TWO SEQUENTIAL PCR AMPLIFICATIONS FOR DETECTION OF \textit{Schistosoma mansoni} IN STOOL SAMPLES WITH LOW PARASITE LOAD

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SUMMARY

Schistosomiasis constitutes a major public health problem, with an estimated 200 million individuals infected worldwide and 700 million people living in risk areas. In Brazil there are areas of high, medium and low endemicity. Studies have shown that in endemic areas with a low prevalence of \textit{Schistosoma} infection the sensitivity of parasitological methods is clearly reduced. Consequently diagnosis is often impeded due to the presence of false-negative results. The aim of this study is to present the PCR reamplification (Re-PCR) protocol for the detection of \textit{Schistosoma mansoni} in samples with low parasite load (with less than 100 eggs per gram (epg) of feces). Three methods were used for the lysis of the envelopes of the \textit{S. mansoni} eggs and two techniques of DNA extraction were carried out. Extracted DNA was quantified, and the results suggested that the extraction technique, which mixed glass beads with a guanidine isothiocyanate/phenol/chloroform (GT) solution, produced good results. PCR reamplification was conducted and detection sensitivity was found to be five eggs per 500 mg of artificially marked feces. The results achieved using these methods suggest that they are potentially viable for the detection of \textit{Schistosoma} infection with low parasite load.

KEYWORDS: \textit{S. mansoni}; PCR reamplification; Eggs; Human feces; Diagnosis.

INTRODUCTION

Schistosomiasis is a major public health problem, with an estimated 200 million individuals infected worldwide, of whom 120 million are symptomatic and 20 million have severe forms of the disease. Seven hundred million people are at risk of infection by six species that can infect humans\(^1\).

In Brazil, the only species of medical and sanitary interest is \textit{Schistosoma mansoni}\(^1\). Schistosomiasis mansoni is considered an endemic disease in 19 Federative Units. For this reason, the Brazilian Ministry of Health aims to reduce the occurrence of the severe forms of the disease and the number of deaths caused by it, as well as to reduce the prevalence of infection and the risk of geographic expansion of the disease\(^1\).

Parasitological methods continue to be the gold standard for laboratory diagnosis of this parasitosis. However, despite being less costly and technically simple, these diagnostic techniques lack sensitivity in areas of low prevalence (prevalence below 10\%, with most infected asymptomatic subjects, eliminating less than 96 eggs/gram of feces) of schistosomiasis\(^6,17\).

Thus, diagnosis of schistosomiasis requires the use of more sensitive techniques than the search for eggs in feces, both in areas of low endemicity and for the evaluation of parasitologic cure after treatment. This is important from the epidemiologic view, because the permanence of infected individuals with false-negative results in coproscopic inquiries is enough to continue the risk of transmission, even after suitable interventions are carried out by means of sanitary measures\(^18\).

Extraction of the sample DNA is an important stage when carrying out molecular techniques. There are several different kits for extracting DNA from blood, urine, solid tissues and fecal samples. Among biological samples, feces pose the greatest problems for DNA extraction and require the development of new strategies, especially when searching for \textit{S. mansoni} eggs because they have complex shells that are not easily removed by chemical digestion\(^5\). Hence, the aim of this study is to present the PCR reamplification protocol for the detection of \textit{S. mansoni} in samples with low parasite load (with few numbers of eggs per gram (epg) of feces).

MATERIAL AND METHODS

Laboratory cycle of \textit{S. mansoni}: The biologic cycle of \textit{S. mansoni} is maintained in the Laboratory of Immunopathology of Schistosomiasis (LIM-06) of the Institute of Tropical Medicine of the University of São Paulo, São Paulo, SP, Brazil. E-mails: monica.viviana@usp.br; jrrpinho@usp.br

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DNA extraction from feces artificially marked with *S. mansoni* eggs - The total DNA of 500 mg of feces artificially marked with *S. mansoni* eggs was extracted using the GT® extraction technique, and modified by adding five glass beads in each 2 mL plastic tube.

