

ORIGINAL ARTICLE

DIAGNOSIS OF *Strongyloides stercoralis* INFECTION IN IMMUNOCOMPROMISED PATIENTS BY SEROLOGICAL AND MOLECULAR METHODS

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SUMMARY

Strongyloidiasis is a potentially serious infection in immunocompromised patients. Thus, the availability of sensitive and specific diagnostic methods is desirable, especially in the context of immunosuppressed patients in whom the diagnosis and treatment of strongyloidiasis is of utmost importance. In this study, serological and molecular tools were used to diagnose *Strongyloides stercoralis* infections in immunosuppressed patients. Serum and stool samples were obtained from 52 patients. Stool samples were first analyzed by Lutz, Rugai, and Agar plate culture methods, and then by a quantitative real time polymerase chain reaction (qPCR). Serum samples were evaluated by an enzyme-linked immunosorbent assay (ELISA) using a soluble (AS) or a membrane fractions antigen (AM) obtained from alkaline solutions of the filariform larvae of *Strongyloides venezuelensis*. Of the 52 immunosuppressed patients, three (5.8%) were positive for *S. stercoralis* by parasitological methods, compared to two patients (3.8%) and one patient (1.9%) who were detected by ELISA using the AS and the AM antigens, respectively. *S. stercoralis* DNA was amplified in seven (13.5%) stool samples by qPCR. These results suggest the utility of qPCR as an alternative diagnostic tool for the diagnosis of *S. stercoralis* infection in immunocompromised patients, considering the possible severity of this helminthiasis in this group of patients.

KEYWORDS: *Strongyloides stercoralis*; Parasitological diagnosis; ELISA test; Real-time PCR; Immunocompromised.

INTRODUCTION

Strongyloides stercoralis, an intestinal parasitic nematode, infects 30-100 million people worldwide, and is commonly found in tropical and subtropical regions^{1,2}. Strongyloidiasis can occur without any symptoms or as a potentially fatal hyperinfection or disseminated infection^{1,3,4}, especially in immunosuppressed patients. Studies have shown the association between severe strongyloidiasis and immunosuppression, particularly in patients under corticotherapy, those infected with the human T cell lymphotropic virus-1 (HTLV-1), and to a lesser extent, those infected with the human immunodeficiency virus (HIV)^{3,5,6}.

The diagnosis of strongyloidiasis depends on the identification of larvae in fecal specimens through concentration techniques or cultures⁷. The majority of cases present with chronic low parasite load infections with minimal and irregular larval output⁸. Several studies support the idea

that detection of parasite-specific antibodies may be a useful complement to the parasitological diagnosis of strongyloidiasis⁷. Diagnostic immunological methods include the enzyme-linked immunosorbent assay (ELISA) and the Western blot technique that have already been reported in the context of strongyloidiasis, but they seem to present with variable sensitivity and specificity depending on the antigen and protocols used⁹⁻¹¹.

On the contrary, methods such as the polymerase chain reaction (PCR) that are based on DNA detection have demonstrated reproducible results with high sensitivity and specificity^{12,13}. The serological screening associated with molecular methods is slowly gaining popularity as an option to perform routine diagnosis^{14,15}. However, there are few reports that have investigated the serological and molecular diagnosis of human strongyloidiasis in parallel^{16,17}, especially in immunocompromised patients. This study aimed to evaluate serological and molecular methods for the diagnosis of human strongyloidiasis in samples

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from immunosuppressed patients that were previously analyzed by parasitological methods.

