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APPLICATION OF LOOP MEDIATED ISOTHERMAL AMPLIFICATION TO DETECT SCHISTOSOMA MANSONI DNA IN WILD SNAILS FROM MBITA SUB-COUNTY, KENYA
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# APPLICATION OF LOOP MEDIATED ISOTHERMAL AMPLIFICATION TO DETECT SCHISTOSOMA MANSONI DNA IN WILD SNAILS FROM MBITA SUB-COUNTY, KENYA

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## ABSTRACT

*Background:* **Studies show that wild snail schistosomal infection rates correspond well to the prevalence of human infection and that in the diagnosis of schistosomiasis, Deoxy-Ribonucleic Acid (DNA) detection by Polymerase Chain Reaction (PCR) and Loop-Mediated Isothermal Amplification (LAMP) are superior to snail cercariometry and microscopy. PCR and LAMP are expensive and high-level laboratory tests whereas schistosomiasis is prevalent in rural resource-poor environments.**

*Objective:* **To develop LAMP for use in detecting Schistosoma mansoni in snails in resource-poor field settings.**

*Design, Methods and Materials:* **In a laboratory experiment, field snails were tested for S. mansoni DNA using LAMP and PCR. LAMP employed primers designed from parasite tandem repeat sequences. The amplicon was analyzed using a visible dye which makes the test suitable for resource-poor settings that often lack sophisticated detection equipment.**

*Setting and Study Subjects:* **The experiment was done in the laboratory of the Institute of Tropical Medicine in Nagasaki. 131 wild snails of the Biomphalaria spp. harvested in Mbita Sub-County were infected, dried and stored. 29 were randomly selected for the experiment.**

*Outcome Measures:* **An amplicon detectable by both LAMP and PCR was considered a positive outcome.**

*Results:* **7 specimens were positive. LAMP was sensitive up to a day-old parasite. A naked eye detectable dye was successfully used to aid detection of the amplicon.**

*Conclusions and Recommendations:* **LAMP detected one-day-old infections in wild snails, and this could be visualized with the naked eye which made the test suitable for further development for use in field laboratories.**

## INTRODUCTION

Schistosoma mansoni are mammalian parasitic blood worms which cause the disease schistosomiasis in mammals including humans [1]. By the year 2014, it was estimated that 249 million people worldwide were infected with schistosomiasis [2].

Snails are the intermediate hosts of S. mansoni and other Schistosoma spp. which cause the disease schistosomiasis in humans. The standard detection method for Schistosoma infection in snails is by cercariometry whereby snails are observed for cercarial shedding. Standard diagnosis in humans is made by identification of parasite eggs in stool, urine or tissue biopsy [1]. Stool and urine however often yield few or no eggs even in the presence of a human infection [3] while snail cercariometry is laborious, requires large space, is resource-consuming and has low sensitivity [4]. Faster and more sensitive detection of Schistosoma infection in snails during all stages of infection would therefore be most useful. Newer methods for example immunological tests or parasite nucleic acid detection tests like Polymerase Chain Reaction (PCR) and LAMP have been developed [1]. LAMP technology enables nucleic acid amplification like PCR but it does it at a constant temperature and compared to PCR, LAMP is faster, cheaper and performs better in terms of sensitivity and specificity [5] [6].

Studies have shown that snail infection rates correspond well to the prevalence and average intensity of infections in human communities. Determination of snail infection rates by snail survey is therefore important for schistosomiasis xenomonitoring as part of the public health control of the disease in endemic areas [7].

The study was conducted on snail samples from Mbita Sub-County, South Nyanza region of Kenya, an area which is known to be schistosomiasis endemic and the second poorest region of Kenya with low health, social, economic and demographic indicators [8]. South Nyanza borders Lake Victoria which serves as the water source and economic resource to region's inhabitants. Snail and snail ecosystem studies in Nyanza region have shown an intimate relationship between the Lake Victoria riverine system, the inhabitant's water use behaviour and rates of Schistosoma spp. infection of both snails and humans [9].

The objectives of the study were thus to extract DNA from dried snails, determine an appropriate LAMP protocol to test the snails for the presence of S. mansoni and demonstrate it via field applicable amplicon detection methods.

## MATERIALS AND METHODS

*Study design:* An experimental laboratory assay of field snails from Mbita Sub-County suspected to be infected with S. mansoni. The snails were tested by LAMP and compared to conventional PCR as the control assay. The optimum LAMP and PCR protocols for the study namely those with the best assay ingredient characteristics were first determined using laboratory cultured and infected snails before they were used to test field snails.

