Cross Reactivity And Diagnostic Value Of PCR Amplifying The 121bp Tandem Repeat Of Schistosoma And The 18S rDNA Of Fasciola

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ABSTRACT

Laboratory diagnosis of Schistosoma mansoni (S.mansonii) and Fasciola represents a challenge especially in areas endemic for both like Egypt. Different laboratory techniques developed for diagnosis of these infections may either lack sensitivity as in cases of low intensity infection or specificity. The high sensitivity of PCR has encouraged scientists to use it in the diagnosis of different parasitological infections. Cross reactivity between S.mansonii and Fasciola parasites is known to occur in the immunological tests diagnosing either parasite. We aimed to test for the cross reactivity between the two parasites at the PCR level using primers commonly used for amplification of parts of Fasciola or S.mansonii, and to test for the diagnostic importance of the PCR using these primers. In this study, primers amplifying the 121 bp tandem repeats of S.mansonii and BfrI and DraI primers amplifying the 356 and 263 bp segments of Fasciola 18S rDNA respectively, were used to amplify DNA extracted from pure S.mansonii or Fasciola gigantica strains. They were also used to amplify DNA extracted from human fecal samples obtained from Tookh villages, endemic for both S.mansonii and Fasciola. It was found that PCR amplifying the 121 bp tandem repeats of S.mansonii gave positive results with S.mansonii pure strain and not Fasciola strain indicating its specificity in schistosomiasis diagnosis. On the other hand, PCR using BfrI primers was proved to be positive with both S.mansonii & Fasciola strains and also with 80% and 66.6% of human fecal samples with positive S.mansonii and Fasciola respectively. In addition, PCR using DraI primers was positive for both S.mansonii and Fasciola strains, but with significantly lower DNA yield than with using BfrI primers. It was also positive with 40% and 33.3% of human fecal samples with positive S.mansonii and Fasciola respectively. In conclusion, PCR amplifying the 121 bp tandem repeats of S.mansonii can be used efficiently for diagnosis of human schistosomiasis in areas like Egypt endemic for Both Schistosoma and Fasciola. On the contrary, PCR using BfrI or DraI primers has low validity in the diagnosis of human fascioliasis in areas where both parasites coexist.

INTRODUCTION

The blood fluke Schistosoma mansoni (S.mansonii) and the liver fluke Fasciola are two helminthes belonging to the class Trematoda of the phylum Platyhelminthes (Castro, 1996). Schistosomiasis and fascioliasis have been proven to be important human parasitic diseases that share having a seriously harmful effect on liver (Lambertucci et al., 2000 and Haseeb et al., 2002). Schistosomiasis is endemic in 74 countries and affects some 200 million people worldwide, causing an estimated 200,000 deaths annually (Chistulo et al., 2004). On the other hand, Human Fasciola cases have been reported in countries of the five continents (Esteban et al., 1998). The global prevalence of fasciola infection is in excess of 3 million (Maguire, 2005). Both parasites are endemic in Egypt (El-Khoby et al., 2000 and Curtale et al., 2007), and overlapped areas of geographical distribution have been identified (Esteban et al., 2003). Moreover, in case of human fascioliasis, and since 1980, the number of cases has risen drastically and human infection has been reported in different governorates (WHO, 2007, Soliman, 2008).
Control of either infections requires accurate diagnosis and methodologies to properly delineate the target regions of the control programs. It also requires to get reliable and accurate epidemiological data, plan for mass treatment and to monitor the success of those programs through verifying infection dynamics in a certain environment.

