrated in an immunoassay to detect IgE anti-*Leishmania* (Souza-Atta et al., 1999) and anti-*Paracoccidioides* (Mamoni et al., 2001). In the present study, 20% (10/50) of sera from patients with *S. stercoralis*-confirmed infection by parasitologic diagnoses presented false-negative results in IgE-ELISA. This fact can be attributed to low levels of specific circulating IgE antibodies, which hampers detection by serologic assays. Moreover, in disseminated strongyloidiasis, patients often have low levels of circulating IgE (Atkins et al., 1998; Hayashi et al., 1997; Porto et al., 2001). In conclusion, the results presented in this study show a decrease in the sensitivity and specificity of ELISA for IgG and IgE detection in the absence of *S. stercoralis* glycosylated epitopes. Additionally, previous absorption of IgG from patients' sera improved the sensitivity of ELISA for IgE anti-*S. stercoralis*, increasing the number of infected patients diagnosed.

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