

Optimization of a Loop-Mediated Isothermal Amplification (LAMP) Assay for *Schistosoma Mansoni* (Trematoda: Digenea) Detection in *Biomphalaria* spp. from Endemic Areas for Schistosomiasis in Brazil

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Abstract

Background: Schistosomiasis mansoni is a neglected tropical disease endemic in Brazil caused by *Schistosoma mansoni*, which is transmitted by *Biomphalaria* snails. Among all measures to control and eliminate the disease, accurate mapping and monitoring of snail breeding sites for susceptible and/or infected hosts in endemic areas are recommended. Parasitological methods are frequently used to identify infected snails, although they have many limitations, often providing false-negative results. Loop-mediated isothermal amplification (LAMP) is a promising alternative method for a more sensitive, rapid, and cost-effective diagnosis. However, standardization of LAMP assays is challenging due to the variety of parasites that are co-endemic with *S. mansoni*, and their varying prevalence rates in different areas. In this work, we aimed to optimize a LAMP assay for the detection of *S. mansoni* in *Biomphalaria* snails from endemic areas in the state of Minas Gerais, Brazil.

Methods: A total of 1,001 snails were collected in five municipalities of the Mucuri and Jequitinhonha Valleys. Snails were pooled and squeezed according to the collection site to detect the presence of the larval forms of *S. mansoni* and other trematodes. Pooled snails were submitted to pepsin digestion and DNA extraction. Then molecular assays were performed for the species-specific identification and characterization of the samples. A LAMP assay was optimized and validated using laboratory and field samples.

Results: Using the parasitological method, *S. mansoni* cercariae were detected in snails from two collection sites. *Biomphalaria glabrata*, the main snail host of *S. mansoni* in Brazil, was detected in 72.2% of the collection sites by PCR-RFLP. Multiplex PCR, LS-PCR, and conventional PCR allowed the detection of positive snails in four additional sites. The optimized LAMP assay was effective in detecting the presence of *S. mansoni* infection with 100% sensitivity, 91.66% specificity, and a Kappa index of 0.88, when compared to LS-PCR and conventional PCR.

Conclusions: Our findings suggest that LAMP is a good alternative for the detection and monitoring of transmission foci of *S. mansoni*, as it enabled the detection of infection three times more than the parasitological examination and is more applicable directly in the field when compared to other molecular approaches.

1 Background

Schistosomiasis is a parasitic disease that affect nearly 240 million people in the world. The disease is highly associated with low sanitation conditions and poverty, which lead people to use contaminated water for work and leisure (1). It is estimated that over 25 million people live in areas with a high-risk of schistosomiasis in the Americas. In Latin America, approximately 7.1 million people are infected with the etiological agent *Schistosoma mansoni* Sambon, 1907, with 95% of them living in Brazil (2), where the Northeast and Southeast regions are the most affected (3). *Biomphalaria* (Preston, 1910) snails are essential for maintenance of the parasite life cycle. Eleven *Biomphalaria* species and one subspecies

have been reported in Brazil, and three of them – *Biomphalaria glabrata* (Say, 1818), *Biomphalaria tenagophila* (d'Orbigny, 1835) and *Biomphalaria straminea* (Dunker, 1848) – have been found naturally infected with *S. mansoni* (4). The presence of the susceptible snail hosts in water bodies is crucial for parasite development, and determines the distribution of the disease (5). Knowledge regarding the geographic distribution of *Biomphalaria* snails in Brazil is being progressively updated and demonstrates that intermediate host species are spreading to new locations (3).

Among the control measures available to eliminate schistosomiasis, the surveillance of potential and active transmission foci, together with snail control measures, are highly recommended (6). Traditionally, the detection of infected snail hosts is performed by either inducing cercarial shedding through artificial light exposure (7) or using the shell-crushing method, both followed by stereomicroscope examination to detect either cercariae or sporocysts found in snail tissue (8, 9). However, many factors can limit the effectiveness of these parasitological methods. Inducing cercarial shedding does not detect the early stages of snail infection, and can result in misidentification, as the larvae of other trematode species are morphologically similar to each other, requiring an experienced person for their accurate identification. The shell-crushing method does not allow the specific identification of sporocysts and can damage the cercarial tissue hindering the observation of differential morphological characters. In addition, neither methods can be performed using dead snails (10–13).