Both infections have the same diagnostic dilemmas. Their diagnosis depends mainly on coprologic techniques that aim to find the characteristic egg in stool samples but these techniques lack adequate sensitivity (Mas-Coma et al., 1999, Berhe et al, 2004). Also, the developed immunological techniques detecting antibodies are of limited diagnostic value (Doenhoff et al., 1993, Espino et al., 1990). Moreover, antigen detection assays are not sensitive more than direct coprologic techniques in case of schistosomiasis (Van Lieshout et al., 1995), while in case of human fascioliasis, their results are tightly linked to the infection dynamics and the endemicity degree (Espino et al., 1998), or just recently applied on human cases in the research field after many trials on experimental animals (Ubeira et al., 2009, Demerdash et al., 2009) but not commercially offered yet. So, in Egypt, we have a problem of two endemic parasites, both affect the Egyptian livers which are indeed robustly hurt by hepatitis C & B viruses (Kamal, 2011, Selim et al., 2011), coexist in the same areas and their control depends on unreliable diagnostic techniques. The situation is further worsened by the common phylogenetic origin which is expressed in the diagnostic field by a cross reactivity (Maghraby et al., 2009) that hinders specific diagnosis of both parasites.

Polymerase Chain Reaction (PCR) was first used in the diagnosis of S.mansoni parasite DNA in the sera and stool samples of human by Pontes et al.(2002) using primers that amplify the 121 bp tandem repeat of S.mansoni. Since that, It has been evaluated and improved in several studies (Pontes et al., 2003, Sadek et al, 2008, Allam et al., 2009, Gomes et al., 2009, Oliveira et al., 2010 & Eraky et al., 2010). It has been proved to be highly sensitive and highly specific technique (Pontes et al., 2003), but during those studies, and to our knowledge, it has not been tested for cross reactivity against Fasciola except for 2 cases assessed by Sadek et al.(2008). On the other side, PCR has not been used yet for diagnosis of human Fasciola infection either in the sera or stool samples. Nevertheless, PCR was used efficiently for the detection of the parasite in the snail intermediate host (Caron et al., 2011) and also for species differentiation (Alasaad et al., 2011).

These promising results were the trigger of the current study that aimed to assess for proper use of this advanced technique, the PCR, in solving the problem of human fascioliasis and schistosomiasis diagnosis in Egypt and areas where both of Fasciola and Schistosoma parasites coexist. Therefore, we had to evaluate the potential cross reactivity between both helminthes at the PCR level and the diagnostic value of PCR, using primers amplifying the 356 & the 263 segments of 18s rDNA of Fasciola (Karimi, 2008) and the 121bp tandem repeat of S.mansoni, mutually tested against the pure parasite strains and clinical fecal samples.

MATERIALS AND METHODS

This study was carried out at Molecular Biology and biotechnology Unit and Medical parasitology department research lab., Faculty of Medicine-Benha University. Also, the Thermo Scientific NanoDrop™ 2000 Spectrophotometer present in Clinilab laboratory-Egypt was used for the measurement of the concentration of extracted DNA and the amplified DNA products of S.mansoni and Fasciola strains.

I - PCR performed on S.mansoni and Fasciola strains

Parasite strains

Adult Fasciola gigantica and S.mansoni eggs were obtained from Schistosome Biological Supply Center (SBSC) and Parasitology Laboratory, Theodor Bilharz Research Institute(TBRI), Giza, Egypt to be used as pure strains for both parasites. Each parasite was then put in nuclease free water then frozen at - 80°C until time of genomic DNA extraction.
Genomic DNA extraction

DNA was extracted from 200 µl S. mansoni ova solution and from the adult Fasciola after cutting it into small pieces using QIAamp DNA minikits supplied by Qiagen according to the manufacturer instructions. 200 µl of the DNA was eluted. The concentration of DNA 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 260 nm to the absorbance at 280 nm. The absorbance was measured using Thermo Scientific NanoDrop™ 2000 Spectrophotometer. The extracted DNA was then stored at -20°C until further processing.

DNA Amplification

Primers described by Hamburger et al. (1991) were used for amplification of 121bp tandem repeat DNA sequence of S. mansoni. The sequence of these primers were 5’- GATCTGAATCCGACCAACCG-3’ for the forward primer and 5’- ATATTAACGCC CACGCTTC-3’ for the reverse primer. These primers were expected to amplify multiple DNA bands and the main band was expected to be 110 bp long.