*Study Variables:* Study was based on LAMP compared to PCR as the control assay. The following variables were also considered: -

- Age of parasite
- Source of parasite
- Amplicon endpoint detection method

*Study Location and Cost:* Study was done in the Parasitology Laboratory of Institute of Tropical Medicine (NEKKEN), Nagasaki University and was fully sponsored by Nagasaki University.

*Duration of Study:* Study was conducted between February and July 2018.
*Data collection and analysis:* The study data was mixed quantitative and pictorial. Data was collected by way of a laboratory journal which was used to store all findings of the experiments.
*Ethical considerations:* The study was done on non-human non-animal subjects. Requisite authorization for the study was obtained from the Kenya Medical Research Institute (KEMRI) Ethics Board and Nagasaki University.

The experiments were done following the laboratory rules and regulations governing biological experiments at the Nagasaki University. At the end of the study, the results and conclusions thereof were handed over to Nagasaki University.
*Limitations of study:* At the time of the study it was based at an advanced laboratory as can only be found an advanced institution like Nagasaki University whereas the need for schistosomiasis diagnosis exists more in remote field laboratory locations of the under-resourced developing world. The need to research on field-location applicable schistosomiasis tests cannot therefore be overemphasized.

## MATERIALS AND METHODOLOGY

***Materials:*** Snails from the laboratory of the Department of Parasitology, NEKKEN and snails from field laboratory sites in Mbita Sub-County were randomly sampled for the study.

1. *Laboratory snails:* A Puerto Rican strain of S.mansoni (NIH-Sm-PR-1) was infected into Biomphalaria glabrata snails in the laboratory for days ranging from 1 day to 5 after which the snails (Figure 1) were harvested dried and DNA extracted from them for analysis. Parasite DNA maturity is shown in Table 1. This DNA was extracted for use in the study.

**Table 1**
*Laboratory cultured B. glabrata snails infected with miracidia of S. mansoni*

| | **Characteristics of Infected Snail** |
|---|---|
| 11 | 5 days after being infected with 5 miracidia |
| 22 | 1 day after being infected with 5 miracidia |
| 33 | 5 days after being infected with 1 miracidium |
| 44 | 1 day after being infected with 1 miracidium |

**Figure 1: - Biomphalaria spp. in a shell**
*Source: K Futami*

2. *Field snails:* 131 field snails of the Biomphalaria spp. from Mbita Sub-County in Kenya were harvested from 3 sites namely Uyoga, Sewage and Tinga Dam (Figure 2). They were observed in the laboratory for evidence of cercarial shedding and then were dried at 60° C in an incubator for 24 hours and were transferred to NEKKEN for the study. 29 dried snails were randomly selected for testing. Confirmation of the species' identity snails was done by physical examination of the snail shells (Figure 1) and by reference to the identification data from NEKKEN's Department of Vector Ecology and Environment.

