

## Detection of *Schistosoma mansoni* and *Schistosoma haematobium* DNA by Loop-Mediated Isothermal Amplification: Identification of Infected Snails from Early Prepatency

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**Abstract.** Monitoring post-control transmission of schistosomes by examining humans becomes less effective as infection rates among humans decrease. Molecular monitoring of prepatent schistosome infection in snails by the polymerase chain reaction (PCR) has been used for studying human-to-snail transmission, and snail prepatent infection rates were found to correspond to infection prevalence and average intensity in human populations contacting the sites studied. We have now developed loop-mediated isothermal amplification (LAMP) assays for identifying *Schistosoma mansoni* and *S. haematobium* to facilitate large-scale evaluation of post-intervention transmission potential. LAMP primers were designed based on the Sm1-7 and DraI repeated sequences of the corresponding schistosomes, and amplification by LAMP of these 121-basepair highly abundant sequences provided a detection sensitivity of 0.1 fg of genomic DNA. When these LAMP assays were applied for examining infected laboratory snails, it was possible to identify infection from the first day after exposure to miracidia. The potential advantages of these assays are discussed.

### INTRODUCTION

The most widely used methods for assessing schistosomiasis endemicity are based on examining the prevalence of *Schistosoma* infection among humans. The standard screening assays rely on detection of excreted eggs in stool or urine, or the use of questionnaires for self-report of characteristic symptoms.<sup>1,2</sup> Such approaches are frequently used for targeting population-based mass drug administration (MDA) in schistosomiasis control campaigns.<sup>3</sup> However, despite MDA efforts, *Schistosoma* transmission usually persists to a greater or lesser extent after delivery of treatment.<sup>4</sup> After MDA, human prevalence decreases and the remaining infections are mostly of light intensity, but because of continuing local transmission and exposure to reinfection, risk remains for patients to redevelop schistosomiasis-related morbidities such as anemia, undernutrition, and decreased performance status.<sup>5</sup> In addition, after MDA, the number of negative stool or urine test results increases and the performance characteristics of standard diagnostic tests are decreased (especially in terms of sensitivity and negative predictive value), and test-to-test variability is increased.<sup>6</sup> Furthermore, where elimination is contemplated, the uncertainty of a zero value poses a programmatic challenge because much larger sample sizes are needed to obtain a high degree of confidence that the observed rate is actually zero.

The detection of schistosome eggs in feces or in urine is particularly insensitive for detection of low-level or light infections,<sup>7</sup> and in primate models, egg counts and circulating antigen tests regularly miss detection of infection intensities of up to 10–20 worm pairs per person.<sup>8</sup> Infection surrogates, such as microhaematuria in *S. haematobium* infection, also lose their diagnostic reliability in low-level infections,<sup>9</sup> and positive antibody test results (indicative of *Schistosoma* exposure) may take an indefinite period of time to convert to negativity after elimination of infection.

For more in-depth assessment of post-intervention *Schistosoma* transmission, various monitoring approaches have been tried to complement or replace human testing. Among these are monitoring of human water use activity,<sup>10,11</sup> estimation of egg contamination of water by human excreta,<sup>12</sup> detection of cercarial shedding by snails,<sup>13</sup> and cercariometry.<sup>14,15</sup> Of these approaches, cercarial shedding has been most extensively used (in combination with human water contact data) to provide estimates of snail-to-human transmission. However, cercarial shedding can be highly focal<sup>16</sup> and of low frequency, even in areas of significant transmission.<sup>17</sup> Where snail shedding rates are low, quite extensive efforts at snail collection and testing are required to accurately assess the impact of intervention on levels of patent snail infection and risk of human re-infection.

