

BRIEF COMMUNICATION

IMMUNODIAGNOSIS OF HUMAN STRONGYLOIDIASIS: USE OF SIX DIFFERENT ANTIGENIC FRACTIONS FROM *Strongyloides venezuelensis* PARASITIC FEMALES

Marcelo Andreetta CORRAL(1,2), Fabiana Martins de PAULA(1,2), Maiara GOTTARDI(1,2), Dirce Mary Correia Lima MEISEL(1,2), Vera Lucia Pagliusi CASTILHO(3), Elenice Messias do Nascimento GONÇALVES(3), Pedro Paulo CHIEFFI(1,4) & Ronaldo Cesar Borges GRYSCHER(1,2)

SUMMARY

The aim of this study was to evaluate six different antigenic fractions from *Strongyloides venezuelensis* parasitic females for the immunodiagnosis of human strongyloidiasis. Soluble and membrane fractions from *S. venezuelensis* parasitic females were prepared in phosphate-buffered saline (SSF and SMF, respectively), Tris-HCl (TSF and TMF, respectively), and an alkaline buffer (ASF and AMF, respectively). Serum samples obtained from patients with strongyloidiasis or, other parasitic diseases, and healthy individuals were analyzed by enzyme-linked immunosorbent assay (ELISA). Soluble fractions SSF, TSF, and ASF showed 85.0%, 75.0%, and 80.0% sensitivity and 93.1%, 93.1%, and 87.5% specificity, respectively. Membrane fractions SMF, TMF, and AMF showed 80.0%, 75.0%, and 85.0% sensitivity, and 95.8%, 90.3%, and 91.7% specificity, respectively. In conclusion, the present results suggest that the fractions obtained from parasitic females, especially the SSF and SMF, could be used as alternative antigen sources in the serodiagnosis of human strongyloidiasis.

KEYWORDS: Strongyloidiasis; Serodiagnosis; Heterologous antigens; *Strongyloides venezuelensis*; Parasitic females.

Human strongyloidiasis is often caused by the nematode *Strongyloides stercoralis*. This parasite is found worldwide but is mainly located in tropical and subtropical regions¹⁷. In humans, *S. stercoralis* can cause an asymptomatic chronic gastrointestinal infection. However, in immunocompromised individuals this parasite can cause a fatal hyperinfection syndrome or disseminated strongyloidiasis¹⁴.

A definitive diagnosis of strongyloidiasis is made through the detection of larvae in feces; however, parasitological methods have low sensitivity because of the irregular and intermittent release of larvae into feces¹⁰. Immunological methods have been widely used in the diagnosis of human strongyloidiasis because of their high sensitivity³. A major limitation of these methods is that it can be difficult to obtain sufficient quantities of *S. stercoralis* larvae for fractionation and analysis. If alternative antigens were available, including those from heterologous species such as *Strongyloides venezuelensis*, more satisfactory results for the immunodiagnosis of human strongyloidiasis might be possible^{2,4,7}.

Antigenic preparations from *S. venezuelensis* filariform larvae are most often used in the standardization and application of immunological techniques^{4,7}. Considering the life cycle of *Strongyloides* spp., it is necessary to study other antigenic sources, such as female parasites,

as they are found in the intestinal mucosa of the host and might elicit an immune response^{5,6}. The aim of our study was to evaluate different antigenic fractions from *S. venezuelensis* parasitic females for their application in the immunodiagnosis of human strongyloidiasis.