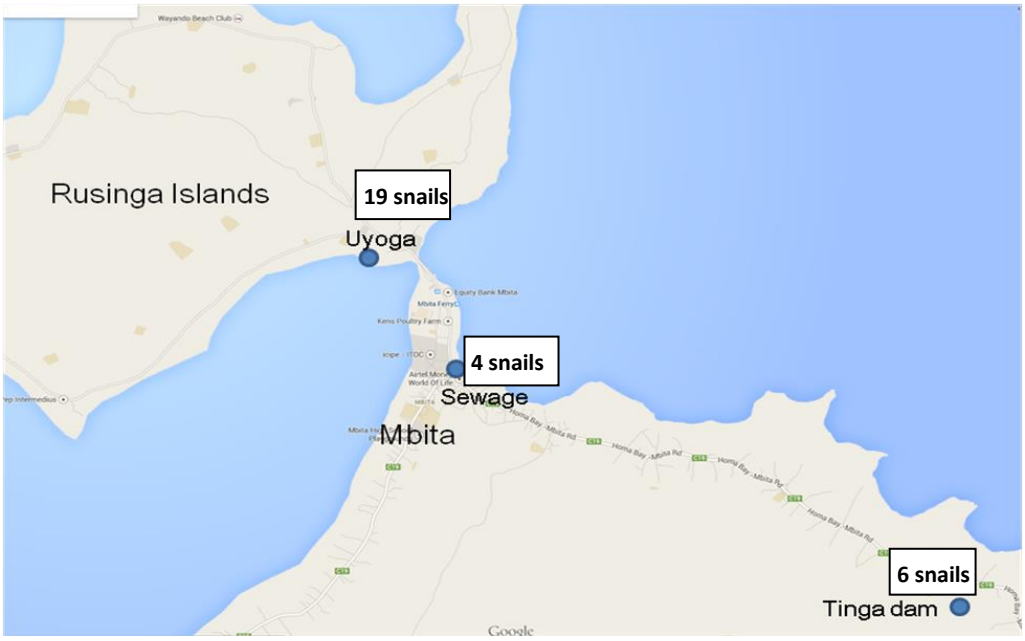



**Figure 2.** *Study area (Mbita Sub-County) with snail collection points shown*
*Source: Google Maps.*

3. *Test and Control Study Samples:* Test samples were snails from the laboratory cultured B. glabrata snails and Biomphalaria spp. snails from Mbita Sub-County in Kenya. The identity of the field snails was confirmed by physical examination of the snail shells and by reference to the snail source data. Control samples were DNA from an adult S.mansoni worm of a Puerto Rican strain of S.mansoni (NIH-Sm-PR-1) as positive control and DNA from non-infected B. glabrata snails as negative control.

4. *Primers:*
   i. *PCR primers:* Primers were based on the sequence for the gene for the mitochondrial cytochrome c oxidase subunit 1 of S. mansoni. The complete sequences this gene was obtained from GenBank (http://www.ncbi.nlm.-nih.gov/Genbank/index.html): S. mansoni (GenBank Accession No.AF216698 [10]. Arising from this the following PCR primers were used (Table 2): -

**Table 2**
*PCR Primers*

| | Primer Name | Source Gene | Sequence | Amplicon Size |
|---|---|---|---|---|
| 1 | SM COI-R | Cytochrome c oxidase Subunit 1 (COX-1) | 5′-3′ AgA TAA AgC CAC CCC TgT gA | 201 |
| 2 | SM COI-F | Cytochrome c oxidase Subunit 1 (COX-1) | 5′-3′ ATg gTT ggC TTT gAT TCg TT | 201 |

*Source: Abbasi et al., 2010*

ii. *LAMP primers:* Four LAMP primers each were designed based on S. mansoni short DNA tandem repeat sequences Sm1-7 [6]. The primer characteristics are summarized in the table 3 below.

**Table 3**

*LAMP Primers*

| | Primer Name | Source Gene | Sequence | Amplicon Size |
|---|---|---|---|---|
| 1 | F3 | Tandem repeated sequences Sm1-7 | 5′-3′ GAT CTG AAT CCG AAC CG | 121 |
| 2 | B3 | Tandem repeated sequences Sm1-7 | 5′-3′ AAC GCC CAC GCT CTC GCA | 121 |
| 3 | FIP: F1c+F2 | Tandem repeated sequences Sm1-7 | 5′-3′ AAATCCGTCCAGTGG TTTTTTTGAAAATCGTTG TATCTCCG | 121 |
| 4 | BIP: B1c+B2 | Tandem repeated sequences Sm1-7 | 5′-3′ AAACCACTGGAC GGATTTTTATTTTTAATCTAAAAC AAACATC | 121 |

*Source: Abbasi et al., 2010*

5. *Reagents:* DNA extraction reagents were used to extract genomic DNA from both laboratory-cultured and field snails. Additionally, DNA amplification reagents were used for both LAMP and PCR assays. Details of these reagents are covered in the detailed description of the methods in the following section.

***Methods:***

1. *DNA Extraction:* Both laboratory-cultured and field snails were harvested and immediately dried in an incubator at 60° C for 24 hours. They were then stored at -20° C awaiting DNA extraction. The following generic protocol for DNA extraction from dried snails was designed and used. To extract DNA, each dried snail was separately crushed to powder and transferred to a 1.5ml microtube. 1ml CMLB lysis solution containing 40mM Sucrose, 1% Triton X, 5mM $MgCl_2$, 4mM Tris-HCl (pH 7.6) in DDW water was added. The following steps were then followed to extract DNA.

