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An improved DNA isolation technique for PCR detection of *Strongyloides stercoralis* in stool samples

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ABSTRACT

Strongyloides stercoralis is a nematode that causes severe infections in immunocompromised patients. The low parasitic burden of chronically infected patients makes diagnosis difficult to achieve by conventional methods. Here, an in-house (IH) method for the isolation of parasite DNA from stools and a PCR assay for the molecular diagnosis of *S. stercoralis* were optimized. DNA yield and purity improved with the IH method which included a step of incubation of stool samples with a glycine–SDS buffer and mechanical disruption prior to DNA extraction. For the PCR assay, the addition of bovine serum albumin was required to neutralize inhibitors present in stool. The analytical sensitivity of the PCR using DNA as template, isolated with the IH method, was superior to the commercial one. This study demonstrates that a combined method that adds the step of glycine–SDS buffer incubation plus mechanical disruption prior to DNA isolation with the commercial kit increased PCR sensitivity to levels of the IH method. Finally, our assay was tested on 17 clinical samples. With the IH method for DNA isolation, a *S. stercoralis* specific band was detected by PCR in the first stool sample in all patients (17/17), while with the commercial kit, our *S. stercoralis*-specific band was only observed in 7 samples. The superior efficiency of the IH and combined methods over the commercial kit was demonstrated when applied to clinical samples with low parasitic burden. These results show that the DNA extraction procedure is a key to increase sensitivity of the *S. stercoralis* PCR assay in stool samples. The method developed here could help to improve the molecular diagnosis of *S. stercoralis.*

1. Introduction

The diagnosis of *Strongyloides stercoralis*, endemic in tropical and subtropical regions, is difficult to achieve owing to its life cycle. This nematode has alternate parasitic and free-living life styles. Soil or auto-infective filariform larvae (L3) penetrate the skin, enter the bloodstream and finally get into the small intestine to dwell as parthenogenetic females. Autoinfection is responsible for chronic infections in people living out of endemic areas for decades. In these persons, larvae excretion is fluctuating and low. Most chronic infections are asymptomatic, sometimes with eosinophilia as the only laboratory finding. Immunocompromised people may experience accelerated autoinfection causing hyperinfection or dissemination syndromes where mortality reaches roughly 80% (Siddiqui and Berk, 2001; Keiser and Nutman, 2004; Concha et al., 2005). These patients can suffer bacteraemia, bacterial meningitis, bacterial abscesses, diarrhea and pneumonia due to the erratic migration of enterobacteria-carrying autoinfectant stage L3. In this setting, larvae are easily detected in samples. These clinical presentations can be observed following immunosuppressive therapy (e.g. corticosteroids) as in transplant recipients or autoimmune disease patients. Accordingly, current practice guidelines recommend the screening for *Strongyloides* infection before transplantation or immunosuppressive therapy (Tomblyn et al., 2009; Ramanathan and Nutman 2008; Roxby et al., 2009). However, physicians out of endemic areas do not routinely check for

Abbreviations: APC, agar plate culture method; BSA, bovine serum albumin; IAC, internal amplification control; IH, in-house.

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S. stercoralis in asymptomatic patients missing opportunity of diagnosis (Boulware et al., 2007). To date, patients with suspected strongyloidosis are controlled by direct observation of larvae in stool. Those with negative results, but having eosinophilia and history of residence in endemic areas, receive ivermectin as empiric treatment (Tomblyn et al., 2009). Nevertheless, recent studies have raised concerns on the efficacy of the current therapy dosage (Bisoffi et al., 2011; Ramanathan and Nutman, 2008). Direct observation of larvae in stools has low sensitivity, and serology may provide false negative or indeterminate results thus limiting the use of these methods as screening tests (Keiser and Nutman, 2004; Segarra-Newnham, 2007; Vadlamudi et al., 2006). Agar plate culture (APC) is currently the most sensitive method without reaching 100% sensitivity. It is laborious and requires at least 7-day culture follow-up for best results (Segarra-Newnham, 2007; Repetto et al., 2010).

Molecular methods can be useful for the early diagnosis and etiological treatment of strongyloidosis in those asymptomatic patients that will undergo immunosuppressive therapy (Verweij et al., 2009; Gordon et al., 2011). However, optimization of DNA extraction and PCR assays for the detection of intestinal nematodes in stools are challenging due to the complexity of nematode cuticle and inhibitors that can be present in stools (Wilson, 1997; Al-Soud Waleed and Rådstrom, 2000). Thus, the aim of this study was to develop an in-house (IH) method for nematode DNA isolation to improve the accuracy of *S. stercoralis* diagnosis by PCR.