Antigenic fractions from *S. venezuelensis* parasitic females were evaluated by samples of immunocompetent individuals. Serum samples were obtained from individuals at the *Hospital das Clínicas* of the *Faculdade de Medicina* at the *Universidade de São Paulo* (HCFMUSP). Of these, 20 patients were harboring *S. stercoralis* larvae. Thirty-two patients were infected with other parasites: hookworm (n = 4); *Ascaris lumbricoides* (n = 2); *Blastocystis* spp. (n = 3); *Enterobius vermicularis* (n = 1); *Endolimax nana* (n = 3); *Giardia intestinalis* (n = 3); *Hymenolepis nana* (n = 1); *Schistosoma mansoni* (n = 9); hookworm and *H. nana* (n = 1); *S. mansoni*, *A. lumbricoides*, *Entamoeba coli*, *Blastocystis* spp., and *E. nana* (n = 1); *G. intestinalis* and *E. nana* (n = 1); *A. lumbricoides* and *Blastocystis* spp (n = 1); *E. nana*, *S. mansoni*, and *Blastocystis* spp (n = 1); hookworm, *Escherichia coli*, *Entamoeba dispar/histolytica*, and *Schistosoma mansoni* (n = 1). The remaining 40 samples were from seemingly healthy volunteers based on their clinical observation, without evidence of contact with *S. stercoralis* or previous history of strongyloidiasis, and negative in all parasitological diagnostic methods.

(1) Universidade de São Paulo, Instituto de Medicina Tropical de São Paulo, São Paulo, SP, Brasil.

(2) Universidade de São Paulo, Faculdade de Medicina, Hospital das Clínicas, Departamento de Moléstias Infecciosas e Parasitárias (LIM/06) São Paulo, SP, Brasil.

(3) Universidade de São Paulo, Faculdade de Medicina, Seção de Parasitologia da Divisão de Laboratório Central do Hospital das Clínicas, São Paulo, SP, Brasil.

(4) Santa Casa de Misericórdia de São Paulo, Faculdade de Ciências Médicas, São Paulo, SP, Brasil.

Correspondence to: Ronaldo Cesar Borges Gryschek, Universidade de São Paulo, Faculdade de Medicina, Hospital das Clínicas, Instituto de Medicina Tropical, Laboratório de Investigação Médica (LIM06), Av. Dr. Enéas de Carvalho Aguiar 470, 05403-000 São Paulo, SP, Brasil. Tel: +55 11 3061 8220. E-mail: rcbgry@usp.br

For each group, one stool sample was analyzed by the techniques described by LUTZ¹² and RUGAI *et al.*¹⁹ and an agar plate culture method¹⁸. The study received approval (protocol 266.046) from the Research Ethics Committee of the *Universidade de São Paulo* (Sao Paulo, Brazil).

S. venezuelensis parasitic females were obtained from Wistar rats (*Rattus norvegicus*) that were experimentally infected using protocol CPE-IMT 2011/126. To recover parasitic females, rats were euthanized on day 14 post-infection. The small intestines were then removed as described by NAKAI & AMARANTE¹⁵. Briefly, the intestine was longitudinally sectioned and placed on a sedimentation chalice in contact with saline solution for four h at 37 °C; the females were then counted and stored at -20 °C until use.

For antigenic extraction, approximately 5,000 parasitic females (F) were incubated with three different extraction buffers: 10 mM phosphate-buffered saline (PBS, pH 7.2), 25 mM Tris-HCl (pH 7.5), or 0.15 M NaOH. All the extraction buffers were supplemented with protease inhibitors (Sigma-Aldrich, St. Louis, MO, USA). Each extraction buffer was used to generate soluble (S) and membrane (M) antigenic fractions. For the soluble fractions, parasitic females were added to PBS (S), Tris-HCl (T), or NaOH (A) and disrupted in an ice bath using a tissue homogenizer. The suspensions were centrifuged at 12,400 × g for 30 min at 4 °C and supernatants collected. The soluble fractions in PBS, Tris-HCl, and NaOH were designated SSF, TSF, and ASF, respectively. For membrane fractions, SSF and ASF pellets were resuspended in 1% sodium dodecyl sulfate (SDS), boiled for five min at 100 °C, and centrifuged (12,400 × g, 30 min, 4 °C); subsequently, the supernatants were collected. These membrane fractions were designated SMF and AMF. The TSF pellets were resuspended in 7 M urea, 2 M thiourea, and 2% CHAPS ([3-cholamidopropyl] dimethyl-ammonio]-1-propanesulfonate); then, they were disrupted in an ice bath using a tissue homogenizer. The samples were centrifuged (12,400 × g, 30 min, 4 °C) and supernatants were collected; these fractions were designated TMF. All fractions were analyzed for protein content according to the method of LOWRY *et al.*¹¹, subdivided into aliquots, and stored at -20 °C until use. Fractions were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described by LAEMMLI⁹ under reducing conditions. Each of the antigenic fractions (SSF, TSF, ASF, SMF, TMF, and AMF) was electrophoresed using a 12% acrylamide separation gel. The gels were then analyzed using PlusOne Coomassie table PhastGel Blue R-350 (GE HealthCare Bio-Sciences AB, Piscataway, NJ, USA).