To address this issue, we have focused on the development of molecular monitoring of early prepatent (non-shedding) plus patent (shedding) snail infections as a tool for large-scale tracking of residual transmission. We developed polymerase chain reaction (PCR) assays for amplification of the Sm1-7 repeated sequence of *S. mansoni*,<sup>18,19</sup> and of the DraI repeated sequence of *S. haematobium*.<sup>20</sup> These PCRs have proven to be highly sensitive and specific tools for detecting the respective schistosomes in biological materials. By virtue of the fact that snails at all stages of prepatency and patency are detected, we found it possible to detect surprisingly high (30–50%) rates of snail infection persisting at sites where community-based control had reduced human prevalence but not transmission of *S. haematobium*.<sup>21</sup> Cercarial shedding rates did not correspond to infection rates in the human population, whereas rates of snail PCR positivity did.<sup>21,22</sup> Molecular detection of snail infection rates appears to be a suitable marker of persisting infection in the human population. This approach resembles those used for assessing transmission of lymphatic filariasis by PCR testing of insect vectors.<sup>23</sup> Both methods are examples of xenomonitoring, i.e., monitoring infection in the intermediate host for obtaining evidence on infection in humans.

In rural areas of developing countries, large-scale implementation of molecular transmission monitoring will require tools that are more user-friendly than PCR. The PCR is dependent on complex technology and on specialized training in molecular biology. As an alternative, the loop-mediated isothermal

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amplification (LAMP) technique may provide the answer to program monitoring needs. The LAMP, initially described in 2000,<sup>24</sup> has rapidly gained acceptance for detection of a variety of infectious agents including *Plasmodium falciparum* and *S. japonicum*.<sup>25,26</sup> This technique does not require complex equipment for DNA amplification or for amplicon detection,<sup>27</sup> and is potentially suitable for molecular monitoring in basic laboratory facilities.<sup>28</sup> In the present study, we describe the development of two new LAMP assays for detecting *S. haematobium* and *S. mansoni* DNA in biological materials and their sensitivity for detecting infected snails.

## MATERIALS AND METHODS

**Snails and schistosomes.** Snails of the Puerto Rican strain of *Biomphalaria glabrata* maintained at Tel Aviv University were exposed individually, when approximately 6 mm in size, to 10 miracidia of an Egyptian strain of *Schistosoma mansoni*. *Biomphalaria glabrata* obtained from the Natural History Museum (London, United Kingdom) (NHM no. 3056), were individually exposed to 10 miracidia of *S. mansoni*, obtained from Besoum, Cameroon. *Bulinus wrighti*, Oman (NHM No. 3053) were individually exposed, when approximately 3 mm in size, to 5 miracidia of *S. haematobium* isolated at Ouro Doukoudje, Cameroon. The various snails were preserved in absolute ethanol after survival for 1 day, 6–7 days, or 14 days post-exposure. These specimens were then used for LAMP assay development and standardization.

**Adult worms.** *Schistosoma haematobium* adult worms from Zanzibar were preserved in liquid nitrogen as part of the schistosome collection at the Natural History Museum (London, United Kingdom). They were preserved in absolute ethanol for shipment to Jerusalem and for subsequent storage. *Schistosoma mansoni* adult worms were harvested from infected mice by perfusion<sup>29</sup> and preserved in ethanol.

**DNA extraction.** DNA from adult worms was prepared as previously described. Briefly, the worms were kept in lysis buffer (0.1 M EDTA, pH 8.0, 0.1 M Tris-HCl, pH 7.5, 0.2 M NaCl, 1% sodium dodecyl sulfate, 0.2% β-mercaptoethanol, and 100 μg of proteinase K), at 65°C for 1–2 hours. Lysis was followed by phenol-chloroform extraction.

**DNA from snails.** Laboratory-infected snails and uninfected snails, which were kept in ethanol, were washed with TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) diluted 1:10, and dried on tissue paper for few seconds to remove excess liquid. This procedure was followed by pulling out the body of the snail from the shell by using a fine needle. Tissue of each snail was kept in separate 1.5-mL tubes and covered with 200 μL of lysis buffer (DNeasy Blood and Tissue Kit; Qiagen, Valencia, CA). DNA extraction was conducted according to the manufacturer's instructions. Extracted DNA was eluted in 200 μL of TE buffer, and a 5-μL aliquot was tested in each LAMP reaction. DNA extraction from snails was also carried by using the High Pure PCR Template Preparation Kit (Roche Diagnostics, Basel, Switzerland). Similar results were obtained.

