

## Evaluation of Loop-Mediated Isothermal Amplification Suitable for Molecular Monitoring of Schistosome-Infected Snails in Field Laboratories

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**Abstract.** We previously described loop-mediated isothermal amplification (LAMP) for detection of *Schistosoma haematobium* and *S. mansoni* DNA in infected snails. In the present study, we adapted the LAMP assay for application in field laboratories in schistosomiasis-endemic areas. Isolation of DNA was simplified by blotting snail tissue (extracted in NaOH/sodium dodecyl sulfate) onto treated membranes, which enabled preservation at ambient temperatures. A ready-mix of LAMP reagents, suitable for shipment at ambient temperature and storage in minimal refrigeration, was used. Local survey teams without experience in molecular biology acquired operational expertise with this test within a few hours. Fifty-four field-caught snails were tested locally by LAMP and 59 were tested at similar conditions in Jerusalem. The LAMP results were consistent with those of a polymerase chain reaction; only four samples showed false-negative results. Results indicate that LAMP assays are suitable for detection of *S. haematobium* and *S. mansoni* in low-technology parasitology laboratories in which schistosomiasis elimination activities are undertaken.

### INTRODUCTION

Large-scale schistosomiasis control efforts are now being applied in Africa and elsewhere by means of mass drug administration (MDA) regimens that are assigned based on morbidity and infection intensity criteria. In China, schistosomiasis japonica control efforts have included MDA, snail control, and modification of snail habitat.<sup>1</sup> These efforts have led to significant reduction in morbidity and infection intensity and, in some areas, to profound changes in transmission ecology resulting in low-intensity residual levels of schistosomiasis or even to local elimination. As control project succeed, it becomes increasingly difficult to monitor remaining transmission by testing previously treated human populations for infection or symptoms.<sup>1,2</sup> This difficulty is even more evident in populations where elimination has been briefly accomplished but where resurgence of transmission is later observed.<sup>1</sup> Therefore, a change in approach is needed for monitoring residual post-control transmission, to assess both short-term and long-term impacts of control activities. Monitoring of snails for *Schistosoma* infection was thus proposed as a valuable new focus for assessing residual transmission.<sup>1,3,4</sup>

Because it is now possible to determine whether a snail has been penetrated by a schistosome miracidium by detecting schistosome DNA,<sup>3,5,6</sup> the term infected snail acquires a new epidemiologic meaning regardless of whether it will eventually shed cercariae. Thus, the infection rate for snails, as determined by polymerase chain reaction (PCR) or loop-mediated isothermal amplification (LAMP),<sup>3,7</sup> represents patent and prepatent infections and can thus provide a measure of transmission from human to snail, and serve as a marker of residual human infection.<sup>4</sup> Thus, PCR- or LAMP-positive snails can provide information on whether control efforts have been successful in significantly reducing the num-

ber of schistosome ova that reach the key local water-bodies harboring host snails.

This approach has already been implemented by using PCR for post-elimination testing for *S. haematobium* transmission in Morocco (Abbasi I and others, unpublished data), and the development of LAMP assays for future post-eradication testing for *S. japonicum* was recently reported.<sup>8,9</sup> More recently, the Zanzibar Enterprise and Sustainable Tourism Project for elimination of *S. haematobium* transmission in Zanzibar is planning to include PCR for surveillance of post elimination transmission (Rollinson D, unpublished data).

The question of which DNA detection tool should be used for post-control surveillance of schistosomiasis is now being examined by those involved in development and validation of relevant monitoring and evaluation tools.<sup>10</sup> In a recent report,<sup>7</sup> we presented molecular components and amplification conditions required for LAMP amplification of *S. haematobium* and of *S. mansoni* DNA, and for detection of laboratory infected snails. The sensitivity of the LAMP assay was shown to be 10 times higher than that of PCR (i.e., < 1 fg), and the ability of LAMP to detect laboratory-infected snails as soon as one day after exposure to miracidia was also shown. The present report describes the adaptation of these *S. haematobium* and *S. mansoni* LAMP assays for application by local technologists in parasitology laboratories located near endemic disease transmission sites.

### MATERIALS AND METHODS

**Snails and parasites.** Laboratory snails (*Bulinus* and *Biomphalaria*) were exposed to different numbers of miracidia and were tested at different times post exposure. Individual *Bulinus nasutus* snails (F<sub>2</sub> progeny of snails collected in the field and cultured in the Msambweni field laboratory on the southern coast of Kenya) were exposed to one or three miracidia of *S. haematobium*, hatched from eggs recovered from patient's urine, and were kept alive for 1, 3, 5, 7, and 10 days before their transfer into 70% ethanol where they were kept until DNA extraction. Similarly, *Biomphalaria glabrata* snails were exposed to two or five miracidia of *S. mansoni* (Egyptian

