Molecular Diagnosis of *Schistosoma mansoni* infection in human serum and feces by using Polymerase Chain Reaction

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Abstract: A Fast, sensitive and specific detection of cercariae of human schistosomes is required for better definition of risk of infection. So, a novel method for the detection of *Schistosoma mansoni* in human samples that is based on the amplification of a highly repeated DNA sequence has been developed. By use of simple DNA extraction techniques and a rapid 2-step polymerase chain reaction (PCR), it was possible to amplify *S. mansoni* DNA in human fecal and serum samples. The high sensitivity of the approach enabled the detection of the parasite DNA in fecal samples containing as few as 3 eggs per gram of feces, which makes it 10 times more sensitive than the Kato-Katz examination. A detection limit of 1 fg of *Schistosoma* sp. DNA was determined when pure DNA was used as PCR template. The amplification reaction showed to be specific giving no cross-reaction with DNA from other helminths. The PCR assay developed in this study may constitute a valuable alternative for the diagnosis of the *Schistosoma* sp. infection.

[Samir H. Haggag and Saleh M. Abdullah. **Molecular diagnosis of *Schistosoma mansoni* infection in human serum and feces by using Polymerase Chain Reaction**. Life Science Journal. 2011;8(2):398-404] (ISSN:1097-8135). http://www.lifesciencesite.com.

Key words: *Schistosoma mansoni*, molecular Diagnosis, DNA, PCR, serum, feces.

Introduction

Human schistosomiasis, or blood flukes, currently infects several hundred million people globally. The World Health Organization estimates that hundreds of thousands of people die of schistosomiasis each year and about a billion people are at risk of exposure. Schistosomes also infect livestock and cause serious economic hardship in many third world nations, including the eastern Mediterranean and Egypt[1]. The disease is characterized by the presence of adult worms, or blood flukes, within the host's vascular system. These worms, living as male/female pairs, can survive for many years during which time the female produces hundreds of eggs per day. The study and development of new diagnostic techniques for schistosomiasis are still necessary in view of the difficulties to evaluate infection patterns accurately and to control the disease.

Definitive diagnosis of *S. mansoni* infection requires the demonstration of eggs in feces. The Kato-Katz technique [2] is currently the method of choice for parasitological diagnosis of *Schistosomiasis mansoni*, as it is relatively inexpensive and simple.[1] However, it is observed that the sensitivity of parasitological methods diminishes when prevalence and intensity of infection are low, making these methods less appropriate for low-endemic areas and in post treatment situations. In addition, parasitological methods are not sufficient for diagnosing recent infections in which worms have not yet started to produce eggs (the prepatent period).

To address these shortcomings, antibody detection methods have been evaluated as adjuncts to fecal examinations. Comparative studies of parasitological and serological methods confirmed higher sensitivity of the latter [3]. Several authors have approached diagnosis through the specific detection of antigens or antibodies [4]. The existence of cross-reactivity with other helminthic infections, however, and its low specificity after treatment, due to the slow reduction of specific antibody levels, constitute great disadvantages of the immunodiagnostic methods.

One possible solution to this problem could be the search for circulating antigens rather than antibodies. Several circulating antigens assays have been described in different laboratories [5-7]. The high specificity is the main advantage that the detection of circulating antigens offers as compared with the antibodies' determination and the disadvantages are low sensitivity in light infections and dependence on the production of monoclonal antibodies [8,9].

Polymerase chain reaction (PCR) has shown its usefulness in the clinical approach of a wide variety of pathogenic infections, such as human immunodeficiency viruses,[10,11] *Legionella pneumophila* [12], *Plasmodium falciparum* [13,14], and *Trypanosoma cruzi* [15,16]. In the study of *Schistosoma* sp., it has also been successfully used for sex

determination of the cercariae [17], for the cloning and sequencing of specific genes, [18,19] in the determination of genetic variability and population structure of *Schistosoma* sp. strains and species [11,20], and in the development and application of new techniques to generate expressed sequence tags [11]. Hamburguer and others [21] developed a PCR protocol that was based on the amplification of a highly repetitive DNA sequence of the parasite for monitoring *S. mansoni* infestation in water [22]. In the present article, we describe, for the first time in Egypt, the usefulness of the PCR for detecting *S. mansoni* DNA in human fecal and serum samples.

