Ion-exchange protocol to obtain antigenic fractions with potential for serodiagnosis of strongyloidiasis

H. T. GONZAGA[1], C. VILA-VERDE[1], D. S. NUNES[1], V. S. RIBEIRO[1], J. P. CUNHA-JÚNIOR[2] *and* J. M. COSTA-CRUZ[1]*

[1] *Laboratório de Diagnóstico de Parasitoses, Instituto de Ciências Biomédicas, Universidade Federal de Uberlândia, Uberlândia, Minas Gerais, Brazil*
[2] *Laboratório de Imunoquímica e Imunotecnologia, Instituto de Ciências Biomédicas, Universidade Federal de Uberlândia, Uberlândia, Minas Gerais, Brazil*

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SUMMARY

The aim of this study was to fractionate and partially characterize the antigenic extract of filariform larvae of *Strongyloides venezuelensis* in ion-exchange resin diethylaminoethyl sepharose (DEAE), to obtain antigenic fractions potentially applicable in immunoassays. Somatic antigen (SA) and its fractions DEAE S1 and DEAE S2 - which interacted with the resin - were evaluated by 1-dimensional electrophoresis to obtain protein profiles. SA and its fractions were tested in serum samples for IgG detection by ELISA. Serum samples ($n = 155$) were analysed: 50 from strongyloidiasis patients (G1), 55 from patients with other parasitic infections (G2) and 50 from healthy volunteers. Sensitivity (Se), specificity (Sp), area under curve (AUC) and likelihood ratios (LR) were calculated. The DEAE S2 fraction provided a high diagnostic value for IgG detection (Se 92·0%, Sp 91·4%, AUC 0·981, LR+ 10·75, LR− 0·09). In conclusion, the DEAE S2 fraction would probably be a source of immunodominant polypeptides for IgG detection in human strongyloidiasis serodiagnosis.

Key words: strongyloidiasis, serodiagnosis, ion exchange, heterologous antigens.

INTRODUCTION

Strongyloidiasis is caused by *Strongyloides stercoralis*, an intestinal nematode which is mainly endemic in tropical and subtropical regions (Grove, 1996; Paula and Costa-Cruz, 2011) and affects 30–100 million people in the world (Siddiqui and Berk, 2001). The parasite causes chronic asymptomatic infections in immunocompetent human hosts and systemic invasion in immunocompromised patients, developing into a fatal hyperinfection syndrome (Marcos *et al*. 2011).

Clinical diagnosis is uncertain because most cases are oligo- or asymptomatic or present as pulmonary and intestinal symptoms common to other parasitic diseases (Agrawal *et al*. 2009). Parasitological diagnosis presents low sensitivity, due to an intermittent larval shedding (Uparanukraw *et al*. 1999; Blatt and Cantos, 2003; Carvalho *et al*. 2012).

Immunological methods, such as enzyme-linked immunosorbent assay (ELISA), have been widely used in the diagnosis of human strongyloidiasis due to its applicability, safety, availability of reagents, and high diagnostic parameters, being considered the best test for serological screening (Mangali *et al*. 1991; Atkins *et al*. 1999; Schaffel *et al*. 2001; Siddiqui and Berk, 2001; Koosha *et al*. 2004; Feliciano *et al*. 2010; Gonzaga *et al*. 2011*b*).

As strongyloidiasis immunodiagnosis continues to be a challenge because of the increasing difficulty in obtaining sufficient quantities of *S. stercoralis* larvae (Rossi *et al*. 1993; Costa-Cruz *et al*. 1998) alternative antigens, including heterologous ones from *Strongyloides venezuelensis* have also been used, with satisfactory results, to diagnose human strongyloidiasis (Sato *et al*. 1995; Machado *et al*. 2008; Ribeiro *et al*. 2010; Gonçalves *et al*. 2012).

Recent research studies have shown that fractions of total saline extract of *S. venezuelensis* filariform larvae, obtained by different separation principles, can be used to detect IgG in *S. stercoralis* infection (Rigo *et al*. 2008; Feliciano *et al*. 2010; Gonzaga *et al*. 2011*a*).

The aim of this study was to fractionate, for the first time, the antigenic extract from filariform larvae of *S. venezuelensis* in ion-exchange resin diethylaminoethyl sepharose (DEAE), to obtain antigenic fractions potentially applicable in immunoassays.