As regards the Fasciola species, DraI primers were used to amplify a DNA fragment of 263 bp of the 18S rDNA gene. The sequence of these primers were 5’- CATATGCTTGTCTCAGAGATTAAGCC-3’ for the forward primer DraI sense and 5’- CGATCAGTGAAGTTATCCAGAGTC-3’ for the reverse primer DraI antisense. Also BfrI primers were used to amplify a DNA fragment of 356 bp of the 18S rDNA gene. The sequence of these primers were 5’- CGAAGACGATCAGATACCGTCCAGAGTC-3’ for the forward primer BfrI sense and 5’- AGCAGGCCAGAGTCTCGTTC-3’ for the reverse primer BfrI antisense. These primers were described by Karimi (2008).

Amplification was done using Dream Taq Green PCR Master Mix (2x) supplied by Fermentas, Germany. The PCR mixtures used to amplify the 121bp tandem repeat DNA sequence of S. mansoni and each of the fragments of the 18S rDNA gene were the same, with the same starting DNA concentration. Each contained 25 µl of Taq PC master Mix 2X, 2.5 µl (0.5µM) of the forward primer, 2.5 µl (0.5µM) of the reverse primer, 100 ng of the template DNA and completed with nuclease free water to reach a final volume of 50 µl. Amplification was done in the 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 min., 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 min. then hold at 4°C. On the other hand, the program for amplification of the fragments the 18S rDNA gene was done as follows: initial denaturation at 95°C for 3 min., 35 cycles of denaturation at 95°C for 1 min., annealing at 51.5°C for 1 min. and extension at 72°C for 2 min., followed by final extension at 72°C for 10 min. then hold at 4°C.

Three PCR reactions using each set of primers were done for each parasite. The concentration of the amplified DNA was measured using Thermo Scientific NanoDrop™ 2000 Spectrophotometer. The mean values of the concentrations of the amplified DNA using each set of primers for each parasite were calculated.

Agarose gel electrophoresis

10 µl of each amplified DNA & DNA ladder (Gene RulerTM 100bp plus DNA ladder, 100-3000 bp supplied by Fermentas, 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.

II - PCR performed on stool samples

In order to support the results of PCR performed on the parasite strains, and in order to evaluate the diagnostic value of PCR amplifying the segments of 18S rDNA in human schistosomiasis in comparison to PCR amplifying the 121 bp tandem repeat and in human fascioliases, a clinical study was performed on stool samples. These stool samples were obtained from subjects living in Tookh villages, endemic for both Fasciola and S. mansoni. A written informed
consent was obtained from each adult subject and from the parents of minors. All the stool samples were taken in sterile containers and examined by direct smear, simple precipitation and 3 Kato-Katz smears (Katz et al., 1972) for S. mansoni, Fasciola or any other helminthic infections. Part of each stool sample was stored in 3 eppendorf tubes at -80°C for further DNA extraction and PCR. According to the results of the Kato-Katz smears and the PCR amplifying the 121 bp tandem repeats of S. mansoni in the stool samples, the study subjects were chosen to be categorized into 3 groups:

- **Group A**: composed of 10 subjects positive for S. mansoni infection but negative for Fasciola infection.
- **Group B**: composed of 6 subjects negative for S. mansoni infection but positive for Fasciola infection.
- **Group C**: composed of 10 subjects negative for both S. mansoni and Fasciola infections.

In the 3 groups, the positive S. mansoni infection was evidenced by positive Kato-Katz for S. mansoni eggs and PCR amplifying the 121 bp tandem repeat or PCR alone if Kato-Katz was negative. The negative S. mansoni infection was evidenced by negative Kato-Katz for S. mansoni eggs and PCR amplifying the 121 bp tandem repeat. However, positive or negative Fasciola infection was evidenced by positive or negative Kato-Katz smears for Fasciola eggs. In addition, subjects with any other helminthic infections were excluded from the study.

