Diagnostic value of PCR in the detection of *Schistosoma mansoni* DNA in stool of clinically suspected individuals

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ABSTRACT

Schistosomiasis is a major health problem in Egypt with high socioeconomical impact. For effective *Schistosoma mansoni* (*S. mansoni*) control, sensitive and specific methods of diagnosis are required. The aim of the present study was to detect the prevalence of *S. mansoni* in one of the endemic areas, Tookh villages, using Kato-Katz technique and to evaluate the diagnostic value of polymerase chain reaction (PCR) as an alternative tool for diagnosing schistosomiasis in stool specimens from individuals at high risk of infection by *S. mansoni*. A total of 370 stool samples were tested for *S. mansoni* infection using 3 Kato-Katz smears. Of them 49 samples were tested using primers targeted to a highly repeated 121-base pair sequence of *S. mansoni*. The samples were divided into 2 groups according to parasitic status of the individual as follows: 10 individuals with active schistosomiasis and free from other parasitic infections as proved by Kato-Katz thick smear and 39 individuals with negative parasitological examination. The results revealed a prevalence of 2.7% (10 out of 370) for *S. mansoni* infection using Kato-Katz technique. They also showed a high sensitivity for PCR as *S. mansoni* DNA was detected in 48.72% (19/39) of the samples with negative Kato-Katz and in 100% (10/10) of the samples with positive Kato-Katz analyzed. In conclusion, PCR is an important tool for detecting *S. mansoni* infection in individuals excreting few eggs in feces and Kato-Katz is not enough to diagnose positive *S. mansoni* cases in individuals at high risk of infection.

**KEY WORDS:** Schistosomiasis, PCR, Kato-Katz, stool.
INTRODUCTION

Schistosomiasis, caused by infection with Schistosoma species, remains a public health problem in tropical and subtropical areas of the world. It is currently estimated that 207 million people harbor the parasites and about 779 million people are at risk of being infected with schistosomes (Steinmann et al., 2006). Diagnosis of Schistosoma mansoni (S. mansoni) traditionally rely on the detection of parasite eggs in stool using Kato-Katz technique (Doenhoff et al., 1993). However, the sensitivity of this technique decreases when the prevalence and intensity of infection are low (Ebrahim et al., 1997). Thus, this method is less efficient in areas of low endemicity, in post-treatment situations, and in the control of transmission. Other diagnostic alternatives include immunologic methods, such as detection of parasite-specific antibodies and circulating antigens. Antibody detection assays have been shown to be more sensitive than the parasitological examination (Hamilton et al., 1998), but generally lack specificity and are unable to discriminate between active and past S. mansoni infections (Doenhoff et al., 1993). The detection of circulating antigens is a highly specific assay (Attallah et al., 1999). However it shows a similar sensitivity to stool examination for low infection intensity (Doenhoff et al., 1993 and Van Lieshout et al., 1995). Polymerase chain reaction (PCR) was reported to be a useful, sensitive and specific technique for the detection of S. mansoni in human feces and serum (Pontes et al., 2002 and Pontes et al., 2003). However, the application to the detection of S. mansoni is relatively few compared to its known impact on humans life quality (King, 2010), and despite a recommendation of the World Health Organization (WHO) that a major focus of research on schistosomiasis should be on the development and evaluation of new strategies and tools for control of the disease (WHO, 2004). Egypt is a cradle of civilization, but has been plagued by
schistosomiasis since at least the Middle Kingdom period (1,500 BC) (Abdel-Wahab, 1982). However, and as most of developing countries, diagnosis of schistosomiasis infection mainly relies on the coprology based assays. Recently in Egypt, molecular techniques have proved a promising efficiency regarding sensitive and specific diagnosis of infection in sera of infected patients (Sadek et al., 2008). The aim of the present study was to detect the prevalence of *S. mansoni* in one of the endemic areas, Tookh villages, using Kato-Katz technique and to evaluate PCR performed on stool samples as an alternative non invasive technique to Kato-Katz. for diagnosing schistosomiasis in individuals at high risk of infection by *S. mansoni*.