MATERIAL AND METHODS

Study population and ethical approval

This study received approval from the Research Ethics Committee of the *Universidade de São Paulo*, state of *São Paulo*, Brazil (protocol no. 0123/10). To evaluate the performance of serological and molecular methods in immunocompromised patients, serum and stool samples from 52 patients treated at the *Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo (HC-FMUSP)* were obtained. Patients between 10 to 60 years of age, of both genders were included as follows: HIV-positive (n = 20) and HTLV-1-positive (n = 32) patients, assuming that the underlying diseases conferred some degree of immune dysfunction. The diagnostic techniques were previously evaluated on samples from immunocompetent individuals. Serum and stool samples from 83 immunocompetent individuals, of both genders, aged between 10 to 60 years, who were attended at the *HC-FMUSP* showed 20 patients harboring *S. stercoralis* larvae; 30 patients with other parasites [mono-infections: hookworm (n = 3); *Ascaris lumbricoides* (n = 2); *Blastocystis* spp. (n = 2); *Enterobius vermicularis* (n = 1); *Endolimax nana* (n = 3); *Giardia intestinalis* (n = 3); *Hymenolepis nana* (n = 1); *Schistosoma mansoni* (n = 9); and poly-infections: hookworm and *H. nana* (n = 1); *S. mansoni*, *A. lumbricoides*, *Entamoeba coli*, *Blastocystis* spp. and *Endolimax nana* (n = 1); *G. intestinalis* and *Endolimax nana* (n = 1); *A. lumbricoides* and *Blastocystis* spp. (n=1); *Endolimax nana*, *S. mansoni* and *Blastocystis* spp. (n = 1); hookworm, *Entamoeba coli*, *Entamoeba dispar/histolytica* and *S. mansoni* (n = 1)]; and 33 apparently healthy, non-parasitized individuals based on their clinical features, and medical histories having no evidence of previous *S. stercoralis* infection or previous treatment for strongyloidiasis. All of the stool samples were analyzed according to methods described by Lutz¹⁸ and Rugai *et al.*¹⁹, and by agar plate culture²⁰.

Parasites and antigenic fractions

For the antigen extraction, *Strongyloides venezuelensis* filariform larvae (L3) were obtained from charcoal cultures of experimentally infected *Rattus norvegicus* (Wistar) feces (ethical approval CPE-IMT 2011/126). Approximately 400,000 L3 were added to NaOH (0.15 M) containing a protease inhibitor cocktail (P8340; Sigma-Aldrich, St. Louis, MO, USA), and lysed in an ice bath using a tissue homogenizer with 5 cycles of 20 pulses. The suspensions were centrifuged at 12,400 × g for 30 min at 4 °C and the supernatant was collected (soluble fractions, AS). The pellets were re-suspended in 1% SDS, heated to 100 °C for 5 min, centrifuged at 12,400 × g for 30 min at 4 °C, and the supernatants were collected (membrane fraction, AM). The protein quantification was performed as described by Lowry *et al.*²¹, and the antigen fractions were stored at -20 °C until required.

Enzyme-linked immunosorbent assay (ELISA)

ELISA was performed according to a previously described method⁹. Briefly, polystyrene microplates were coated with the antigenic fraction at a concentration of 5 µg/mL in carbonate-bicarbonate buffer (0.06 mol/L, pH 9.6) followed by an overnight incubation at 4 °C. After incubation,

the plates were washed three times for 5 min with phosphate-buffered saline (0.01 mol/L, pH 7.2) (PBS) containing 0.05% Tween 20 (PBS-T), and blocked with PBS-T plus 3% nonfat milk (PBS-TM) for 45 min at 37 °C. Serum samples were diluted (1:200) in PBS-TM before addition and incubated for 45 min at 37 °C. Peroxidase-conjugated goat anti-human IgG Fc-specific antibodies (Sigma, St. Louis, MO, USA) were added diluted at 1:30,000 in PBS-TM and the solution was incubated for 45 min at 37 °C. The enzyme substrate, ortho-phenylenediamine with 0.03% hydrogen peroxide in 0.1 mol/L citrate phosphate buffer (pH 5.5) was then added to the plate and incubated in the dark for 15 min at room temperature. The reaction was stopped by the addition of 2N H₂SO₄. Optical densities were determined at 492 nm in an ELISA reader (Thermo Fischer Scientific, Waltham, MA, USA). The ELISA index (EI) was calculated according to the following formula: EI = OD/cutoff. Values of EI > 1 for each of the antigen fractions were considered positive. The diagnostic parameters (e.g., cut-off, sensitivity, and specificity) were established by analyzing the samples from immunocompetent individuals, using a ROC curve analysis.

Molecular diagnosis – real time PCR (qPCR)