This was followed by performing PCR on the extracted DNA from the stool samples of all the 3 groups, amplifying the 2 segments of the 18S rDNA using DraI and BfrI primers.

**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 µl of the DNA was eluted. The extracted DNA was then stored at -20°C until further processing.

**DNA Amplification**

Amplification of the 121bp tandem repeat DNA sequence of S. mansoni and of the 263 bp and 356 bp DNA fragments of the 18S rDNA gene in the stool samples, was done according to the same protocols done to the pure parasites in this work.

DNA extracted from stool samples obtained from breast fed 1 month old baby of one of the colleges were taken as negative control and included in each run to detect any cross contamination. In addition, the nuclease free water containing S. mansoni ova or Fasciola strain were used as positive controls.

**Agarose gel electrophoresis**

It was performed as already described

**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 mean, standard deviation, percentage, tests of significance including freedman test, paired T test & Student T test. P ≤ 0.05 was considered significant. P ≤ 0.001 was considered highly significant.

**RESULTS**

I - Study results of PCR performed on S. mansoni and Fasciola strains

As regard the PCR amplifying the 121 bp tandem repeats, our study showed positive results for S. mansoni eggs and negative results for the Fasciola adult worm (Figure 1).

On the other hand, the study results of the PCR amplifying the 18S rDNA revealed the presence of cross reaction between the 2 parasites (Figure 2).

On quantitation of the amplified PCR products of S. mansoni & Fasciola (Table 1), it was revealed that the DNA yield of S. mansoni using BfrI primers was significantly higher than with using DraI primers or primers amplifying the 121 bp tandem repeat. However, it was significantly lower using DraI primers than with using primers amplifying the 121 bp tandem repeat. On the
other hand, the DNA yield of *Fasciola* using BfrI primers was significantly higher than with using Dral primers.

When comparing the DNA yields of *S.mansoni* and *Fasciola* using BfrI or Dral primers, the results showed that the yield of *S.mansoni* was significantly higher than that of *Fasciola* using either of the 2 sets of primers.

![Figure (1): Gel electrophoresis of the amplified products of 121bp tandem repeat DNA sequence. Lane 2 shows 100bp DNA ladder. Lane 1 shows negative PCR using DNA extracted from the *Fasciola* adult worm. Lane 3 shows the amplification products of the DNA of *S.mansoni* (multiple bands).](image1)

![Figure (2): Gel electrophoresis of the amplified products of 18S rDNA. Lane M shows 100bp DNA ladder. Lanes 1, 2 & 3 show PCR products using BfrI primers with lane 1 showing the negative control revealing negative PCR & lanes 2 & 3 showing positive 356 bp DNA fragments of *S.mansoni* & *Fasciola* respectively. Lanes 4, 5 & 6 show PCR products using Dral primers with lane 4 showing the negative control revealing negative PCR & lanes 5 & 6 showing positive 263 bp DNA fragments of *Fasciola* & *S.mansoni* respectively.](image2)
Table (1): Concentrations of the amplified DNA products of *S.mansoni* and *Fasciola* using the primers amplifying the 121 bp tandem repeat of *S.mansoni*, BfrI or DraI primers