**MATERIALS AND METHODS**

**Study design**

The study was carried on 370 subjects randomly selected from 20 villages located in Tookh (Qualubia governorate), highly endemic for *S. mansoni* infection. They were evaluated for the presence of *S. mansoni* infection and other helminthic infections using Kato-Katz technique repeated for 3 times. Of them 49 subjects were chosen and divided into 2 groups: 1- Group A: Consisted of the only 10 subjects detected with positive *S. mansoni* eggs in stool (≥ 6.67 eggs/gram of feces) and free from other helminthic infections. 2- Group B: Consisted of 39 subjects with no *S. mansoni* eggs or any other helminthic infections in stool and complaining of different manifestations as abdominal colic, abdominal distention, headache, dizziness, dysentery and fatigue. 7 subjects of group A and 30 subjects from group B, had history of receiving previous treatment of schistosomiasis with praziquantel.
Sample collection and storage

Stool samples were taken from the study group in sterile containers after written informed consent from each adult subject and from the parents of minors. Part of each sample was examined by Kato-Katz technique and the other part was further stored at –80°C for further DNA extraction and PCR.

For PCR, stool samples obtained from breast fed 1 month old baby of one of the colleges were taken as negative control. In addition, nuclease free water containing bilharzial ova obtained from Theodor Bilharz research institute and stored at -80°C was used as a positive control.

Stool examination by the Kato-Katz technique

Fecal samples were evaluated for the presence of S. mansoni eggs by the quantitative Kato-Katz parasitological technique. Three glass slides (50 mg of feces per slide) were prepared for analyzing each sample, and the arithmetical mean number of eggs per gram of feces was the final result.

Genomic DNA extraction and amplification

Genomic DNA extraction: DNA was extracted from 200 mg stool using QIAamp DNA stool mini kit - supplied by Qiagen according to the manufacturer's instructions following the protocols of isolation of DNA from stool for pathogen detection. 200 ul of the DNA was eluted. The DNA concentration in the elute was measured by absorbance at 260 nm. On the other hand, the DNA purity was determined by calculating the ratio of the absorbance at 260nm to the absorbance at 280 nm. The extracted DNA was then stored at - 20°C until further processing. DNA from bilharzial ova was extracted using QIAamp DNA minikits-supplied by Qiagen according to the manufacturer's instruction.
**DNA Amplification:** Primers were cited for amplification of 121bp tandem repeat DNA sequence of *S. mansoni* described by Hamburguer et al. (1991). The sequence of these primers were 5`-GATCTGAATCCGACCAACCG-3` for the forward primer and 5`-ATATTAACGCCACGCTCTC-3` for the reverse primer; supplied by Eurofins MWG Operon. These primers were expected to amplify multiple DNA bands and the main band was expected to be 110 bp long.

Amplification was done using *Dream Taq Green PCR Master Mix (2x)* supplied by Fermentas, Germany. The PCR mix used to amplify the 121bp tandem repeat DNA sequence of *S.mansoni* contained 25 ul of *Taq* PCR master Mix 2x, 2.5 ul (0.5μM) of the forward primer, 2.5 ul (0.5μM) of the reverse primer, 5 ul of the template DNA and 15ul of nuclease free water to reach a final volume of 50 ul. Amplification was done in G storm thermal cycler UK. Amplification of 121bp tandem repeat DNA sequence of *S.mansoni* was done according to the following program: initial denaturation at 95 °C for 3 mins, 35 cycles of denaturation at 95 °C for 45 sec., annealing at 51.5 °C for 45 sec. and extension at 72 °C for 1.5 min, followed by final extension at 72 °C for 10 mins then hold at 4 °C. Fecal samples obtained from the breast fed 1 month old baby were included in each run as negative control to detect any cross contamination.