MATERIALS AND METHODS

Serum samples

Serum samples were collected from 155 subjects attending the Laboratory of Clinical Analysis at the Clinical Hospital (Groups 1 and 2) and from the

* Corresponding author: Laboratório de Diagnóstico de Parasitoses, Instituto de Ciências Biomédicas, Universidade Federal de Uberlândia, Uberlândia, MG, Brazil, Avenida Pará 1720, 38400-902, Uberlândia, MG, Brasil. Tel: +55 34 3218 2187. Fax: +55 34 3118 2333. E-mail: costacruz@ufu.br

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Laboratory of Parasitology (Group 3) of the Federal University of Uberlândia in the State of Minas Gerais, Brazil. Group 1 (G1) consisted of 50 patients living in an endemic area and with confirmed parasitological diagnosis of strongyloidiasis using the Baermann-Moraes (Baermann, 1917; Moraes, 1948) method, based on positive larval thermo-hydrotropism, and the method of Lutz (1919), a gravity sedimentation technique.

Group 2 (G2) consisted of 55 patients who harboured other parasites: hookworm (*Necator americanus* or *Ancylostoma* spp.): ($n=16$; hookworm only ($n=12$); co-infected patients ($n=4$): (hookworm + *A. lumbricoides* + *T. trichiura* ($n=2$); hookworm + *A. lumbricoides* ($n=1$); and hookworm + *Hymenolepis nana* ($n=1$)), *Ascaris lumbricoides* ($n=6$), *Trichuris trichiura* ($n=2$), *A. lumbricoides* + *T. trichiura* ($n=1$), *Entamoeba histolytica* ($n=1$), *Enterobius vermicularis* ($n=5$), *Giardia lamblia* ($n=6$), *Hymenolepis nana* ($n=5$), *Schistosoma mansoni* ($n=5$), *Taenia* sp. ($n=8$; taeniasis ($n=5$) and cysticercosis ($n=3$)).

Group 3 (G3) consisted of 50 healthy volunteers based on their clinical profile. Although they live in an endemic area for strongyloidiasis, none had had household contact with *S. stercoralis* infection or any previous clinical related history of strongyloidiasis. Group 3 individuals underwent 3 faecal samples which were negative by Lutz (1919) and Baermann-Moraes (Baermann, 1917; Moraes, 1948). This study was approved by the Research Ethics Committee of the Federal University of Uberlândia.

Larvae obtention

S. venezuelensis third-stage larvae (L3) were obtained from charcoal cultures of feces from experimentally infected rats (*Rattus norvegicus*; L-2 strain; 1500 L3 larvae; abdominal subcutaneous inoculation). Fecal samples were mixed with equal parts of charcoal, moistened with water, spread in uniform layers on Petri dishes, and incubated at 28 °C for 72 h. Infective larvae were collected and concentrated using a Rugai apparatus (Rugai *et al.* 1954). L3 were washed 5 times in phosphate-buffered saline (PBS), 0·01 M, pH 7·2, and stored at −20 °C in PBS until use in antigen preparation.

Somatic antigen (SA) of Strongyloides venezuelensis *filariform larvae*

Somatic antigen was prepared according to the method of Gonzaga *et al.* (2011*a*) with minor modifications. Briefly, *S. venezuelensis* filariform larvae (300 000) were re-suspended in PBS containing protease inhibitors (ethylenediaminetetraacetic acid 1 mmol/L, benzamidine 1 mmol/L, phenyl methyl sulfonyl fluoride 1 mmol/L, aprotinin 1 μg/mL, and leupeptin 2 μg/mL) and disrupted by 10 cycles of freezing (1 min, −196 °C) and thawing/sonication (5 min, 40 kHz, 37 °C) (Thornton, Inspec Eletrônica, São Paulo, Brazil). After a 2-h incubation period at 4 °C under gentle shaking, the suspension was centrifuged (12 400 ***g***, 30 min, 4 °C) and the supernatant was analysed for protein content according to the method of Lowry *et al.* (1951), and then stored at −20 °C until use.