**LAMP primer design.** Sets of forward and backward external primers (F3 and B3) and forward and backward internal primers (FIP and BIP) were designed for *S. haematobium* and for *S. mansoni* on the basis of respective tandem repeated sequences DraI<sup>20</sup> and Sm1-7,<sup>18</sup> each 121 basepairs. Selection of primers used required a number of preliminary LAMP

experiments for optimization. Selected LAMP primers are shown in Table 1, and their locations within the respective target repeated sequences are shown in Figure 1.

**LAMP assays.** *Reaction mixture (25 μL) and amplifications.* The reaction mixture contained primers (40 pmol of FIP and BIP and 5 pmol of F3 and B3 outer primers), DNA polymerase, 8 units of *Bst*I large fragment, 1 mM dNTPs, 0.8 M betaine; 1× reaction buffer (20 mM Tris-HCl, pH 8.8, 10 mM KCl, 10 mM (NH<sub>4</sub>)<sub>2</sub> SO<sub>4</sub>, 8 mM MgSO<sub>4</sub>, and 1% Tween 20), and target DNA from *S. haematobium* or *S. mansoni*. The reaction was incubated at 63°C in a water bath for 2 hours.

*Amplicon detection.* Detection of LAMP-amplified products was performed by using SYBR Green I stain (Invitrogen, Carlsbad, CA). One microliter of 1:50 diluted SYBR Green I was added to the reaction mixture (25 μL). Amplified DNA was seen by exposure to ultraviolet light at 320 nm and subjected to black and white photography. When using SYBR Green at a dilution of 1:10, amplicons could be detected directly by the unaided eye because the color of the reaction solution changed from orange to green in the presence of LAMP amplicon. Amplicon analysis by standard gel electrophoresis was carried out in parallel with SYBR Green staining for quality control.

## RESULTS

**LAMP amplification of *S. haematobium* DNA for determining detection sensitivity.** Serial 10-fold dilutions of *S. haematobium* genomic DNA were amplified by LAMP using DraI LAMP primers (Table 1 and Figure 1A). The results of this amplification experiment are shown in Figure 2A after amplicon examination by agarose gel electrophoresis and staining with SYBR Green I and exposure to ultraviolet light (Figure 2B). Detection sensitivity of this assay was 0.1 fg, which was 10-fold higher than the detection sensitivity achieved by using PCR.<sup>19,20</sup>

**LAMP amplification of *S. haematobium*-infected snails.** For identification of snails infected with *S. haematobium*, we applied LAMP by using *S. haematobium* DraI LAMP primers. The results of amplicon detection in these assays by agarose

TABLE 1

Loop-mediated isothermal amplification primer sets used for *Schistosoma haematobium* and *S. mansoni* DNA amplification\*

Primer set	Primer position	Primers sequence (5'→3')
<i>S. haematobium</i> (DraI)	F3	gat ctc acc tat cag acg
	B3	GTC ACC AAT AAT ATG AAAC
	FIP: F1c + F2	CCACCAACAATTTAAA <b>TTTT</b> ATCAGACGAAAC AAAGAAAAT
	BIP: B1c + B2	GGTCGTATCGTTGTGAAA <b>TTTT</b> CACCAATAATA TGAAACAAT
<i>S. mansoni</i> (Sm1-7)	F3	GAT CTG AAT CCG ACC AAC CG
	B3	AAC GCC CAC GCT CTC GCA
	FIP: F1c + F2	AAATCCGTCCAGTGGTTT <b>TTTT</b> GAAAATCGTTGT ATCTCCG
	BIP: B1c + B2	CCGAAACCCTGGACGGA <b>TTTT</b> TATTTTAAATCT AAAACAAAC ATC

\*F3 = forward external primer; B3 = backward external primer; FIP = forward internal primer composed of the F1c and F2 primers (Figure 1) connected by a TTTT hinge (**bold**); BIP = backward internal primer composed of the B1c and B2 primers (Figure 1) connected by a TTTT (**bold**) hinge.

