DifferENTiation of Schistosoma Haematobium from Related Schistosomes by PCR Amplifying an Inter-Repeat Sequence

IBRAHIM ABBASI,* CHARLES H. KING, ROBERT F. STURROCK, CURTIS KARIUKI, ERIC MUCHIRI, AND JOSEPH HAMBURGER

Kuvin Center, Hebrew University, Hadassah Medical School, Jerusalem, Israel; Center for Global Health and Diseases, Case Western Reserve University, School of Medicine, Cleveland, Ohio; London School of Hygiene and Tropical Medicine, London, United Kingdom; Division of Vector Borne Diseases, Ministry of Health, Nairobi, Kenya; Kenya Medical Research Institute, Nairobi, Kenya

Abstract. Schistosoma haematobium infects nearly 150 million people, primarily in Africa, and is transmitted by select species of local bulinid snails. These snails can host other related trematode species as well, so that effective detection and monitoring of snails infected with S. haematobium requires a successful differentiation between S. haematobium and any closely related schistosome species. To enable differential detection of S. haematobium DNA by simple polymerase chain reaction (PCR), we designed and tested primer pairs from numerous newly identified Schistosoma DNA repeat sequences. However, all pairs tested were found unsuitable for this purpose. Differentiation of S. haematobium from S. bovis, S. mattheei, S. curassoni, and S. intercalatum (and not from S. margegrewi) was ultimately accomplished by PCR using one primer from a newly identified repeat, Sh110, and a second primer from a known schistosomal splice-leader sequence. For evaluation of residual S. haematobium transmission after control interventions, this differentiation tool will enable accurate monitoring of infected snails in areas where S. haematobium is sympatric with the most prevalent other schistosome species.

INTRODUCTION

Schistosoma haematobium, which causes urinary schistosomiasis in humans, afflicts ~150 million people in 53 countries in Africa and the Middle East. In many areas, S. haematobium is sympatric with related schistosome parasites (most of other mammals) (i.e., S. bovis, S. mattheei, S. curassoni, S. intercalatum, and S. margegrewi), and these related species are known to develop in the same bulinid intermediate snails that transmit S. haematobium. Because S. haematobium and its related schistosome species often infect snails inhabiting the same bodies of water, the assessment of the transmission of S. haematobium by examining cercariae or infected snails is confounded by the need to differentially identify S. haematobium. Of the schistosome species related to S. haematobium, S. bovis is the most widespread, with reported distribution in the Middle East, the Mediterranean basin, and most parts of Africa. The other species of schistosomes examined in this study, S. mattheei, S. margegrewi, and S. curassoni, are also present in various parts of Africa, although less prevalent. In addition, S. intercalatum, which causes mixed intestinal and urinary schistosomiasis in humans, can also be found in transmission foci in Africa that are co-inhabited by S. haematobium. Previously, the differential identification of snails infected by S. haematobium needed careful examination and testing of shed cercariae. Given that cercariae belonging to the S. haematobium–related species are not readily distinguishable morphologically from others within the group, a variety of approaches have been taken for differential identification, with the standard method being infection of laboratory animals and subsequent parasite species identification based on the morphology of the adult worms. The importance of discriminating S. haematobium from other species later led to the development of molecular approaches, which have included isoenzyme profiling.

Southern blot analysis employing rDNA gene probes, randomly amplified DNA, and polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) analysis of the ITS2 region. Amplification by simple PCR using defined primers, considered more suitable for large-scale screening, has not been accomplished for this purpose thus far.

We have recently accomplished identification of cercariae and of snails infected with S. haematobium by using primers designed from a repeated sequence, DraI, in a PCR assay. DraI was not found in S. mansoni or S. japonicum by dot hybridization. However, because the DraI repeat is present in DNA of other schistosomes belonging to the S. haematobium group, the identification of S. haematobium–infected snails by a simple PCR assay has been limited, thus far, to areas where these other S. haematobium–related schistosome species are absent or very rare.

PCR, unlike other methods previously used for identifying snails with prepatent infection, has enabled detection of snail infection from its very earliest stages. It thus identifies the entire population of infected snails, regardless of whether they eventually shed cercariae, representing in quantitative terms the direct outcome of human contamination of water bodies. This approach has been applied to large-scale monitoring of infection in field snails at transmission sites in Coast Province, Kenya. Because differential identification of snails infected with S. haematobium is important for further evaluation of integrated control approaches in other endemic regions, we have undertaken a search for DNA sequences from which primers can be designed for differential identification of S. haematobium by means of simple PCR techniques.