Ion-exchange chromatography

Fractions of SA were obtained by ion-exchange chromatography developed in microtubes as follows. Briefly, 1200 μg of SA was loaded onto a 200 μl diethylaminoethyl Sepharose-DEAE resin (GE Healthcare Life Sciences), previously equilibrated with 10 volumes of PBS by 3 cycles of centrifugation (2000 ***g***, 2 min). The suspension SA/resin was maintained under gentle mixing for 20 min at 4 °C. After, the suspension had been centrifuged as described, the supernatant was recovered and considered as the non-binding resin fraction (DEAE S1). Resin was washed by centrifugation with 10 volumes of PBS, and the retained proteins (DEAE S2) were eluted using PBS supplemented with 0·5 M NaCl. The obtained fractions were analysed by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under non-reducing conditions, as described by Laemmli (1970). The proteins were visualized in SDS-PAGE by silver staining (Friedman, 1982). Gel analysis was performed using a graphical method to generate a protein lane profile plot of each antigenic fraction, by Image J version 1.44 software (National Institutes of Health, Bethesda, EUA); and then peaks referring to bands were compared with a protein standard marker (Real Biotech, RECOM™ Blue Wide Range Prestain Marker, Banqiao City, Taiwan) to estimate their relative molecular weights.

*ELISA to detect IgG anti-*Strongyloides

Preliminary experiments were carried out to determine the optimal conditions for ELISA, through block titration of reagents (antigens, sera and conjugate). ELISA was carried out as described by Gonzaga *et al.* (2011*b*) with modifications. Polystyrene microplates (Interlab, Brazil) were coated with each antigen (SA, DEAE S1 and DEAE S2) at a concentration of 5 μg/ml in carbonate-bicarbonate buffer, pH 9·6, and incubated overnight at 4 °C. Microplates were washed 3 times with PBS containing 0·05% Tween 20 (PBS-T). Then serum samples were diluted 1:160 in PBST containing 3% skimmed milk (PBST-M) and incubated for 60 min at 37 °C. After another step of washing, the immunoenzymatic conjugate (peroxidase-goat anti-human IgG, Fc specific; Sigma) was added diluted 1:2000 in PBS-T and incubated for 60 min at 37 °C. The assay was developed after an additional washing procedure by

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adding the enzyme substrate consisting of hydrogen peroxide and orthophenylenediamine (OPD) in 0·1 M citrate phosphate Na_2HPO_4 buffer, pH 5·5, for 15 min. The reaction was interrupted by addition of 25 μl per well of 2M H_2SO_4. Optical densities (OD) were determined at 492 nm in an ELISA reader (Titertek Plus, Flow Laboratories, USA).

Statistical analyses

Analyses were performed using GraphPad software package 5.0 (GraphPad Software Inc., San Diego, USA). Cut-off points were established using a two-graph receiver operating characteristic curve (TG-ROC) (Greiner *et al*. 1995) based on ELISA results from patients positive for *S. stercoralis* in stool samples (positive controls) and individuals from the other 2 groups (strongyloidiasis negative controls). The ELISA reactivity index (RI) was obtained by the ratio between OD and cut-off. Values of RI greater than the optimum point of reaction for each extract were considered positive (RI >1). Sensitivity (Se) and specificity (Sp) were calculated according to the formulae: $Se = a/(a+b)$ and $Sp = d/(c+d)$ where a = is true positive, b = false positive, c = false negative and d = true negative (Youden, 1950).

Friedman (F) and *post-hoc* Wilcoxon Signed-Rank tests (W), with Bonferroni-Holm (Holm, 1979) correction for a multiple comparison situation, were conducted to evaluate statistical differences of IgG levels using the 3 antigenic fractions among studied groups.

Receiver operating characteristic curves (ROC) were built to describe the tests (Martinez *et al*. 2003). The area under the ROC curve (AUC), an overall index of diagnostic accuracy, was calculated, values close to 1 indicate an informative test; and close to 0·5 indicate an uninformative test (Hanley and McNeil, 1982). An AUC comparison between antigenic preparations, which were derived from the same sera panel, was made using the method described by Hanley and McNeil (1983). The likelihood ratio (LR), an efficiency diagnostic parameter independent of prevalence (Greiner *et al*. 1995) was calculated, as follows: $LR+ = Se/(1-Sp)$, which indicates how likely patients with strongyloidiasis are to have a positive test result compared with other patients; and $LR- = (1-Se)/Sp$, defined as the probability of having a negative test result for strongyloidiasis patients. Probability (P) values of <0·05 were regarded as significant and 95% confidence intervals (CI) were provided to Se, Sp and AUC statistical calculations.