In order to overcome these limitations, several alternative methods for the xenomonitoring of human schistosomes have been described. Molecular approaches, such as conventional polymerase chain reaction (PCR) (14–17), low-stringency polymerase chain reaction (LS-PCR) (18), restriction fragment length polymorphism (PCR-RFLP) (19), nested PCR (17), multiplex PCR (20–22), real-time quantitative PCR (qPCR) (23, 24), DNA sequencing (25), and loop-mediated isothermal amplification (LAMP) (26–30) have all proved to be more accurate and sensitive alternatives than the traditional microscope-based methods. Despite the high sensitivity and specificity of molecular methods, their cost and requirement of laboratory infrastructure have limited their usage in surveillance. In this context, LAMP assay stands out as a promising method for the direct detection of *S. mansoni* infection in the field, as it does not require laboratory equipment, such as a PCR machine or electrophoresis apparatus (27).

The estimated annual cost of schistosomiasis in Brazil is over US\$41 million, with more than 90% of this economic burden being related to indirect costs (e.g., loss of productivity and wages due to sick-leave, hospitalization, and premature death) (31). This high economic burden, and the persistence of schistosomiasis transmission in many areas in Brazil, highlight the need for additional tools to control and eliminate the disease. By the optimization of the LAMP assay performed in this study, we provided a rapid, accurate, specific, and sensitive isothermal method as an alternative approach for mapping and monitoring *S. mansoni* infection in *Biomphalaria* snail hosts.

2 Methods

2.1 Study area and malacological survey

The study was conducted in the municipalities of Franciscópolis (-17.9579, -42.0079) and Malacacheta (-17.84379959, -42.11119843) located in the Mucuri Valley region, and Jequitinhonha (-16.4355, -41.0033), Joáima (-16.653889, -41.030833) and Ponto dos Volantes (-16.752778, -41.503889) located in the Jequitinhonha Valley. Both regions are endemic areas for schistosomiasis and located in the state of Minas Gerais, Brazil (Additional File 1).

One-thousand-and-one snails were collected between July and August 2019 by team members of the Helminthology and Medical Malacology Research Group (René Rachou Institute / Fiocruz-Minas). All collection sites were georeferenced using global positioning system (GPS) technology (Additional File 2). The snails were transported to Fiocruz Minas, and after all the analyses reported here, part of the collected snails were deposited in the Medical Malacology Collection (Fiocruz-CMM).

2.2 Parasitological examination and morphological identification of trematode larvae

In the Lobato Paraense Mollusk Room (LPMR) at the René Rachou Institute / Fiocruz-Minas, snails were separated into pools according to their collection site and then submitted to the shell-crushing method to detect natural infection with *S. mansoni* or other trematodes. The squeezed material was examined under a stereomicroscope to detect the presence of cercariae and/or sporocysts. The detected cercariae were isolated and then observed under an optical microscope using non-permanent preparations for morphological identification. The morphological identification step was carried out according to the identification keys and descriptive works of different authors (32–35). Cercariae were preserved in ethanol for future analysis.

2.3 Pepsin digestion and DNA extraction

The squeezed snail pools were transferred to 50 mL centrifuge tubes labelled with a collection site code, and submitted to pepsin digestion following the protocol of Wallace & Rosen (36), and sedimentation by the Baermann-Moraes method. The sediment was centrifuged for 20 min at 5,000 *g*, the supernatant was removed, and the remaining pellet was cryopreserved at -80°C until DNA extraction.

DNA extraction from the digested pool of snails was performed using the Wizard® Genomic DNA Purification Kit (Promega, Madison, USA), according to the manufacturer's instructions. The DNA from the cercariae isolated from snails was extracted using the DNeasy Blood and Tissue Kit (Qiagen, MD, USA), again following the manufacturer's protocol.

2.4 PCR-RFLP for species-specific molecular identification of snails

Genomic DNA (gDNA) obtained from snail samples from all collection sites were used as template for a species-specific identification using a PCR-RFLP assay. The species-specific profiles generated after the digestion of the amplified ITS fragment by the *Dde*I restriction enzyme (Promega, Madison, USA) were

used to identify the snails present in each pool, using as a reference the profiles previously described by Caldeira et al (19). The results were visualized on silver-stained 6% polyacrylamide gels.