MATERIALS AND METHODS

Parasites. Adult worms, preserved in liquid nitrogen as part of the schistosome collection at the Natural History Museum, London, UK, were preserved in ethanol and shipped, and kept in ethanol until subjected to DNA extraction in Jerusalem. These included S. haematobium from Cameroon, Mauritius, and Zanzibar; S. bovis from Tanzania and Senegal; S. margegrewi from Zambia; S. curassoni from Senegal; S.
from Zambia and Malawi; and S. intercalatum from Cameroon and Zaire.

**Normal and infected snails.** Bulinus nasutus field snails were collected during 2001 from transmission sites in the Msambweni area of Kwale District, Coast Province, Kenya. Details of the study area have been described elsewhere. For additional genomic libraries

Briefly, the worms were kept in lysis buffer containing (0.1 mol/L Ethylenediaminetetraacetate, pH 8.0, 0.1 mol/L Tris-HCl, pH 7.5, 0.2 mol/L NaCl, 1% SDS, 0.2% 2-mercaptoethanol, and 100 μg Proteinase K) at 65°C for 1–2 hours. This was followed by phenol and chloroform extraction.

DNA from snails was extracted by the hexadecytrimethylammonium bromide (CTAB) extraction method as previously described. Briefly, snail bodies were removed from their shell and incubated in 0.6 mL of lysis buffer (100 mmol/L Tris base, 20 mmol/L EDTA, 1.4 mol/L NaCl, 0.2% β-mercaptoethanol, and 2% CTAB) and Proteinase K. After incubation for 3 hours at 60°C, nucleic acids were extracted twice by chloroform and precipitated with ethanol.

**Construction of genomic DNA libraries.** Genomic libraries were prepared from DNA of all the schistosome species included in this study. Materials for recombinant DNA technology, including enzymes used for restriction, ligation, and dephosphorylation, were from MBI Fermentas (Vilnius, Lithuania). Genomic DNA was partially digested by Sau3A restriction endonuclease, followed by phenol/chloroform extraction and ethanol precipitation. Digested DNA was ligated into the BamHI site of the Bluescript plasmid (Stratagene, La Jolla, CA), by standard methods. For additional genomic libraries of S. haematobium, DNA was digested by Alul or RsaI, and digestion products ligated into the Smal site of the Bluescript plasmid. Ligation products were transformed into Escherichia coli, strain XL1-Blue, and were plated for color selection of recombinants by standard methods.

**Selection of repetitive DNA clones.** The selected white recombinant colonies were transferred onto nylon filters and probed with 32P-labeled total S. haematobium genomic DNA. Colony lysis, DNA denaturation, and fixation onto the nylon filter were carried out in a microwave oven as previously described. Clones initially selected were those that exhibited strong hybridization signals with the homologous total DNA probe, indicating the presence of repeated sequences. These were transferred to a grid on a nylon filter, and a second level of selection was applied by hybridization with the DraI repeat of S. haematobium, shared by all of the schistosome species studied in this paper. Colonies not hybridizing with the DraI probe underwent a third level of selection involving cross-hybridization with total DNA from the other species and selection of those giving hybridization signals with S. haematobium DNA only. A fourth level of selection of candidate clones followed sequence analysis, with selection of those that exhibited the following features: 1) they were not similar to the DraI repeat; 2) they were not similar to known sequences from the other schistosomes included in this study nor to schistosomal sequences present in GenBank; and 3) when aligned with other sequences of the same species, sequences thereof were found to be represented more than once in the same species, suggesting a relatively high copy number.

**Dot-blot and Southern blot analysis.** Comparative dot-blot hybridization was performed to estimate the abundance of the sequence amplified by applying PCR using the selected primers (see below). Various concentrations of S. haematobium genomic DNA (ranging from 0.01 to 10 ng) and DNA of the said amplification product, ranging between 0.1 and 10 pg, were dotted onto a nitrocellulose membrane using a dot-blot apparatus (BioRad, Hercules, CA). These underwent denaturation and neutralization by standard procedures and were finally hybridized with radiolabeled total DNA probe and with the cloned amplification product probe.

