Diagnosis of Schistosoma haematobium by Detection of Specific DNA Fragments from Filtered Urine Samples

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Abstract. Definitive diagnosis of Schistosoma haematobium infection in adult patients is a clinically important challenge. Chronically infected adults pass few eggs in the urine, which are often missed when current diagnostic methods are used. In the work presented here, we report on an alternative diagnostic method based on presence of the S. haematobium-specific Dra 1, 121 bp repeat fragment in human urine. A novel method of collecting the urine specimens in the field and filtering them through heavy Whatman No. 3 paper was introduced. After drying, the samples remained viable for several months at room temperature. To test the potential utility of this method, 89 urine specimens from school children in Kollo District, Niger, were examined. In all, 52 of 89 (58.4%) were positive for hematuria, 4 of 89 (49.4%) were positive for eggs, and 51 of 89 (57.3%) showed parasite-specific DNA. These were compared with 60 filtered urine specimens obtained from random samples of adults from two study sites in Nigeria, one endemic and one non-endemic for S. haematobium. In the 30 patients from the endemic site, all 10 samples with detectable eggs and 7 of the 20 egg-negative samples were DNA positive. It was concluded that the urine filter paper method was sufficiently sensitive to detect low and cryptic infections, that DNA detection was more sensitive than egg detection, and that the filtration method facilitated specimen collection and transport from the field.

INTRODUCTION

The gold standard for diagnosis of schistosomiasis is the microscopic detection of parasite eggs present in urine or stool; however, parasitological diagnosis of schistosomiasis in adults is difficult, particularly among persons who have chronic infections and pass only small numbers of eggs. This fact has resulted in clinicians resorting to rectal biopsy for chronic infections and pass only small numbers of eggs. This is difficult to set anything like a “gold” standard in areas with variable diagnostic performance of these techniques is variable and it is difficult to set anything like a “gold” standard in areas with variable S. haematobium prevalence. Recently, polymerase chain reaction (PCR) assays have shown potential as an effective method for the detection of parasite DNA in saliva and urine. The PCR was used to detect S. mansoni DNA in human fecal samples. It was based on amplification of DNA sequences from S. mansoni cercariae. In Ghana, Obeng and others examined urine specimens taken from children 9–14 years of age to detect circulating cathodic antigen, and then transported frozen specimens to Holland where they performed real-time PCR using ITS2 sequences for S. haematobium. They showed that when compared with eggs in the urine, the PCR test was 100% sensitive, yet specificity was low. The detectable product was dependant on the number of eggs passed in the specimen and it was postulated that the template DNA was derived from the eggs. In other studies, the use of PCR has been reported for diagnosing female genital S. haematobium infection, low intensity of Schistosoma japonicum infections in stool samples, and for detection of schistosomes such as S. mansoni when present with other parasitic co-infections.

Schistosomes have extensive repeat sequences in their genome, a feature that was exploited by Hamburger and others in the development of an PCR-based test for S. haematobium cercariae in snails. The 121 bp Dra 1 tandem repeat sequence constitutes ~15% of the entire S. haematobium genome and primers designed to amplify this repeat by PCR showed use in the detection of S. haematobium DNA from

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free-living cercariae and infected snails. This information, and evidence that detection of *Plasmodium* DNA in body fluids has been demonstrated, suggested that the *Dra* 1 tandem repeat could serve as a target for the detection of low-level *S. haematobium* infections in humans. Because of the high level of *Dra* 1 repeats in the parasite genome, we postulated that these may be detectable in urine by use of specific primers.

In this study, we adapted the 121 bp *Dra* 1 tandem repeat PCR developed by Hamburger and others to detect *S. haematobium* DNA in urine collected on dried filter paper. In specimens from both children and adults the *Dra* 1 fragments were detected when schistosome eggs were seen in the specimens and when no eggs were detected. The *Dra* 1 fragments were not seen in specimens from people living in non-endemic areas. The process of filtration as described greatly facilitates collection, storage, and transport of urine specimens.

**MATERIALS AND METHODS**

**Urine samples.** Specimens were collected from children under the auspices of the Schistosomiasis Control Initiative (SCI) in Kollo District, Niger. After informed consent from parents and teachers, 89 urine specimens were collected from school children between 10 and 15 years of age. Additionally, we obtained 30 specimens from adults in a high transmission area in Nigeria, and 30 specimens from a low transmission area as part of a study carried out in Ogun Province, Nigeria. These individuals were between 20 and 59 years of age. The demographics of the patients are outlined in Table 1.