Enzyme-linked immunosorbent assays (ELISAs) were performed according to CORRAL *et al.*², with some modifications. Briefly, the wells of polystyrene microplates (Corning-Costar, New York, NY, USA) were coated with SSF, TSF, ASF, SMF, TMF, or AMF (5 µg/mL) in carbonate-bicarbonate buffer (0.06 mM, pH 9.6) and incubated overnight at 4 °C. After incubation, plates were washed (3 × 5 min) with PBS containing 0.05% (v/v) Tween 20 (PBS-T), and blocked with PBS-T supplemented with 3% (w/v) nonfat milk (PBS-TM) for 45 min at 37 °C. Serum samples were diluted 1:400 and 1:200 in PBS-TM for the soluble and membrane fractions, respectively. Fc-specific goat anti-human IgG conjugated to peroxidase (Sigma-Aldrich, St. Louis, MO, USA) was diluted 1:30,000 in PBS-TM. Color was developed by adding enzyme substrate (4 mM orthophenylenediamine, 0.03% H₂O₂, 0.1 M citrate phosphate buffer, pH 5.5) and incubated at room temperature for 15 min in the dark. The

reactions were stopped by adding 2 N H₂SO₄. The optical density (OD) was determined at 492 nm using an ELISA reader (Thermo Fischer Scientific, Waltham, MA, USA).

Statistical analyses were performed using GraphPad Prism version 5.0 (Graph Pad Software Inc. San Diego, USA). The ELISA index (EI) was calculated according to the following formula: EI = OD/cutoff. An EI value greater than one for a sample was considered positive. Tests were evaluated by calculating sensitivity, specificity, accuracy, kappa index (κ), and likelihood ratio (LR). Any *p*-value below 0.05 was considered statistically significant.

To our knowledge, the present study is the first to use different antigenic fractions from *S. venezuelensis* parasitic females for the immunodiagnosis of human strongyloidiasis. We used six antigenic fractions from parasitic females; three fractions were soluble and three were membrane-derived. The concentration of proteins from SSF, TSF, and ASF were 0.174, 0.174 and 0.708 mg/mL, respectively. Membrane fractions showed protein concentration of 0.631 (SMF), 0.300 (TMF), and 0.261 mg/mL (AMF). Although differences were observed in protein concentration, there was no change in the pattern of electrophoretic migration for the six fractions investigated, presenting bands of 25 and 150 kDa, and almost all samples presenting a band of approximately 23 kDa (Fig. 1).

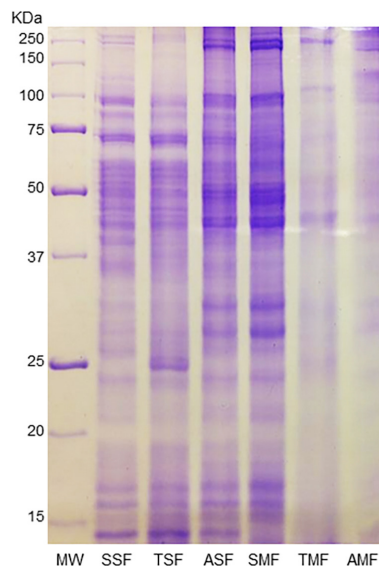


Fig. 1 - Electrophoretic profiles of soluble (SSF, TSF, and ASF) and membrane (SMF, TMF, and AMF) fractions from *Strongyloides venezuelensis* parasitic females, obtained using 12% SDS-PAGE and Coomassie blue staining. MW, molecular weight standard in kilodaltons (kDa).