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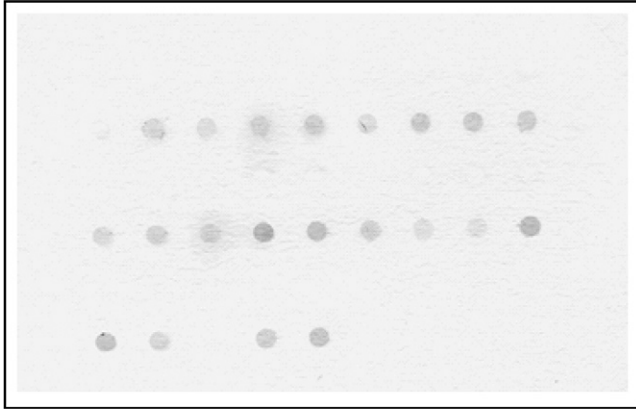


FIGURE 1. Snail extracts bound to a treated glass membrane by using a dot-blotting manifold.

strain) and kept alive for two and six days, then transferred to 70% ethanol. Snails and parasites were provided by Dr. Fred Lewis (Schistosomiasis Resource Center, National Institute of Allergy and Infectious Disease, Bethesda, MD). In both cases, non-exposed snails were used as negative controls in tests of DNA preparation and amplification by LAMP, PCR, or real-time PCR.

Field snails were collected from sites where schistosome-infected snails had been detected in Kwale County, Kenya. *Schistosoma haematobium* host snails (*B. globosus* or *B. nasutus*) were identified morphologically and stored in 70% ethanol until DNA harvesting. Snails were collected by team members of the Division of Vector Borne and Neglected Tropical Diseases, Ministry of Public Health and Sanitation, who are based at Msambweni District Hospital laboratories on the southern coast of Kenya. Snails were collected at the following sites: Kinango Pond (3.8182°S, 39.18620°E), Nimbodze Dam (4.28318°S, 39.27070°E, where *B. nasutus* is the host of *S. haematobium*), and from Kwatelengo Mamduli Pond (3.90947°S, 39.52976°E, where *B. globosus* is the host of *S. haematobium*). *Biomphalaria pfeifferi* host snails of *S. mansoni* were collected in the Kibwezi area of Kenya near the Mombasa-Nairobi highway.

**Extraction and purification of snail DNA.** The LAMP procedure (including DNA extraction) was composed of steps involving operational blocks that enable breaks for man-

agement of other tasks common in a field-station parasitologic laboratory.

**Snail lysis.** DNA extraction from snail tissue was performed by removing excess ethanol by blotting on a tissue paper. The snail tissue was then extracted from the shell by using a fine needle, and macerated by wooden sticks in a 1.5-mL Eppendorf (Hamburg, Germany) tube after adding 250  $\mu$ L of snail lysis solution (1 N NaOH, 0.1% sodium dodecyl sulfate [SDS]) for a snail up to 5 mm in size and 500  $\mu$ L for larger snails. Lysis was carried out at 60°C for 1 hour or at room temperature for 1–4 days (depending on time availability). This procedure was followed by adding concentrated (36%) HCl solution (approximately 18  $\mu$ L for 250  $\mu$ L of lysis solution and approximately 35  $\mu$ L for 500  $\mu$ L of lysis solution) to reach a pH of 5–7, as measured by pH detection strips. This step required only one or two pH tests for each batch of lysis solution. Removal of debris was performed by high-speed centrifugation in a microfuge for 5–10 minutes. After lysis of snail tissue, DNA was further purified by using NaOH-treated glass filters as described below.

**Preparation of glass fiber membranes for DNA harvesting.** Glass fiber membranes (GF/C; Whatman, Piscataway, NJ) were treated by soaking in a 0.2 N NaOH solution. Several membranes were soaked in a suitable glass tray containing 20 mL of NaOH solution at 35°C for two hours, and then left overnight at room temperature. The treated membranes were washed four times with 20 mL of double-distilled water and three times with 20 mL of acetone. The membranes were air dried for 20 minutes, then oven-dried for 1 hour at 100°C. The dried membranes can be stored for months and shipped at room temperature if kept dry and clean.

**DNA purification of snail DNA by using glass membranes.** For each NaOH/SDS-treated snail, a 1:1 volume of 6 M guanadinium chloride was added and the mixture was dot-blotted onto the treated glass membranes by using a suction manifold (Bio-Rad, Hercules, CA). The membrane-bound DNA was washed twice with 0.5 mL of 80% ethanol containing 50 mM Tris, pH 7.0, air-dried, and the spotted dots subsequently punched off by using a card punching tool that produced circular holes (diameter = 4.5 mm) and corresponding discs (Kangaro Industries, Ludhiana, India). The filter discs were incubated at 40°C for approximately 10 minutes for DNA elution in separate 1.5 mL Eppendorf tubes containing 200  $\mu$ L of double-distilled water. The membrane-bound snail extract spots could be kept dry at room temperature for