- Mix by vortexing while incubating for 1 hour at room temperature.
- Centrifuge at 8,000 rpm for 3 minutes at room temperature.
- Discard the supernant, add 600ul of lysate solution (CMLB) in the tube and mix well by vortexing.
- Centrifuge at 8,000rpm for 3 minutes at room temperature.
- Discard the supernant and add 250ul of NMLB-T solution to the plates and mix well.
- Add NMLB-T contained Guanidine Thiocyanate, 12mM Tris-HCl (pH7.6), 12mM EDTA-2Na (pH 8.0), 375mM NaCl, 0.5% Na-N-Lauroyl Sarcosinate, 0.1M b-Mercaptoethanol mixed in DDW water.
- Next steps were to incubate for 20 minutes at 56°C; to spin down and transfer supernant to a new tube; to add 625ul (2.5 volumes) of 2-propanol and gently mix until separate white fibres (DNA) appeared.
- Then centrifuging at 12,000rpm for 3 minutes at room and discard the supernant and add 800ul of Buffer AW1 ( Buffer AW1 contained 20% 2-propanol

and 50% Ethanol); then another centrifuging at 12,000 rpm for 3 minutes.

- From this point the supernant was discarded and pellets disrupted to form a powder mixture to which 800ul of Buffer AW2 containing 70% Ethanol was added.
- The last step was to centrifuge at 12,000 rpm for 3 minutes, discard the supernant, remove the extra AW2 by a pipette and dry the DNA powder at room temperature. The DNA was then dissolved in 200 ul Tris-EDTA buffer containing 0.1mM EDTA (pH8.0) and 10mM Tris-Cl (pH 8.0) and labelled and stored at -20° C awaiting experiments.

2. *DNA amplification by LAMP and PCR:*
A final elution of 200 ul of DNA was obtained and for each sample, its DNA concentration was measured then labelled and stored at refrigeration of -20° C. Samples were whenever needed, thawed to 4°C and used at room temperature while placed on ice.

*i) PCR Assay*

To begin the experiments, PCR assays were done on laboratory-cultured snail DNA as preliminary tests to determine the optimum PCR protocol and to set up PCR as the study's control assay. The generic PCR protocol described by Takara Biotechnology (Dalian) Company of Japan, which is described here below, was used as the primary reference protocol from which this study protocol would be developed. To establish this study protocol, variables like age of snail post-infection and PCR premix ingredients and their concentrations as well as the thermocycling conditions were variously varied and titrated against each other until the best protocol was determined.

The following study protocol was selected to be used for this study's PCR assays.

- For each test sample, a PCR premix was made containing 2.5 µl of 10 x Ex Taq Buffer (20mM Mg2+ plus), 2.0 µl of dNTP Mixture (2.5mM) (all from Takara Bio Inc. Japan), Japan), 2.0 µl of 10mM x Primer SM COI Forward Primer for S. mansoni, 2.0 µl of 10mM x Primer SM COI Reverse Primer for S. mansoni (both from G-net, Japan), 0.05 µl of 25mM x Magnesium Chloride (Roche, Germany) and 13.3 µl of DDW water.
- 3 µl of test DNA sample (Snail, infected snail or S. mansoni) was added for each PCR assay. 0.15 µl of Taq Polymerase enzyme, TaKaRa Ex Taq HS (Takara Bio Inc. Japan). Japan) was added to the premix to make a final 25 µl test mixture which was then run in the PCR thermo-cycler.
- For thermocycling, PCR cycles were run serially thus:- 1 cycle of 95°C for 2 minutes; 35 cycles of 94°C for 30 seconds, 62°C for 30 seconds and 72°C for 50 seconds; and finally 72°C for 10 minutes.
- The amplicon was analyzed by Ethidium Bromide enriched 3% Agarose Gel Electrophoresis to visualize and characterize the amplified DNA under UV light.

*ii) LAMP Assay*

Similar to the PCR assay protocol, preliminary LAMP assays were first done on laboratory cultured snail

DNA to determine the optimum protocol which was then employed to test field snail samples. Here as in the previously described PCR assays, the same study variables were variously varied and titrated against each other using the generic LAMP assay protocol developed by New England BioLabs Company, which is described here below, until the optimum study protocol was determined.