2.5 Multiplex PCR for family-specific molecular identification of trematodes.

In order to investigate the presence of trematode infection, the gDNA obtained from snail samples from all collection sites were used as template for a trematode-family-specific multiplex PCR according to Mesquita et al (37). The gDNA from isolated cercariae were also used to confirm morphological identification. In order to compare the size of the amplified DNA fragments obtained from the field material, various positive controls were included using gDNA from cercariae belonging to the following trematode families: the Clinostomidae Lühe, 1901, the Echinostomatidae Lühe, 1901, the Schistosomatidae Stiles & Hassall, 1898, and the Strigeidae Railliet, 1919. These samples were provided by the Laboratory of Trematode Biology, Department of Parasitology, Federal University of Minas Gerais, Brazil. Negative controls with no DNA were included in each reaction. The resulting PCR products were visualized on silver-stained 6% polyacrylamide gels.

2.6 LS-PCR for molecular detection of the presence of *Schistosoma mansoni* infection in snails.

The gDNA obtained from snail samples from all collection sites were used as template for the LS-PCR to detect *S. mansoni* infection in snails using the primers for the minisatellite region- mtDNA and protocol described by Jannotti-Passos et al (18). A sample from *S. mansoni* (10 ng/μl) was included as a positive control, and as a negative control no DNA was used. The amplification profile of the positive control was used as a standard for the amplification profile obtained from the unknown DNA samples.

2.7 Conventional PCR for molecular detection of the presence of *Schistosoma mansoni* in snails.

The outer primers F3 and B3 designed by Fernández-Soto et al (38) were used in conventional PCR to amplify a 203 bp mitochondrial fragment from the *S. mansoni* samples from the Mucuri and Jequitinhonha Valleys. Positive (10 ng/μl of *S. mansoni* gDNA) and negative controls (no DNA) were included. The reaction was carried out in a final volume of 25 μl containing: 1X PCR Buffer (Invitrogen, USA), 1.5mM MgCl₂ (Invitrogen, USA), 0.25 mM each dNTP (Invitrogen, USA), 2 pmol/μl of each primer (F3 and B3), 1.5 U of Platinum™ *Taq* DNA Polymerase (Invitrogen, USA) and 2 μl of the DNA. The reaction was set up as follows: (i) initial denaturation at 94°C for 1 min, followed by (ii) 30 cycles of 20 s at 94°C, 20 s at 60°C and 30 s at 72°C, and then (iii) a final extension at 72°C for 10 min. The PCR products were run and visualized on silver-stained 6% polyacrylamide gels.

2.8 Optimization of a LAMP assay for specific detection of *Schistosoma mansoni* infection in snails.

In order to detect *S. mansoni* infection, gDNA obtained from pooled snails were used as a template for the LAMP assay using the primers described by Fernández-Soto et al (38) targeting the minisatellite region of the mtDNA, with optimization of the established protocol. Briefly, the optimized reaction was carried out in a final volume of 25 µl as follows: 1X Isothermal Amplification Buffer (20 mM Tris-HCl at pH 8.8, 50 mM KCl, 10 mM (NH₄)₂SO₄, 2 mM MgSO₄, 0.1% Tween20; New England Biolabs, Massachusetts, USA), 8 mM of supplementary MgSO₄ (New England Biolabs, Massachusetts, USA), 1.4 mM of each dNTP (Invitrogen, USA), 40 pmol/µL of each inner primer (FIP/BIP), 5 pmol/µL of each outer primer (F3/B3), 1 M Betaine (Sigma, USA), 8 U of *Bst* 2.0 Warm-Start DNA polymerase (New England Biolabs, Massachusetts, USA), and 2 µl of the DNA. The reaction tubes were incubated at 65°C for 50 min, and then heated at 80°C for 5 min to stop the reaction. The amplification product was detected using silver-stained 6% polyacrylamide gels. The result was also visualized by color change after the addition of 2 µl of a SYBR Green I 1,000 X (Life Technologies, California, USA) by the naked eye (positive: yellow-green, negative: orange) and by UV light exposure (positive: fluorescent, negative: non-fluorescent).