TABLE 1

Primer sets used for *Schistosoma haematobium* and *S. mansoni* DNA amplification by LAMP, PCR, and real-time PCR methods\*

<i>Schistosoma</i> species	Amplification system	Primer	Sequence, 5'→3'	Reference
<i>S. haematobium</i> (based on DraI sequence)	LAMP	F3	GATCTCACCTATCAGACG	7
		B3	GTCACCAATAATATGAAAC	
		FIP	CCACCAACAATTTTAAATTTTATCAGACGAAACAAAGAAAAT	
		BIP	GGTCGTATCGTTGTGAAATTTTCACCAATAATATGAAACAAT	
	PCR and qPCR	DraI forward DraI reverse	TCACAACGATACGACCAAC GATCTCACCTACAGACGAAAC	6
<i>S. mansoni</i> (based on Sm1-7 sequence)	LAMP	F3	GATCTGAATCCGACCAACCG	7
		B3	AACGCCACGCTCTCGCA	
		FIP	AAATCCGTCCAGTGGTTTTTGAATAATCGTTGTATCTCCG	
		BIP	CCGAAACCACTGGACGGATTTTATTTTAATCTAAACAAACATC	
	PCR and qPCR	Sm1-7D Sm1-7R	GAAAATCGTTGTATCTCCG CATAAAAATCCGTCAGTGG	5

\*LAMP = loop-mediated isothermal amplification; PCR = polymerase chain reaction; F3 = forward external primer; B3 = backwards external primer; FIP = forward internal primer; BIP = backwards internal primer; qPCR = quantitative PCR.

shipment and long term preservation (Figure 1). Although the preservation end point was not determined, preservation for six months was observed when membranes were kept dry and sealed in a nylon wrap.

**LAMP reagents ready-mix suitable for field laboratory conditions.** The prepared ready mixture was composed of two parts. The first part was a solution of ready-mix reagents, and the second part was a ready mix of freeze-dried primers, as outlined below.

**LAMP ready-mix reagents solution.** This mixture consisted of a solution containing 2× concentrations of the following reagents, which enabled their shipment at ambient temperature and storage for months. The components were eight units of *Bst* DNA polymerase (New England Biolabs, Ipswich, MA), 200 mM dNTPs (Fermentase, Vilnius, Lithuania), 2 M betaine (Sigma, St. Louis, MO), 2× LAMP buffer (20 mM Tris-HCl, pH 7.5, 7 mM MgCl<sub>2</sub>, 150 mM KCl), and stabilizers (17.5% sucrose). It is noteworthy that *Bst* DNA polymerase, which is usually shipped and kept at −20°C to avoid denaturation, can be shipped and preserved for months at these conditions. Although the preservation end point was not determined, the ready mix was preserved for a minimum of three months at room temperature, which implies that it may have a longer preservation time, especially when kept at cold conditions.

**Ready-mix primers.** One microliter of each of the four LAMP primers (5 pmoles of F3 and B3 and 40 pmoles of FIP and BIP) is considered a primer dose for a single LAMP reaction as indicated in the original protocol (Table 1). Primer mixture was kept lyophilized in batches sufficient for small (approximately 10) numbers of reactions. This feature enabled elimination of the need for storing and shipping the primers under deep-freeze conditions. For actual use, the dried primers were reconstituted in double-distilled water and added to the reaction mixture.

**LAMP for detection of infected snails.** For a LAMP test, 12.5 µL of the 2× concentrated ready-mix solution was combined with 1 µL of each of the 4 primers (or with 4 µL of the ready-mix of primers), added to 5 µL of the target DNA preparation and 3.5 µL of double-distilled water (RNA/DNA free). The LAMP samples were incubated at 63°C in a water bath for 2 hours.

Detection of LAMP products was performed by using SYBR Green I stain (Invitrogen, Carlsbad, CA). One microliter of 1:10-diluted SYBR Green I was added to the reaction tube (25 µL) to enable direct detection of LAMP product with the unaided eye because the color of the reaction changed from orange to yellow-green in the presence of LAMP amplicon (Figure 2). Amplicon analysis by standard gel electrophoresis was conducted in parallel to SYBR-Green staining for quality control. The stained reaction tubes were photographed by using a cell phone camera on location to enable long distance delivery of results. For comparison, photographs of PCR results have been combined with images of the SYBR Green read-outs of LAMP reactions (Figures 3 and 4). The comparison of LAMP results with results of *Dra*I PCR (previously used successfully for molecular monitoring of infected snails<sup>3</sup>) is presented to show that both methods have similar detection sensitivities.