RESULTS

Eletrophoretical characterization

Figure 1 shows the eletrophoretical profile of each antigenic fraction after 12% SDS-PAGE. SA showed several proteic compounds with molecular weights ranging from <15 to 240 kDa. In the DEAE S1 fraction an enriched fraction of proteins with molecular weight from <15 to 21 kDa was observed. Otherwise, the analysis of DEAE S2 revealed a wider range of protein profile throughout the major antigens of 21 to 50 kDa.

IgG detection by ELISA using SA, DEAE S1 and DEAE S2

All serum samples were tested by ELISA using SA and its fractions obtained after ion-exchange chromatography (DEAE S1 and DEAE S2). At G1, 88% (44/50) of positivity for SA, 80% (40/50) and 92% (46/50) for DEAE S1 and DEAE S2 was observed, respectively. By analysis of detection patterns in G2, positivity rates of 9% (5/55) were observed when using SA and DEAE S1, and 5% (3/55) to DEAE S2. At G3 positivity was 20% (10/50), 26% (13/50) and 12% (6/50) for SA, DEAE S1 and DEAE S2, respectively. Differences in positivity were accompanied by a statistically significant difference in IgG antibody levels ($F > 21·71$; $P < 0·0001$) according to each antigenic fraction used (Fig. 2); notably the DEAE S2 fraction presented the highest RI median in G1 (1·924; SA *x* DEAE S2, $W = -769·0$, $P = 0·0002$; DEAE S1 *x* DEAE S2, $W = -973·0$, $P < 0·0001$) and the lowest in G2 (0·512; SA *x* DEAE S2, $W = 1134·0$, $P < 0·0001$; DEAE S1 *x* DEAE S2, $W = 950·0$, $P < 0·0001$) and G3 (0·687; SA *x* DEAE S2, $W = 845·0$, $P < 0·0001$; DEAE S1 *x* DEAE S2, $W = -1099·0$, $P < 0·0001$).

Cross-reactivity at G2 (RI >1) was observed when testing serum samples from patients infected with hookworm only (3/12 SA, DEAE S1 and DEAE S2), *E. vermicularis* (1/5 SA and DEAE S1), *G. lamblia* (1/6 SA) and *A. lumbricoides* (1/6 DEAE S1).

According to the cut-off point established by TG-ROC, sensitivity and specificity were 88% and 85·7% for SA; 80% and 82·9%, respectively for DEAE S1. When DEAE S2 was used an antigenic fraction having the best diagnostic performance with the highest values for sensitivity (92%) and specificity (91·4%) (Fig. 3) for IgG detection was observed.

Analysis of the ROC curve (Fig. 3), demonstrated that the DEAE S2 fraction efficiently distinguished patients with strongyloidiasis from control groups (G2 and G3). Test performance, indicated by AUC, showed that IgG detection using the DEAE S2 fraction (0·981) had the closest to the maximum value (1·00) of efficiency. Otherwise, when considering AUC values from SA (0·957) and DEAE S1 (0·915), efficiency was lower. Comparing AUC by the method of Hanley and McNeil (1983) it was observed that the DEAE S2 fraction achieved a value statistically better than SA ($z = -3·464$; $P = 0·0008$) and DEAE S1 ($z = -3·509$; $P = 0·0005$).

