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Comparison between PCR and larvae visualization methods for diagnosis of *Strongyloides stercoralis* out of endemic area: A proposed algorithm

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ABSTRACT

Underdiagnosis of chronic infection with the nematode *Strongyloides stercoralis* may lead to severe disease in the immunosuppressed. Thus, we have set-up a specific and highly sensitive molecular diagnosis in stool samples. Here, we compared the accuracy of our polymerase chain reaction (PCR)-based method with that of conventional diagnostic methods for chronic infection. We also analyzed clinical and epidemiological predictors of infection to propose an algorithm for the diagnosis of strongyloidiasis useful for the clinician. Molecular and gold standard methods were performed to evaluate a cohort of 237 individuals recruited in Buenos Aires, Argentina. Subjects were assigned according to their immunological status, eosinophilia and/or history of residence in endemic areas. Diagnosis of strongyloidiasis by PCR on the first stool sample was achieved in 71/237 (29.9%) individuals whereas only 35/237(27.4%) were positive by conventional methods, requiring up to four serial stool samples at weekly intervals. Eosinophilia and history of residence in endemic areas have been revealed as independent factors as they increase the likelihood of detecting the parasite according to our study population. Our results underscore the usefulness of robust molecular tools aimed to diagnose chronic *S. stercoralis* infection. Evidence also highlights the need to survey patients with eosinophilia even when history of an endemic area is absent.

1. Introduction

The soil-transmitted nematode *Strongyloides stercoralis* is estimated to infect at least 30–100 million people worldwide. The prevalence has been increasing, mainly in southern, eastern and central Europe, the Caribbean, Southeast Asia, Latin America, and sub-Saharan Africa. Worldwide, it is mostly diagnosed in immigrants from endemic regions. Argentina is regarded as an endemic country. However, owing to its vast size and considerable latitude range, the northern subtropical regions offer better conditions for the nematode life cycle than the southern colder regions (Schär et al., 2013b; Puthiyakunnon et al., 2014; Buonfrate et al., 2015b).

S. stercoralis has alternate parasitic and free-living life styles. Soil or auto-infective filariform larvae (L3a) penetrate the skin, access the bloodstream and finally get into the small intestine to dwell as parthenogenetic females. Autoinfection is responsible for chronic infections in people living for several years out of endemic areas. Most chronic infections are asymptomatic, with eosinophilia being sometimes the only laboratory finding, while larvae excretion fluctuates at very low levels (Concha et al., 2005; Brigandi et al., 1997).

However, immunosuppressed patients, such as those receiving steroid therapy, can develop hyperinfection or dissemination syndromes (severe infection) due to accelerated autoinfection and migration of auto-infectant filariform larvae (L3) towards different body locations. These patients can suffer bacteraemia, bacterial meningitis, bacterial abscesses, diarrhea and pneumonia due to the erratic migration of enterobacteria-carrying L3 larvae (Siddiqui and

Abbreviations: AIDS, acquired immunodeficiency syndrome; APC, agar plate culture; CI, confidence interval; EA, endemic area risk; Eo, eosinophil cell; HIV, human immunodeficiency virus; HTLV-1, human T lymphotropic virus type 1; IC, immunocompromised patient; IRIS, immune reconstitution inflammatory syndrome; L3 larvae, filariform larvae; NPV, negative predictive value; PCR, polymerase chain reaction; PPV, positive predictive value.

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Berk, 2001; Concha et al., 2005). In such clinical situations, the high parasite burden allows diagnosis to be easily performed by detecting of L3 larvae in tissue samples. In spite of this, mortality reaches nearly 80% (Siddiqui and Berk, 2001; Concha et al., 2005).

The link between steroid therapy and strongyloidiasis has been extensively reported. It has been associated with a two- to three-fold increase in the risk of severe infections because of its suppressive effects on the major mediators of the immune response to *S. stercoralis* larvae (Fardet et al., 2006). It has also been suggested that steroids may have a direct effect on the parasite by accelerating molting of rhabditiform to invasive filariform larvae or by rejuvenating adult females (Keiser and Nutman, 2004). In this report, patients on steroid treatment also presented higher risk of severe infection compared with those untreated. In endemic areas, *S. stercoralis* is often detected among AIDS patients but severe clinical forms are rare (Viney et al., 2004). Indeed, considering their immune status, cases of severe infection are not as frequently reported as those with other immunosuppressive conditions (Tanaka et al., 1999).

Early diagnosis is important to interrupt transmission and to avoid severe complications in immunosuppressed patients. As parthenogenetic females lay embryonated eggs that hatch inside the intestine, only rhabditiform larvae are passed out in the feces. Thus, current detection methods are based on microscopic observation of larvae in stools (Siddiqui and Berk, 2001). However, the diagnostic sensitivity value of a single stool sample is around 30% because of the intermittent larval excretion during chronic infections (Siddiqui and Berk, 2001). The test most frequently performed is the formalin-ether concentration technique although it lacks high sensitivity. Other authors reported that 3 stool samples are required to increase the diagnosis value while 7 consecutive cultures roughly reach 100% (Siddiqui and Berk, 2001; Nielsen and Mojon, 1987; Campo Polanco et al., 2014). The Baermann funnel concentration and the Harada-Mori filter paper method are much more sensitive than the single direct fecal smear. There are also different nutrient agar plate methods (e.g. the Koga agar culture) that have been specifically developed for detecting strongyloidiasis (Koga et al., 1991; Becker et al., 2013). A *meta*-analysis reported that the agar plate culture (APC) has better diagnosis performance (89% sensitivity) than the Baermann technique (Campo Polanco et al., 2014; Buonfrate et al., 2015a). Still, this result could be biased by the lack of standardization of the techniques across different studies (e.g. variations in incubation times and amounts of stool used).

Serological tests have high sensitivity in patients with chronic infection but are less reliable among immunocompromised as well as in people living in endemic areas where they cannot distinguish present from past infections (Marcos et al., 2011; Olsen et al., 2009). Regarding laboratory findings, eosinophilia, when present, may be the unique marker of nematode infection.