Material and Methods

Sample preparation: *Schistosoma mansoni* eggs were obtained from livers of mice (Swiss albinos) 45 days after infection with 100 cercariae and stored at -20◦C in 0.9% saline until use. Ten microliters of egg-saline solution containing ~2,000 eggs were diluted in 90 µL of water and vortexed for 5 min in order to break the eggs and release the miracidia, the larval form that infects the snail host. The entire volume was then used for DNA extraction.

The DNA from other parasite species: The DNA from 4 related helminthic parasites (*Ascaris lumbricoides*, *Ancylostoma duodenale*, *Taenia solium*, and *Trichiuris trichiuria*).

Artificial mixtures of eggs and feces: Four positive fecal samples (five replicates) were artificially prepared by the following procedure: 0.1 g of *S. mansoni* positive feces containing 216 eggs per gram of feces (determined by Kato-Katz stool examination) were mixed to 0.9 g of negative feces, resulting in a preparation of ~21.6 eggs per gram of feces. Tenfold dilution were subsequently performed, producing 2 more samples with estimated concentrations of ~ 2.16 and 0.216 eggs/g, respectively. The samples were then analyzed by the Kato-Katz method and stored at -70°C until use. Approximately, 100 mg of feces were diluted in 400 µL of water and vortexed for 5 min to break the *S. mansoni* eggs and release the miracidia. Insoluble material and large debris were separated by decantation, leaving the samples for 2 min at room temperature. One hundred microliters of the supernatant were used for DNA extraction.

Patient samples: Patient samples were obtained from the endemic area of Kafer El-Shakh, Egypt, or from the laboratory staff (Informed consent was obtained from all adult participants and from parents or legal guardians of minors).

Stool examination: All fecal samples used in this work were evaluated for the presence of *S. mansoni* eggs by the quantitative parasitological method of Kato-Katz.

Patient feces: Two hundred patient fecal samples provided feces that were previously analyzed by Kato-Katz method and contained 98–910 eggs/g. All fecal samples were stored at -70°C until use. The samples were treated in the same manner described for the artificial mixture of eggs and feces and then used for DNA extraction.

Serum: Two hundred positive serum samples were taken from infected patients containing 96 and 216 eggs/g. The laboratory staff provided 5 negative serum samples. All serum samples were stored at -20°C until use. Serum samples needed no previous preparation and were used directly in DNA isolation.

Free-circulating DNA was isolated, purified from 200 µL of serum with the QIAamp DNA Blood Mini Kit (QIAGEN) according to the manufacturer's instructions.

Extraction of DNA, feces, *S. mansoni* eggs, and related helminthes: The fecal and egg DNA were extracted through a modification of the ROSE method [24].

Briefly, 100 µL of the fecal supernatant, or of the solution containing disrupted eggs, were diluted in 200 µL of slightly modified ROSE buffer (10 mM Tris, pH 8.0; 270 mM ethylenediamine tetraacetic acid [EDTA], pH 8.0; 1% lauryl sulfate; 1% polyvinylpolypyrrolidone). This mixture was incubated at 95°C for 20 min, with one rapid manual agitation after 10 min of incubation. The sample was then cooled on ice for 1 min and centrifuged at 8,000 × *g* for 10 min at room temperature. After that, 200 µL of the supernatant was collected in a separate tube and mixed with 500 µL of frosty ethanol (-20°C) and 10 µL of cold 3 M sodium acetate (4°C), pH 5.3, and incubated overnight at -20◦C or -70°C for 2 hr. The samples were then centrifuged at 8,000 × *g* for 15 min, the supernatant was discarded, and the pellet washed once with 500 µL 70% ethanol (25°C) for a final 10 min at 8,000 × *g* centrifugation. Finally, the supernatant ethanol was discarded, and the pellet was left to dry at 37°C for 15 min, then resuspended with 100 µL of TE (10 mM Tris, pH 8.0; 1 mM EDTA, pH 8.0).