All specimens were processed and filtered in the field in the following manner. The urine sample was taken between the hours of 10:00 and 14:00 for optimum egg passage and tested with a Hemastix (Bayer Corp., USA) test strip to detect hematuria. Approximately 50 mL urine was passed through a 12.5 cm Whatman No. 3 (Whatman International, Maidstone, England) filter paper, folded in a cone. This grade of paper was selected because it is coarse, maintains a cone shape when folded, and it retained both schistosome eggs and DNA fragments from urine during filtration. The cone was set in a plastic cup to collect the filtered urine. The cups were used only once and care was taken to avoid contamination. Folded cones only present half of the surface area to particulate matter, hence the exposed area is marked and these quadrants are used for egg detection and DNA extraction. Inner quadrants were used for replication.

After filtration, the paper disc was opened and allowed to dry in a fly-proof box before being packed with desiccant in individual plastic sleeves. The specimens were kept under desiccant after drying and maintained as such until use. They were used for DNA extractions after ~3 months of storage. For analysis, the central 2 cm portion of the filter was excised and divided into quadrants.

**Egg detection and DNA extraction.** One exposed quadrant of paper was stained with 0.2% ninhydrin in ethanol and the eggs counted. The solution was sprayed on the filter paper and allowed to develop in the dark for at least 15 min before examination under a dissecting microscope.

For DNA extraction, one quadrant of the 2 cm disc was cut in half, and one such segment was sliced into 2–3 mm squares that were placed into a 1.5 mL eppendorf tube containing 600 μL nuclease-free water. This was incubated at 95°C for 10 min and then shaken on a rotator at room temperature overnight (12 hrs). The tube was then centrifuged at 3,000 rpm for 10 min and the water transferred to a second sterile 1.5 mL tube. The DNA was precipitated and concentrated using the Qiagen QiAmp mini-kit (Qiagen Sciences, MD) according to the manufacturer’s protocol. The DNA concentration was measured using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, USA).

**PCR amplification.** The PCR was carried out by employing specific primers (forward: 5′-GATTCACCTATCGAGCAGAAC-3′ and reverse: 5′-TCACACGATAACGC AAC-3′) previously designed by Hamburger and others, for specific amplification of *Dra* 1 repeats of *S. haematobium*. The reaction volume of 25 μL consisted of 1.25 units Taq DNA polymerase, 2.5 μL 10x buffer, 1.5 mM MgCl2, 200 μM (each) of dATP, dCTP, dGTP, and dTTP, 1 μM of each of the amplification primers and 5 μL (3 ng) of template DNA. There was an activation/denaturing step of 15 min at 95°C, followed by 33 cycles of 95°C for 30 sec, annealing temperature of 53°C for 1.5 min, and expansion at 72°C for 1 min, followed by a final extension step at 60°C for 5 min. Amplification was conducted in 0.2 mL PCR tubes in a thermal cycler. The products were analyzed on a 2% agarose gel stained with ethidium bromide (10 mg/mL) and visualized with UV light. The size marker 100 bp ladder was used to estimate band sizes.

**RESULTS**

The results of preliminary experiments using *S. haematobium* genomic DNA as template showed that the 121 bp amplicon and the higher-order forms of the *Dra* 1 repeat could be readily visualized on an agarose gel (Figure 1, lane 2 positive control). A similar amplicon pattern was seen when the DNA isolated from the urine of egg-positive specimens was used as the template (Figure 1, lanes 3–7). Because it was consistent in all egg-positive specimens, except one, it was deemed consistent and indicative of *S. haematobium* infection even in the absence of detectable eggs.

![Figure 1](https://example.com/figure1.png)
Comparison of sensitivity and specificity of polymerase chain reaction (PCR), parasite eggs, and hematuria in children from Kollo District, Niger*

<table>
<thead>
<tr>
<th>Condition</th>
<th>PCR</th>
<th>Eggs</th>
<th>Hematuria</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>43</td>
<td>37</td>
<td>0</td>
</tr>
<tr>
<td>−</td>
<td>1</td>
<td>8</td>
<td>37</td>
</tr>
</tbody>
</table>

Total | 51 | 52 | 37 |
SN | 98% | 100% | 82% |
SP | 82% | 84% | 45% |

Table 2

We have shown that there is an important disparity between PCR results and presence of detectable eggs in both groups of endemic individuals. In Tables 2 and 3 data from both groups are presented for comparison to show the sensitivity and specificity of the three tests. When children were tested there was no real difference between hematuria, presence of eggs, or Dra 1 DNA. Comparison between DNA positivity in adults and children from endemic areas was significantly different by χ² (P = 0.0001).