Over the last few years, DNA-based methods have been developed aimed to detect intestinal parasites in stool samples, increasing the sensitivity and the specificity of the diagnosis (Verweij et al., 2009). In this regard, we have recently reported the setup of a DNA isolation technique from fresh stool samples and a PCR that improves the performance of *S. stercoralis* diagnosis (Repetto et al., 2010, 2013). This method allows the detection of one larva per gram of stool without cross-reactions with DNA from other nematodes or intestinal pathogens.

In the present study, we compared the performance of this molecular diagnostic method with the reference larvae detection techniques in a blind study. For this, 237 participants currently residing in the metropolitan area of Buenos Aires (Argentina) were recruited and stratified according to their previous residency in an endemic area, eosinophil counts and immunological status. These characteristics as well as HIV infection and steroid therapy were considered in an attempt to determine diagnosis predictors or prognosis markers of infection and disease outcome.

In view of the scarcity of data and the lack of established guidelines in the literature concerning the prevention and management of *S. stercoralis* infection in immunosuppressed patients, we propose an algorithm to improve the early diagnosis of this parasitic infection in patients at risk of severe infection.

2. Materials and methods

2.1. Study population and data collection

Individuals older than 18 years were eligible for the study if they had history of residency in endemic areas and/or eosinophilia and/or immunosuppressed status and/or symptoms suggestive of *S. stercoralis* infection: abdominal pain, acute diarrhea, chronic diarrhea, dermatological manifestations, gram-negative bacilli bacteraemia, Löeffler's syndrome and septic shock.

Demographic information, current and past occupation, history of residence in endemic areas, underlying illnesses, eosinophils counts and risk of recent infection or reinfection were recorded from every participant by the physician using an individual questionnaire in a standardized data form. This questionnaire also requested details on clinical manifestations attributable to *S. stercoralis* infection explained above. Upon data collection, clinical forms were categorized as asymptomatic, intestinal and severe disease (hyperinfection and disseminated forms).

Previous residency in endemic area was considered when patients resided in Argentinean northeastern and northwestern endemic regions or in tropical and subtropical Latin-American and worldwide countries (Fig. 1).

Eosinophilia was defined as a peripheral blood eosinophil count equal to or over 450/mm^3. The immunological status was defined according to the presence of chronic illnesses, immunosuppressive or steroid therapy, hematologic malignancies, HIV infection, HTLV-1 infection (Human T lymphotropic virus type 1), transplantation or connective tissue disease. HIV and HTLV-1 infections were screened by enzyme immunoassays (EIA) and confirmed by Western blot.

Exclusion criteria involved: individuals with exogenous infection risk for the last five years and pregnancy.

2.2. Study design

A prospective, descriptive and observational study was designed aimed to compare between the diagnosis of *S. stercoralis* diagnosis by PCR and its detection by standard microbiological methods in Buenos Aires city, Argentina. This temperate region of the country is not currently considered as an endemic area for this parasite.

Subjects were recruited by sentinel clinicians of the Infectious Diseases Division, Hospital de Clínicas José de San Martín (Universidad de Buenos Aires) among patients attending the hospital with signs suggestive of strongyloidiasis identified and referred to the study. Healthy individuals from group V and VI were recruited at the vaccination center and among blood donors at the Hospital de Clínicas José de San Martín. Parasitological methods were performed at the Diagnostic Parasitology Laboratory, from the Instituto de Investigaciones en Microbiología y Parasitología Médica (Universidad de Buenos Aires-Consejo Nacional de Investigaciones Científicas y Técnicas) from January 2009 to February 2012.

S. stercoralis diagnosis was evaluated in 237 patients assigned to the following groups:

Group I (n: 53): Immunosuppressed patients with eosinophilia and risk of *S. stercoralis* infection (previous residency in an endemic area).