2. Materials and methods

This study was approved by the Ethics 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. All stool samples were obtained from patients above 18 years old.

2.1. S. stercoralis L3 larvae

L3 larvae were isolated from stool samples of patients diagnosed by microscopic examination. Stools were seeded in charcoal and L3 larvae, recovered by the Baerman method, and washed repeatedly with PBS (Viney and Lok, 2007; Lok, 2007). Purified larvae were stored at −20 °C until use (Lok, 2007; Verweij et al., 2009).

2.2. Stool samples

Negative control samples were obtained from healthy volunteers with negative parasitological tests, who never lived in endemic areas ($n = 20$). Fresh stools were also obtained from patients infected with other pathogens such as protozoa: (*Giardia intestinalis* $n = 4$, *Cystoisospora belli* $n = 1$, *Cryptosporidium spp* $n = 4$, *Blastocystis hominis* $n = 5$), helminths: (*Taenia* spp. $n = 2$, *Hymenolepis nana* $n = 2$, *Ascaris lumbricoides* $n = 3$, *hookworms* $n = 4$, *Trichuris trichiura* $n = 2$), bacteria: (*Salmonella* spp. $n = 1$, *Staphilococcus aureus* $n = 1$, *Pseudomonas aeruginosa* $n = 1$), fungi: (*Candida albicans* $n = 1$) and rotavirus ($n = 1$).

2.3. Positive stool samples

Stool samples ($n = 17$) were obtained from asymptomatic patients with suspicion of strongyloidosis referred from the Medical School Hospital for diagnosis. Strongyloidosis was confirmed by APC on stool samples obtained at weekly intervals (Repetto et al., 2010). Stools were stored at −20 °C for DNA isolation. All samples were obtained before treatment with ivermectin (Segarra-Newnham, 2007).

2.4. In-house method of DNA isolation

One gram of stools from negative control samples diluted in 10 ml of PBS was individually spiked with known amounts of purified L3 larvae to obtain *S. stercoralis* experimentally spiked stools. For DNA isolation by the IH method, 500 μl of the PBS-diluted stools were incubated overnight with 500 μl GTES buffer (100 mM glycine, 0.05% SDS, 100 mM Tris/Cl, and 1 mM EDTA) at 37 °C (Dorris, 1999; Dorris et al., 2002). Next, samples were subjected to 3 freeze–thaw cycles followed by sonication (3 cycles of 30-s at 40 Hz at 0 °C) and incubated in nematode lysis buffer (100 mM EDTA, 100 mM NaCl, 100 mM Tris pH 7.5, 0.05% SDS, proteinase K 100 μg/ml) for 12 h more at 37 °C. Then, samples were extracted with one volume of phenol/chloroform/isoamyl alcohol (25:24:1). It was performed two times and then, DNA was precipitated with 500 μl of isopropanol followed by 1 ml of 100% ethanol, washed with 300 μl of 70% ethanol and resuspended in 200 μl of Tris/EDTA buffer (Harris and Szalanski, 2004; Castaño Zubieta et al., 2005). *S. stercoralis* DNA was obtained from 400 L3 larvae resuspended in 500 μl of PBS as mentioned above. DNA was also extracted from stool using a commercial method (*QIAamp DNA Stool Mini Kit*, Qiagen, Argentina) according to the manufacturers' instructions. DNA samples were stored at −20 °C until use.

2.5. S. stercoralis-specific PCR

S. stercoralis specific primer pairs from the 18S rRNA gene sequence (Dorris and Blaxter, 2000), named A and B, were used. Primers A were reported by Verweij et al. (2009) and primers B were designed using the Primer BLAST software of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov) (Table 1). PCR conditions were optimized in a final volume of 20 μl as follows: 0.4 μM dNTPs, 0.01 U/μl Taq polimerase (Hot Start Fermentas), 0.5 μM of each primer, 0.1 μg/μl BSA, 3 mM $MgCl_2$ and 4 μl of DNA as template. Cycling conditions were: 3 min at 95 °C, 35 cycles of 45 s each at 95 °C, 1 min at 55 °C (for primers A) or 66 °C (for primers B) and 45 s at 72 °C with a final elongation step of 5 min at 72 °C in a T-18 model thermocycler (IVEMA Desarrollos). Nuclease free water was used as negative control and DNA from stool samples spiked with 50 L3 as positive control. 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).