<table>
<thead>
<tr>
<th>Parasite</th>
<th>Concentration of the amplified DNA product (ng/µl) (detected on the NanoDrop™ 2000 Spectrophotometer)</th>
<th>Freedman test</th>
<th>Paired T- test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Using BfrI primers</td>
<td>using DraI primers</td>
<td>Using primers amplifying the 121bp tandem repeat DNA sequence</td>
</tr>
<tr>
<td><em>S.mansoni</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1st reading</td>
<td>1129.9</td>
<td>164.4</td>
<td>1003</td>
</tr>
<tr>
<td>2nd reading</td>
<td>1141.8</td>
<td>165.2</td>
<td>1005</td>
</tr>
<tr>
<td>3rd reading</td>
<td>1137.8</td>
<td>169</td>
<td>998</td>
</tr>
<tr>
<td>Mean±SD</td>
<td>1136.5 ± 6.1</td>
<td>166.2 ± 2.5</td>
<td>1002 ± 3.6</td>
</tr>
<tr>
<td><em>Fasciola</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1st reading</td>
<td>67</td>
<td>13.2</td>
<td></td>
</tr>
<tr>
<td>2nd reading</td>
<td>67.4</td>
<td>14.5</td>
<td></td>
</tr>
<tr>
<td>3rd reading</td>
<td>69.9</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>Mean±SD</td>
<td>68.1 ± 1.6</td>
<td>12.9 ± 1.8</td>
<td>29.5 (P4 = 0.001)</td>
</tr>
</tbody>
</table>

**Student T test**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group A</th>
<th>Group B</th>
<th>Group C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male / female</td>
<td>5/5</td>
<td>4/2</td>
<td>6/4</td>
</tr>
<tr>
<td>Age (Years):</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>29.1 ± 10.2</td>
<td>28.8 ± 16.3</td>
<td>32.8 ± 12.6</td>
</tr>
<tr>
<td>Range</td>
<td>11 - 45</td>
<td>15 - 53</td>
<td>16 - 52</td>
</tr>
</tbody>
</table>

**II - Study results of PCR performed on stool samples**

The clinical study involved 30 subjects divided into 3 groups (A,B&C). The age & sex data are shown in table (2).

Table (2): Age & sex of the subjects of the clinical study groups.
The number of *S.mansoni* eggs in group A estimated by the 3 Kato-Katz smears in a positive stool sample ranged from 6.7 to 80 eggs/gram of feces, while that of *Fasciola* eggs in group B ranged from 6.7 to 33.3 eggs/gram of feces. Negative 3 kato-Katz smears for either *S.mansoni* or *Fasciola* meant that the number of eggs/gram of feces was less than 6.7.

The results of Kato-Katz and PCR for the study groups are shown in table (2).

**Table (2):** Kato-Katz and PCR results for the different studied groups.

<table>
<thead>
<tr>
<th>Sample n.</th>
<th>3 Kato-Katz results <em>(S.mansoni egg number/gram of feces)</em></th>
<th>PCR amplifying 121bp tandem repeats</th>
<th>PCR using BfrI primers</th>
<th>PCR using DraI primers</th>
<th>Sample n.</th>
<th>3 Kato-Katz results <em>(Fasciola egg number/gram of feces)</em></th>
<th>PCR using BfrI primers</th>
<th>PCR using DraI primers</th>
<th>Sample n.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>- (&lt; 6.7)</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>1</td>
<td>33.3</td>
<td>+</td>
<td>+</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>- (&lt; 6.7)</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>2</td>
<td>6.7</td>
<td>+</td>
<td>-</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>- (&lt; 6.7)</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>3</td>
<td>13.3</td>
<td>-</td>
<td>-</td>
<td>3</td>
</tr>
<tr>
<td>4</td>
<td>- (&lt; 6.7)</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>4</td>
<td>26.7</td>
<td>+</td>
<td>+</td>
<td>4</td>
</tr>
<tr>
<td>5</td>
<td>+ (6.7)</td>
<td>+</td>
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<td>+</td>
<td>-</td>
<td>5</td>
</tr>
<tr>
<td>6</td>
<td>+ (20)</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>6</td>
<td>13.3</td>
<td>-</td>
<td>-</td>
<td>6</td>
</tr>
<tr>
<td>7</td>
<td>+ (80)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>7</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>+ (60)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>8</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>+ (33.3)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>9</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>+ (46.7)</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>10</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Total number of positive cases</th>
<th>6</th>
<th>10</th>
<th>8</th>
<th>4</th>
<th>6</th>
<th>4</th>
<th>2</th>
<th>2</th>
<th>1</th>
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<tr>
<td>Percentage (%)</td>
<td>60</td>
<td>100</td>
<td>80</td>
<td>40</td>
<td>100</td>
<td>66.7</td>
<td>33.3</td>
<td>20</td>
<td>10</td>
</tr>
</tbody>
</table>