The DNA extracted from S. *mansoni* eggs and from pure total extracts of other related helminthes was quantified by spectrophotometric readings at 260 and 280 nm. All DNA samples were stored at -20°C until use.

Free-circulating DNA was purified from 200 µL of serum with the QIAamp DNA Blood Mini Kit

(QIAGEN) according to the manufacturer's instructions.

Polymerase Chain Reaction:

Primers were designed to amplify the 121-bp tandem repeat DNA sequence of *S. mansoni* described by Hamburguer and others [22] were modified with our team of research (Figure 1). For amplification of egg-derived DNA, 1 µL of the extracted material was used as initial template. For stool amplification, DNA samples were diluted 100 times, and 1 µL was used as template. For serum amplification, 2 µL of undiluted extracted DNA was used as template. All reactions were carried out in a Gen Amp 9700 Thermal cycler PCR in a 10-µL mixture containing: 20 mM Tris-HCl (pH 8.4), 50 mM KCl buffer; 1.5 mM $MgCl_2$; 0.5 µM of each primer; 200 µM dNTPs and 0.75 U of *Taq* DNA polymerase.

GATCTGAATC CGACCAACCG TTCTATGAAA ATCGTTGTAT
CTAGACTTAG GCTGGTTGGC AAGATACTTT TAGCAACATA

CTCCGAAACC ACTGGACGGA TTTTTATGAT GTTTGTTTTA
GAGGCTTTGG TGACCTGCCT AAAAATACTA CAAACAAAAT

GATTATTTGC GAGAGCGTGG GCGTTAATAT AAAACAAAGAA
CTAATAAACG CTCTCGCACC CGCAATTATA TTTTGTTTCTT

Primer -1: 5'-GATCTGAATC CGACCAACCG-3' (Forward -20mer)

Primer -2: 5'-CCACGCTCTC GCAAATAATA-3' (Backward -20mer)

Figure 1. The DNA sequence of the *Schistosoma mansoni* tandem repeat unit used as target and the primers used for amplification. The PCR primers are internally located and are marked by underline letters. The main DNA band is expected to be 100 bp long.

The 2-step amplification cycle consisted of denaturation at 95°C for 45 sec and an annealing step at 63°C for 30 sec. A total of 35 cycles was performed. The denaturation step was prolonged for 5 min in the first cycle, and the last cycle included an extension step of 2 min. After amplification, 10 µL of the sample was electrophoresed on a 6% polyacrylamide gel, and the amplified sequences were visualized via silver staining.[25] The amplification products had their identity confirmed by DNA sequencing performed by standard techniques.

Results:

Sensitivity of *S. mansoni* eggs DNA detection: To assess the sensitivity of the PCR, *S. mansoni* egg DNA was quantified and tested for amplification after serial dilutions. Where the minimum detectable DNA quantity was found to be 1 fg.

Specificity of PCR. The specificity of the PCR in amplifying *Schistosoma* DNA was evaluated by means of equal quantities of DNA from other related parasites under the same reaction conditions; *S. mansoni* DNA was used as positive control for the amplification reaction. As shown in **Fig. 2**, DNA amplification was not achieved for any of the other helminthic parasites evaluated.

Comparison of PCR and Kato-Katz methods on the detection of *S. mansoni* eggs added to noninfected feces: The sensitivity of the PCR and Kato-Katz methods in detecting *S. mansoni* in artificially prepared positive fecal samples (see sample preparation in Materials and Methods) was compared. **Fig. 3** shows the results obtained. The first 2 samples (216 and 21.6 eggs/g) were counted by Kato-Katz method as 216 eggs/g and 48 eggs/g, respectively, and were also positive by PCR. The last 2 samples were negative by the Kato-Katz examination, but the PCR was able to detect the *S. mansoni* DNA, also in the third sample, estimated to contain 2.16 eggs/g.

Detection via PCR of *S. mansoni* in human clinical samples: *Fecal samples*. Fresh fecal samples from *S. mansoni*- positive patients were examined by the Kato-Katz and PCR methods. As shown in **Fig. 4**, PCR was able to detect the *S. mansoni* DNA in fecal samples containing different numbers of eggs per gram, giving equally strong amplification signals.