The following LAMP protocol was selected as optimum and used to test field samples:

- For each test sample, a LAMP premix was made containing 2.5 µl of 10 x Bst Isothermal Amplification buffer, 2.5 µl of 10mM x dNTP Mixture, 1.5µl of 100mM x Magnesium Sulphate (All from New England BioLabs Inc.), 0.4 µl of 100mM x Primer FIP, 0.4 µl of 100mM x Primer BIP, 0.5 µl of 10mM x Primer forward internal (F3), 0.5 µl of 10mM x Primer reverse internal (B3) (All from G-Net, Japan), 4.0 µl of 5M x Betaine (Sigma Life Sciences, USA) and 8.7 µl of DDW water.
- 3 µl of test DNA sample (Snail, infected snail or S. mansoni) was added for each LAMP assay.
- µl of 8 units/ µl Bst-DNA polymerase enzyme, (New England BioLabs Inc.) was added to the premix to make 25 µl test mixture.
- The LAMP mixture was run in the thermo-cycler at a constant temperature of 63°C for 2 hours.

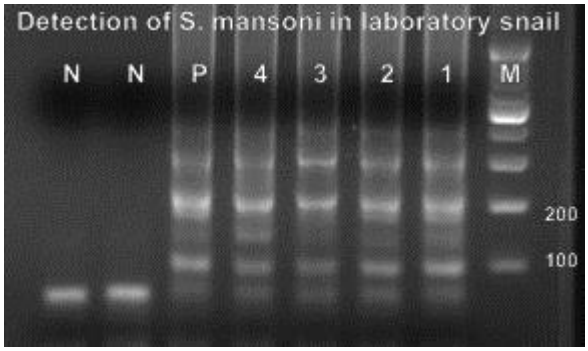
3. *Endpoint / Amplicon Detection:*

Both the LAMP and PCR amplicons were analyzed by Ethidium Bromide enriched 3% Agarose Gel Electrophoresis and visualized under UV light. Additionally, for LAMP assays, SYBR®-Green I dye (Lonza Rockland, Inc., USA) was added to the test tubes and the amplified products were visualized by the naked eye under natural light. The dye was diluted 10-fold with Tris-EDTA (ET) buffer containing Tris Hydrochloric Acid and 0.1M EDTA (Sigma Life Sciences, USA) at a pH of 8.0 and 2 µl was added to the amplicon tube. Positive samples produced a green colour while negative samples remained orange.

## RESULTS

***DNA Extraction and Assay Protocol Development:***

S. mansoni DNA was successfully extracted from dried snails and amplified by LAMP and PCR. A maximum detection sensitivity level of one miracidium one day old was achieved. Figures 3a, 3b and 4 show the agarose gel electrophoresis (AGE) and SYBR®-Green-I-dye results.




*LAMP amplicon size = tandem repeats of 121

**Figure 3a: AGE of LAMP of S. mansoni from laboratory snails. All samples are positive.**

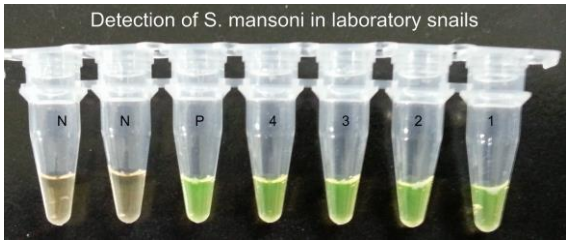



**Figure 3b: SYBR®-Green dye-LAMP detection of S. mansoni from laboratory B. glabrata snail**


*PCR amplicon size = 201 bp.

**Figure 4: AGE of PCR of S. mansoni from laboratory snails. All samples are positive.**

**Key (Figures 3a, 3b and 4): -**

1 = Snail + 5 miracidia after 5 days
2 = Snail + 5 miracidia after 1 day
3 = Snail + 1 miracidium after 5 days
4 = Snail + 1 miracidium after 1 day
P = Positive Control (*S.mansoni*)
N = Negative Control (*B. glabrata*)
N = Negative Control (Water)
M = 100 base pair (bp) Molecular Weight Marker
Orange Dye = Nagetive Result
Green Dye = Positive Result

***Interpretation***

All the laboratory snail DNA samples that were tested by both LAMP and PCR were positive including the sample from snails one day after being infected with a single miracidium. This result showed that the LAMP and PCR protocols were working well, and it set the sensitivity level of the study. The SYBR®-Green dye LAMP assays were also positive in conformity with electrophoresis. The conclusion made was that the protocols were good and could therefore be applied to test field samples.

***DNA Extraction and Testing of Field Snails.***

Out of 29 field samples, 7 samples were positive by LAMP assay and 4 by PCR (Table 3) also proving that LAMP is more sensitive than PCR. Separate DNA sequencing confirmed these results. LAMP and PCR amplicon or endpoint detection was done by ultraviolet light visualized gel electrophoresis. In addition, LAMP amplicon was demonstrable by use of a normal light visible SYBR®-Green I dye which could be used in resource-poor field laboratories.