The extraction was carried out in two stages: 1) each sample was resuspended in 500 µL of ASL Buffer from the QIAamp® DNA Stool Mini Kit. Five glass beads were added and homogenized for five minutes. Subsequently, 400 µL of the supernatant was removed and added to another tube containing 200 µL of AL Buffer and 30 µL of proteinase K (QIAamp® DNA Stool Mini Kit). The samples, prepared as described, were incubated at 37 °C for 12 hours. 2) 300 µL of GT and 50 µL of chloroform at -20 °C were added to each sample, followed by homogenization and incubation for 10 minutes and centrifugation at 13,200 rpm. The supernatant was transferred to another tube and precipitated at 300 µL of isopropanol at -20 °C. Once the DNA was precipitated, the isopropanol was discarded and 300 µL of ethanol absolute was added, followed by centrifugation at 13,200 rpm. Finally, the ethanol was discarded and the samples were incubated at 95 °C for one minute and resuspended in 100 µL of DEPC-treated Milli-Q water. The extracted DNA was stored at -20 °C.

Amplification of the *S. mansoni* DNA - Once the DNA extraction was carried out the samples were submitted to two sequential amplifications by PCR.

Primers that amplify a 121 bp tandem repeat DNA fragment of the *S. mansoni* genome were used for the amplification of this region previously cited by PONTES et al. Bovine Serum Albumin (BSA) (SIGMA, USA) was also added in order to neutralize the action of any inhibitors that could be present at the fecal samples.

The first PCR reactions were performed in a final volume of 40 µL under the following conditions: 1X Buffer (10X), 2.5 mM MgCl2, 0.4 mM of each dNTP (10 mM), 0.6 pmol/µL of each primer, 0.1 µg/µL BSA, 2.0 U of Platinum Taq DNA polymerase (Invitrogen TM Life Technologies, Carlsbad, CA, USA) and 10 µL of DNA. The second PCR was performed under the same conditions plus 7 µL of amplified DNA from the first reaction. The PCR program used had been described by GOMES et al., 2009. The detection of the amplified DNA was performed by electrophoresis in 2.5% agarose gel, which was stained with ethidium bromide and Low Mass Ladder in order to check the size of the amplicon generated.

A 120 bp fragment of the gene encoding human beta actin (ACTB) was amplified, as an internal control of the DNA extraction with the previously described primers Aco1 and Aco2. The amplification conditions were performed according to GOMES et al., 2010.

Purification and sequencing of the positive control - The amplified DNA from the positive control (sample with 1,000 eggs/µL) was purified using the ChargeSwitch® PCR Clean-Up Kit. The sequencing was then carried out in the ABI Prism® 3100 Automatic Sequencer (Applied Biosystems, Foster City, CA, USA) using deoxynucleotide triphosphates (dNTPs) with fluorescent markers (Big Dye® Terminator v3.1 Cycle Sequencing Ready Reaction kit - Applied Biosystems, Foster City, CA, USA). In order to obtain the consensus sequence, the forward and reverse sequences obtained were initially analyzed using the Phred-Phrap-Consed
The development of laboratory tools for the diagnosis of infections with low parasite load is one of the priorities in the schistosomiasis research agenda of the World Health Organization’s control programs. Among these tools, the research agenda points to the development and standardization of molecular methods for the surveillance of infections in humans and snails which can be applied to individual and public health studies.

In the present study, a new DNA extraction technique was implemented, followed by PCR reamplification (two sequential amplifications of the same PCR fragment), with the detection of the amplified fragment in 2.5% agarose gel. This is a sensitive method, its detection limit was 5 eggs/500 mg of artificially marked feces, which represents the equivalent of 0.4 eggs/40 mg of feces, according to the Kato-Katz method or an estimate of 9.6 eggs/gram of feces.