Serum sample: Serum samples taken from patients with positive stool examination were tested by PCR, and these also gave positive results, as shown in **Fig. 5**. No bands were seen with the negative control serums.

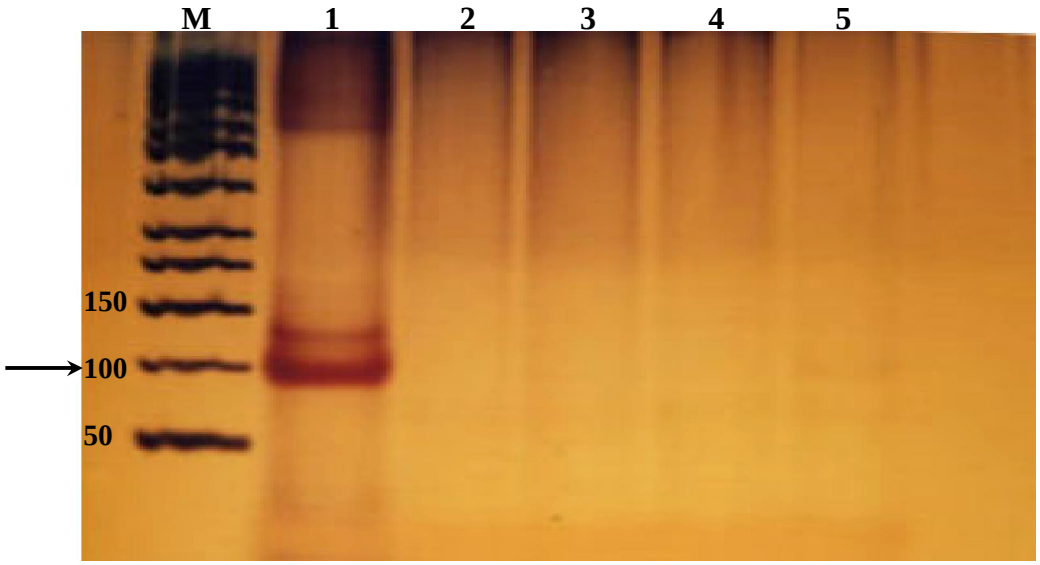

Figure 2. Specificity of the *Schistosoma mansoni* polymerase chain reaction (PCR); 0.1 ng of DNA from several related parasite species were tested for amplification in the same described PCR conditions. **Lane M,** molecular weight marker (Gene Ruler ™ **50** bp DNA Ladder [Fermentas]). **Lane 1,** *S. mansoni.* **Lane 2,** *Ascaris lumbricoides.***Lane 3,** *Ancylostoma duodenale.* **Lane 4,** *Taenia solium.* **Lane 5,** *Trichiuris trichiuria.*