2.6. Exogenous internal amplification control (IAC)

Axenic culture epimastigotes (1×104) from the protozoa *Trypanosoma cruzi*, irrelevant to chronic intestinal infections, were added to each stool sample before DNA isolation. *T. cruzi*-specific PCR was performed on all clinical and *S. stercoralis* experimentally spiked stool samples in parallel in order to identify false-negative PCR results. *T. cruzi* DNA was amplified with primers: TCZ1: 5′CGAGCTCTTGCCCACACGGGTGCT′3 and TCZ2: 5′CCTCCAAGCAGCGGATAGTTCAGG′3 (expected product size

Table 1
Select primers sets for *S. stercoralis* PCR (target sequence *S. stercoralis* 18S rRNA gene).

Primers	Sequence	Size of PCR product (bp)
A[a]	5′-GAATTCCAAGTAAACGTAAGTCATTAGC-3′ 5′-TGCCTCTGGATATTGCTCAGTTC-3′	101
B	5′-GAAGGCAGCAGGCGCGAAAA-3′ 5′-GCTGGCACCAGACTTGCCCTTT-3′	149

[a] Verweij et al. (2009).

Table 2
Nucleotide sequences of *S. stercoralis* 18S rRNA gene PCR products.

Primers A*	CGAG**GAATTCCAAGTAAACGTAAGTCATTAGC**TTACATTGATTACGTCCCTGCCCTTTGTACACACCGCCCGTCGCTGCCCG-**GAACTGAGCAATATCCAGAGGCA**GGA
Primers B	AAG**GAAGGCAGCAGGCGCGAAAA**TTACCCAATTTTAGTTAAAAGAGGTAGTGACGAAAAATGACAACCAAATATTATTAT-TAATATTTGGATTGAAAATCTTCAAGTTTAAATAACCTTGTTGGTAAAGG**AAAGGGCAAGTCTGGTGCCAGC**AGC

* Primers A (Verweij et al., 2009) and B (Genbank ID, AF279916.2). Primers A and B sequences are highlighted.

188 bp; Moser et al., 1989). PCR was carried out in a final volume of 20 μl as follows: 0.4 μM dNTPs, 0.01 U/μl Taq polimerase (Hot Start Fermentas), 0.5 μM of each specific primer, 3 mM MgCl2 and 4 μl of DNA as template. Cycling conditions were: 3 min at 95 °C, 35 cycles of 45 s each at 95 °C, 1 min at 55 °C and 45 s at 72 °C with a final elongation step of 5 min at 72 °C in a T-18 model thermocycler (IVEMA Desarrollos).

2.7. Statistics

Data was expressed as mean ± standard deviation. Mann–Whitney test was used for paired comparisons to determine significance of differences ($P < 0.05$). All tests were performed using the Prism 5 GraphPad software (San Diego, CA, USA).

3. Results

3.1. Optimization of an IH method for DNA isolation of stools

Different protocols of DNA extraction of purified *S. stercoralis* L3 larvae were assayed. The DNA yield was significantly higher ($P < 0.01$) with the IH method, where samples were incubated with GTES followed by mechanical disruption (2.13 ± 1.34 μg/ml; $n = 7$), compared to those where this step was omitted (0.25 ± 0.20 μg/ml; $n = 7$). Moreover, by adding this step, the A260/280 ratio rose from values below 1.2 to 1.8 ± 0.02 reflecting an enhanced DNA purity. When the IH method was applied to stools from healthy donors experimentally spiked with L3 larvae, DNA consistently displayed an A260/280 ratio above 1.8 and ran as a single band of high molecular weight DNA on 0.8% agarose gels.

3.2. Specificity of S. stercoralis PCR

PCR amplification using DNA extracted from stools is usually challenging due to the presence of polymerase inhibitors (Wilson, 1997; Al-Soud Waleed and Rådstrom, 2000; Mc Orist et al., 2002). DNA amplification using primer pairs A and B was only achieved when 0.1 μg/μl BSA was added to the PCR reaction. Sequencing of PCR products confirmed the amplification of fragments of the 18S rRNA gene sequence (Table 2). The specificity of the PCR using primer pairs A and B was evaluated and no cross-reaction with related nematodes or other pathogens was observed (Fig. 1). Similarly, no amplification products were obtained using DNA from stools of healthy volunteers (data not shown). The exogenous internal amplification control (IAC) added to all samples to rule out in parallel PCR inhibition, detected a single band of *T. cruzi* specific 188 bp product (Fig. 1).