Radiolabeling with 32P of genomic DNA, repetitive DNA clones, or PCR products used as probes was done by the Random Primer DNA Labeling Kit (Biologic Industries Co., Beit Haemek, Israel). Analysis of the dot hybridization signals was performed using the public domain NIH Image program (developed at the Research Services Branch at the US National Institute of Mental Health and available on the Internet at http://rsb.info.nih.gov/nih-image/about.html).

Southern blot analysis was performed, according to standard protocols for determining the distribution of the amplified DNA sequence (assumed to represent the DNA segment stretching between the primer sequences used). S. haematobium genomic DNA (1 μg) was partially digested by RsaI or Alul restriction enzymes followed by Southern transfer to nitrocellulose filter and probing by the corresponding amplification product.

**Filter hybridization.** This was done according to standard procedures. The filters were incubated for 2 hours at 65°C in a prehybridization solution (6× sodium chloride, sodium citrate [SSC], 10× Denhardt solution, 0.1% SDS, and 100 μg/mL denatured salmon sperm DNA). The radiolabeled probe was directly added to the filters in the prehybridization solution at 60°C for overnight. The filters were washed once in 2× SSC at room temperature for 30 minutes, followed by two washes with 2× SSC, 0.1% SDS at 60°C for 15 minutes each time. Filters were air-dried and exposed to x-ray film.

** Primer design and PCR assay.** Primers were designed by using the PRIME software version 9.0, of the Wisconsin Package (Genetic Computer Group [GCG], Madison, WI) based on the sequence information of the newly described repeated sequences of S. haematobium selected for these experiments. The primer combination ultimately enabling specific amplification of S. haematobium DNA included one primer (Sh110: 5′ TTCCTCCAACTACCATCTTCCTTC) based on a newly identified S. haematobium repetitive sequence termed Sh110 (Figure 1A), and a second primer (Sm-SL: 5′AACGCACGTTTATCTGGT) based on the highly abundant S. mansoni splice leader sequence. The degree of similarity of the S. mansoni splice leader sequence to that of S. haematobium and of other schistosomes is likely to be high, given that the same splice leader primer sequence is...
found (with complete identity) in protein mRNA sequences of *S. japonicum*, as shown by a Blast search in the GenBank.

PCR assays for sensitivity and specificity studies were carried out in a total volume of 100 µL containing 200 µmol/L of each dNTPs, 25 pmol of the two amplified primers, 2.5 units of *Taq* polymerase (MBI Fermentas, Vilnius, Lithuania), and the target DNA. *Taq* polymerases from various other sources were also used for establishing reproducibility. These included Red Hot *Taq* Polymerase (Advanced Biotechnologies, Epsom, UK), a Sigma *Taq* Polymerase (Sigma-Aldrich, St. Louis, MO) and a *Taq* Polymerase from an unspecified source. The reaction buffer consisted of 10 mmol/L tris-HCl, pH 8.3, 25 mmol/L KCl, and 3.5 mmol/L MgCl$_2$. A thermal cycler (MJ Research, Watertown, MA) was used with a thermal profile involving 5 minutes at 95°C, followed by 35 cycles each of 1 minute at 95°C, 1 minute at 50°C, followed by 1 minute at 72°C, and a final elongation step at 72°C for 10 minutes.

For detecting *S. haematobium*–infected snails by PCR, preliminary experiments were carried out for establishing DNA extraction conditions and PCR conditions by testing snail bodies spiked with schistosomal DNA. Further examinations were carried out with infected and uninfected field-collected snails. Conditions for snail PCR have been described elsewhere.$^13$

### Nucleotide sequencing

Plasmid Isolation Kit (Quiagen, Chatsworth, CA) was used according to the instructions of the manufacturer for obtaining DNA from recombinant plasmids selected for sequence analysis. Nucleotide sequence analysis was carried out using Dye Terminator Cycle Sequencing kit (Perkin Elmer, Wallington, UK) according to the instructions of the manufacturer. An ABI PRISM 377 DNA Sequencer and ABI Sequencing Analysis software (Perkin Elmer, Foster City, CA) were used. The thermal profile was 10 seconds at 96°C, 5 seconds at 50°C, and 4 minutes at 60°C for 25 cycles. Consensus sequence was analyzed by employing the Wisconsin Package (Genetic Computer Group, Madison, WI).