To ensure that positive LAMP results were not caused by excess snail DNA, LAMP products were examined by agarose gel electrophoresis (Figure 2B). This procedure was different

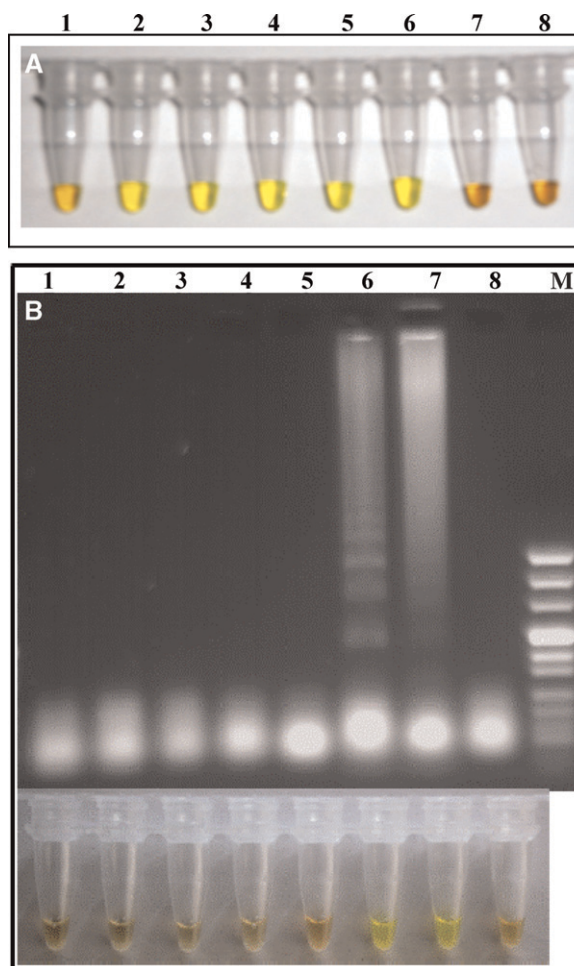


FIGURE 2. **A**, Loop-mediated isothermal amplification (LAMP) results immediately after adding SYBR Green after amplification of *Schistosoma haematobium* DNA from field snails. 1 µL of 1:10 diluted SYBR green stain was added to 25 µL of LAMP reaction. Lanes 1–6: field snails; lanes 7 and 8: negative controls. Note the weak orange LAMP-positive reaction in lane 1 (see text). **B**, Agarose gel electrophoresis of *S. haematobium* LAMP-amplified products of uninfected snails (lanes 1–5) and of infected snails (lanes 6 and 7) compared with LAMP product detection after the addition of 1 µL of 1:10 diluted SYBR green I dye to a total of 25 µL of LAMP reaction. Lane 8: no DNA; lane M: size marker.

from examination of *Dra*I PCR results by agarose gel electrophoresis (Figures 3 and 4).

**Polymerase chain reaction.** The PCRs were conducted in a volume of 25 µL by using ready mix PCR tubes (Syntezza, Jerusalem, Israel). Each reaction was performed by mixing 1 µL of the direct and reverse primers (20 pmoles) and 18 µL of double-distilled water (DNA/RNA free). Twenty microliters was added to the ready mix tubes, which contained Taq polymerase, buffer, and dNTPs. Five microliters of target DNA (extracted snail DNA) was then added. Primer sequences and recommended thermocycler profiles for *S. mansoni* and *S. haematobium* detection were as described (Table 1).<sup>3</sup>

**Quantitative real-time PCR.** A real-time hot-start PCR was performed by using the Absolute Blue qPCR Kit (Thermo Scientific, Surrey, United Kingdom) based on SYBR green detection using the real-time PCR machine Rotor-Gene 6000 (QIAGEN, Hilden, Germany). The quantitative PCR (qPCR) (total volume = 20 µL) was prepared by mixing 10 µL of the 2×