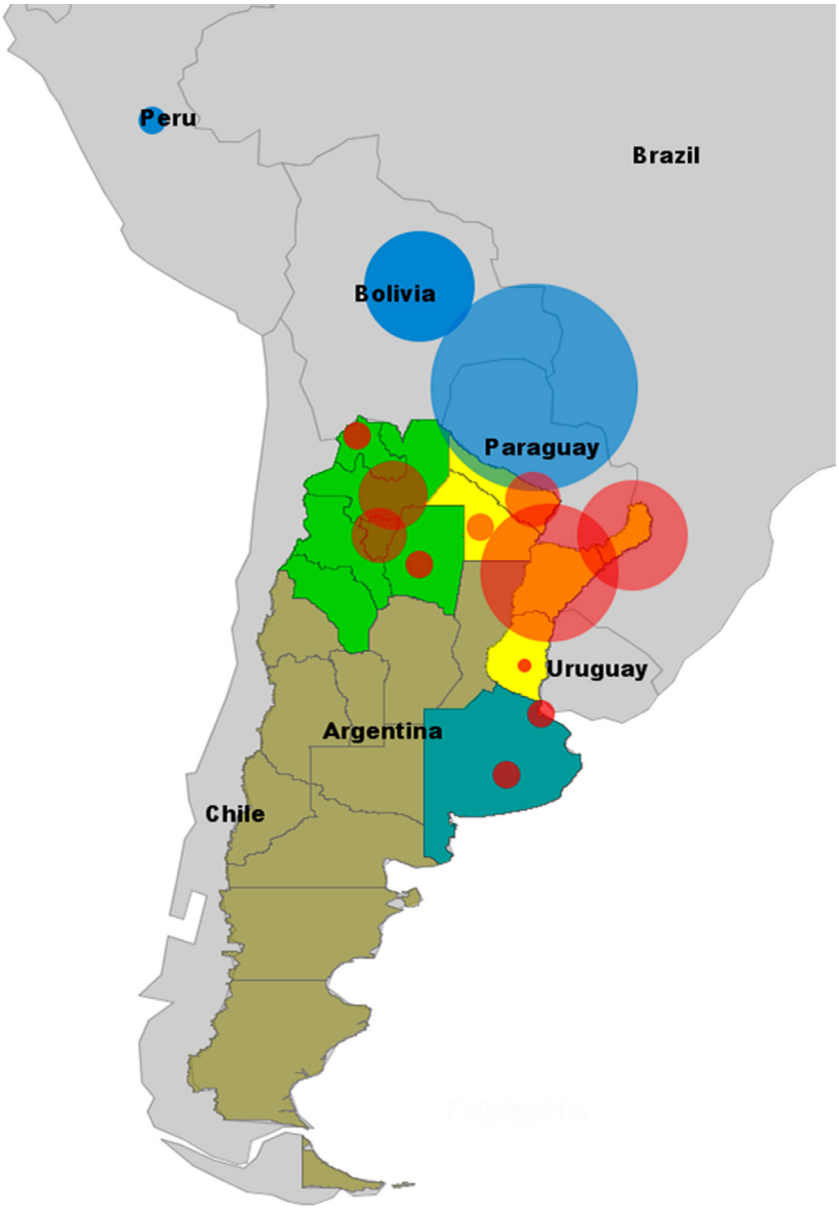

Fig. 1. Areas of origin of patients infected with *S. stercoralis* in the current study. Geographic origins were mapped based on Argentinean provinces or countries reference coordinates on a. kml format and mapped by gvSIG software (http://www.gvsig.com/). Dots are shown in the map according to the number of patients (size) and isolate origin (Color). Blue: Patients from Peru (n: 2), Bolivia (n: 8) and Paraguay (n: 15). Red: Argentinean patients distributed in 11 provinces (n: 43). Three patients were not included due to their geographic distance: Africa (n: 1), India (n: 1) and Dominican Republic (n: 1). Argentinean endemic regions are indicated in green (Northwestern Argentina) and yellow (Northeastern Argentina). Buenos Aires province is indicated in turquoise as the geographic origin of four sporadic infection events. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Group II (n: 30): Immunocompetent individuals with eosinophilia and risk of *S. stercoralis* infection (previous residency in an endemic area).

Group III (n: 30): Immunocompetent individuals with eosinophilia and without risk of *S. stercoralis* infection.

Group IV (n: 51): Immunosuppressed patients without eosinophilia and with risk of *S. stercoralis* infection (previous residency in an endemic area).

Group V (n: 29): Healthy individuals: Immunocompetent individuals without eosinophilia and with risk of *S. stercoralis* infection (previous residency in an endemic area).

Group VI (n: 44): Healthy individuals: Immunocompetent individuals without eosinophilia and without risk of *S. stercoralis* infection.

2.3. Ethics statement

This study was approved by the Ethic Committees of both, the School of Medicine and the Medical School Hospital (Hospital de Clínicas José de San Martin) at University of Buenos Aires. Informed consents were signed by all participants before sample collection.

2.4. Stool samples

2.4.1. Stool collection

Formalin-preserved stool samples: Participants collected stool samples for seven consecutive days in 5% formaldehyde aqueous solution into a single collection flask (Buonfrate et al., 2015a).

Table 1
Characterization of the individuals included in the study.

Group (individuals)	I (53)	II (30)	III (30)	IV (51)	V (29)	VI (44)
Endemic area	+	+	–	+	+	–
Eosinophilia	+	+	+	–	–	–
Immunocompromised	+	–	–	+	–	–
Infected individuals *n*, (%)[a]	32 (61.5)	12 (40)	4 (13.3)	14 (27.4)	9 (31)	0 (0)
Non-infected individuals *n*, (%)[a]	21 (38.5)	18 (60)	26 (86.6)	37 (72.6)	20 (69)	44 (100)
Eosinophils counts of infected individuals Median mm^3 (IQR)[b]	1970.0 (2236)	1868.0 (1357)	3128.0 (19627.50)	180.0 (245.6)	293.0 (116.5)	–
Eosinophil counts of non-infected individuals Median mm^3 (IQR)[b]	659.0 (525)	1278.0 (1294)	874.0 (493)	153.5 (200)	220.5 (121)	134.0 (114)
S.s diagnosis by larvae detection *n* (%)[a]	21 (65.6)	6 (50)	1 (25)	6 (42.8)	1 (11.1)	0 (0)
S.s diagnosis by PCR in stool *n* (%)[a]	32 (100)	12 (100)	4(100)	14 (100)	9 (100)	0 (0)

[a] Percentages (%) were calculated based on the total number of individuals in each group.
[b] IQR: interquartile range.

Fresh stool sample: On the seventh day, a fresh sample was also obtained from each participant. Aliquots of fresh stool samples were stored at −20 °C for molecular studies (Repetto et al., 2013).

Those participants whose formalin- preserved or fresh stool sample tested negative for strongyloidiasis by microscopic diagnosis (described in the next section), were asked to repeat the collection of fresh stool samples at weekly intervals for up to four weeks.

Aliquoted formalin-preserved and fresh stool samples obtained from each patient were submitted to the Diagnostic Parasitology Laboratory, where current reference methods were performed (presence of larvae by direct microscopic diagnosis and/or APC). Up to four different samples were analyzed before ruling out *S. stercoralis* infection. When positive, patients were treated with ivermectin as described below.

PCR was performed on the first fresh stool sample after all samples were evaluated by reference methods. Samples were coded and blind testing was carried out. Reference methods and PCR were performed by different investigators to exclude test interpretation bias.

Samples were considered positive for strongyloidiasis when rhabditoid/filariform *S. stercoralis* larvae were observed and/or *S. stercoralis*-specific PCR was positive (Repetto et al., 2013).