### Results

#### Search for repeated sequences and primers specific for *S. haematobium*

An extensive preliminary search (Table 1) failed to identify repeated DNA sequences that were suitable, by themselves, for differentiation of *S. haematobium* by direct PCR (unpublished data).† We next examined an alternative possibility to accomplish this goal. This involved attempts at inter-repeat amplification using mixed primer pairs—one end, a primer designed from newly identified *S. haematobium* repeat sequences, and on the other end, a primer based on known *Schistosoma* splice-leader sequence (taken from *S. mansoni* sequence$^19$ and common to *S. japonicum*). A total of 52 such mixed primer pairs were used in this part of the search (Table 1). The primer designed from the newly identified repeated sequence clone Sh110 (Figure 1A) in combination with the splice leader primer, Sm-S1, (Figure 1B), were selected for further testing for differential PCR identification of *S. haematobium* based on an initial demonstration of differentiation from *S. bovis*.

### Structure, abundance, and arrangement of the Sh110 repeated sequence

This 120-bp sequence, derived from a Sau3AI library of *S. haematobium* (Figure 1A), is flanked by a GAT-rich region with GAT repeated 50 times (results not shown). Although our study detected the presence of this repeated sequence in *S. haematobium*, its relation to any particular repeated gene or non-coding sequence of *S. haematobium* is yet unknown.

The abundance of the Sh110 sequence has not been directly determined but can be inferred, with some approximation, from the abundance of the amplified sequence (see below).

The arrangement of the Sh110 repeated sequence was determined by Southern blot analysis using a Sh110 clone as probe. Several bands of different sizes were obtained (results not shown), as was also found with the Sm-S1/Sh110 PCR product (see below), but there was no ladder banding pattern typical of tandem repeated sequences.$^{19,20,21}$ These results suggested that the Sm110 repeat sequence is dispersed in the genome.

### Structure, abundance, and arrangement of the Sh110/Sm-S1 PCR product

The DNA amplification by PCR using the Sh110 and Sm-S1 primers yielded a 525-bp product (Gen-

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Table 1

<table>
<thead>
<tr>
<th>Species included</th>
<th>Library constructed by</th>
<th>NOCs selected$^a$</th>
<th>NOCs sequenced (%)$^b$</th>
<th>NOCs selected for Sh-diff</th>
<th>NOCs tested for Sh-diff</th>
<th>NOP** tested in intra-repeat PCR$^c$</th>
<th>NOP** used with the Sm-S1 primer</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. haematobium</em></td>
<td>Sau3AI</td>
<td>68</td>
<td>7 (10.3)</td>
<td>5</td>
<td>5</td>
<td>15$^d$</td>
<td>10</td>
</tr>
<tr>
<td><em>S. haematobium</em></td>
<td>AluI</td>
<td>147</td>
<td>55 (37.4)</td>
<td>15</td>
<td>9</td>
<td>18$^e$</td>
<td>18</td>
</tr>
<tr>
<td><em>S. haematobium</em></td>
<td>RsuI</td>
<td>236</td>
<td>95 (40.2)</td>
<td>4</td>
<td>4</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td><em>S. matthei</em></td>
<td>Sau3AI</td>
<td>103</td>
<td>21 (20.4)</td>
<td>4</td>
<td>2</td>
<td>6$^f$</td>
<td>4</td>
</tr>
<tr>
<td><em>S. bovis</em></td>
<td>Sau3AI</td>
<td>43</td>
<td>20 (46.5)</td>
<td>6</td>
<td>4</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td><em>S. intercalatum</em></td>
<td>Sau3AI</td>
<td>88</td>
<td>18 (20.4)</td>
<td>4</td>
<td>2</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td><em>S. margrebowiei</em></td>
<td>Sau3AI</td>
<td>64</td>
<td>17 (25.6)</td>
<td>4</td>
<td>2</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td><em>S. curassoni</em></td>
<td>Sau3AI</td>
<td>64</td>
<td>17 (25.6)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>813</td>
<td>250</td>
<td>42</td>
<td>28</td>
<td>63</td>
<td>52</td>
</tr>
</tbody>
</table>

$^a$ Hybridization with homologous schistosomal DNA yielded strong hybridization signals, characteristic of highly repetitive sequences.

$^b$ These clones exhibited cross hybridization with DNA of *S. haematobium* but not with DNA of the other *Schistosoma* species, nor with the *D. rotundum* repeated$^{14}$ sequence.

$^c$ Most of the primers were used in both intra and inter repeat amplifications.

$^d$ For some clones more than one pair of primers were designed;

NOC$^*$ = number of clones; NOP** = number of primers.