**Agarose gel electrophoresis:** 10 μl of each amplified DNA & DNA ladder (Gene Ruler™ 100bp plus DNA ladder, 100-3000bp supplied byFermentas-Germany) were separated on 2% agarose gel containing 0.3 μg/ml of ethidium bromide .The bands were visualized using UV transilluminator (254nm), photographed & analyzed.
Statistical analysis

The collected data were computed and statistically analyzed using SPSS version 9 software for windows (SPSS inc., chicago). Suitable statistical techniques were calculated as range, mean, standard deviation, percentage, sensitivity, specificity, PPV, NPV and accuracy.

RESULTS

Our study involved 370 subjects, 183 males and 187 females. Their age ranged from 10 to 55 years with mean value 31.6 ± 14.5. The prevalence of *S. mansoni* infection, using 3 Kato-Katz smears was 2.7% (10 out of 370) (Table 1). The number of eggs estimated by the 3 Kato-Katz smears in a positive stool sample ranged from 6.7 to 100 eggs/gram of feces.

<table>
<thead>
<tr>
<th>Results of 3 Kato-Katz smears</th>
<th>Frequency</th>
<th>Percentage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive <em>S. mansoni</em> infection</td>
<td>10/370</td>
<td>2.7</td>
</tr>
<tr>
<td>Negative <em>S. mansoni</em> infection</td>
<td>360/370</td>
<td>97.3</td>
</tr>
</tbody>
</table>

PCR was performed on stool samples from 49 subjects; 18 males and 31 females divided into 2 groups (A&B). Group A included the 10 subjects with positive *S. mansoni* eggs in stool; 3 males and 7 females. Their age ranged from 19 to 45 years with mean value 35 years ± 7.5. Group B included 39 subjects (15 males and 24 females) with no *S. mansoni* eggs in stool. Their age ranged from 11 to 49 years with mean value 33 years ± 11.8. The minimum detection limit of 3 Kato-Katz smears is 6.7 eggs/gram of feces, meaning that the samples containing less than this number of
eggs will be missed by 3 Kato-Katz smears. So group B may contain < 6.7 eggs/gram of feces (it may be negative or with low intensity of infection).

The PCR results in the two groups of the study are shown in table (2).

Table (2): PCR results performed on stool samples in the study groups.

<table>
<thead>
<tr>
<th>Group according to Kato-Katz result</th>
<th>Number of cases</th>
<th>PCR results</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Positive No (%)</td>
</tr>
<tr>
<td>Group A (Positive <em>S. mansoni</em>)</td>
<td>10</td>
<td>10 (100%)</td>
</tr>
<tr>
<td>Group B (Negative <em>S. mansoni</em>)</td>
<td>39</td>
<td>19 (48.72%)</td>
</tr>
</tbody>
</table>

The sensitivity, specificity, PPV and NPV of PCR, considering Kato-Katz technique as a reference gold standard diagnostic test for *S. mansoni* are shown in table (3).

Table (3): Sensitivity, specificity, PPV and NPV of PCR, considering Kato-Katz technique as a reference test for *S. mansoni* diagnosis.

<table>
<thead>
<tr>
<th>AUC</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>PPV</th>
<th>NPV</th>
<th>Accuracy</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.76</td>
<td>100%</td>
<td>51%</td>
<td>34.5%</td>
<td>100%</td>
<td>75.6%</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

The results of gel electrophoresis of the PCR products in figure (1) show multiple bands of 121 bp tandem repeats with the common band is 110bp in positive PCR reactions.
Figure (1): gel electrophoresis of amplified products of 121bp tandem repeat DNA sequence of *S. mansoni*. Lane 1 shows 100bp DNA ladder. Lane 2 shows negative control from baby stool. Lane 3 shows amplification products of bilharzial DNA strain. Lane 4 & 5 shows amplification products of 2 stool samples with positive Kato-Katz. Lanes 6 & 7 shows amplification products of 2 stool samples with negative Kato-Katz. Lane 8 shows negative PCR in a stool sample with negative Kato-Katz.

**DISCUSSION**

Schistosomiasis is a major health problem in Egypt. *S. mansoni* is a liver fluke that results in periportal fibrosis, pre-sinusoidal portal hypertension, ascitis, splenomegaly, hypersplenism, esophageal varices and hemorrhage (El-Zayadi, 2004). Therefore proper diagnosis and thus treatment of *S.mansoni* infection are essential to avoid its bad sequele.