2.5. S. stercoralis *diagnosis*

Microscopic diagnosis: One gram of fresh stools was homogenized in phosphate-buffered saline (PBS) and centrifuged. Pellets were analyzed by light microscopy with and without Lugol's iodine solution. Stools preserved in formalin were studied by the Ritchieís method. Briefly, stools were homogenized, filtered through folded gauze and centrifuged for 1 min at 2500 rpm. The pellet was suspended in 5 ml saline water and 5 ml of 0.1% formalin and centrifuged for 1 min at 2500 rpm. The sediment was observed by light microscopy (Silva Anécimo et al., 2012). Samples were considered positive for strongyloidiasis when larvae were detected. All stool samples were analyzed in triplicate.

Larvae or eggs of other helminths and cysts of protozoa detected in the formalin-fixed and/or in the fresh stools samples were recorded as well. Kinyoun staining was used for the diagnosis of *Cryptosporidium spp*., *Cystoisospora belli* and *Cyclospora cayetanensis* (Repetto et al., 2010, 2013).

Agar plate culture (APC): This diagnostic test was performed as reported previously (Repetto et al., 2010). Briefly, three grams of fresh stools/plate were seeded in the center of agar plates in triplicate. They were incubated at 37 °C for up to seven days and examined daily under a stereomicroscope in search of larvae or bacteria colony tracks caused by larvae migration. Morphology of *S. stercoralis* worms was confirmed by microscopic examination (Repetto et al., 2010, 2013).

2.6. *Molecular diagnosis*

DNA isolation and *S. stercoralis*-specific PCR were performed on the first stool sample of each participant as follows:

i) DNA isolation: DNA extraction was performed using the Combined Method standardized in our laboratory (Repetto et al., 2013). Briefly, one gram of stool was suspended in 500 μl of Glycine-Tris-EDTA buffer overnight followed by mechanical disruption prior to DNA isolation by a commercial kit procedure (QIAamp DNA Stool Mini Kit, QIAGEN, USA) following the manufacturer's instructions.

   Axenic culture epimastigotes (1×10^4) from the protozoa *Trypanosoma cruzi* were added to each stool sample before DNA isolation as exogenous internal amplification control for the extraction process (Repetto et al., 2013; Moser et al., 1989).

ii) *S.stercoralis*-specific PCR: Amplification of a 101 bp hypervariable region of *S. stercoralis* 18S small subunit rRNA gene (GenBank accession N° AF279916) was performed using the following primers: Stro 18S-1530F 5′ GAATTCCAAGTAAACGTAAGTCATTAGC3′ and Stro 18S-1630R 5′TGCCTCTGGATATTGCTCAGTTC 3′ (Verweij et al., 2009).

   Nuclease free water and DNA isolated from stool samples of Group VI were used as negative controls. DNA from each sample spiked with 50 *S. stercoralis* filariform larvae was used as positive control. PCR was performed as reported previously (Repetto et al., 2013).

PCR products were run in 3% (w/v) agarose gel stained with GelGreen™ Nucleic Acid Gel Stain. Images were acquired by the Molecular Imager Gel Doc XR System (BioRad laboratories).

iii) Exogenous internal amplification control: *T. cruzi*-specific PCR was performed on all clinical samples. *T. cruzi* DNA was amplified with primers: TCZ1 5′CGAGCTCTTGCCCACACGGGTGCTí3′ and TCZ2: 5′CCTCCAAGCAGCGGATAGTTCAGGí3′ (expected product size 188 bp) (Repetto et al., 2013; Moser et al., 1989). PCR was carried out as reported previously (Repetto et al., 2013).

2.7. Pharmacological treatment

Patients with strongyloidiasis diagnosis by larvae visualization received oral ivermectin 200 μg/ kg/day once a day for two days and a repeated dose after two weeks (Vadlamudi et al., 2006; Segarra-Newnham, 2007).

The first two doses of ivermectin were directly administered by the physician and the remaining doses were observed by a companion of the participant.

After unmasking the blind study, 36 patients whose diagnosis was performed only by PCR, were contacted. Thirteen of them returned for parasitological treatment.

2.8. Follow up

Treated patients were re-evaluated for the presence of symptoms compatible with strongyloidiasis, as well as for eosinophilia and parasite detection, one month after treatment.

2.9. Statistical analysis

Results of reference tests and PCR of stool samples were used to analyze the agreement between these two diagnostic methods and to calculate statistical indicators in order to assess the performance of PCR in stool samples. Sensitivity, specificity, negative predictive value (NPV) and likelihood ratio were calculated using EPIDAT 3.1.

Both descriptive and inferential statistical analyses were performed using SPSS software package for Windows (version 21) and GraphPad Prism software package (version 5.03). A p-value lower than 0.05 was considered statistically significant for the inferential tests. Cohen's Kappa index was used to assess reliability between diagnostic tests. For continuous variables, data distribution was checked for normality using Shapiro or Wilk's W-tests. Mean, median, and confidence interval (CI) were estimated. Non-parametric statistical tests (Wilcoxon signed-rank test, Mann-Whitney test and Kruskal Wallis with Dunns post-test) were used in the events where data distribution was not normal. Statistical associations among categorical variables were analyzed using the Pearson's chi-square test and presented as observed frequencies and proportions. The probability of finding the outcome of interest was calculated as odds ratio. For multivariate analysis, variables of clinical importance and those with significant associations confirmed by univariate analyses were introduced into a forward selective set of logistic regression models predicting each outcome separately.

3. Results

3.1. Study population profile

The age of the patients ranged from 18 to 78 years (mean 44.00-95% CI 40.37–47.83). Of them, 42.3% were women. None was at risk of recent *S. stercoralis* infection as they did not return to an endemic area for at least 5 years before their inclusion in the study. The main features of the participants that were assigned according to their endemic area history, eosinophilia and immunological status are described in Table 1. Strongyloidiasis was detected in 71 out of 237 (30%) individuals. None of the healthy individuals were infected (Group VI). Regarding the geographical origin of the infected individuals, most of them came from northern Argentina (60.56%) and from Paraguay (21.12%), a neighboring country (Fig. 1).