The results of gel electrophoresis of some PCR products of the 18S rDNA extracted from the stool samples of the different studied groups are shown in figure (3).

![Figure (3): Gel electrophoresis of the amplified products of 18S rDNA in the stool samples in the different study groups: a) using BfrI primers (356 bp DNA fragments) B) using DraI primers (356 bp DNA fragments). Lanes M show 100bp DNA ladder. Lanes 1 show the negative control (baby stool sample) revealing negative PCR using both sets of primers (BfrI & DraI). Lanes 3 & 5 show stool samples with negative PCR using both sets of primers. Lanes 4 & 6 show stool samples with positive PCR using both sets of primers. Lanes 7 show positive PCR using BfrI primers & negative PCR using DraI primers. Lanes 2 show negative PCR using BfrI primers & positive PCR using DraI primers.](image-url)
DISCUSSION

Cross-reactions in immunodiagnosis occur using schistosomal soluble egg antigen and Fasciola adult worm antigen in cases with human fascioliasis, and schistosomiasis. False positive reactions occur in 10% of cases with fascioliasis when using schistosomal soluble egg antigen, while, these false positive reactions occur in 25% of cases with schistosomiasis using Fasciola crude antigen (Hassan et al., 1989).

Our study demonstrated that the PCR amplifying the 121 bp tandem repeats showed positive results for S. mansoni eggs and negative results for the Fasciola adult worm strains indicating no cross reaction between the 2 parasites using the primers amplifying the 121 bp tandem repeats. Other studies demonstrated the absence of cross-reaction with DNA from many other helminthes including Ascaris lumbricoides, Ancylostoma duodenale, Taenia Solium, Trichiuris trichiuris (Pontes et al., 2002). Strongyloides stercoralis, Hymenolepis diminuta, Hookworms, Giardia intestinalis, Entamoeba histolytica, Iodamoeba butschlii, Entamoeba coli, Blastocystis hominis and Endolimax nana (Oliveira et al., 2010). This together with the absence of cross reaction with Fasciola DNA detected in our study indicates that the PCR amplifying the 121 bp tandem repeats is highly specific for S. mansoni. Moreover, studies reported its high sensitivity in the diagnosis of S. mansoni (Pontes et al., 2003, Sadek et al., 2008, Allam et al., 2009, Gomes et al., 2009, Oliveira et al., 2010 & Eraky et al., 2010). Also, high DNA yield was detected following amplification of S. mansoni strain in our study. So, PCR amplifying the 121 bp tandem repeat is valuable as a highly specific and sensitive test for S. mansoni diagnosis. Therefore, it can be used for the diagnosis of S. mansoni in areas endemic for both S. mansoni and Fasciola.

On the other hand, cross reaction was detected between S. mansoni strain and Fasciola gigantica strain using BfrI and Dral primers. This cross reactivity between Fasciola and S. mansoni is fully understood in view of common phylogenic origin of the two parasites (Baguñà and Riutort, 2004). The genus Fasciola includes the species Fasciola hepatica and Fasciola gigantica (Garcia, 2007). In Egypt and many other areas of Asia and Africa both Fasciola hepatica and Fasciola gigantica species coexist (Lotfy et al., 2002, Karimi, 2008). Both species are usually difficult to be discriminated from each other morphologically on parasitological examination and clinical, pathological and immunologival analysis cannot differentiate between them (Marcilla et al., 2002, Karimi, 2008). Karimi (2008) reported that PCR using either BfrI or Dral primers gave positive bands with both Fasciola hepatica and Fasciola gigantica with no difference in length of bands in the 2 parasites. Therefore the Fasciola detected in the clinical stool samples by PCR using BfrI or Dral primers can be either one of them.