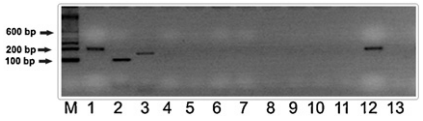

Fig. 1. Specificity of the PCR assay. Agarose gel showing PCR results using DNA from stools with *S. stercoralis*, related nematodes and other pathogens. Lane M, 100 bp DNA ladder (Invitrogen); lane 1, IAC (188 bp); lane 2, *S. stercoralis* (primers A, 101 bp); lane 3, *S. stercoralis* (primers B, 149 bp); lanes 4 and 5, *A. lumbricoides*; lanes 6 and 7, *T. trichiura*; lanes 8 and 9, hookworms; lanes 10 and 11, *C. albicans*; lane 12, IAC; lane 13, negative control (nuclease-free water). Lanes 1, 4, 6, 8, and 10: PCR assay using primers A. Lanes 5, 7, 9 and 11: PCR assay using primers B.

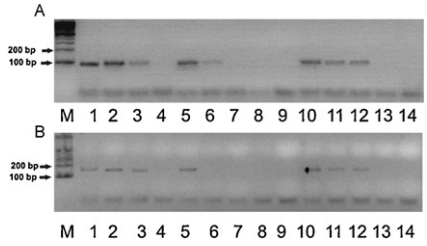

Fig. 2. Analytical sensitivity *S. stercoralis* PCR assay. DNA from L3 *S. stercoralis*-spiked stools (one L3 larva/g stool) was purified by different methods. PCR using tenfold serial dilutions of these DNA was performed. (A) PCR assay using primers A (101 bp). (B) PCR assay using primers B (149 bp). Lane M, 100 bp DNA ladder (Invitrogen); lanes 1–4, isolation DNA by IH method; lane 1, one larva; lanes 2, 3 and 4, 10^{-1}, 10^{-2} and 10^{-3} dilutions, respectively; lanes 5–8, isolation DNA by commercial kit; lane 5, one larva; lanes 6, 7 and 8, 10^{-1}, 10^{-2} and 10^{-3} dilutions, respectively; lane 9, negative control (nuclease-free water); lanes 10–13, DNA isolation by combined method; lane 10, one larva; lanes 11, 12 and 13, 10^{-1}, 10^{-2} and 10^{-3} dilutions, respectively; lane 14, negative stool.

3.3. Sensitivity of S. stercoralis PCR

PCR sensitivity was determined to detect *S. stercoralis* DNA obtained by the different methods. First, ten-fold dilutions of DNA were used as template isolated from *S. stercoralis* experimentally spiked stools (one L3 larva/g stool) by the IH, commercial and combined methods. The latter applied the GTES buffer incubation followed by mechanical disruption prior to the commercial kit procedure for DNA isolation. With primers A and B, specific bands were identified (101 bp and 149 bp respectively) up to the 10^{-2} dilution of DNA purified by the IH and combined methods. Using the commercial kit, a specific band was detected up to the 10^{-1} dilution with primers A and only with the undiluted DNA using primers B (Fig. 2A and B). Next, the sensitivity of PCR was measured to detect *S. stercoralis* in DNA isolated by the IH method from experimentally spiked stools with known amounts of L3 larvae. PCR was useful to detect one larva/g stool with both primers A and B (Fig. 3).

3.4. Application of S. stercoralis PCR to clinical samples

Detection of *S. stercoralis* was evaluated by PCR on clinical samples for which DNA was isolated by the IH, commercial

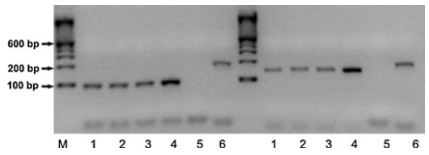

Fig. 3. Sensitivity of the *S. stercoralis* PCR assay using DNA from experimentally spiked stools. Negative stools were spiked with known amounts of *S. stercoralis* L3 larvae before DNA isolation by the IH method. Lane M, 100 bp DNA ladder (Invitrogen). Using primers A (101 bp): lane 1, one larva; lane 2, ten larvae; lane 3, fifty larvae; lane 4, one hundred larvae; lane 5, negative control (nuclease-free water) and lane 6, IAC. Using primers B (149 bp): lane 7, one larva; lane 8, ten larvae; lane 9, fifty larvae; lane 10, one hundred larvae; lane 11, negative control (nuclease-free water) and lane 12, IAC (188 bp).