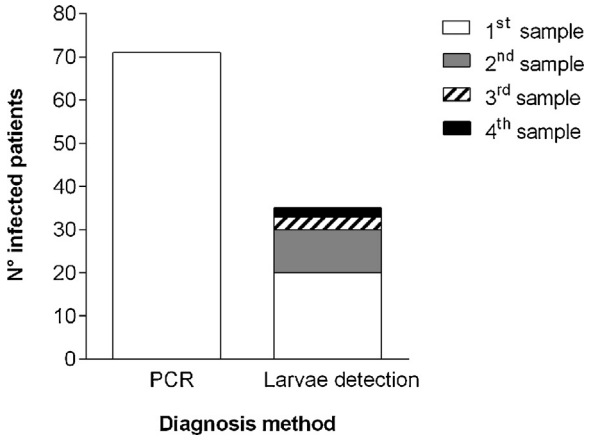

Fig. 2. *S.stercoralis*-specific PCR in the first stool sample. Temporal comparison between the methods used for the diagnosis of *S. stercoralis* in stool samples of infected patients (n = 71): Polymerase chain reaction (PCR) or microscopic detection of larvae in stool (Larvae detection).

Table 2
Comparison between PCR in stool and reference test for *S.stercoralis* diagnosis.

		Reference Test (Larvae detection)[a]		
		Positive	Negative	**Total**
PCR in stool	Positive	35	36	**71**
	Negative	0	166	**166**
	Total	**35**	**202**	**237**

[a] Larvae visualization by direct smear, Ritchie's method or APC.

3.2. Diagnostic performance of the PCR-based method

By conventional methods of larvae observation, 20 individuals were diagnosed with *S. stercoralis* in the first stool sample. Of these, 14 were positive by examination of fresh stool sample, Ritchie's method and APC, 3 of them were positive by Ritchie's method and APC, one patient was positive by fresh stool sample and APC. Finally, 3 of them were positive only by APC. Diagnosis by APC was extended to 15 more patients but required up to four samples to confirm infection.

In contrast, PCR in stool tested positive in the first sample in all infected individuals (Fig. 2). Thus, infection was only detected by a PCR-based method in 36 out of 71 patients (50.7%). The performance comparison between PCR and reference methods is shown in Table 2. According to this analysis, the sensitivity of PCR was 100%. The calculated NPV was 100% (CI 95%: 97.74–100) and the PPV was 49.3% (CI 95%: 38–60.66). The test accuracy was 84.81% (CI 95%: 79.69–88.82). The Cohen's Kappa index of 0.577 indicated a moderate agreement among tests.

3.3. History of residency in an endemic area or eosinophilia independently increased the likelihood of S. stercoralis infection

Among infected individuals (N = 71) with history of living in endemic areas, those without eosinophilia (Groups IV and V) had a lower frequency of strongyloidiasis (30.2%) in comparison with patients with eosinophilia (62%) (Groups I and II). Interestingly, *S. stercoralis* was also detected in four individuals lacking a history of

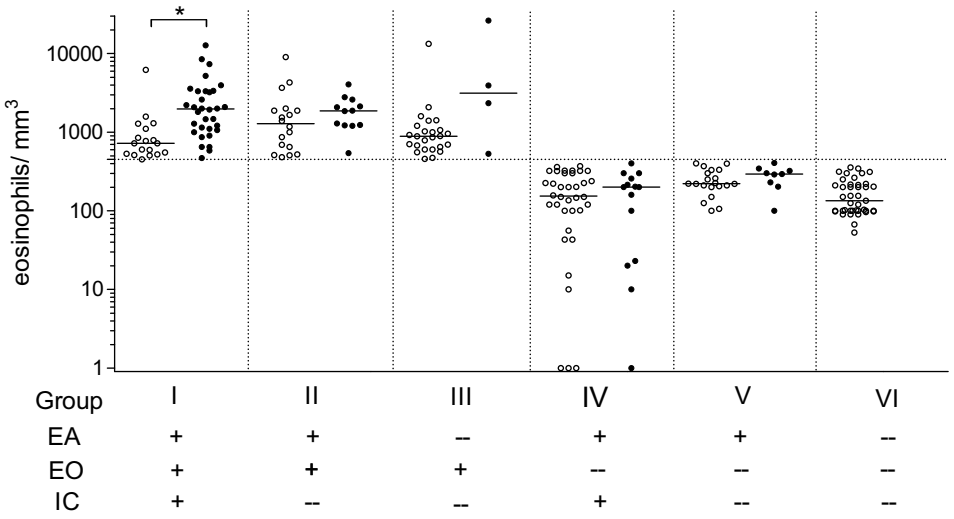

Fig. 3. Eosinophil levels of *S. stercoralis*-infected and non-infected patients. Individual eosinophil counts, as well as the median eosinophil counts of each group of infected (filled circle) and non-infected (open circle) patients were recorded. Individuals in study were segregated in groups according to eosinophilia (Eo), endemic area risk (EA) and immunocompromised status (IC). The doted horizontal line indicates the accepted normal threshold of eosinophils counts (450 Eo/mm^3). * $p < 0001$ Kruskal-Wallis, Dunns post test.

Table 3
Clinical presentation of strongyloidiasis and its association with steroid therapy and HIV co-infection.