The DNA yield of PCR for S. mansoni strain using BfrI primers was significantly higher than that amplifying the 121 bp tandem repeat or PCR using Dral primers, using the same starting extracted DNA concentration. This indicates that PCR using BfrI primers can be highly sensitive for the diagnosis of S. mansoni. However, the cross reactivity detected between the 2 parasites using BfrI primers, together with the difficult problematic diagnosis and thus exclusion of Fasciola by microscopy and immunological methods used for Fasciola diagnosis, restricts the utilization of PCR using BfrI in the diagnosis of S. mansoni in areas where the 2 parasites coexist. Stool examination by microscopy shows scanty number of Fasciola eggs, and fewer than 35% of the cases of chronic fascioliasis are diagnosed by parasitic stool tests (Taheri et al., 2007). Serology although useful for the diagnosis of acute Fasciola infection before eggs become detectable in stool and sensitivities of more than 90% for ELISA were reported, the specificity of ELISA may be less owing to cross-reactivity with other helminthes (Maguire, 2005).

Moreover, the PCR we performed using BfrI primers in group A missed 2 cases of S. mansoni in group A (20%) that were positive by Kato-Katz and PCR amplifying the 121 bp tandem repeat or by the PCR only. This means that its detection rate for S. mansoni infection (80%) was lower than...
the detection rate of PCR amplifying the 121 bp tandem repeat when applied on clinical samples in

group A. This make the PCR amplifying the 121 bp tandem repeat of S.mansoni better for use in the
diagnosis of S.mansoni.

In groups B and C of the clinical part of the study, we needed to exclude S.mansoni infection
in order to evaluate the diagnostic value of PCR using BfrI or Dral primers in the diagnosis of
human fascioliasis. For proper exclusion of S.mansoni infection we should have made rectal snip or
 sigmoidoscopy, but most of the study subjects refused to perform such invasive techniques.
 Therefore we performed for them only 3 Kato-Katz smears and PCR amplifying the 121 bp tandem
 repeat on their stool samples. The minimum detection limit for the PCR amplifying the 121 bp
tandem repeat was reported to be 2.4 eggs/gram of feces by Pontes et al. (2002). Therefore, the
cases of groups B and C most probably true negative cases for S.mansoni infection. However, false
negative S.mansoni infection with egg number < 2.4 eggs/gram of feces cannot be excluded.

Our study detected higher detection rate of positive Fasciola or S.mansoni in stool samples
using BfrI primers than with using Dral primers in all the groups A, B and C indicating higher
detection rate of PCR using BfrI primers for either Fasciola or S.mansoni infection. This coincides
with our results on either of the 2 parasite strains, which revealed higher DNA yield using BfrI
primers than with using Dral primers in spite of same starting extracted DNA concentration.

Moreover, our study reported that the DNA yield of S.mansoni was significantly higher than
that of Fasciola using either of BfrI or Dral primers. This coincides with the higher detection rate of
S.mansoni (80%) than that of Fasciola (66.7%) using BfrI primers in groups A and B respectively.
This also coincides with the higher detection rate of S.mansoni (40%) than that of Fasciola
(33.3%) using Dral primers in groups A and B respectively. It worth noting that the DNA yield of
S.mansoni strain using BfrI primers was 1136.5 ± 6.1 compared to 68.1 ± 1.6 of Fasciola, and that
the DNA yield of S.mansoni strain using Dral primers was 166.2 ± 2.5 compared to 12.9 ± 1.8 of
Fasciola.