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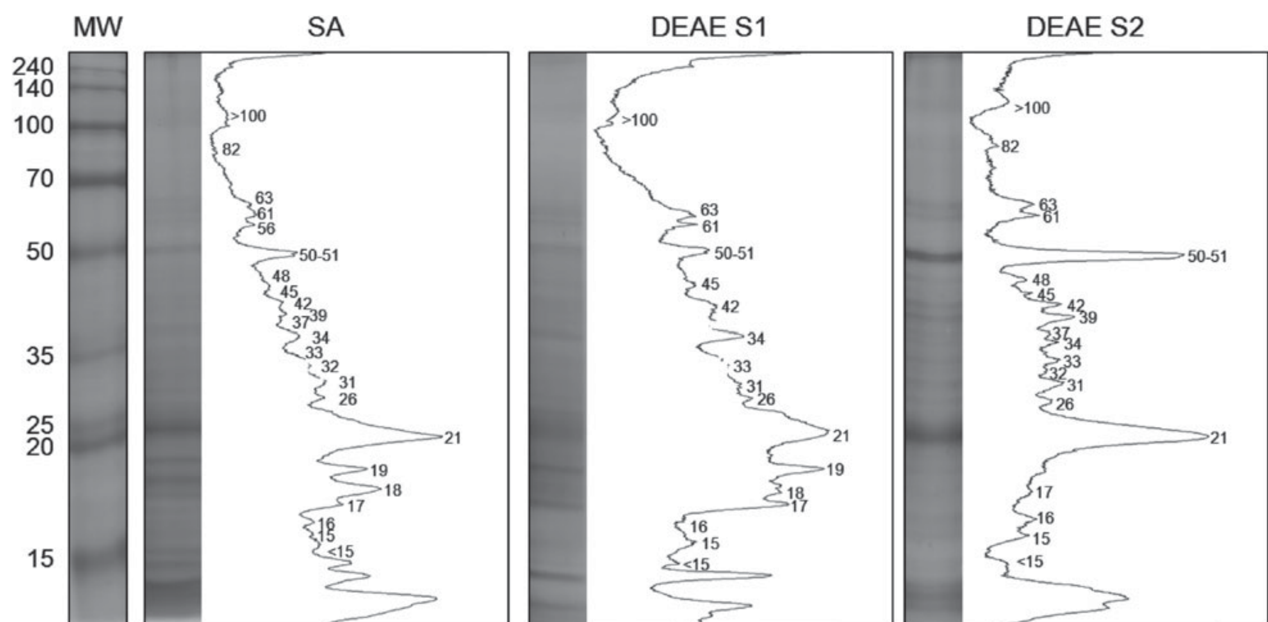

Fig. 1. Electrophoretic profiles of somatic antigen (SA) of *Strongyloides venezuelensis* filariform larvae and fractions obtained in ion-exchange diethylaminoethyl Sepharose resin – DEAE (DEAE S1 and a DEAE S2, that interacted with the resin) and Image J software analysis, in 12% SDS-PAGE, silver stained are shown. MW, molecular weight standard in kilodaltons (kDA).

LR+ to SA was 6·17 and for its fraction DEAE S1 was 4·67, indicating a moderate and small probability of a true positive strongyloidiasis, respectively. However, for the DEAE S2 fraction, a value of LR+ (10·73) points to an efficient test. The LR− values for SA (LR− = 0·14) and DEAE S1 (LR− = 0·14) indicate that both tests had a moderate effect on diminishing the probability of disease, while a low LR− (< 0·1) for DEAE S2 (LR− = 0·09) virtually excludes the chance that a patient is infected.

DISCUSSION

Considering the difficulties regarding to *S. stercoralis* immunodiagnosis, mainly in the cross-reactivity with other parasitic infections and normal endemic sera, efforts to achieve a reliable diagnostic test are needed. The present study was the first attempt using ion-exchange chromatography to obtain fractions potentially applicable in human strongyloidiasis diagnosis. Several studies have demonstrated the partial fractionation of *Strongyloides* antigenic extracts using other chromatographic approaches, including: gel filtration by S-200 (Mangali *et al.* 1991), hydrophobic interaction by octyl-Sepharose® (Rigo *et al.* 2008) and carbohydrate-affinity interaction by concanavalin-A-agarose resin (Gonzaga *et al.* 2011*a*). In the present study, electrophoretical analysis of DEAE S1 and S2 fractions showed a complementary profile when compared with SA. In the DEAE S1 fraction, an enrichment of proteins with low molecular weight was detected, including polypeptides from 21 to <15 kDa, indicating that these molecules do not interact with the DEAE resin. On the other hand, the DEAE S2 fraction is mainly enriched with proteins from 21 to 50 kDa that interact tightly with cationic diethylaminoethyl group. These findings suggest that proteins belonging to DEAE S2 display a negative liquid charge under pH physiological conditions.

DEAE resin was applied as a first fractionation step of *Strongyloides* larval extract to evaluate ELISA protocols. Despite having lower ELISA diagnostic parameters, the DEAE S1 fraction included regions of immunodominant proteins recently described: a 10–30 kDa region (Rigo *et al.* 2008), and a 28 kDa band (Feliciano *et al.* 2010) of *S. venezuelensis* larval preparations were detected by IgG from infected patients, even after different detergent fractionations. Other studies have demonstrated reactivity with 10, 17, 19 kDa proteic bands in the sera of *S. stercoralis* patients (Atkins *et al.* 1999; Silva *et al.* 2003).