Clinical forms	Signs and symptoms	Total	Steroid therapy		HIV co-infection	
			–	+	–	+
Asymptomatic N: 42	With eosinophilia	34	21	13	29	5
	Without eosinophilia	8	8	0	8	0
Intestinal	Mild abdominal pain	7	5	2	4	3
N:11	Acute diarrhea	1	1	0	1	0
	Chronic diarrhea	3	2	1	1	2
Severe	Severe abdominal pain	1	0	1	1	0
Infection[a]	Acute diarrhea and bacteriaemia	5	2	3	5	0
N:18	Löffler's syndrome	3	0	3	3	0
	Dermatological manifestations	1	0	1	1	0
	Septic shock	5	2	3	4	1
	Abscess	1	1	0	0	1
	Bowel obstruction	2	2	0	2	0
Total N:71		71	44	27	59	12

[a] Severe infection included hyperinfection and dissemination forms of the parasitic disease. (Siddiqui and Berk, 2001; Keiser and Nutman, 2004). None of the HIV- *S. stercoralis* coinfected patients were on under steroid therapy.

residence in an endemic area but displaying eosinophilia (Group III-5.6%). In this group diagnosis was positive by PCR, but only in one subject larvae were also detected by APC. Furthermore, the median eosinophil counts among infected people from this group were higher than those from other groups with eosinophilia ($p < 0.0001$; Kruskal-Wallis, Dunns post test) (Fig. 3). Accordingly, multivariate analyses identified that two independent factors had increased the likelihood of *S. stercoralis* infection: eosinophilia (OR 4.95, $p < 0.006$) and history of residence in an endemic area (OR 7.70, $p < 0.0001$).

3.4. *Underlying conditions and clinical manifestations of* S. stercoralis- *infected individuals*

The comorbidities most frequently identified were: HIV co-infection (17.1%), hematological malignancies (12.9%) and rheumatological disorders (10%). Only one patient was found to be co-infected with HTLV-1. Forty-eight of the 71 infected individuals were immunocompromised (67.6%). Forty-two S. stercoralis-infected patients (59.9%) were asymptomatic and 34 of them displayed eosinophil counts above 450/mm^3 (Table 3 and Fig. 3). Among the asymptomatic patients, the diagnosis was performed in 16 (38.1%) by larvae detection while in the remaining 26 (61.9%) the diagnosis was achieved only by PCR.

Presence of symptoms of strongyloidiasis and high eosinophil counts were associated with increased likelihood of parasite observation (odds ratio 1.7; $p < 0.02$). The median eosinophil count in patients diagnosed by microscopic larvae visualization was 1812 Eo/mm^3 (IQR 2962) while in those diagnosed by PCR it was 556.5 Eo/mm^3 (IQR 1637.2).

Among the 71 infected individuals, 13 reported gastrointestinal symptoms, 16 presented severe strongyloidiasis and 27 were on steroid therapy (Table 3).

Mixed infections with other intestinal parasites were observed in 10 patients (*Blastocystis hominis*, n: 5; *Entamoeba coli*, n: 2; *Cyclospora cayetanensis*, n: 1; *Entamoeba histolytica*, n: 1; *Ascaris lumbricoides*, n:1).

3.5. *Profile of immunocompromised patients infected with* S. stercoralis

Patients on corticosteroid therapy or with HIV co-infection presented increased eosinophil levels compared with individuals with the same underlying conditions but without strongyloidiasis (Fig. 4). In contrast, individuals on steroid therapy presented an increased risk of severe disease (odds radio: 4.35, Pearson's Chi-square test, $p < 0.01$).

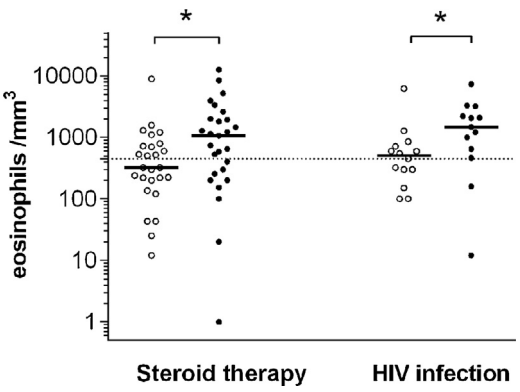

Fig. 4. Association between eosinophilia and *S. stercoralis* infection in patients on steroid therapy and those infected with HIV. Single eosinophils values and the median for *S. stercoralis* infected (black circle) and non-infected (open circle) individuals from each group are shown. The doted horizontal line indicates the accepted normal threshold of eosinophils counts (450 Eo/mm^3). * $p < 0.05$, Mann-Whitney test.

No association between HIV co-infection and severity of symptoms of *S. stercoralis* infection was found (odds radio: 0.54, Pearson's Chi-square test, $p = 0.63$).

3.6. *Eosinophil levels decreased after* S. stercoralis *specific treatment*

Forty-nine *S. stercoralis*-infected individuals (diagnosed by conventional methods and/or by PCR) were treated with ivermectin. Thirty days after treatment, eosinophil counts (Eo) were evaluated. As shown in Fig. 5A, Eo levels decreased significantly (pre-treatment median = 1474 Eo/mm^3, IQR 2006; post-treatment median = 436 Eo/mm^3 IQR 566.5, Wilcoxon signed rank test $p < 0.001$) except for a patient with neutropenia (Group IV). The group of individuals whose diagnoses were achieved only by PCR (13/49) also displayed a significant reduction in eosinophil counts (pre-treatment median = 1144 Eo/mm^3, IQR 1641; post- treatment median = 416 Eo/mm^3 IQR 320.5, Wilcoxon signed rank test $p < 0.001$) (Fig. 5B). The same trend was observed among individuals from group III (presence of eosinophilia without a history of living in endemic areas, 4/49) (Fig. 5C).

3.7. *Proposed algorithm for the diagnosis of strongyloidiasis*

Based on our results, we propose an algorithm for the diagnosis of strongyloidiasis. We strongly recommend performing PCR after the first negative result obtained from the fresh stool sample when available. This strategy provides quick access to the diagnosis since a single sample is referred to the laboratory. In the events where performing a PCR is not feasible, at least three samples should be examined by APC method (Fig. 6).