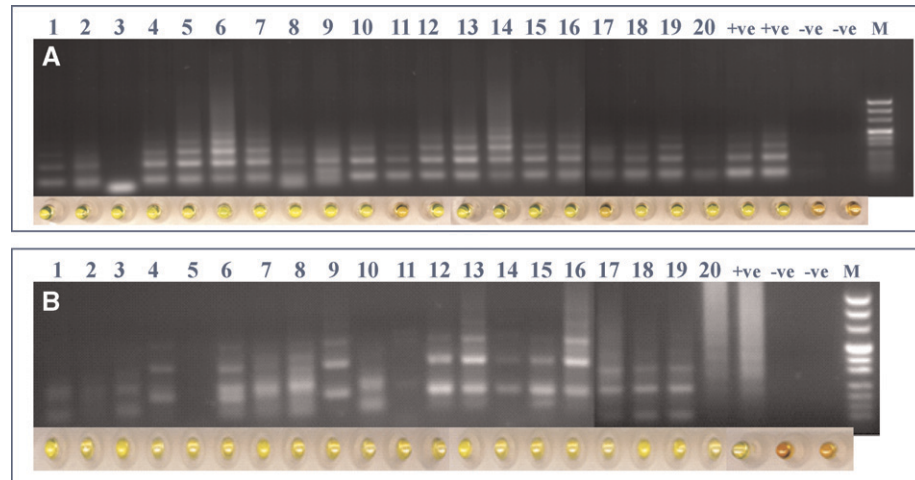


FIGURE 3. Agarose gel electrophoresis of polymerase chain reaction (PCR) products (DraI PCR or Sm1-7 PCR) and corresponding loop-mediated isothermal amplification (LAMP) (SYBR Green staining) of amplicons from infected snails exposed to different numbers of miracidia, then tested at different times thereafter. **A**, DraI PCR results of *Bulinus nasutus* exposed to *Schistosoma haematobium*. Lanes 1 and 2, 1 miracidium after 1 day; lanes 3 and 4, 1 miracidium after 3 days; lanes 5 and 6, 1 miracidium after 5 days; lanes 7 and 8, 1 miracidium after 7 days; lanes 9 and 10, 1 miracidium after 10 days; lanes 11 and 12, 3 miracidia after 1 day; lanes 13 and 14, 3 miracidia after 3 days; lanes 15 and 16, 3 miracidia after 5 days; lanes 17 and 18, 3 miracidia after 7 days; lanes 19 and 20, 3 miracidia after 10 days. +ve = positive controls (*S. haematobium* DNA); -ve = negative controls (no DNA). Lane M, size marker. Note the weak orange LAMP-positive reaction in lanes 11 and 17 (see text). **B**, Sm1-7 PCR products of *Biomphalaria glabrata* snails exposed to different numbers of *S. mansoni* miracidia and tested after different times thereafter. Lanes 1–5, 2 miracidia after 2 days; lanes 6–10, 2 miracidia after 6 days; lanes 11–15, 5 miracidia after 2 days; lanes 16–20, 5 miracidia after 6 days. +ve = positive controls (*S. mansoni* DNA); -ve = negative controls (no DNA). Lane M, size markers.

concentrated absolute blue solution with 1  $\mu$ M of each direct and reverse primer, DraI sequence-based for *S. haematobium*, or Sm1-7 sequence-based for *S. mansoni* (Table 1). Template DNA (2  $\mu$ L) from each snail tested was added to each reaction. For fluorescence signal acquisition, time and temperature profile was set as follows: a holding step at 95°C for 15 minutes for enzyme activation; then amplification of 40 cycles, starting with a denaturation step at 95°C for 10 seconds; then annealing at 58°C for 10 seconds; and lastly an extension step at 72°C for 10 seconds. The test results were analyzed by using real-time PCR machine Rotor-gene 6000 series software version 1.7 (QIAGEN).

**LAMP technology transfer.** Field snails were tested in the Division of Vector Borne and Neglected Tropical Diseases Laboratory at Msambweni District Hospital in Coast Province, Kenya. The local team included a technologist and a student towards a Master of Science degree. They were first provided with a stepwise protocol for ensuring that all required supplies and equipment were available and in operating condition. Subsequently, they carried out LAMP tests with two of the investigators (JH and IA) present. The first local LAMP

session involved direct assistance, the second session involved only supervision by the investigator team, and the third session involved independent operation by the local team. The last session was carried out independently, three months later, by the local team.

## RESULTS

**Adaptation of DNA harvesting and LAMP assay for field laboratory conditions.** A summary of new procedures used for facilitating molecular detection of *Schistosoma*-infected snails using DNA amplification by LAMP in field laboratories is shown in Table 2. The advantages of these procedures in facilitating preservation and shipment of materials, as well as the ease of data recording and delivery, are indicated. The result of simultaneous dot-blotting of multiple samples onto treated membrane as part of the first step of the LAMP assay is shown in Figure 1. This product is suitable for long-term preservation and for mail shipment. The dots are then punched out and processed for DNA elution as indicated in

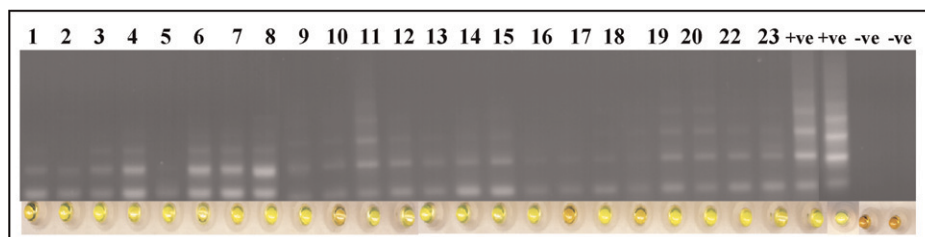


FIGURE 4. Comparison of DraI polymerase chain reaction products and loop-mediated isothermal amplification amplicons from *Schistosoma haematobium*-infected field-collected *Bulinus* snails. Positive snails were selected for presentation. +ve = positive; -ve = negative.