The Kato-Katz parasitological technique is currently the recommended and the most widely used method for diagnosing *S.mansoni* infection. This is because it is of high specificity, quantitative simple technique that requires relatively unsophisticated equipment and, in areas of high endemicity, personnel with only basic training. Therefore it could be of lowest cost when technical assistance is plentiful. However, the sensitivity of this technique decreases when the intensity of infection is low. In Kato–Katz tests, as little as 50 mg of material are examined, which gives a sensitivity cut-off of 20 eggs/gram of feces which is the detection limit of the Kato-Katz examination. The factors that contribute to inaccuracy include uneven distribution of eggs in solid excreta, large day-to-day variations in infected individuals, and the operators’ technical competence (Pontes et al., 2003 and Doenhoff et al., 2004). In our study, we performed Kato-Katz to 3 times on 50 mg sample each time allowing the detection limit to be 6.7 eggs/gram of feces and the staff who performed it were well trained as Egypt is endemic of schistosomiasis and thus the sensitivity and accuracy
were increased. In agreement with the importance of repetition of Kato-Katz in our study in ensuring the presence or absence of *S. mansoni* eggs in stool, is a study reporting that the prevalence of *S. mansoni* infection calculated with one, two, or three Kato-Katz examinations were 25.3, 29.4, and 30.9%, respectively. Moreover, among 12 patients whose samples were negative by three Kato-Katz examinations for *S. mansoni* eggs, but positive by one PCR survey, five (41%) were positive by the Kato-Katz method after at least four additional repeated Kato-Katz examinations (Pontes et al., 2003).

Our study revealed a prevalence of *S. mansoni* infection of 2.7% (10 out of 370) in Tookh villages using 3 Kato-Katz smears. This prevalence is coincident with the National Schistosomiasis Control Project during which the prevalence of *S. mansoni* in Egypt declined from 14.8% in 1993 (the first year of the project) to 2.7% in 2002 (when the project was closed) and continued to decline thereafter, reaching 1.5% in 2006 (The world bank, 2008). However the sensitivity of Kato-Katz is low in detecting the infection.

In spite of increasing the sensitivity of Kato-Katz by multiple examinations by well qualified personnel, our study revealed positive PCR in 48.72% of stool samples with negative Kato-Katz. It is possible that those individuals with positive PCR had low intensity of infection (egg outputs less than 6.7 eggs/gram as we increased the sensitivity of Kato-Katz by the above mentioned measures). This low intensity of infection may be due to introduction of control measures that are effective in reducing transmission, early- or late-stage infections or post treatment (Doenhoff et al., 2004 and Ebrahim et al., 1997). Our study reported previous treatment with praziquantel in most of group B subjects. The high sensitivity of the PCR technique using the primers used in our study reported by Pontes et al. (2002) can explain why high percentage
(48.72%) of Kato-Katz negative patients with low intensity of infection give positive PCR. It can detect the parasite DNA in fecal samples containing as few as 2.4 eggs /gram of feces, which makes it 10 times more sensitive than the Kato-Katz examination. A detection limit of 1 fg of Schistosoma DNA was determined when pure DNA was used as PCR template (Pontes et al., 2002). The high sensitivity of PCR amplifying 121bp tandem repeat DNA sequence is due to the high copy number (600,000/cell) of the target sequence which comprises 10% of the S.mansoni genome (Hamburguer et al., 1991). This high copy number allowed the detection of fractions of a single S.mansoni individual cell, instead of the entire eggs needed for microscopic detection.