4. Discussion

Our results confirm the superior performance of PCR over the reference method for the diagnosis of *S. stercoralis* in subjects residing out of endemic areas. The PCR-based method did not miss any case of strongyloidiasis confirmed by the reference tests reaching a sensitivity of 100%. Moreover, it was able to identify 36 new cases that were negative by reference tests. Having a higher sensitivity than the "gold standard' method, the cross-table calculation provides a relative low specificity (84.81%) and PPV (49.3%). This artificially reduced specificity is caused by the high number of discrepant results provided by reference tests (Conraths and Schares, 2006). These so called 'false positives' are confirmed cases since participants diagnosed exclusively by PCR and with eosinophilia who received ivermectin treatment significantly decreased their eosinophil values (Segarra-Newnham, 2007; Conraths and Schares, 2006). These patients were not reactive for toxocariasis and had no other epidemiologically relevant parasitic diseases to justify the decrease in eosinophil counts after ivermectin treatment.

Labor intensive and low sensitivity traditional diagnostic methods based on larvae visualization are being replaced by molecular methods (Saugar et al., 2015; Becker et al., 2015). Indeed, PCR strategy proved to be highly specific and more sensitive for the diagnosis of strongyloidiasis. However, false-negative PCR results may occur due to the presence of inhibitors (bacterial proteases, nucleases, cell debris and bile acids), low parasite load in stool samples, and low recovery of DNA due to variations in extraction techniques. In this context, different molecular methods for the detection of *S. stercoralis* in stool samples have been described using conventional or real time-based approaches (Verweij et al., 2009; Schär et al., 2013a; Sitta et al., 2014; Saugar et al., 2015). These methods consistently reach sensitivity values below 94% compared to conventional methods (e.g. Baermann method/agar culture). The sensitivity of these methods exhibits further decrease when applied to asymptomatic individuals (Schär et al., 2013a). Here, the PCR method achieved parasite detection in all subjects with asymptomatic eosinophilia and showed 100% sensitivity when compared to the gold standard methods. As previously reported by our group, a key step for the increased sensitivity is the DNA extraction strategy that involves the combination of GTES buffer and a strong mechanical disruption (Repetto et al., 2013). The "in house" or the combined DNA isolation methods (GTES buffer and mechanical disruption prior to DNA isolation with a commercial kit) raised the sensitivity of the PCR applied to clinical samples to 100% when compared to DNA isolation using the commercial kit protocol (Repetto et al., 2013). The DNA extraction method described by us is critical for the detection of larvae DNA in stool samples (Repetto et al., 2010). The present study and other recent results (Sharifdini et al., 2015) used this method to perform nested-PCR and qPCR for the diagnosis of strongyloidiasis and reinforced this fact. The increase in sensitivity they reported using the nested-PCR is expectable considering the successive amplification and the primers designed against mitochondrial genes which are present in higher copy numbers than ribosomal RNA (Sharifdini et al., 2015).

Molecular methods are usually far more expensive than standard methods and appear to require higher initial budget than the conventional ones. Laboratory accreditation requirements, qualified personnel, specialized equipment, special work space and laboratory practices to avoid cross contamination are needed. In spite of this, the implementation of molecular technologies is cost-effective as they improve the diagnostic performance and reduce the cost of managing clinical complications. In high complexity diagnostic centers where molecular approaches are already applied, the application of this technique should be less laborious than that of other specialized methods such as parasite enrichment and culture. Our PCR-based method allowed us to diagnose *S. stercoralis* infection three to four weeks earlier than reference methods. Both physicians and patients will benefit from a reduced amount of testing time required to establish its diagnosis (Yang and Rothman, 2004).

The prevalence of strongyloidiasis is underestimated worldwide owing in part to the low sensitivity of reference methods. High-endemicity areas are reported in tropical and subtropical regions of America, Asia and Africa. Prevalence over 20% is reported in regions of Ecuador, Venezuela, Peru, Brazil and Argentina (Buonfrate et al., 2015b). Argentina is a vast country where most reported cases are related to the present or past residence in subtropical northern areas. However, no countrywide epidemiological surveys have been conducted aimed to evaluate the prevalence of *S. stercoralis*.

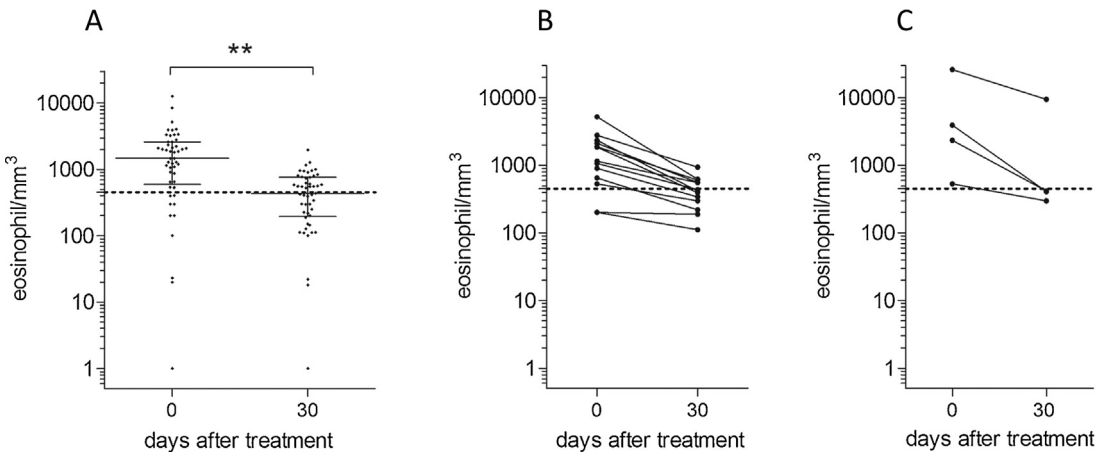

Fig. 5. Decrease in eosinophil counts one month after ivermectin treatment. Forty-nine patients were treated with ivermectin after diagnosis by reference methods or PCR. Individual and median (IQR) eosinophil counts were plotted at day 0 and 30 after treatment initiation. One patient with neutropenia is represented by the point with value of 1 Eo/mm^3 (A). Eosinophil counts of those patients diagnosed by PCR only (B) and those from group III (C) are plotted before and after therapy. The doted horizontal line indicates the accepted normal threshold of eosinophil counts (450 Eo/mm^3). ** $p < 0.001$. Wilcoxon signed rank test.