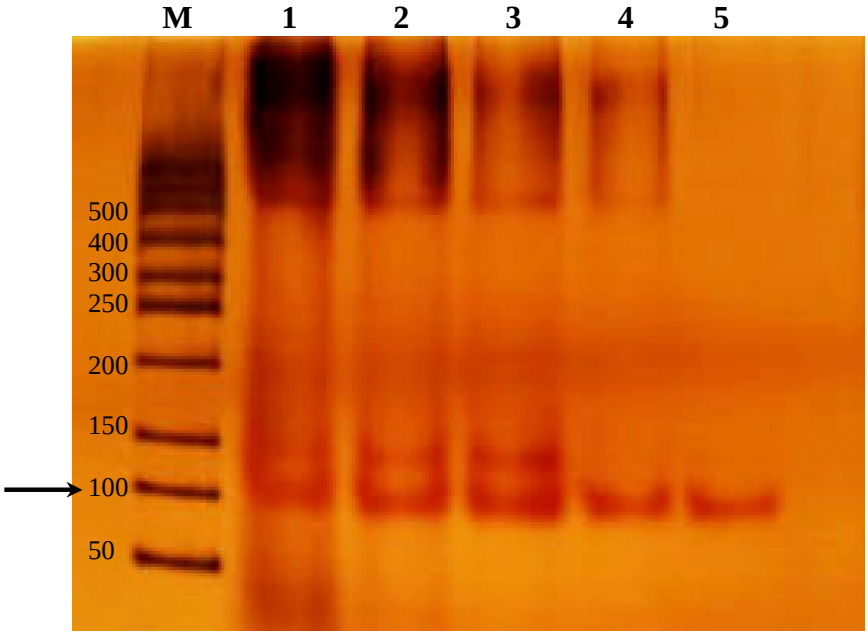

Figure 3. Compared sensitivity between polymerase chain reaction (PCR) and parasitological examination in the diagnosis of *Schistosoma mansoni* on human feces. The ability of PCR and Kato-Katz technique to detect the parasite was evaluated by means of artificially positive feces (see Materials and Methods) with low numbers of egg per gram of feces. **Lane M,** molecular weight marker (Gene Ruler™ **50** bp DNA Ladder [Fermentas]). **Lane 1,** positive control (*S. mansoni* egg DNA). **Lanes 2–5,** artificially infected feces containing approximated values of 216, 21.6, 2.16, and 0.216 eggs per gram of feces. Although the parasitological examination was positive only for the first 2 samples, the PCR was positive for the first 3 samples because it was 10 times more sensitive.

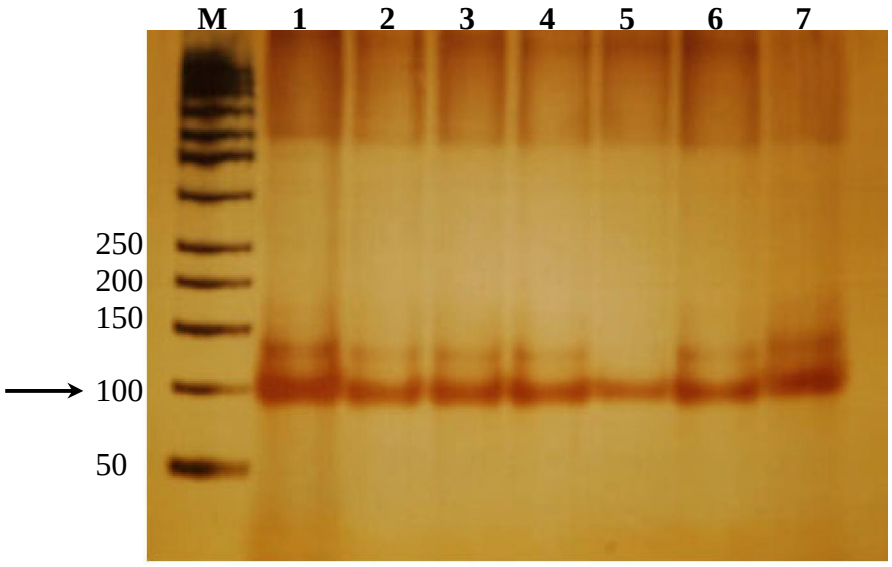

FIGURE 4. *Schistosoma mansoni* detection in feces via polymerase chain reaction (PCR). Six human clinical feces, previously examined by Kato-Katz, were tested by PCR. **Lane M,** molecular weight marker (Gene Ruler™ **50** bp DNA Ladder [Fermentas]). **Lanes 1,** positive control (*S. mansoni* egg DNA). **Lanes 2–7,** fecal samples containing 270, 445, 323, 98, 822, and 910 eggs per gram of feces.