In order to access the specificity of the optimized assay, gDNA from trematodes commonly found parasitizing *Biomphalaria* snails in the Neotropical region were used as templates for the reaction described above. Cercariae samples of the following trematode families were used for this purpose: the Clinostomidae, the Echinostomatidae, the Strigeidae, the Spirorchiidae Stunkard, 1921, the Diplostomidae Poirier, 1886 and the Notocotylidae Lühe, 1909. These samples were provided by the Laboratory of Trematode Biology, Department of Parasitology, Federal University of Minas Gerais, Brazil. *Biomphalaria glabrata* positive and negative for *S. mansoni* infection, as well as adult worms of *S. mansoni*, were also used (samples provided by the Fiocruz-CMM).

The analytical detection limit of the LAMP assay was determined by serial dilutions of *S. mansoni* from 10 ng/µl to 0.1 fg/µl.

2.9 Validation of optimized LAMP assay using laboratory and field samples.

The validation step using laboratory samples evaluated the capacity of the assay to detect different stages of infection, and in pools having a different proportion of negative and positive snails. For that, *B. glabrata* snails were obtained from the LPMR and the following squeezed samples prepared: single individual snails obtained at either 1, 7, 14 or 28 days post-infection (dpi); a single pool containing 20 negative snails and 1 snail in the pre-patent period of infection; and another single pool containing 20 negative snails and 1 snail shedding cercariae. The extracted gDNA were used as templates for the optimized LAMP assay.

For validation with field samples, the gDNA from snails collected at the Mucuri and Jequitinhonha Valley regions were used as a template for the optimized LAMP assay.

2.10 Statistical analysis

To analyze the agreement between diagnostic tests, the Kappa index and its 95% confidence interval were calculated using the GraphPad online tool (www.graphpad.com/quickcalcs/kappa1/). The Landis and Koch (39) scale of agreement was used to analyze the data

The sensitivity and specificity of the LAMP assay were calculated using a combination of the results from the LS-PCR and conventional PCR as references and the following formulas: sensitivity = (number of LAMP-positive results/ number of infected snails) x 100; specificity = (number of LAMP-negative results/number of non-infected snails) x 100.

3 Results

3.1 Parasitological examination and morphological identification of trematode larvae.

Three-hundred-and-ninety-nine snails were collected from 13 collection sites in the Mucuri Valley. In the Jequitinhonha Valley, 602 snails were collected from five collection sites (Fig. 1). Observation under a stereomicroscope after the shell-crushing examination demonstrated the presence of *S. mansoni* cercariae in snails from the collection sites MV 41 and JV 04. The presence of cercariae belonging to the Spirorchiidae family was observed in snails from the collection site JV 03.

3.2 Species-specific molecular identification of the snails

Biomphalaria glabrata ITS-Dde I restriction profile was observed in snails from the collection sites MV 03, MV 16, MV 40, MV 41, MV 45, MV 49, MV 52 and MV 65 from the Mucuri Valley, and all collection sites from the Jequitinhonha Valley. *Biomphalaria kuhniana* ITS-Dde I restriction profile was observed in snails from the collection sites MV 07, MV 20, MV 34, MV 37 and MV 39 from the Mucuri Valley.

3. Molecular approaches for the detection of the presence of *Schistosoma mansoni* and infection with other trematodes in the field-collected *Biomphalaria* snails

3.3.1 Molecular investigation of the presence of four different families of trematodes in *Biomphalaria* snails

A multiplex PCR was conducted to confirm the morphological identification of the cercariae observed at the parasitological examination, and to detect the presence of the infection of parasites from Clinostomidae, Echinostomatidae, Schistosomatidae, and Strigeidae families in the field-collected snails. Cercariae isolated from the snails collected at the MV 41 site in the Mucuri Valley, and JV 04 in the Jequitinhonha Valley, had their identification confirmed as belonging to the Schistosomatidae family. No amplification was observed in snails collected at JV 03 (Fig. 2A and B). Using the gDNA extracted from

pooled snails, it was possible to detect the presence of cercariae belonging to the Strigeidae and Schistosomatidae families in snails from the collection site MV 03, the Echinostomatidae family in snails from the collection site MV 20, and the Schistosomatidae family in snails from the collection sites MV 41, MV 45, MV 52, MV 65, JV 04 and JV 05 (Figs. 2C and D).