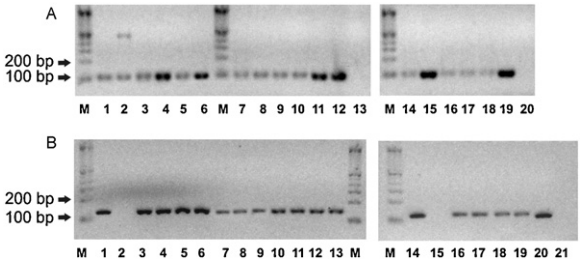

Fig. 4. Application of *S. stercoralis* PCR to clinical samples. PCR assay using primers A (A) and primers B (B). (A) lanes 4, 11 and 19, positive controls (*S. stercoralis* spiked stools); lanes 1–3, 5–10, 12–18, patients with strongyloidosis; lane 13, negative control (nuclease-free water); lane 20, healthy volunteer. (B) lanes 1, 10 and 14 positive controls (*S. stercoralis* spiked stools); lanes 3–9, 11–13, 16–20 patients with strongyloidosis; lanes 2 and 15 negative controls (nuclease-free water); lane 21, healthy volunteer; lane M, 100 bp ladder (Invitrogen). The nonspecific ~500 bp band on lane 2 disappeared when $MgCl_2$ was increased to 4 mM in the PCR reaction.

and combined methods. For this, a retrospective analysis of the performance of conventional parasitological diagnosis (APC) was conducted with that of *S. stercoralis*-specific PCR. With the APC, nine patients were found positive from the first stool sample. The remaining patients required a second (7/17) or even a third (1/17) stool sample collection with one week interval to become positive. When DNA was isolated by the IH and combined methods, PCR with both primers A and B detected *S. stercoralis* DNA in the first stool sample in all patients (Fig. 4). With the commercial kit, nematode DNA was only detected in the first stool sample from seven patients. Moreover, five patients positive by APC in the first sample were negative by PCR using this extraction method. Finally, the eight patients that needed a second or a third stool sample to become positive by APC were all *S. stercoralis* positive by PCR using the IH and combined methods of DNA isolation, and only 3 using the commercial one.

4. Discussion

The results presented here show that *S. stercoralis* DNA obtained from stool samples by an IH method is suitable for a PCR technique, and could improve the sensitivity of diagnosis. A combination of GTES plus a strong mechanical disruption was required for successful DNA recovery. Stools may contain bacterial proteases, nucleases, cell debris and bile acids which can inhibit DNA amplification by PCR. The ability of BSA to bind and neutralize PCR inhibitors has been demonstrated previously (Wilson, 1997; Al-Soud Waleed and Rådstrom, 2000; Mc Orist et al., 2002). Our results suggest that this molecule acts as a PCR amplification facilitator in our study. The *S. stercoralis* PCR developed here proved to be specific since no cross-reactions were observed with other pathogens tested. PCR was more sensitive and faster for the diagnosis of strongyloidosis than the APC which requires between 3 and 9 agar plates (3 g of stools per plate) to detect, in most cases, only one larva after one week of culture (Repetto et al., 2010). All DNA isolation methods tested proved to be useful to detect 10^{-1} larva/g stool by PCR. However, the superior efficiency of the IH and combined methods over the commercial kit was demonstrated when applied to clinical samples. This is important for the parasitological diagnosis due to the likelihood of false negative results. The DNA isolation method described here might also be suitable for the molecular diagnosis of other nematodes. In our hands, this isolation method was effective for the detection of DNA by PCR obtained from adult *A. lumbricoides*, L3 *Toxocara canis*, as well as L3 hookworms (unpublished results).

Previous reports using quantitative PCR methods proved to be more sensitive and specific for the detection of strongyloidosis infection than direct larvae detection (Verweij et al., 2009; Basuni et al., 2011). Our results support the fact that conventional qualitative PCR is much more sensitive than direct parasitological methods for the detection of *S. stercoralis* provided that the appropriate method of DNA isolation is used. In summary, the results shown here suggest that the IH and combined methods of DNA isolation could increase the sensitivity of the molecular diagnosis based on a conventional PCR. This improved molecular diagnosis could benefit asymptomatic chronically infected patients that will undergo an immunosuppressive therapy leading to high risk of hyperinfection. Further studies are being conducted to validate the diagnostic sensitivity and specificity of this PCR assay in a greater number of patients.

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Conflict of interest

All authors declare no conflict of interest.

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