DNA extraction was performed according to a previously described method¹². Briefly, approximately 500 mg of stool samples preserved in 70% ethanol were washed twice in PBS. The resultant pellet was used for DNA extraction using the QIAamp stool mini kit (Qiagen, Hilden, Germany), following the manufacturer's instructions with some modifications, including an initial incubation at 56 °C overnight, after addition of the lysis buffer containing proteinase K. The resultant DNA was eluted in 100 µL of buffer and was quantified in a NanoDrop ND-1000 UV-VIS spectrophotometer v.3.2.1 (NanoDrop Technologies, Wilmington DE, USA). qPCR was performed according to a previously described protocol¹³ in a 12.5 µL reaction containing 3 µL DNA, 6.25 µL TaqMan Universal PCR Master Mix (Life Technologies Applied BioSystems, Foster City, CA, USA), 1.5 pmol/µL of each primer (18S ribosomal RNA gene) (forward 5'-GAATTCCAAGTAAACGTAAGTCATT AGC-3' and reverse 5'-TGCCTCTGGATAT TGCTCAGTTC-3') and 2.5 pmol/µL of the probes (FAM-5'-ACACACCGCCGTCG CTGC-3'-BHQ1). qPCR was performed on the ABI 7300 real-time PCR System (Applied BioSystems, Life Technologies, Foster City, CA, USA). The amplification conditions were set at 50 cycles of 95 °C for 15 min, 95 °C for 15 sec and 60 °C for 1 min. The TaqMan exogenous internal control kit (Applied BioSystems, Life Technologies, Foster City, CA, USA) was used as the reaction control. The qPCR plate included a negative control (PCR mixture without DNA template), and a positive control (DNA from filariform larvae of *S. stercoralis*). To determine the sensitivity of qPCR, a 10-fold dilution series was performed in triplicate using DNA samples obtained from the filariform larvae of *S. stercoralis*. The qPCR result was considered negative when the Ct value was more than 39.2 or when no amplification curve was observed²².

Data analysis

Statistical analyses were performed using the GraphPad Prism software version 5.0 (GraphPad Software Inc. San Diego, USA). The results of each method were compared with those of the parasitological methods and the degree of agreement was determined by the Kappa coefficient (κ). Statistical significance was set at *p* < 0.05.

Table 1
Diagnostic parameters of different methods applied to strongyloidiasis

Method	Cut-off	Se	Sp	LR+	LR-	DE	κ
ELISA-SA	0.362	85.0	93.6	13.4	0.16	91.6	0.773
ELISA-MA	0.276	90.0	95.2	18.9	0.10	94.0	0.838
qPCR	Ct < 39.2	85.0	87.3	6.7	0.17	86.7	0.666

Cut-off (optimum point of reaction); Se (sensitivity); Sp (specificity); LR (likelihood ratios); DE (Diagnostic efficiency); κ (kappa index); Ct (threshold cycle) value range.

RESULTS

An initial ELISA evaluation performed with samples from immunocompetent individuals resulted in 85% and 90% of sensitivity, and 93.6% and 95.2% of specificity using the AS and the AM antigens, respectively (Table 1). Cross-reactivity was observed in serum samples of patients infected with *S. mansoni* (1/9 in AS and 1/9 in AM), hookworm (1/4 in AS) and poly-infections (1/6 in AM). The same evaluation was performed for the qPCR assay (Table 1). Amplification of *S. stercoralis* DNA was observed in 17 samples of *S. stercoralis* larvae-positive patients (range Ct range 27.8-39.2), in three patients with other parasites (Ct range 33.5-37.1), and in five samples from apparently healthy individuals (Ct range 36.3-38.4).

After establishing the diagnosis parameters, the samples from immunosuppressed patients were tested by ELISA and qPCR. IgG-ELISA was positive in 3.8% of the HIV and HTLV-1 patients, and in 1.9% of the HIV patients using the AS and AM antigens, respectively (Table 2). qPCR showed *S. stercoralis* DNA amplification in seven samples (13.5% of positivity), including four HIV-positive (range 23.0-38.9) and three HTLV-1-positive (Ct range 37.7-39.2) samples. Two other samples from HIV and HTLV-1 patients presented with a Ct > 39.2 (Ct 43.6 and 45.0). The remaining 43 samples did not present any Ct value (Table 2). Table 2 compares the findings of the parasitological, serological, and molecular methods performed on samples from immunosuppressed patients. Considering at least one parasitological method, only three samples from HIV-positive patients presented larvae in the feces (5.8%), and these samples were also positive by qPCR (Ct 23.0-37.9). The patients that were found to be positive by the ELISA test using the antigenic fraction AS were negative by the parasitological and qPCR methods, whereas the positive patients by the antigenic fraction AM, also showed positive results by other methods.

DISCUSSION

S. stercoralis remains one of the most neglected and under-reported helminthic infections¹. Considering that Brazil is a tropical country and that the chronicity and autoinfection characteristic of this parasite can result in severe forms of hyperinfection or disease dissemination, strongyloidiasis constitutes a medically important and socially neglected problem². The present study is the first performed in Brazil that aimed to evaluate parasitological, serological, and molecular methods for the diagnosis of strongyloidiasis in immunosuppressed individuals.