3.3.2 Molecular detection of *Schistosoma mansoni* infection in snails using LS-PCR and conventional PCR

The amplification pattern for *S. mansoni* generated after the LS-PCR, as established by Jannotti-Passos et al (18), was observed in snails from the collection sites MV 41, MV 45 and MV 52 in the Mucuri Valley, and JV 02, JV 04, and JV 05 in the Jequitinhonha Valley (Fig. 3A and B). The conventional PCR amplification of a mitochondrial fragment using the outer primers described by Fernández-Soto et al (38) in the conditions standardized in the current study (Additional file 3), gave the same results as observed using LS-PCR. *Schistosoma mansoni* infection was detected in snails from the collection sites MV 41, MV 45 and MV 52 from the Mucuri Valley, and JV 02, JV 04 and JV 05 from the Jequitinhonha Valley (Figs. 3C and D).

3.3.3 Optimized LAMP assay for the high-precision detection of *S. mansoni* infection in *Biomphalaria* host snails

We found that with some modifications to the Fernández-Soto et al (38) protocol, the optimized LAMP assay was effective in detecting *S. mansoni* infection in snails, with no cross-reactivity to other trematode species that also parasitize *Biomphalaria* spp. (e.g. the families Clinostomidae, Diplostomidae, Echinostomatidae, Notocotylidae, Spirorchiiidae and Strigeidae) (Fig. 4A). The assay had a detection limit of 0.1 ng of the parasite DNA (Fig. 4B). The assay was also able to detect infection by *S. mansoni* in laboratory samples when polyacrylamide gels or SYBR Green I were used to visualize the amplification products. When the visual inspection by SYBR Green I was used, the infection could be detected as early as 7 days after the exposure of the snail to the parasite (Fig. 4C).

3.3.4 Applicability of the optimized LAMP assay to field-collected *Biomphalaria* snail hosts

Snails collected in the Mucuri and Jequitinhonha Valleys were examined using the optimized LAMP assay. The LAMP amplification product was detected in snails from the collection sites MV 03, MV 41, MV 45, MV 52, JV 02, JV 04 and JV 05 when visualized using silver-stained 6% polyacrylamide gels. After the addition of 2 µl of SYBR Green I 1,000X, the color change reaction was detected in snails from the same collection sites, except MV 03 (Fig. 5) (Additional file 4).

Considering LS-PCR and conventional PCR as the reference tests, the optimized LAMP assay presented a sensitivity of 100% and specificity of 91.66%. Kappa statistics showed an “almost perfect” agreement of 0.88 between the LAMP assay and the other molecular methods evaluated in this study. Therefore, our

results showed that either LS-PCR, conventional PCR, and/or the optimized LAMP assay could be used for xenomonitoring of transmission areas (Table 1), and that *S. mansoni* infection could be detected by all three methods in collection sites MV 41, MV 45, MV 52, JV 02, JV 04 and JV 05 (Fig. 1).

Table 1
Comparison between the results of LS-PCR + conventional PCR, and the LAMP assay.

| | | LAMP assay | | |
|------------------------------|----------|-------------------|----------|-------|
| | | Positive | Negative | Total |
| LS-PCR + conventional PCR | Positive | 6 | 0 | 6 |
| | Negative | 1 | 11 | 12 |
| | Total | 7 | 11 | 18 |
| | | Kappa index: 0.88 | | |

4 Discussion

Our work has identified active foci of *S. mansoni* transmission in six collection sites from the municipalities of Franciscópolis, Jequitinhonha, Joáima, Malacacheta, and Ponto dos Volantes, confirming that these are endemic areas in the state of Minas Gerais. Molecular approaches enabled the detection of infected snails with higher accuracy than parasitological methods, reinforcing the need for additional tools to precisely map and monitor endemic areas, and, in the future, achieve schistosomiasis control and elimination.