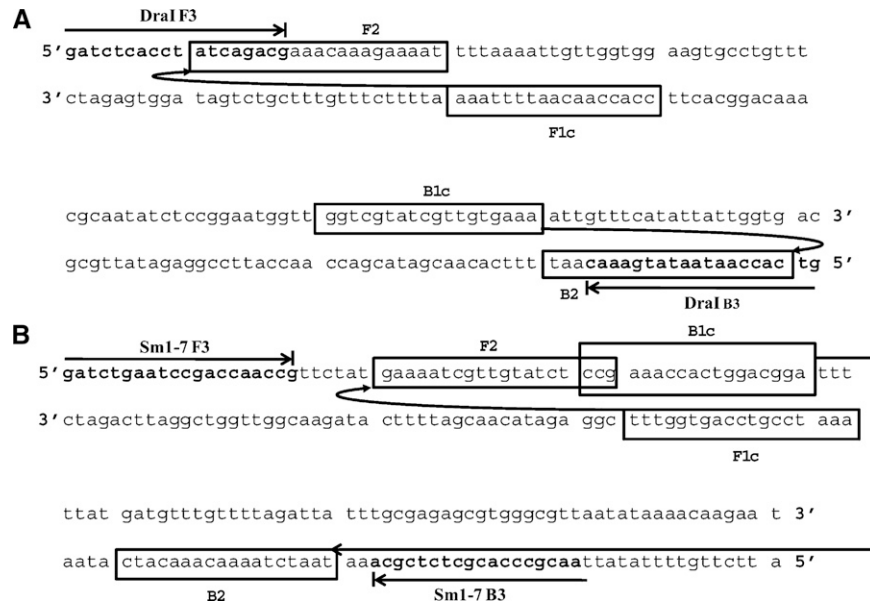


FIGURE 1. Location of the loop-mediated isothermal amplification forward (F3) and backward (B3) external primers and of internal forward (FIP) and backward (BIP) primers within the respective sequences DraI of *Schistosoma haematobium* (A) and Sm1-7 of *S. mansoni* (B).

gel electrophoresis are shown in Figure 3. Infected snails were detected from day 1 after exposure to miracidia. Detection by SYBR Green-generated fluorescence was not possible in the case of examining snails because uninfected snails also showed positive fluorescent signals given the amount of snail DNA present in the reaction mixture. In comparison, differential detection of infected snails by using SYBR Green dye, which is less sensitive than fluorescence, was possible (not shown).

Thus, in the presence of amplicon DNA, the DNA level was within the detectable range for SYBR Green, and the color change from orange to green was observed.

**LAMP amplification of *S. mansoni* DNA for determining detection sensitivity.** As described earlier regarding determination of the sensitivity of LAMP for detecting *S. haematobium* DNA, the same process was used for amplifying serial 10-fold dilutions of *S. mansoni* genomic DNA by using a LAMP assay and corresponding Sm1-7 LAMP primers (Table 1 and Figure 1B). The results of this amplification are shown in Figure 4, which shows results of agarose gel electrophoresis (Figure 4A) and parallel results of staining with SYBR Green I

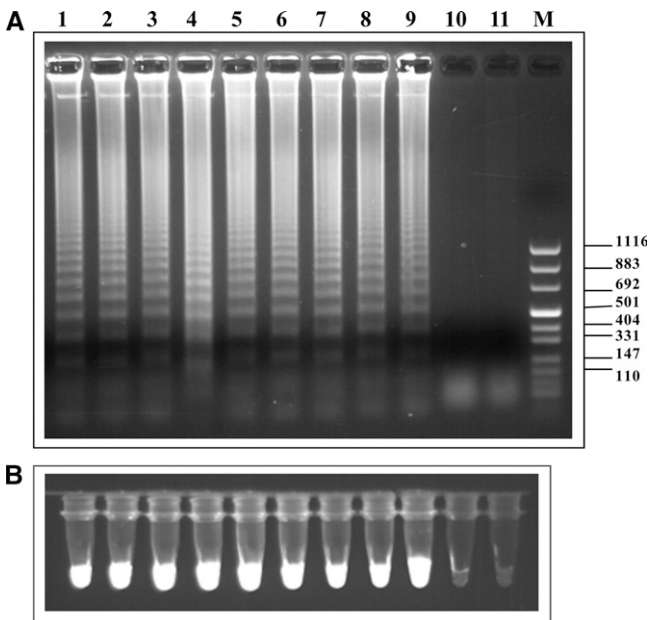


FIGURE 2. **A**, Agarose gel electrophoresis of loop isothermal amplified products (LAMP) from different concentrations of *Schistosoma haematobium* genomic DNA. Ten-fold serial dilutions starting from 10 ng genomic DNA (lane 1) down to 0.001 fg (lane 11) were tested. Lane M, molecular mass marker. **B**, Fluorescence of LAMP products as indicated in **A** using SYBR Green I staining followed by ultraviolet illumination at 320 nm. Values on the right are in basepairs