Failure of PCR using Dral & BfrI primers to detect Fasciola or S.mansoni parasites DNA in
clinical samples of groups A and B in spite of their capability to do that in pure strain samples and
their high DNA yield may be attributed to low intensity infection of missed samples together with
uneven distribution of eggs throughout the clinical sample unlike the pure strain sample in addition
to the possibility of occurrence of partial DNA degradation.

Therefore, the missed 2 cases of S.mansoni using BfrI primers in group A (20%) that were
positive by Kato-Katz and PCR amplifying the 121 bp tandem repeat, may be explained by Low
intensity of infection (egg count was 6.7 and 20 eggs / gram of feces in the 2 cases), uneven
distribution of eggs in feces and partial DNA degradation. Partial DNA degradation could have led to
the degradation of the target sequence of the PCR using BfrI primers but not all the 121bp tandem
repeat DNA sequence which was reported by Hamburger et al. (1991) to have high copy number
(600,000/cell) comprising 10% of the S.mansoni genome.

Also, partial DNA degradation with uneven distribution of eggs in feces and low intensity
infection may explain the case in group A with positive PCR using Dral primers and negative PCR
using BfrI primers in spite of the finding of our work that the DNA yield of S.mansoni strain using
BfrI primers was much significantly higher than with using Dral primers. Partial DNA degradation
could have degraded the 356 bp DNA segment amplified by the BfrI primers and not the 263 bp
DNA segment amplified by Dral primers.

Members of group C of clinical samples were negative for both S.mansoni and Fasciola
parasites. These cases were diagnosed by 3 negative Kato-Katz thick smears. 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 of either Fasciola or S.mansoni were missed by 3 Kato-Katz
smears. Negative S.mansoni, was also diagnosed by the PCR amplifying the 121 bp tandem repeat
which has a detection limit of 2.4 eggs/gram of feces as reported by Pontes et al. (2002). Therefore,
the positive 2 cases of group C using BfrI or Dral primers, were false negative cases and

most probably *Fasciola* cases. However, being *S.mansoni* with egg count less than 2.4 eggs/gram of feces or with egg count < 6.7 eggs/gram of feces due to negative PCR amplifying the 121 tandem repeat of *S.mansoni* because of partially degraded DNA cannot be excluded. Moreover, the remaining negative 8 cases using BfrI or Dral primers, can be true negative cases, but false negative cases with low intensity of infection cannot be excluded.

**CONCLUSION:**

In conclusion, using BfrI and Dral primers amplifying the 356 bp and 263 bp fragments of 18S *Fasciola* rDNA in diagnosing human fascioliasis with this ascertained cross reactivity with *S.mansoni* at the level of both pure strains and clinical samples, in an area where both *S.mansoni* and *Fasciola* parasites are endemic, will lead to erratic diagnosis and thus wrongly directing both patient’s therapeutic regimen and community based control programs. Therefore PCR using these primers cannot be used for the diagnosis of *Fasciola* in individuals exposed to *S.mansoni* infection except after exclusion of infection with this parasite. This means that it can be used for the diagnosis of *Fasciola* in in areas not endemic for bilharziasis in individuals not exposed to infection with Bilharziasis. Conversely, PCR amplifying the 121 bp tandem repeats of *S.mansoni* showed no cross reaction with *Fasciola* together with high sensitivity that was previously detected. Therefore it can be used in the diagnosis of *S.mansoni* in areas endemic for both of *S.mansoni* and *Fasciola*.

It is recommended to further study BfrI and Dral primers on a larger number of *Fasciola* positive and negative cases, in areas endemic for *Fasciola* but not for *Schistosoma*, in order to evaluate their value in the diagnosis of human fascioliasis in these areas. It is also recommended to evaluate other *Fasciola* species primers that can solve the dilemma of cross reactivity with *Schistosoma* species and reach to decisive diagnostic protocol before any widely scaled application of PCR in diagnosis of that parasite in areas like Egypt where the 2 parasites coexist.

**REFERENCES**