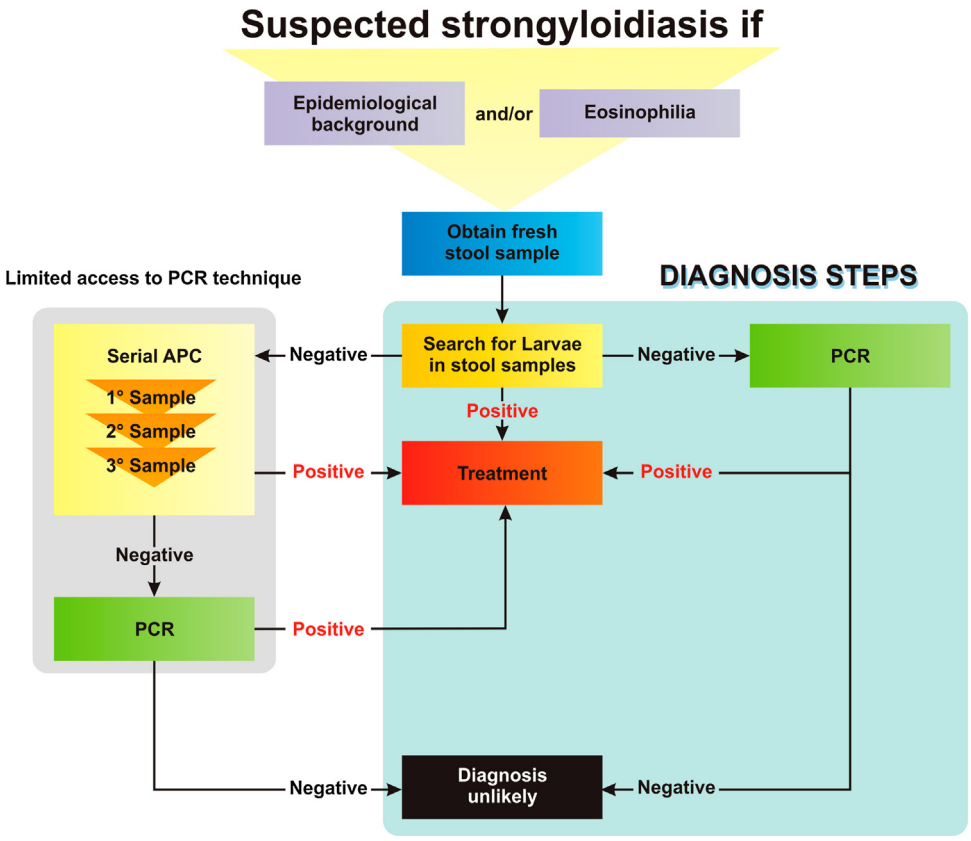

Fig. 6. Diagnostic algorithm for *S. stercoralis* in asymptomatic patients. Asymptomatic patients with epidemiological background compatible with *S. stercoralis* infection and/or eosinophilia should be screened by PCR when no larvae are observed in the first fresh stool sample. When the availability of molecular methods is limited, at least three different stool samples should be examined by the APC method. However, the PCR method is recommended in patients with repeated negative results by APC method.

In our cohort, we detected four infected subjects without apparent risk of infection. These participants denied previous residency in endemic areas as well as any other risk of infection. These findings are in line with previous reported cases in urban centers of temperate regions considered free of local transmission (Ros et al., 2013). The distribution and prevalence of soil-transmitted helminths are heavily influenced by human behavior, migrations and environmental conditions. Transmission can increase in endemic areas or expand to non-endemic ones in response to climate or environmental changes in association with conditions of poor sanitation (Puthiyakunnon et al., 2014). Therefore, it would be relevant to monitor *S. stercoralis* infection in selected patients even when epidemiological risk factors are not present through anamnesis. In fact, in our cohort study, multivariate analyses identified eosinophilia and endemic areas as factors that, independently, increase the likelihood of *S. stercoralis* infection. In the screening of this parasitosis, both variables should be considered separately as predictors of infection.

Strong evidence supports that strongyloidiasis is highly prevalent among HIV-infected individuals (Nabha et al., 2012; Marcos

et al., 2011). Therefore, screening this nematode should be mandatory for HIV patients with epidemiological risk factors. In the present study we found neither increased risk of severe forms nor symptoms of immune reconstitution inflammatory syndrome (IRIS) in co-infected patients. In contrast, concerning patients with non-HIV related immunosuppression; we emphasize the importance of documenting asymptomatic infection as a key to prevent hyperinfection and dissemination syndromes. Although helminthic infections are the common cause of eosinophilia, various reports have described that 20–50% of infected individuals have normal eosinophil counts at the time of diagnosis (Löscher and Saathoff, 2008; Schulte et al., 2002). This strengthens the concept that patients with epdemiological risk and clinical manifestations must be surveyed for *S. stercoralis* regardless of their eosinophil counts (Löscher and Saathoff, 2008). In other words, we discourage the use of eosinophilia as a single criterion for conducting *S. stercoralis* screening in immunosuppressed patients.

From the clinical point of view, strongyloidiasis diagnosis is easily achieved in severe forms. However, there are no suitable tests for the detection of this infection in asymptomatic patients. Serodiagnosis has been reported as a useful approach in many laboratories out of endemic areas but the major disadvantages of this technique are the false-negative results observed in immunosuppressed patients (Siddiqui and Berk, 2001; Buonfrate et al., 2015a). Finally, we suggest an algorithm for the diagnosis of asymptomatic patients. Our rapid PCR strategy could be performed as the first diagnosis step when evaluating high risk patients (e.g. screening before transplantation, initiation of steroid or other immunosuppressive therapies, immunosuppression status).

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