Previous studies that employed homologous and/or heterologous *Strongyloides* antigens showed serum reactivity in a comparable proteic region to that obtained in this study, especially in DEAE S2. Machado *et al.* (2008) identified a 45 kDa immunodominant antigenic fraction, by immunoblot, which was recognized by IgG antibodies using *S. venezuelensis* antigenic extract. Using homologous antigens, other authors have shown IgG reactivity with 21, 26, 31, 32 and 33 kDa bands from *S. stercoralis* antigens (Sato *et al.* 1990; Atkins *et al.* 1999; Sudré *et al.* 2007). Although similar electrophoretic motility does not characterize an absolute measure for polypeptide

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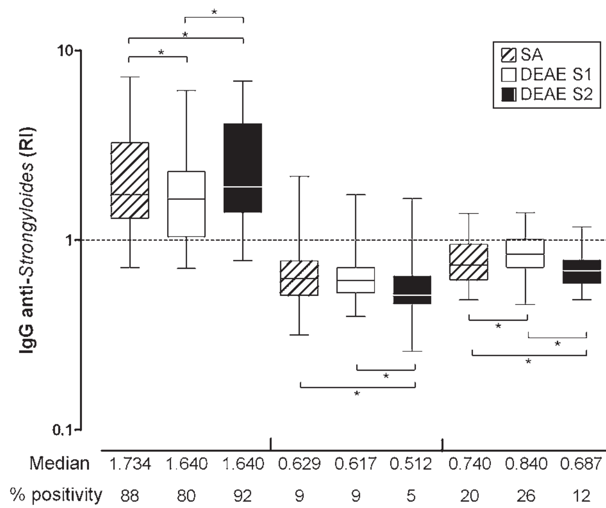

Fig. 2. Detection of IgG anti-*Strongyloides* in serum samples from patients with strongyloidiasis (G1, $n = 50$), other parasitic infections (G2, $n = 55$) and healthy individuals (G3, $n = 50$) analysed by ELISA (enzyme-linked immunosorbent assay) using somatic antigen (SA) of *S. venezuelensis*, and DEAE fractions DEAE S1 (unbound fraction) and DEAE S2 (bound fraction). Data by groups represented in box and whisker plot, with box indicating median and 25–75% percentiles, and whiskers indicating data range. Statistical significance was calculated by the Friedman test and *post-hoc* Wilcoxon Signed-Rank test, with Bonferroni-Holm correction (*$P<0{\cdot}017$).

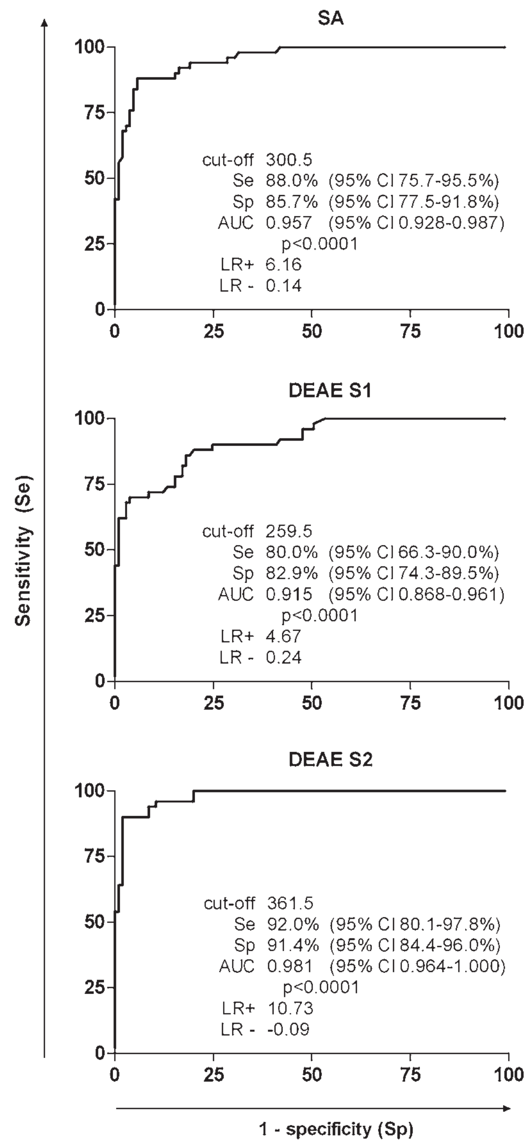

Fig. 3. Receiver operating characteristic curves (ROC) indicating the optimum point of reaction (cut-off), sensitivity (Se), specificity (Sp), area under curve (AUC) and likelihood ratios (LR+ and LR−) for IgG detection in serum samples using somatic antigen (SA) of *Strongyloides venezuelensis* and DEAE fractions (DEAE S1 and DEAE S2).

identity (Gomez-Munoz *et al*. 1996) it is suggested that the bands described are equivalent to the ones found herein, and the partially purified DEAE S2 fraction should be relevant in human strongyloidiasis immunodiagnosis.