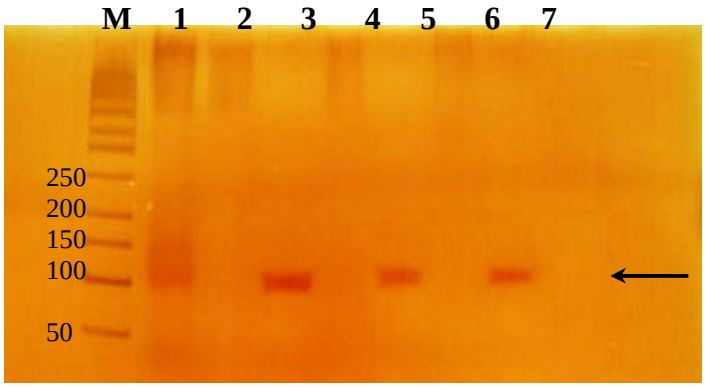

FIGURE 5. *Schistosoma mansoni* polymerase chain reaction (PCR) detection in clinical serum samples. The DNA from 4 serum samples was analyzed by PCR. **Lane M,** molecular weight marker (Gene Ruler™ **50** bp DNA Ladder [Fermentas]). **Lane 1,** positive control (*S. mansoni* egg DNA). **Lanes 2, 4, 6** serum from patients with 0 (negative sample) No bands were visualized in the 3 negative samples, **lane 3** (445), **lane 5** (270), and **lane 7** (98) eggs per gram of feces, respectively.

Discussions:

A consistent diagnosis of schistosomiasis still depends on the parasitological demonstration of the *Schistosoma* sp. eggs in fecal samples, which is well accomplished by the Kato-Katz technique. Despite of the remarkable qualities of this diagnostic method, a more sensitive approach would be of great value in situations such as the presence of light infections. Polymerase chain reaction has been acclaimed as an outstanding sensitive and specific diagnostic tool for many diseases, but to our knowledge, this technique has not been used for demonstrating the presence of *S. manson* DNA in clinical samples.

We tested the possibility of the use of PCR for the detection of *S. mansoni* DNA in human samples of serum and feces. The high sensitivity of the assay was demonstrated by its ability to achieve amplification with minimum amounts of *S. mansoni*

egg template DNA (1 fg). Hamburguer and others [20] used a different set of primers that targets the internal part of the same DNA sequence and obtained very similar results (1.2 fg) when they used adult worm DNA as template. Amplification of the *S. mansoni* tandem-repeated unit generates a ladder of PCR products, which DNA sequence determination proved to be multiples of the 100-bp long sequence (data not shown). The high sensitivity of the assay is certainly due to the unusually high copy number (600,000/cell) of the target sequence, which comprises ~ 10% of the *S. mansoni* genome.[22] The specificity of the test was demonstrated by the absence of amplification when DNA from other 4 helminthes commonly found in the same endemic areas as *S. mansoni* were used as templates (Figure 2). Figures 3 and 4 show the ability of the PCR assay in detecting the *S. mansoni* DNA in human fecal samples.

Particularly interesting was the comparison between the sensitivity of the Kato-Katz method and this new PCR approach (Figure 3). By use of an artificially prepared positive fecal sample, the PCR was able to detect the *S. mansoni* DNA in fecal samples where eggs could no longer be observed by the Kato-Katz examination (2.16 eggs/g). Because of the high number of copies of this repeated region, this technique should enable the detection of fractions of a single *S. mansoni* individual cell, instead of the entire eggs needed for microscopic detection. Polymerase chain reaction in clinical fecal samples gave strong amplification signals, regardless of the difference among the number of eggs per gram of feces (Figure 4).

The possibility of detecting *S. mansoni* DNA in the serum of infected patients is illustrated in Figure 5. These results show that serum samples can be used as a template source for schistosomiasis clinical diagnosis. The serum DNA bands were sharp enough to allow a steady interpretation, although not as strong as those seen in fecal samples, suggesting limited quantities of free-circulating DNA.

The DNA amplification assay developed in this study may constitute an unprecedented alternative to the available diagnostic techniques for the detection of *S. mansoni* infection. The value of this approach in the field needs further study.

It certainly demands a more sophisticated laboratory apparatus and a greater operational effort when compared with the Kato-Katz parasitological examination, which is better than molecular and serological techniques in terms of low costs and ease of operation. Nevertheless, our results indicate that PCR is probably more sensitive than the Kato-Katz technique and the circulating antigen detection that presents the similar sensitivity to stool examination in situation of low worm burden, medication and in low endemic area. The high degree of specificity may make it better diagnostic alternative then serological techniques.

This investigation received technical and financial support from the joint WHO Eastern Mediterranean Region (EMRO), Division of Communicable Diseases (DCD) and the WHO Special Program for Research and Training in Tropical Diseases (TDR): The EMRO/TDR Small Grants Scheme for Operational research in Tropical and other Communicable Diseases."

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3/11/2011