† The structure and other characteristics of the various repeated sequences found in the course of our search are beyond the scope of the present report, which centers on differential identification of *S. haematobium*, and will be detailed in a future publication. Those 27 clones that provided primers for inter-repeat amplification with the Sm-S1 primer were deposited in the GenBank (accession nos. DQ831697–DQ831706).
Bank accession no. DQ831696). The position of the 5' end was inferred from the known position of the *S. mansoni* splice leader sequence. The PCR product's sequence and the corresponding primers are presented in Figure 2. A Blast search in the GenBank identified a high degree of sequence homology between parts of this PCR product (93- to 122-bp-long segments) and a number of schistosomal sequences, including *S. haematobium* acetylcholinesterase gene (accession no. AY167025). However, the amplified sequence, as presented in its entirety (Figure 2), is not represented in the GenBank.

The abundance of the PCR product in the schistosomal genome was determined by comparative dot blot hybridization, as previously described for repeated sequences of *S. mansoni*, *S. haematobium*, and *Echinococcus granulosus*. Signal intensity obtained with 50 ng of total *S. haematobium* DNA was similar to that obtained with 0.1 ng of Sm-Sl/Sh110 PCR product. Thus, the Sm-Sl/Sh110 PCR product constitutes ∼0.002% of the genome. Given the estimated genome size of *S. haematobium*, 2.7 × 10^9 bp, the approximate copy number of Sm-Sl/Sh110 repeat PCR product (525 bases) is 1,026 copies. This figure, however, is much higher than the copy number of the *S. mansoni* splice-leader sequence and may represent a higher number of splice-leader sequences in *S. haematobium* and/or closely related sequences, which could initiate amplification.

The arrangement in the *S. haematobium* genome of the DNA segment amplified by Sm-Sl/Sh110 PCR seems to be dispersed given that several bands of different sizes were exhibited (Figure 3). Bands with corresponding sizes of about 260 bp (Figure 3, lane 1-RsaI cut DNA) and both 260 and 520 bp (Figure 3, lane 2-AluI cut DNA) were exhibited, the latter identical to the size of the amplification product. Given that discrete bands were obtained by Southern hybridization with the corresponding probe (Figure 3), it is unlikely that hybridization occurred with small segments having only partial sequence homology (e.g., parts of the splice leader sequence) but rather with the full sequence of the amplification product or with large parts of it.

**Differential identification of *S. haematobium* by PCR: detection sensitivity and specificity.** Sensitivity of detection by PCR was determined using different concentrations of *S. haematobium* genomic DNA. As shown in Figure 4, 1 pg of *S. haematobium* DNA could be detected in the standard Sm-Sl/Sh110 PCR reaction. For specificity of detection, varying concentrations of genomic DNA from *S. haematobium* and related schistosome species were subjected to PCR, and their products examined by gel electrophoresis (Figure 5). Results indicate that 1 pg of *S. haematobium* DNA was clearly detectable by Sm-Sl/Sh110 PCR, whereas no amplification was exhibited with 1 and 10 ng DNA (1000–10,000 times more DNA) of *S. bovis* or *S. mattheei*. The other species examined exhibited PCR positivity with 10 ng DNA, four orders of magnitude more than was needed to obtain a positive amplification signal with *S. haematobium* DNA. This quantitative difference can enable differentiation of *S. haematobium* but not differential identification of the other schistosome species.
Although the splice leader sequence was not examined in this study, it yielded an ∼S. haematobium sequence that exhibits a second amplification band (of ∼450 bp) clearly distinguishable from that of S. haematobium, as well as from the other species examined. On the other hand, S. margrebowiei, regularly exhibited a single band indistinguishable from that of S. haematobium and therefore the Sm-Sl/Sh110 PCR assay cannot be used to distinguish it from S. haematobium.

Although S. haematobium DNA amplification and amplification of DNA from S. haematobium–infected snails regularly yield a single amplification band (Figures 3–5), a second band of ∼300 bp sometimes appears when ≥10 ng S. haematobium DNA is examined (results not shown). Differentiation between S. haematobium and S. intercalatum, the other species that exhibits a second amplification band (of ∼450 bp in this case), remains possible by the different size of the second band, as well as by the higher amount of DNA required for obtaining amplification products with S. intercalatum DNA.