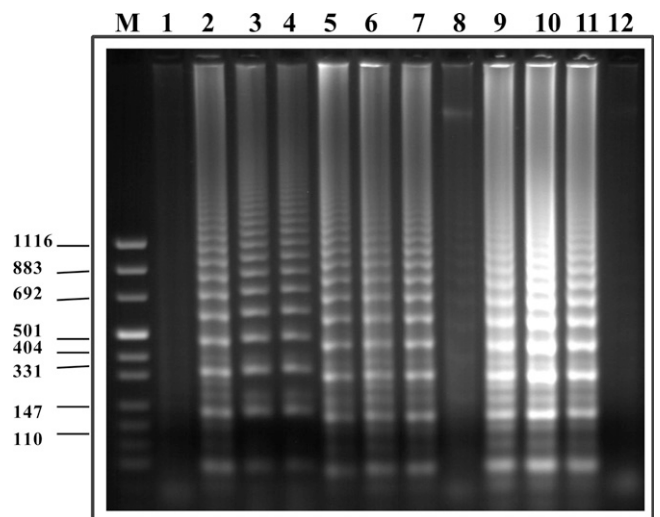


FIGURE 3. Agarose gel electrophoresis of loop-mediated isothermal amplification products after targeting DraI *S. haematobium* repetitive sequence within infected snails. Lanes 1 and 8, uninfected snails; lanes 2-4, snails after one day of infection; lanes 5-7, snails after seven days of infection; lanes 9-11, snails after 14 days of infection; lane 12, negative control (no DNA); lane M, molecular mass marker. Values on the right are in basepairs.



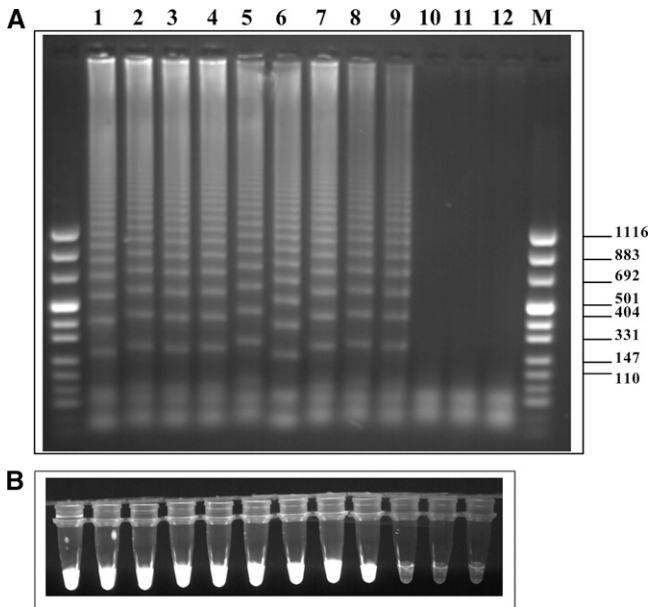


FIGURE 4. **A**, Agarose gel electrophoresis of loop-mediated isothermal amplification (LAMP) products from different concentrations of *Schistosoma mansoni* genomic DNA. Ten-fold serial dilutions starting with 10 ng of genomic DNA (lane 1) down to 0.001 fg (lane 11) were tested. Lane 12, negative control (no DNA); lane M molecular mass marker. **B**, Fluorescence of LAMP products as indicated in **A** using SYBR Green I staining followed by ultraviolet illumination at 320 nm. Values on the right are in basepairs.

and exposure to ultraviolet light at 320 nm (Figure 4B). Detection sensitivity of this LAMP assay was 0.1 fg, which is 10-fold more sensitive than that of the corresponding PCR assay.<sup>18,20</sup>

**LAMP amplification of *S. mansoni*-infected snails.** For identification of snails infected with *S. mansoni*, we applied the LAMP assay and Sm1-7 primers. The results of amplicon detection by agarose gel electrophoresis in these assays are shown in Figure 5. Detection by SYBR Green-generated fluorescence was not possible in this case, but detection by SYBR Green color change was possible (not shown). Infected snails were detected from day 1 after exposure to miracidia.