The transmission of *S. mansoni* has been reported so far in 19 Brazilian states. The state of Minas Gerais includes around 70% of the endemic areas, being the subject of many studies over the years (3, 40–44). The Mucuri and Jequitinhonha Valleys are both very poor regions within the state of Minas Gerais. The Brazilian Institute for Geography and Statistics (IBGE) (45) calculated, in 2010, the human development index adjusted for the context of each municipality (IDHM), and estimated that Franciscópolis, Jequitinhonha, Joáima, Malacacheta, and Ponto dos Volantes had indices ranging from 0.587 to 0.618, which means a low to medium level of socio-economic development, according to the United Nations Development Program (UNDP) (46). Poverty contributes to increased contact of individuals with contaminated water, as populations from underprivileged areas usually seek natural watercourses for work and leisure activities, raising schistosomiasis transmission rates (41, 43). In addition to knowledge regarding socio-economic aspects and habits, biological and ecological features are relevant for understanding the transmission process in each region (47). The temperature from the water body is a determining factor for the development of both the snail and the parasite, and prospective studies have predicted the future impact of climate change on the dynamics of transmission (48). Specific vegetation and parasitism in snails can affect not only their populational density but also cercarial abundance (49). Rain volume has a close relationship with the density of *Biomphalaria* snails and the positivity rate found

among them for *S. mansoni* infection. According to Calasans et al (47), the abundance of *Biomphalaria* snails is negatively related to the rainfall, while *Biomphalaria* infection rises during wet periods of the year. Historically, the Mucuri and Jequitinhonha Valleys have low rainfall levels in all seasons (50). Between July and August 2019, when our surveys were undertaken, the accumulated rainfall ranged from 10–30 mm³ according to the Meteorology National Institute (INMET) (51), being the lowest pluviometric measurements in that year. This indicates that the density of infected *Biomphalaria* snails found in our survey might have been underestimated due to the characteristics of rainfall while it was undertaken.

In this study, a total of 1,001 snails were collected from 18 sites in 5 municipalities of the Mucuri and Jequitinhonha Valleys in the state of Minas Gerais, Brazil. Parasitological examination of the collected snails by the shell-crushing method has enabled detection of trematode larvae in 16.6% (3/18) of the collection sites, with most of them being due to infection with *S. mansoni* (2/3).

PCF-RFLP enabled the identification of *B. glabrata* in 72.2% (13/18) of the surveyed sites, indicating that these are potential areas for the transmission of schistosomiasis, as this snail species is the main intermediate host of the parasite in Brazil due to its high compatibility with *S. mansoni* and wide distribution throughout the country (3, 47, 52, 53). *Biomphalaria kuhniana* (Clessin, 1883) were found in 27.8% (5/18) of the surveyed sites. Although this species does not have importance in the transmission of schistosomiasis, the high morphological similarity showed between *B. kuhniana* and the intermediate host *B. straminea*, highlights the importance of the correct identification of these snails in order to properly map potential foci for schistosomiasis (54–56).

Part of the snails collected in this study was deposited in the Medical Malacology Collection (Fiocruz-CMM). This collection was founded in 1993, and since then has accumulated a large number of representative specimens, especially with regard to *Biomphalaria* snail hosts in Brazil, having currently more than 16,800 snails from all over the world (57, 58). According to the database of Fiocruz-CMM available on the CRIA website (59), the presence of *Biomphalaria* snails has been previously reported in 14 of the 23 municipalities included in the Mucuri Valley. *Biomphalaria glabrata*, *B. straminea*, and *Biomphalaria schrammi* (Crosse, 1864) have been collected in 9, 11, and 2 municipalities, respectively. The last survey in Franciscópolis happened in 2013, and in Malacacheta in 2015. In our study, we have identified for the first time the presence of *B. kuhniana* in Malacacheta and *B. glabrata* in Franciscópolis. The Jequitinhonha Valley comprises 55 municipalities, and *Biomphalaria* snails have been reported in 33 of them. *Biomphalaria straminea*, *B. glabrata*, *B. kuhniana*, and *B. tenagophila* have been previously collected in 21, 18, and one municipality, respectively. The most recent surveys were conducted in Jequitinhonha in 2006, in Joáima in 2014, and in Ponto dos Volantes in 2012. The Jequitinhonha Valley results from our study matches the data obtained from previous ones. We have found that in almost all the areas where we conducted our surveys, the presence of *B. glabrata* has been maintained over the years. Data from Fiocruz-CMM combined with our findings reinforce the importance of constant monitoring of these areas.