TABLE 2

Summary of new procedures for facilitating molecular detection of *Schistosoma*-infected snails at field laboratories by using DNA amplification by LAMP\*

Step/procedure	Description	Advantages
DNA extraction and purification: Blot-dotting snail extract onto treated glass membranes	Extraction by NaOH/SDS dot blotting, then washing and DNA elution	Simple and cheap procedure; possibility of drying the blots for storage and shipment; the washing step ensures removal of inhibitors; the eluted DNA can be tested by PCR, qPCR, and LAMP
Ready reagents for LAMP; ready mix reagent solution; dry primers	Stabilized 2× concentrated reagents mixture that includes <i>Bst</i> DNA polymerase, nucleotides, and suitable amplification buffer. Primers are added separately (see below); lyophilized aliquots of primers enough for 10–20 reactions; reconstitute and add to the ready mix	The stabilized mixture can be stored at room temperature for few months without losing enzyme activity; the mixture can be shipped to field laboratories with no need for cold or freezing conditions; easy to use
Amplicon detection by SYBR Green	1:10 diluted SYBR Green mixture	Used for the on-site detection of the amplified DNA; no need for agarose gel electrophoresis and the use of ethidium bromide
Recording and storage of results	By cell phone photography	No need for gel reader or turbidimeter; results can be delivered to a local or central laboratory

\*LAMP = loop-mediated isothermal amplification; SDS = sodium dodecyl sulfate; PCR = polymerase chain reaction; qPCR = quantitative PCR.

the Materials and Methods. DNA purified by this procedure was also suitable for testing by PCR and qPCR.

**LAMP SYBR Green I and respective PCR electrophoretic results.** The result of a LAMP assay (in this case a limited number of field-collected snails and controls) demonstrating how the reaction tubes look immediately after adding SYBR Green dye is shown in Figure 2A. Electrophoretic results of LAMP products are consistent with tube color change after the addition of SYBR Green I (Figure 2B). This finding eliminates the possibility of false-positive results that may have occurred after interacting of SYBR Green I dye with snail genomic DNA present in the reaction mixture. Subsequent figures show comparative results of LAMP and corresponding *Dra*I PCRs conducted with laboratory snails (Figure 3) or with field snails (Figure 4). It should be noted that the PCR bands in Figure 3B (lanes 5 and 11) were weak and not well reproduced in the photograph. However, their parallel LAMP response was clearly positive, as expected from the higher sensitivity of the LAMP.<sup>7</sup>

**Laboratory snails.** Snails positive by PCR were randomly selected for LAMP assays. As shown in Figure 3, infection by *S. haematobium* or *S. mansoni* can be identified after exposure to 1–2 miracidia and then testing as soon as 1–2 days later. Also, lane 3 in Figure 3 may represent a strong primer-dimer response in the *Dra*I PCR results, rather than a positive result. However, because LAMP is 10 times more sensitive than PCR,<sup>7</sup> the possibility of LAMP-positive results persists. Both *B. globosus* and *B. nasutus* were examined, and similar results were obtained for both species. Results of a four PCR-positive snails tested by LAMP appear as a weak orange color, rather than the yellow-green color typical for positive results. Although such weak orange staining (lanes 11 and 17 in Figure 3A of snails with *S. haematobium*, and as seen in tube 1 in Figure 2) of these few seemingly negative samples can be generally differentiated from clearly negative snails that show a much stronger orange color, we decided to score them as negative to ensure conservative results and avoid misinterpretation of results. It should be noted that when a black and white photocopy is made, weak orange reactions look clearly bright compared with the controls (as can be tested by the reader). For *S. mansoni*, none of the laboratory-infected *B. glabrata* snails positive by PCR were showed a weak orange color by LAMP (Figure 3B).

**Field snails.** Results of comparative LAMP assays and PCR with field-collected *Bulinus* spp. are shown in Table 3. Of the 103 snails tested (47 by the local team), 54 were PCR positive, and 4 of these were seemingly LAMP negative. No false-positive results were observed. All shedding snails (positive controls) were positive by PCR and LAMP.

Approximately 90 field-caught *B. pfeifferi* hosts of *S. mansoni* were examined, and only three were positive by PCR and LAMP. Although the value of the *S. mansoni* LAMP is clearly suggested on the basis of laboratory results (Figure 3B), there is a need for evaluation of the *S. mansoni* LAMP with field-collected snails.

**Examination of snails by real-time PCR.** Representative samples of infected and negative snails were tested by using qPCR and the *Dra*I PCR system (Figure 5). The results of the qPCR can be presented in quantitative terms according to the intensity of fluorescence obtained. All the 54 *Dra*I PCR-positive snails were also tested by qPCR and showed similar results of amplification signal strength relative to that obtained after PCR amplicon analysis by agarose gel electrophoresis.