## DISCUSSION

With the advent of large-scale mass treatment campaigns for schistosomiasis in Africa, the ecology of *S. mansoni* and *S. haematobium* transmission in disease-endemic areas is likely to change. An essential factor in determining the overall effectiveness of population-based treatment is whether such broad treatment coverage to humans can significantly reduce local transmission and reduce the risk for reinfection among high risk subgroups, such as school age children. Consistent with the need for new tools for monitoring schistosomiasis transmission, we previously developed two PCR assays for sensitive detection identification of infected snails,<sup>18–20</sup> and provided evidence that detection of snail infection by PCR enables identification of a large proportion of infected snails even where cercarial shedding (patency) is rare.<sup>21,22</sup> Furthermore, in Coastal Kenya, snail prepatent infection rates (as measured by PCR) were found to correspond to prevalence and average intensity of infection in human communities that had

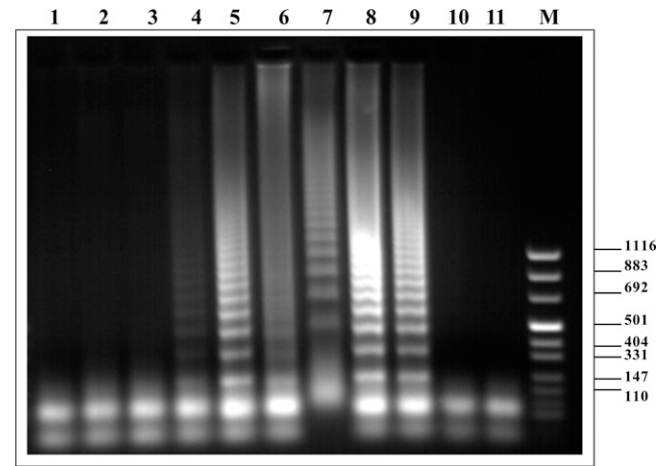


FIGURE 5. Agarose gel electrophoresis of loop-mediated isothermal amplification products after targeting Sm1-7 *Schistosoma mansoni* repetitive sequence from infected snails. Lanes 1–3, uninfected snails; lanes 4–6, snails after one day of infection; lanes 7–9, snails after seven days of infection; lanes 10 and 11, negative control (no DNA); lane M: molecular mass marker. Values on the right are in basepairs.

specific transmission sites.<sup>21,22</sup> This xenomonitoring, which is based on determining snail infection rates, provided a measure of transmission potential (from the human population) that persisted after community-based drug therapy. With the potential advantages of this molecular monitoring in mind, we have developed new, simpler DNA amplification assays for *S. haematobium* and for *S. mansoni* based on the LAMP assay.<sup>24</sup> This assay appears to provide technology that can facilitate large-scale monitoring of prepatent snail infection that will, in turn, enable better assessment of intervention impact on parasite transmission.

The LAMP assay has already been used for amplification of DNA of other microorganisms including parasites, notably *S. japonicum*.<sup>26</sup> Unlike PCR, LAMP does not require amplification cycles by thermocycling or amplicon detection by electrophoresis. Given these features, LAMP is potentially useful for work in the field and has already used in rural laboratories in developing areas for the diagnosis of tuberculosis.<sup>28</sup>

In the present study, we have tested laboratory-infected snails, and some points still need to be addressed in considering LAMP implementation in the field. First, a simple and inexpensive DNA preparation method is required. In the present study, as in most other studies of LAMP development, DNA extraction kits were used for pre-isolation of target nucleic acid material. For achieving field applicability, the expensive DNA extraction kits should be replaced by user-friendly and affordable DNA preparation tools, particularly where large numbers of samples are to be examined. Also, to be applicable in field laboratories, LAMP reaction mixtures will need to be premixed, ready for use, and storable under field-laboratory conditions. Reaction mixtures having these features are now available for PCR<sup>30</sup> and can likely be developed for LAMP. For now, practical use of LAMP in field laboratories for diagnosis of schistosomiasis and for monitoring schistosomiasis transmission will require further system development and validation. This development and validation can be performed in association with ongoing intervention projects and in other areas where schistosomiasis prevalence is decreasing or approaching elimination.