The diagnostic parameters and efficiency of ELISA for the six antigenic fractions are shown in Fig. 2. Among the soluble fractions, SSF had the highest value of sensitivity and specificity (85% and 93.1%, respectively). The value of sensitivity and specificity for the membrane fraction SMF were also high, at 80% and 95.8%, respectively. The κ values ranged from 0.619 to 0.753 for the soluble fractions, and 0.630 to 0.772 for the membrane fractions. Of the six fractions tested, those obtained using PBS provided optimal diagnostic parameters. An LR

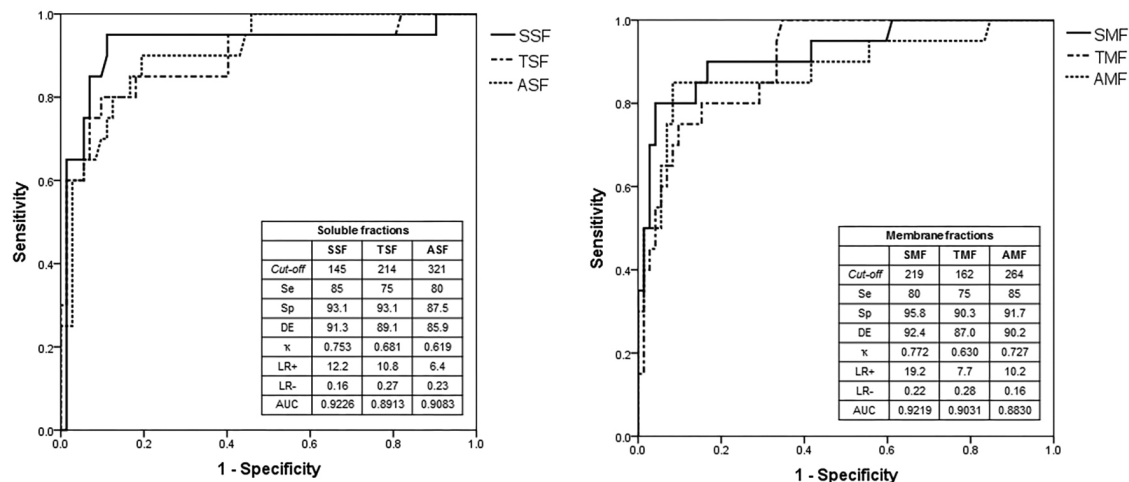


Fig. 2 - ROC curve indicating the optimum point of reaction (cut-off), sensitivity (Se), specificity (Sp), diagnostic efficiency (DE), kappa index (κ), likelihood ratio (LR), and area under curve (AUC) for IgG detection in serum samples using soluble (SSF, TSF, and ASF) and membrane (SMF, TMF, and AMF) fractions from *Strongyloides venezuelensis* parasitic females.

greater than 10 indicates high performance with respect to the detection of specific IgG antibodies in patients with strongyloidiasis⁷. In this study, the antigenic fractions obtained with PBS had high LR (12.2-19.2), indicating that these preparations had high ability to discriminate between strongyloidiasis patients and controls.

Differences among values for diagnosis were observed; it may be due to changes in the action of buffers used for the production of antigenic fractions, which can facilitate the antigen-antibody interaction. In general, there are major concerns regarding the antigenic preparations used in serological tests, especially regarding the evaluation of buffers used for the extraction of proteins, which is fundamental to obtaining antigens⁴. Cross-reactivity was observed for the six antigenic fractions with the following parasites: hookworms (1/4 in SSF and TMF), *Schistosoma mansoni* (2/9 in TSF and TMF), *A. lumbricoides* (1/2 in ASF), *G. intestinalis* (1/3 in AMF), *Blastocystis* spp (1/3 in ASF), and poly-infection with hookworms and *H. nana* (1/1 in SSF, ASF, and AMF).