**Establishment of LAMP in a field laboratory.** The LAMP technology was rapidly acquired by two personnel based at a field laboratory: one, a laboratory technologist (EM) and the other, a student with Bachelor degree who was starting M.astes in Science training (AW). Neither had any previous experience in molecular biology but both had experience in immunologic procedures such as the enzyme-linked immunosorbent assay. The LAMP assay was repeated twice with minimal assistance by using a clear stepwise protocol. This

TABLE 3

Summary of results of testing of infected field snails (*Bulinus* spp.) by LAMP and PCR\*

Snails shedding status (location in Kenya)	No. snails	PCR-positive result	LAMP-positive result
Non-shedders (Kinango)	43	20/43	18/43
Shedders (Kinango)	6	6/6	6/6
Non-shedders (Nimbodze)	5	5/5	4/5
Shedders (Nimbodze)	5	5/5	5/5
Non-shedders (Kwatelengo, 2011)	5	4/5	4/5
Non-shedders (Kwatelengo, 2009)	30	5/30	4/30
Positive snails (shedders)	9	9/9	9/9
Total tested	103	54/103	50/103

\*LAMP = loop-mediated isothermal amplification; PCR = polymerase chain reaction.

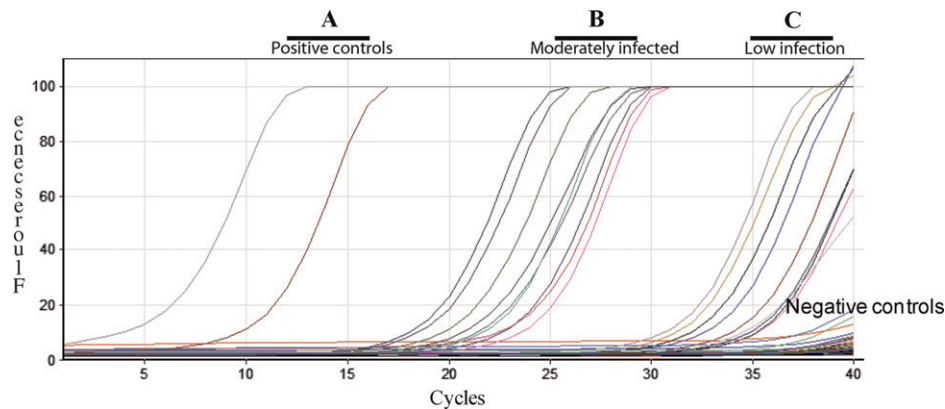


FIGURE 5. Real-time polymerase chain reaction using SYBR green fluorescence dye as a marker. DNA was extracted from representative samples of the field-collected *Bulinus* snails. The amplification was measured according to the amount of SYBR green fluorescence intensity after amplification of target DNA by DraI primers. Group A represents two positive controls (10 ng and 1 ng) of *Schistosoma haematobium* DNA. Group B represents moderately infected snails, Group C represents low infected snails. Any amplification after 35 cycles was considered a negative result.

procedure enabled subsequent independent runs by the local team. Of 103 field snails tested (Table 3), 47 were tested by the local team. The rest of the snails were tested in Jerusalem by the instructors using simulated field-laboratory conditions.

## DISCUSSION

The components required for amplification of *S. haematobium* or *S. mansoni* DNA and for detecting infected snails by LAMP have been reported.<sup>7</sup> These components included identification of the four sets of primers required (external and internal) and their optimal concentrations, the concentration of dNTPs and reaction buffer mixture, as well as the amplification temperature and time. The sensitivity of the DraI LAMP assay versus that of the corresponding DraI PCR was shown to be 10 times higher than that of PCR (0.1 fg instead of 1 fg, respectively), and the ability of LAMP as well as PCR to detect laboratory-infected snails was also demonstrated from early prepatency onward. Those results indicated the potential of the LAMP assay for DNA amplification under conditions that cannot provide machinery such as a thermocyclers for DNA amplification by PCR or qPCR, or the gel electrophoresis needed for PCR amplicon detection and analysis.

Molecular monitoring of infected snails has been shown to provide a superior mechanism for monitoring infected snails than cercarial shedding. In this regard, we have previously identified transmission sites where shedders were present only at low rates (< 1%) or not found at all during a year of snail sampling, although PCR-positive snails were present at a rate of 20–30%.<sup>3</sup> The issue remains whether differentiation of *S. haematobium* from related animal schistosomes remains important in areas where animal schistosomes are abundant (e.g., *S. bovis* and *S. matthei*). Differential molecular monitoring was reported when amplifying the cytochrome oxidase subunit 1 sequence,<sup>11</sup> although with relatively low sensitivity, which still enabled discrimination of *S. haematobium* and *S. bovis* adult worms and larvae but not infected snails. Discrimination of these species was accomplished by amplification of sequences within the ribosomal RNA gene, but restriction fragment analysis was required as an additional step for discrimination.<sup>12</sup> We have previously developed a

simple PCR for direct differentiation of schistosome species within infected snails.<sup>13</sup> However, this differentiation method has not yet been adapted for LAMP, and another molecular tool for differential identification of *S. haematobium* has been developed in our laboratory.