Pictorial results of selected field samples are shown figures 5, 6 and 7 below.


**Figure 5: Agarose Gel Electrophoresis (AGE) - LAMP detection of S. mansoni in field snails. Samples 1, 3, 5 and 10 were positive.**

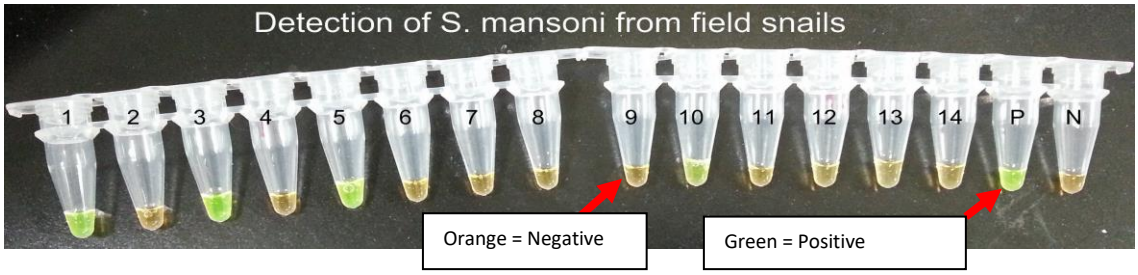



**Figure 6: SYBR®-Green I dye- LAMP detection of S. mansoni in field snails. Samples 1, 3, 5 and 10 were positive corresponding to AGE results.**

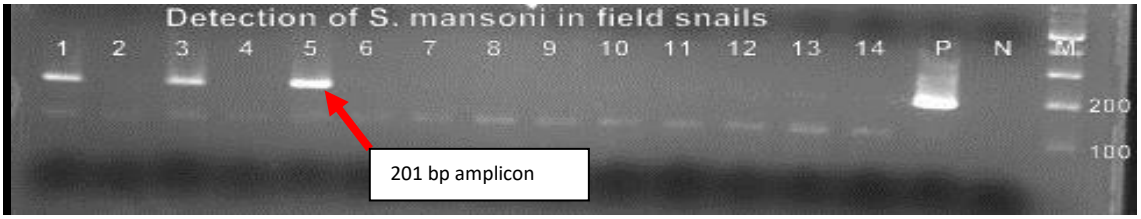



**Figure 7: AGE- PCR detection of S. mansoni in field snails. Samples 1, 3 and 5 are positive.**

**Key (Figure 5, 6 and 7):-**

1-11: Uyoga, 12: Sewage, 13-14: Tinga, P: Positive Control (*S.mansoni*)
N: Negative Control (*B. glabrata*) M: 100 bp Marker *PCR Amplicon size = 201 bp

The table below shows the summary results of all the 29 field samples.

**Table 4**
*Summary of All Field Snails: LAMP and PCR Results*

| Sample Name | Number of Samples | PCR (+) (% Positive) | LAMP (+) (% Positive) |
|---|---|---|---|
| Tinga | 6 | 0 (0.0) | 0 (0.0) |
| Uyoga | 19 | 4 (21.1) | 7 (36.8) |
| Sewage | 4 | 0 (0.0) | 0 (0.0) |
| **TOTAL** | **29** | **4 (13.8)** | **7 (24.1)** |

## DISCUSSION

Study results were significant because LAMP was successfully applied to test field laboratory snails for the presence of S. mansoni. The study results were confirmed by separate experiments that sequenced the entire extracted DNA. The LAMP test endpoint could also be demonstrated using a field laboratory suitable, naked-eye visible dye. Other workers like Hamburger [7] used fresh snails sourced from African countries to detect S. mansoni infection in snails by PCR while Abbasi [11] used LAMP to demonstrate that SYBR®-Green dye can be added to LAMP tubes to a positive test by a naturally visible colour change.

The ability to use a naked eye visible dye removes the need for an ultraviolet light viewing box and agarose gel electrophoresis both which are special equipment that are usually expensive and often unavailable in field laboratory settings like in Mbita. The findings of this study correlate well with those of similar studies done elsewhere in terms of test performance and affirm the equal utility of dried snails.

More studies need to be done to make this method fully usable for resource-poor settings without even needing a thermocycler but perhaps a simple thermostat operated water bath to provide the constant temperature to power the LAMP reaction.

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