The various forms of the *S. venezuelensis* filarial larvae during the parasite life cycle can be easily obtained in murine models¹³. The use of parasitic females has not been widely explored, possibly because of the difficulties involved in obtaining sufficient numbers for antigen production. However, specific proteins are present in parasitic females, and these proteins present new possibilities for antigen production¹⁶. Recently, GONÇALVES *et al.*^{5,6} showed that antigens from *S. venezuelensis* parasitic females could be used in the immunodiagnosis of human strongyloidiasis.

This study is the first to report the use of membrane antigens from *S. venezuelensis* parasitic females. Our results showed high specificity of the membrane antigens compared with those in soluble preparations. The use of membrane fractions from *S. venezuelensis* infective larvae has shown promising results for the immunodiagnosis of human strongyloidiasis².

It is difficult to obtain specific antigenic fractions for the immune diagnosis of strongyloidiasis, and a reliable diagnostic test is urgently required. To produce antigens for the diagnosis of human strongyloidiasis, the use of heterologous species maintained in experimental models has

been proposed. Therefore, female parasites represent a viable antigenic source; however, its use as a source of antigens remains uncommon. GONÇALVES *et al.*^{5,6}, who used an alkaline extract similar to ASF from *S. venezuelensis* parasitic females, reported similar results with respect to sensitivity and specificity as seen in the present study. Other antigenic fractions from the female parasite were not investigated by these authors. In our current study, we have shown that different antigenic extracts from female parasites can be used in the diagnosis of human strongyloidiasis.

The increasing use of recombinant antigens for the serological diagnosis of human strongyloidiasis has shown some promise^{1,8}. However, there are some limitations regarding resources in areas that are endemic for this helminthiasis. This lack of resources makes the application of these methodologies problematic in most regions. If a laboratory has adequate infrastructure and reagents for the production of recombinant antigens, then this technique could be viable. Nevertheless, in countries where the conditions are limited, the antigenic extracts preparations should be based on protocol, lower cost, and with good sensitivity and specificity results. Thus, the use of *S. venezuelensis* parasitic females, especially soluble and membrane fractions extracted with PBS, could provide an alternative source of antigens for the immunodiagnosis of human strongyloidiasis.

RESUMO

Imunodiagnóstico da estrogiloidíase humana: o uso de seis diferentes frações antigênicas de fêmeas parasitas de *Strongyloides venezuelensis*

O objetivo deste estudo foi avaliar seis frações antigênicas diferentes de fêmeas parasitas de *Strongyloides venezuelensis* para o diagnóstico sorológico da estrogiloidíase humana. As frações solúveis e de membrana de fêmeas parasitas de *S. venezuelensis* foram preparadas em solução salina tamponada (SSF e SMF, respectivamente), Tris-HCl (TSF e TMF, respectivamente) e tampão alcalino (ASF e AMF, respectivamente). As amostras de soro obtidas de pacientes com estrogiloidíase, com outras parasitoses e indivíduos saudáveis, foram analisadas pelo ensaio imunoenzimático (ELISA). As frações solúveis SSF, TSF e ASF

apresentaram sensibilidade de 85,0%, 75,0%, e 80,0% e especificidade de 93,1%, 93,1% e 87,5%, respectivamente. As frações de membrana, SMF, TMF e AMF demonstraram sensibilidade de 80,0%, 75,0%, e 85,0%, e especificidade de 95,8%, 90,3% e 91,7%, respectivamente. Em conclusão os presentes resultados sugerem que as frações obtidas a partir de fêmeas parasitas, especialmente o SSF e SMF, podem ser utilizadas como fonte alternativa de antígenos no imunodiagnóstico da estrogiloidiase humana.

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