Another possibility of our high percentage of positive cases by PCR is that they could be false positive results. However, no other helminthic infections were present in our study individuals. Also the specificity of the primers used in our study was tested before against Ascaris lumbricoides, Ancylostoma duodenale, Taenia Solium and Trichiuris trichiuria and revealed no cross-reaction with DNA from these helminthes (Pontes et al., 2002). Moreover, Oliveira et al. (2010) showed that the PCR using primers used in our study was negative in stool samples from healthy individuals with no parasites in stool and in individuals presenting parasites other than S.mansoni including Strongyloides stercoralis, Hymenolepis diminuta, Hookworms, Giardia intestinalis, Entamoeba histolytica, Iodamoeba butschlii, Entamoeba coli, Blastocystis hominis and Endolimax nana. In addition, carry-over contamination could be a cause of false positive results, however it is dislikable in our study as we used negative control using stool samples obtained from breast fed 1 month old baby with every run of PCR performed and strict measures were taken during DNA extraction and PCR to avoid the contamination.
Our study revealed a sensitivity and specificity, PPV, NPP of 100%, 51%, 34.5% and 100% for PCR considering Kato-Katz technique as a reference gold standard diagnostic test for *S. mansoni*. Gold standard test is an accepted reference test that provide a definitive diagnosis of a disease; it can diagnose a disease with certainty detecting all true positive cases and excluding all true negative cases (Knapp & Miller, 1992 and Petrie & Sabin, 2005). Therefore, in our study Kato-Katz cannot be considered as a gold standard diagnostic test due to its low sensitivity as already said. and PCR cannot be compared with it to calculate its validity. Therefore, the detected positive results obtained by PCR (19/39) in group B were considered false positive considering the Kato-Katz as the reference diagnostic test and thus the detected specificity and PPV were low. However these cannot be considered as false positive cases as already discussed.

Our study reported positive PCR in 48.72% of stool samples of individuals with 3 negative Kato-Katz. In comparison, positive PCR was reported in 11.9% of stool samples with three negative Kato-Katz examination in endemic population in Brazil (Pontes et al., 2003) and in 23% of stool samples with 3 negative Kato-Katz in hypo endemic area in Egypt (Allam et al., 2009). Gomes et al. (2009) applied modifications to PCR using our sets of primers to increase the PCR sensitivity and evaluated it in endemic area in Brazil. PCR was positive in 15 out of 39 stool samples (38.46%) with negative Kato-Katz.

In addition, our study revealed positive PCR in 100% of cases with positive Kato-Katz technique. On the contrary, other studies reported missing of some cases with positive Kato-Katz by PCR using our primers. One study revealed positive PCR in 58 out of 60 patients (96.7%) with positive Kato-Katz. It reported that the negative 2 cases were misdiagnosed by PCR and cannot be false positive cases as Kato-Katz.
have 100% specificity and they could be due to many factors, such as inhibition of the amplification reaction by feces compounds and/or DNA degradation during transportation from the field (Pontes et al., 2003). QIAamp DNA stool mini kit used for DNA extraction in our study uses inhibit X tablets which adsorbs the PCR inhibitors in stool which was not available in the above study. Also stool samples used for PCR were put at -4°C for about 2 hours and transported on ice for about 1/2 an hour till preservation at -80°C.

Another study reported positive PCR in 73.5% (25/34) of infected individuals diagnosed by Kato-Katz [56.3% (9/16) in individuals excreting less than 10 eggs/gram of feces and 88.9% (16/18) in individuals excreting more than 10 eggs/gram of feces]. Thus PCR missed 9 cases with positive Kato-Katz (two of them excreting more than 10 eggs/gram of feces) (Oliveira et al., 2010). We can explain these missed cases by inhibition of the amplification reaction by feces compounds as this study also didn't use inhibit X tablets as we used in our study. However in this study, negative samples were spiked with 10 fg S. mansoni DNA as a control and amplification was detected in all samples implicating that it is not the issue of inhibition that caused the 9 cases of false negative results but it may be the reduced egg output and uneven distribution in feces that caused these false negative results especially that 7 cases out of 9 missed cases were excreting less than 10 eggs/gram of feces. In agreement, Gomes et al. (2009) reported that PCR missed two samples positive by the Kato-Katz technique in spite of application of modifications to PCR using our sets of primers to increase the PCR sensitivity. They used QIAamp DNA stool mini kit, added BSA to the PCR mix which neutralizes the contaminating inhibitors and tested all samples for amplification for the human beta actin gene (ACTB) as an internal quality control measure for the DNA isolation procedure and
to test for the possible presence of PCR reaction inhibitors. The two PCR missed samples gave positive results for the amplification of that gene. Therefore DNA degradation and PCR inhibition by inhibitors in stool were excluded. Moreover, these two patients had very low egg outputs (4 and 8 eggs per gram of feces) and thus low egg output and uneven distribution in feces is most probably the cause of the 2 PCR missed cases.