Analysis of the DEAE S2 fraction performance within groups, patients with strongyloidiasis had 4 sera with false-negative results, possibly because these patients did not reach detectable levels of antibodies. Other parasitic infections group (G2) is representative of the general population, especially in developing countries where parasitic diseases are highly prevalent (Grove, 1996; CDC, 2012). Since serum samples from *Toxocara*-infected patients were not available, especially due to the low occurrence, it was not possible to determine cross-reactivity indices for this parasite. In G2 cases of cross-reactivity occurred in patients infected with hookworm, who also were false-positive in the SA and DEAE S1 fraction. In addition, hookworm is one of the main contributors to cross-reactivity in strongyloidiasis serology. There were endemic normal sera (G3) with false-positive results, and it is suggested that these patients only had detectable IgG anti-*Strongyloides* titres due to constant antigenic exposure. These false-positive results in G2 and G3 may also possibly be the result of individuals who previously had an asymptomatic and undetected strongyloidiasis infection and therefore may have had antibodies against *Strongyloides*. In this way, it could be hypothesized that the difference at the composition of the DEAE S1 and DEAE S2 fractions actually affected test performance. The use of DEAE S2 contributed to an increase in G1 IgG detection with possible selection of more sensible epitopes and with a decrease in antibody median of other groups with less cross-reactivity.

ROC and TG-ROC curve analysis provided an optimal cut-off point for each antigenic fraction studied. Information obtained from ELISA showed a different IgG detection profile for each fraction tested. DEAE S1 diagnostic parameters were inferior if compared with those from DEAE S2, which

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exhibited an increased sensitivity, specificity and a higher AUC value. Therefore, it is suggested that different epitopes could be recognized (Atkins *et al*. 1999; Gonzaga *et al*. 2011*a*); however, IgG possibly has a higher affinity for proteic epitopes with selected anionic residues, once the DEAE resin is positively charged. According to Jaeschke *et al*. (1994), values of likelihood ratio LR+ greater than 10, practically confirm the diagnosis, and LR− below 0·1 exclude diagnosis. Therefore, the LRs showed that the DEAE S2 fraction had a remarkable diagnostic performance to detect IgG in patients with strongyloidiasis, reaching values of LR+ = 10·73 and LR− = 0·09.

A simple step to partial purification, as accomplished with DEAE anion-exchange chromatography, already showed appreciable results in immunoassay tests in other parasitic species studies to detect serum antibodies in *Wuchereria bancrofti* filariasis (Chenthamarakshan *et al*. 1996) or for localization of fractionated antigen in *Paragonimus* infection (Lee and Chung, 2001); and also for *Trichinela spiralis* tubulin purification (Martínez-González *et al*. 1998). Regarding diagnosis, the unbound fraction obtained in DEAE from heterologous filarial antigen provided better results in IgG detection for bancroftian filariasis (Chenthamarakshan *et al*. 1996), in contrast with our results.

A detailed immunochemical characterization of the DEAE S2 fraction, would be considerable using other approaches, for example, sequential gel filtration chromatography as previously used for diagnostic purposes in other parasite infections (de Graaf *et al*. 1993; Camargo *et al*. 2004; Revilla-Nuín *et al*. 2005; Kaushal *et al*. 2009). However, it should be considered that a high-level purification of parasitic antigens may be related to an increase in specificity accompanied by a reduction in sensitivity indices (Chenthamarakshan *et al*. 2001). Thus, a defined antigenic fraction, such as DEAE S2, that contains more antigenic polypeptides could ensure an appropriate ELISA system.

In conclusion, the DEAE S2 fraction, obtained from *Strongyloides venezuelensis* somatic antigen fractionated on DEAE ion-exchange resin, showed high diagnostic parameters thereby suggesting its potential for strongyloidiasis diagnosis and as a source of immunodominant polypeptides for IgG detection.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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