DISCUSSION

For this study, a novel urine filtration method was used to extract DNA from 149 filter papers and to detect schistosome eggs trapped during the filtration process. The volunteers were mainly school children in Niger, but samples were augmented by 60 specimens taken from adults in Western Nigeria. There was concurrence between hematuria, presence of eggs, and DNA, however, presence of schistosome-specific DNA was the foremost indicator of infection. It was detected in 17 adult specimens where eggs were present in only 10 and in 51 specimens from children of whom eight were without eggs. The DNA recovery pattern was not dose dependent on the number of eggs seen.

Diagnosis of S. haematobium infection is not always straightforward. Passage of eggs through the urine can be complicated by both host and parasite interaction. The parasite responds to circadian rhythms of the host15 as well as the individual immune response and nature of pathological damage in the bladder wall. Diagnostic procedures must depend on the specific needs of epidemiological or clinical studies. For instance, the use of the Hemastix test in an epidemiological situation when speed, sensitivity, and low cost are important and specificity can be considered a secondary factor. In these situations the populations under study are usually children where the correlation between hematuria and parasitemia is high. However, in the clinical situation this may not be optimum, particularly when considering adults where the need for high sensitivity and specificity is very important. This was demonstrated by Koukounari and colleagues6 who evaluated the efficacy of different diagnostic tools including serology, antigen capture, microscopy, ultrasound scans of the bladder, and detection of hematuria. Their study showed that it was difficult to define a “gold” standard for diagnosis of this parasite in adults. There is need for a test with high sensitivity and specificity that is simple to use and that avoids the need for bulk storage of specimens and even the need for freezing and expensive transport.

There is added cost involved in employing DNA-based technology, but the facilities needed are being introduced progressively in endemic countries and can be used if necessary.
Comparison of sensitivity and specificity of polymerase chain reaction (PCR), parasite eggs, and hematuria in adults from Ogun Province, Nigeria

<table>
<thead>
<tr>
<th>Condition test</th>
<th>+</th>
<th>-</th>
<th>Total</th>
<th>SN</th>
<th>SP</th>
<th>PCR</th>
<th>+</th>
<th>-</th>
<th>Total</th>
<th>SN</th>
<th>SP</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR</td>
<td>+</td>
<td>10</td>
<td>7</td>
<td>17</td>
<td>100%</td>
<td>86%</td>
<td>-</td>
<td>0</td>
<td>43</td>
<td>43</td>
<td></td>
</tr>
<tr>
<td>Eggs</td>
<td>+</td>
<td>10</td>
<td>10</td>
<td>20</td>
<td>100%</td>
<td>80%</td>
<td>-</td>
<td>0</td>
<td>40</td>
<td>40</td>
<td>94%</td>
</tr>
<tr>
<td>Hematuria</td>
<td>+</td>
<td>10</td>
<td>10</td>
<td>20</td>
<td>100%</td>
<td>80%</td>
<td>-</td>
<td>0</td>
<td>40</td>
<td>40</td>
<td>94%</td>
</tr>
</tbody>
</table>

* SN = sensitivity; SP = specificity. Presence of eggs was tested against Dra 1 and hematuria. PCR fragment was tested against eggs and hematuria.

Obeng in Ghana\textsuperscript{10} and Lier in China\textsuperscript{12} both used real-time PCR to detect molecular evidence of schistosome infection, although this technique is expensive and requires specialized equipment. As a means to detect the presence of blood-borne pathogens, the concept of isolating diagnostic DNA from urine has been applied to malaria\textsuperscript{2} and conceptually this could also be applied to the detection of schistosome DNA fragments in urine. The repeat nature and volume of schistosome DNA, as shown by Hamburger and others,\textsuperscript{3} suggested that the Dra 1 fragment would be a good target and this appears to be the case described here.

Although it is difficult to distinguish between DNA fragments in urine and those extracted from eggs, the important thing is that Dra 1 fragments were detected in specimens where eggs were found and in specimens where no eggs were seen. The presence of Dra 1 also appears independent of the number of eggs passed in specimens. Thus, it is likely but not yet proven that the DNA was present free in the urine. The method does appear to be useful both clinically and in the field and the filtration technique facilitates transport and storage of urine specimens required for this analysis. Additional studies are ongoing to expand this work in adult populations in Nigeria.

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REFERENCES