Diagnosis of schistosomiasis mansoni infection in humans by using Sm1-7 sequence amplification for copro-PCR,<sup>31</sup> or plasma-PCR<sup>32</sup> has already been accomplished and used as a tool for detection of low-grade infections, with high detection sensitivity. Using LAMP assays for this purpose should add the inbuilt operational facility of LAMP and another order of magnitude of detection sensitivity, namely 0.1 fg (Figures 2 and 4) compared with 1 fg detection sensitivity of the corresponding PCR assays.<sup>18,20</sup> The high detection sensitivity of the LAMP assays should facilitate examination of pooled snails for monitoring larger areas or in areas where transmission has reached low values, which requires testing of many snails. Such a pooling strategy has facilitated detection of infected filarial vectors.<sup>33-35</sup>

The specificity of the LAMP assay depends largely on the primers used. We have used primers designed from the Sm1-7 repeated sequence of *S. mansoni* and the DraI sequence of *S. haematobium*. These repeated sequences are 121 basepairs and are shorter than sequences typically targeted for LAMP, (e.g., the LAMP primer design site [http://primerexplorer.jp/v4\_manual/02.html], which provides primer design details only for sequences  $\geq 200$  basepairs). The independent primer design of our relatively short sequences, as shown in Figure 1, required multiple trials for optimization. The high abundance of these sequences, their tandem arrangement in large arrays, and their well-studied specificity provided the reasoning for developing corresponding LAMP assays based on these relatively short sequences. We have found that avoiding excessive overlap between sequences of the internal and external primers was important for successful amplification. It should be mentioned that the *S. japonicum* retrotransposon SjR2 has been recently targeted for diagnosis by LAMP,<sup>26</sup> and retrotransposons of other schistosomes may also prove suitable for this purpose if providing suitable detection sensitivity and specificity.

Regarding specificity, detection of *S. haematobium* DraI sequence, which is group specific,<sup>20</sup> can provide a basis for monitoring *S. haematobium* transmission in areas where animal schistosomes, that share this sequence, are rare. Other groups have used other sequences for schistosome species differentiation, such as cyclooxygenase I<sup>36</sup> and internal transcribed spacer 1.<sup>37</sup> We have used the Sh110/SmSL inter-repeat sequence of *S. haematobium* for designing primers capable of differentiating *S. haematobium* from related animal schistosomes by using PCR<sup>38</sup> with a detection sensitivity of 10 pg, which is higher than that described for other tests designed for this purpose.<sup>36,37</sup> Preliminary results obtained by a LAMP assay based on this inter-repeat sequence indicate that its detection sensitivity can reach an even higher range. Considering that *S. bovis* and *S. mattheei*, the major animal schistosomes related to *S. haematobium*, are not detected by Sh110/SmSL-PCR,<sup>38</sup> a corresponding LAMP assay is not expected to produce amplicons and should therefore enable a clear differentiation of *S. haematobium* DNA from DNA of these abundant animal schistosomes.

Given that major differences can exist in snail-schistosome compatibility in different areas,<sup>39</sup> snail prepatency rates (as determined by snail molecular monitoring) cannot be expected to serve as a direct indicator of snail-to-human transmission potential. However, changes in snail prepatency rates over time after an intervention can be expected to reflect changes in the force of transmission from humans to snails in the study

area. This finding, in turn, is likely the outcome of changes in prevalence and mean intensity of infection among humans in the same area, as we have previously found in Coastal Kenya.<sup>22</sup> However, this concept has to be tested in various areas of defined levels of endemicity. Examination of this monitoring approach will be greatly facilitated if the tests can be carried out in field laboratories close to the transmission sites, rather than employing PCR-based testing in remote central laboratories. Using LAMP-based assays will thus enable making use of field laboratories often located in areas endemic for schistosomiasis, without requiring expensive instruments and without having to train special cadres of molecular technologists. The relevance of snail molecular-monitoring by LAMP-based assays will be even more pronounced once area-wide validation will evolve into large-scale application alongside control campaigns.

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