Detection of S. haematobium–infected snails by PCR. Bulinus nasutus snails, known to be either infected or uninfected with schistosomes based on Drai-PCR testing, were subsequently examined by Sm-Sl/Sh110 PCR. Infected and uninfected snails were clearly distinguished (Figure 6), and sensitivity for S. haematobium DNA detection in snail extracts was further established by addition of DNA to extracts of uninfected snails (results not shown are similar to Figure 4).

DISCUSSION

PCR primers were designed for differential identification of S. haematobium DNA content based on a newly identified repeated sequence of S. haematobium (Sh110; Figure 1A) and a second primer derived from the known splice leader sequence of S. mansoni.19 Although the splice leader sequence analog in S. haematobium was not characterized in the present study, it is assumed that it is closely similar to that of S. mansoni and other schistosomes, given that sequences identical to the splice leader primer were identified in the GenBank among numerous protein mRNA sequences of S. japonicum. There is a high likelihood that the same splice leader primer sequence is present in the genomes of the other species studied. We assume that the presence or absence of the Sh110 sequence and differences in its relative location constitute the basis for the differential identification we have observed in our studies.

Of the S. haematobium–related schistosome species examined in this study, S. bovis is the most widespread and prevalent,7,8 and thus has the widest potential geographical overlap with S. haematobium. The other species examined are less widely distributed. These are the animal schistosomes, S. mattheei, found in southern Africa,7 S. margrebowiei, found in a relatively small area in west and southern Central Africa7,8,9 S. curassoni, found primarily in western Africa,7,23 S. intercalatum, belonging to the same group and causing sporadic schistosomiasis in humans, is found in foci in central Africa within Equatorial Guinea, Sao Tome, Nigeria, and Mali.9 S. leiperi, another animal schistosome, the distribution of which overlaps that of S. margrebowiei in southern Central Africa,7 was not examined in this study.

This report describes the differential identification of S. haematobium from the related schistosomes S. bovis, S. mattheei, S. curassoni, and S. intercalatum by simple PCR. In contrast, the much less common S. margrebowiei was not successfully differentiated from S. haematobium, as it yielded an amplification product of the same size (Figure 5). In practical terms, S. haematobium–infected snails can now be clearly differentiated from snails carrying the most prevalent, related
animal schistosomes in Africa (i.e., S. bovis and S. mattheei), as well as from S. curassoni in areas where it is found alongside S. haematobium. These results will allow extension of large-scale PCR monitoring approaches for snails infected with S. haematobium, which had been previously limited to areas where cross-reacting (DrAl containing) animal schistosome species were absent or very rare.

In addition to differentiation between S. haematobium and its related species, specific identification of S. interalatum (Figure 5) could facilitate better study of the distribution of this species and may also help to better define the effects of competition between S. interalatum and S. haematobium in sympatric endemic areas. The fact that S. curassoni can be differentiated from S. haematobium, as well as from other species tested, may help to resolve doubts about the identity of S. curassoni as a distinct species.

Further validation of the assay is needed for field studies because only a few field-collected snails were examined in this study. Such validation should include a whole range of infected and uninfected snails with different signal intensities (as detected by DrAl PCR) to assess the range of sensitivity among field-collected snails. Although PCR has been shown to be practical for large-scale monitoring of S. haematobium transmission in affected communities, a more inexpensive and robust detection method will greatly facilitate implementation of molecular monitoring of transmission potential in large-scale control projects.

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Authors’ addresses: Ibrahim Abbasi and Joseph Hamburger, Department of Parasitology, Kuvin Center for the Study of Infectious and Tropical Diseases, Hebrew University, Hadassah Medical School, PO Box 12272, Jerusalem 91120, Israel, Telephone: 972-2-6758087, Fax: 972-2-6757425, E-mail: ibrahim@md.huji.ac.il. Charles H. King, Center for Global Health and Diseases, Wolstein 4126, CWRU School of Medicine, 10900 Euclid Avenue, Cleveland, OH, 44106-7286, Telephone: 216-368-4818, Fax: 216-368-4825, E-mail: chk@po.cwru.edu. Robert F. Sturrock, 92 Brennand Road, Dongara WA6525, Australia, E-mail: sturrock@iprimus.com.au. Curtis Kariuki and Eric Muchiri, Division of Vector Borne Diseases, Ministry of Health, PO 20750, Nairobi, Kenya, Telephone: 254-20-725853, Fax: 254-20-720030, E-mail: schisto@wananchi.com.

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