In the present study, several technical points were addressed for enabling application of LAMP in field laboratories. First, a simplified DNA harvesting procedure based on snail digestion by NaOH was applied based on the method we have used.<sup>13</sup> Simultaneous processing of multiple samples was accomplished by dot-blotting snail extracts onto a treated glass membrane, followed by elution of the DNA for LAMP testing. This procedure replaced commercial kits that we have used for DNA extraction from snails for application in the LAMP assay.<sup>7</sup> Use of the NaOH extraction method or the cetyltrimethylammonium bromide method<sup>14</sup> without further purification with a kit or membrane did not enable amplification of the DNA by LAMP (Abbasi I and others, unpublished data). The newly adapted LAMP assay also included use of ready to use reagent mixtures as described above.

We demonstrated that LAMP can detect infected snails from early prepatency after exposure to 1–2 miracidia (Figure 3), which provides the sensitivity needed for large-scale monitoring.<sup>3</sup> Few false-negative results were observed with LAMP (Figures 2 and 3 and Table 2), but further refinement of DNA isolation procedures is needed to avoid LAMP inhibition. Use of turbidimetry for detection of LAMP amplicons can also be assessed.<sup>14</sup> However, this approach would require use of further instrumentation and would be more relevant in areas where LAMP is also being used for monitoring of other pathogens, as for infection of tuberculosis.<sup>15</sup>

The timing sequence of the field laboratory-adapted LAMP procedure involves operational time blocks that enable breaks for managing other tasks common in a field laboratory. Such procedural breaks are possible after snail digestion, dot-blotting of extract onto a membrane, and elution of DNA before testing. Also, the first step of DNA extraction (removing snail bodies from shells and processing them with NaOH-SDS solution) is considered manageable by field technologists trained in snail collection and storage in ethanol. After 1–4 days, tubes with digested snail tissue can be transferred to a laboratory for DNA purification and subsequent amplification. This



potential division of labor can be helpful in handling multiple samples and strengthening the contribution of less skilled community workers.

When considering molecular approaches for monitoring post-control *Schistosoma* transmission, the decision to choose LAMP versus PCR will require examination of various factors. The basic assumption is that in most cases, PCR can be conducted in laboratories that are relatively well equipped, well connected to supply chains, and have suitably trained personnel. In comparison, on the basis of experience gained in the present study, the LAMP assay can be conducted in less advanced field laboratories near water bodies from which snails are collected and with much less intensive training of local laboratory personnel. When large numbers of examinations are undertaken in a central laboratory, use of LAMP can save time and expenses on thermocyclers and electrophoresis instrumentation.

Real-time PCR (Figure 5) was also performed to demonstrate that this technology can support a high throughput examination of infected snails in laboratories, and that amplicon detection by electrophoresis would no longer be required when using this method. Thus, as soon as qPCR instrumentation is introduced and staff members are trained in applying this technology, it can be used for molecular monitoring of infected snails. In any event, availability of the reported DNA harvesting procedure should facilitate contact between main and field laboratories because shipment of membranes should facilitate quality control and handling large test volumes. Continued support of a main laboratory for field laboratories for supplies (e.g., membranes, ready-mix reagents, and primer mixture), technical support, and periodical quality control can potentially enable ongoing LAMP assay operation in field laboratories, thus strengthening local operational independence.

Although use of molecular monitoring of infected snails is increasingly recognized as a potential cost-effective approach for assessing low endemicity and eradication, it should be recognized that at this stage of schistosomiasis control there are some intermediate-transmission settings where molecular monitoring would be helpful. Thus, many national programs have identified problem villages in which infection prevalence does not decrease despite intensive school- or community-based treatments.<sup>16</sup> In this situation, molecular monitoring would help identify locations with contamination and infected snail abundance because additional intervention in terms of water management, snail control, sanitation, and behavioral modification will be needed in these villages. For example, Kwatelengo Mamduli Timboni, a large abandoned quarry with year-round rain water accumulation in eastern Kenya, is an example of a successful imposed avoidance of contact with water bodies (behavior modification) and resulting low rate of snail infection (Table 3). Although the results with snails from Kwatelengo collected in 2011 suggests a recent cessation of contact avoidance, the matter of the effect of behavior modification on snail infection rates requires a controlled study with sufficient numbers of snails. The LAMP can potentially enable periodic monitoring of compliance/efficiency in similar behavior modification programs. Other control activities may also require more rapid evaluation of impact.

Implementation of large-scale LAMP-based molecular monitoring in field laboratories is expected to become effective on the basis of factors such as level of persistent post-control endemicity, its distribution, degree of monitoring required, its

duration, urgency of molecular monitoring data required, and cost. Given the possibility that even intensive MDA can leave pockets of continued transmission or post-eradication resurgence of transmission,<sup>17</sup> strengthening district level management and decision making becomes most relevant for successful schistosomiasis control. The present demonstration of a DNA amplification tool suitable for field laboratories warrants a wider examination of its efficacy in various epidemiologic settings.

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