Performing several techniques on a single sample may enhance the detection of parasites since different techniques vary in their sensitivities for different parasite species²³. Therefore, in this study, more than

Table 2

Results of the parasitological, serological and molecular methods after analysis of samples from immunosuppressed patients

	Method			Molecular n (%)
	Parasitological* n (%)	Serological n (%)	AM	
Positive	3 (5.8)	2 (3.8)	1 (1.9)	7 (13.5)
Negative	49 (94.2)	50 (96.2)	51 (98.1)	45 (86.5)
Total	52 (100)	52 (100)	52 (100)	52 (100)

*Parasitological tests: Lutz, Rugai, and the agar plate culture method.

one parasitological method was used to diagnose intestinal parasitic infections, despite the difficulty of obtaining three or more stool samples from the same patient. It is reported that the combination of methods allows the detection of 95% of infections caused by *S. stercoralis*²⁴. In the present study, 5.8% of immunocompromised patients were found to be positive by parasitological methods, and all of them were HIV-positive. Other studies have demonstrated a prevalence of 10-11% of *S. stercoralis* in HIV-positive patients using parasitological methods^{25,26}. Stool samples from HTLV-1-positive patients showed negative results by parasitological methods. In a previous study²⁷, 12.1% of positivity was observed in HTLV-1-infected patients by means of three techniques (spontaneous fecal sedimentation, Baermann larval searching, and Harada-Mori larval cultivation).

Most published studies addressing the diagnosis of human strongyloidiasis in immunocompromised individuals are based on serological and epidemiological surveys, and they have shown that serological methods may be used as screening tests in immunocompromised patients²⁸⁻³⁰. Nevertheless, in the present study, the positivity of parasitological methods was the same found for the ELISA (considering both antigenic fractions). In our study, we obtained 85-90% of sensitivity and 93.7-95.2% of specificity. Alkaline extracts of *S. venezuelensis* were used in other studies and different results were observed regarding the sensitivity (92.5-93.3%) and the specificity (86.1-93.8%)^{10,31}. On the contrary, Bisoffi *et al.*³² showed sensitivities varying from 75.4 to 85.1% and specificities varying from 94.8 to 100%, using recombinant antigens. Detection of IgG antibodies by ELISA in serum samples from immunosuppressed individuals using the antigen soluble fraction showed a higher positivity rate than using the membrane fraction. However, the membrane fraction was more specific for the detection of *S. stercoralis* since positive results by this antigenic fraction were

confirmed by parasitological and molecular methods. It is known that the sensitivity of the serological method is lower in samples from severely immunocompromised patients^{14,30,33}. Therefore, other researchers have proposed a systematic screening of *Strongyloides* infections, using both, serology and stool culture, in all HIV-positive immigrants coming from endemic areas²⁶. Recently, it has been reported that the detergent fraction of *S. venezuelensis* is effective for the detection of anti-*S. stercoralis* IgG antibodies in serum samples from immunocompromised individuals with strongyloidiasis³³.

Molecular techniques are highly useful in epidemiological studies on intestinal parasitic infections, as well as in routine diagnosis. PCR has been demonstrated to generate reproducible results with high sensitivity and specificity^{13,17}. The results obtained in this study evaluating the presence of *S. stercoralis* DNA in stool samples confirm that qPCR is a useful tool for diagnosis, as previously reported¹³. In particular, qPCR can detect and quantify a small amount of parasite DNA³⁴. In the present study, the positivity of qPCR was 2.3 times higher than that of parasitological and serological methods. The routine use of qPCR for the diagnosis of *S. stercoralis* does not eliminate the need for a careful interpretation of results. Particular attention should be given to the diagnostic value of quantitative amplification regarding the Ct values³⁴. Therefore, we determined a cut-off for the qPCR reaction, to avoid non-specific amplification, as described by Paula *et al.*²². However, in the particular case of *S. stercoralis* infection, the molecular diagnosis has not yet demonstrated to have an optimal sensitivity, and this parameter is particularly required for this parasite, for which even very low parasite load infections are relevant and must be detected and treated³⁵.

The potential limitation of this study may be a result of examining only a single fecal sample from each patient by parasitological methods, which may have contributed to the reduced number of positive samples for *S. stercoralis*, even after using a combination of techniques. However, the positive results emphasize the need for further evaluation, by using parasitological, serological, and/or molecular methods mainly because strongyloidiasis has no gold standard for the diagnosis, and this detection is of urgency in immunosuppressed patients.

The results of the present study suggest that qPCR should be considered as an alternative diagnostic tool for the detection of *S. stercoralis* in immunocompromised patients, especially considering the severity of this helminthiasis in this group of patients.

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