CONCLUSION:

In conclusion, PCR is an important tool for detecting *S. mansoni* infection in individuals excreting few eggs in feces and Kato-Katz is not enough to diagnose positive *S. mansoni* cases in areas where individuals are at high risk of infection. Therefore, it is recommended to perform PCR to DNA extracted from stool samples in individuals at high risk of bilharzial infection who showed negative result with Kato-Katz technique. This is especially in those without bilharzial liver fibrosis on ultrasound to prevent the development of bilharzial liver fibrosis and to decrease and maybe eliminate the need for use of invasive techniques as sigmoid scope and rectal snip. It is also recommended to correlate the results of PCR technique on stool with results of sigmoidoscopy and rectal snip in order to determine if PCR can replace these invasive techniques and in order to determine the sensitivity and the specificity of PCR in comparison to Kato-Katz in true positive cases of *S. mansoni* infection diagnosed by sigmoid scope and rectal snip as sure diagnosis depend on these invasive techniques. In addition, the search for sensitive and specific methods to detect *S.mansoni* infections must be continued.

REFERENCES


القيمة التشخيصية لتفاعل البلمرة المتسلسل في الكشف عن الحمض النووي للبلهارسيا المعوية في براز الأفراد الذين يشتبه بهم سريرياً

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الملخص العربي

يمثل مرض البلهارسيا مشكلة صحية كبيرة في مصر ذات مردود كبير على الحالة الاجتماعية والاقتصادية. و من أجل تحقيق السيطرة الفعالة على عدد البلهارسيا المعوية ، فإن هناك حاجة إلى طرق تشخيصية حساسة ومحددة. وقد كان الهدف من هذه الدراسة الكشف عن مدى انتشار البلهارسيا المعوية في بعض من المناطق المشبوهة. قرى طوخ، وذلك باستخدام تقنية الكاتو - كاتز. وكذلك تقييم القيمة التشخيصية لتفاعل كادا بديلة تتشخيص مرض البلهارسيا في عينات البراز من الأفراد في بعض من الأفراد الذين يشتبه بهم. بدأ هذا بمقابلة العينات المعوية باستخدام تقنية الكاتو - كاتز. حيث تم اختبار ما مجموعه 370 عينة من البراز من الأفراد الذين يشتبه بهم في مرض البلهارسيا المعوية باستخدام تقنية الكاتو - كاتز. حيث تم اختبار 44 عينة من البراز مع إيجابية للإيجابية، التي تم استخدامها في الفحص السلبي للعديد من الأمراض الطفيلية الأخرى كما ثبت من اختبار المسحة الصغيرة. وقد تم تقسيم العينات إلى مجموعة واحدة يمكن نظرة على النحو التالي: 39 فرد بفحص إيجابي للعديد من الأمراض التي تم الحصول عليها من عينات البراز من مجموعة الكاتو - كاتز. وأظهرت النتائج التي تم الحصول عليها من عينات البراز من مجموعة الكاتو - كاتز. كما أظهرت حساسية عالية لتفاعل البلمرة المتسلسل، حيث تم الكشف عن الحمض النووي للبلهارسيا المعوية في 100٪ (10/10) من العينات الإيجابية التي تم تحصيلها بالـ كاتو - كاتز. وفي الختام ، يمكن استخدام فحص البلمرة المتسلسل في الأفراد الذين يشتبه بهم في مرض البلهارسيا المعوية. و مناطق المعرضة لخطر الإصابة بالعدوى.