The PCR analytical sensitivity was evaluated by 5-fold dilutions extracted from 500 mg stool samples each labeled as having artificially added 5, 10, 20, 30 and 50 eggs/mL of S. mansoni.

The detection limit was 5 eggs/500 mg, approximately 43 fg of genomic DNA extracted from the eggs of S. mansoni.

More recently, other studies have been trying to improve the sensitivity of molecular methods. GOMES et al., 200913, achieved a detection limit of 3 fg of DNA from S. mansoni in feces. OLIVEIRA et al.13 obtained sensitivity using the conventional PCR of an egg in a suspension of 300 µL of feces. Eventually, GOMES et al., 201016, obtained a sensitivity of 0.15 eggs/gram of feces (1.3 fg of DNA), using the PCR-ELISA detection system. Although these studies may report DNA detection values in lower concentrations, it is important to consider that the observation of the bands of DNA concentration may not be suitable for use in routine diagnosis of this infection.

It is important to highlight that the PCR reamplification may show a high level of amplicon contamination when it is not performed under suitable laboratory conditions. Similarly, one must bear in mind that for the diagnosis of the infection the amplified DNA band in the gel, as a final product of the test, should be sufficiently intense in order to ensure the presence of the pathogen in the sample. Thus the DNA bands obtained in this study showed increased DNA concentration, which can justify its use in routine assessments.

Finally, since other factors may compromise results (e.i. stool consistency, medication use, stool conservation, the presence of other parasites in the samples, etc.), further studies are necessary to evaluate our protocol in the future.

CONCLUSIONS

In conclusion, our results have indicated that the extraction technique, which mixed glass beads with the GT, produced good results in the DNA extraction of S. mansoni eggs. With regard to PCR reamplification, despite its limitations, the use of two sequential amplifications per PCR to detect S. mansoni in feces may be a promising new tool.

RESUMO

Duas amplificações sequenciais por PCR para detecção de Schistosoma mansoni em amostras de fezes com baixa carga parasitária

A esquistossomose constitui grande problema de saúde pública, sendo que estimativas apontam para 200 milhões de pessoas infectadas no mundo e 700 milhões de pessoas em áreas de risco. No Brasil, existem áreas de alta, média e baixa endemicidade. Estudos demonstram...
que nas áreas endêmicas de baixa prevalência da infecção, a reduzida sensibilidade dos métodos parasitológicos torna-se evidente. Isto dificulta o diagnóstico, pela presença de resultados falso-negativos. O objetivo deste estudo foi a padronização de um protocolo de reamplificação da PCR (Re-PCR) para a detecção de *Schistosoma mansoni* em amostras com menos de 100 ovos por grama (opg) de fezes. Foram utilizados três métodos para ruptura dos envoltórios dos ovos de *S. mansoni* e duas técnicas de extração de DNA foram aplicadas. O DNA extraído foi quantificado e os resultados sugerem que a técnica de extração de melhor produtividade foi a que associa esferas de vidro a uma solução de isotoxicanato de guanidina/fenol/cloroformio (GT). Aplicou-se a Re-PCR, que demonstrou sensibilidade para a detecção de cinco ovos/500 mg de fezes artificialmente marcadas. Assim, essas novas ferramentas são potencialmente aplicáveis nas infecções por *S. mansoni* com baixa carga parasitária.

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**AUTHOR CONTRIBUTIONS**

MCCES participated in the design of the study, in the extraction and cleaning of *S. mansoni* eggs, standardization of the DNA extraction method and in the two sequential PCR amplifications. MVAM participated in the design of the study, the standardization of the DNA extraction method and in the two sequential PCR amplifications. PLSP participated in the design of the study and in the extraction and cleaning of *S. mansoni* eggs. FJC participated in the design of the study. MCCES, MVAM, JRRP and RCBG participated in the elaboration of the manuscript. All authors read and approved the final manuscript.

**REFERENCES**