The presence of trematode infection in snails was investigated using a multiplex PCR protocol that enables the differentiation of four important families commonly found parasitizing *Biomphalaria* snails (37, 60–62). Schistosomatidae species were detected in 44.4% (8/18) of the study sites, while Echinostomatidae and Strigeidae were each found in 5.5% (1/18). Snails from the collection sites MV 41 and JV 04 that were found shedding *S. mansoni* cercariae in the parasitological examination had their infection confirmed by multiplex PCR due to the amplification of 140 bp target corresponding to the Schistosomatidae family. In five further sites from the Mucuri Valley, and one from the Jequitinhonha Valley, the presence of Schistosomatidae infection in snails was also detected. Even though the amplification of Schistosomatidae DNA does not necessarily mean the presence of *S. mansoni* itself, this result raises concern that these areas might be potential foci for schistosomiasis. As expected, no amplification was observed in the snails collected at the JV 03 site, since the set of primers used does not cover the Spirorchiidae family isolated from this location during the parasitological examination of snails. As the primers used by the multiplex PCR only amplify four trematode families, it is not possible to confirm that snails from the remaining collection sites are not infected by other trematode families.

LS-PCR and conventional PCR were both able to detect the presence of *S. mansoni* in 33.3% (6/18) of the surveyed sites. The optimized LAMP assay developed in this work revealed the presence of in snails from 38.8% (7/18) of the collection sites, when amplification was visualized using polyacrylamide gels, having an “almost perfect” Kappa agreement with LS-PCR and conventional PCR, with 100% sensitivity, and 91.66% specificity. When the chosen method to check the result was visual inspection of reaction tubes after the addition of an intercalating dye, amplification was detected in six collection sites, presenting the same result obtained when LS-PCR and conventional PCR were used. A very weak amplicon corresponding to the Schistosomatidae family was detected in snails from the collection site MV 03 but no amplification was detected with LS-PCR and conventional PCR using this sample as a template. The apparent LAMP product from this sample, when visualized using a polyacrylamide gel, raises the hypothesis that the LAMP assay was more sensitive than LS-PCR and conventional PCR in detecting *S. mansoni* infection in snails. However, the limit of detection of each method indicates that this is not the case, since LS-PCR can detect up to 1 pg of *S. mansoni* DNA (18), conventional PCR up to 0.01 pg (Additional file 3) and optimized LAMP assay presented a limit of detection of 0.1 ng. Therefore, LAMP is less sensitive than the other evaluated methods, suggesting that snails from the site MV 03 are not infected with *S. mansoni*. Although several trematode samples have been used to test the specificity of the optimized LAMP assay, samples from other species that belong to the Schistosomatidae family have not been used. The analysis of the results from the LAMP assay combined with multiplex PCR suggests cross-reactivity between members of the same family. In the Brazilian context, other schistosomes do not have much relevance to human health, as only *S. mansoni* causes schistosomiasis in this country. Avian schistosomes have been reported causing cercarial dermatitis in countries from the Northern Hemisphere, but the occurrence of this condition has not been reported so far in Brazil (63–65). As an alternative to overcome this false-positive result, we suggest that the visual inspection of reaction tubes by the naked eye should be prioritized, instead of running the LAMP products in gels. This visualization strategy not

only reduces the possibility of false results, but also makes the assay more applicable directly in the field in low infrastructure conditions.

The LAMP assay was first described in 2010 (66), and since then this technique has been used to detect many pathogens, including *S. mansoni*, but mostly in human samples (38, 67–71). The applicability of LAMP for screening snails to characterize transmission areas is very promising (49), and has been tested by several authors (26–30). Molecular techniques can detect the presence of *S. mansoni* even when snails are not shedding cercariae, which would provide valuable information for surveillance services, as in many endemic areas collected snails rarely shed cercariae even though schistosomiasis transmission remains present. This failure to find cercarial shedding can be misleading, often giving the false impression of low or even absent transmission (49). Although molecular methods such as conventional PCR, DNA sequencing, and qPCR can properly fulfill this gap, these techniques are inappropriate for laboratories with limited resources, as they require expensive machinery and technical expertise, raising the associated costs of each reaction. Among all advantages of the using isothermal assays, the possibility of performing the test directly in the field in laboratories with limited infrastructure is undeniable. Our group optimized the LAMP assay using the primers described by Fernández-Soto et al (38). When following the exact conditions described by Gandasegui et al (29), non-specific amplification was detected in trematode samples belonging to the families Diplostomidae and Spirorchiidae (Additional file 5). Even though the optimization resulted in a reduction in the analytical limit of detection (from 1 fg to 0.1 ng), our findings confirmed that the amount of *S. mansoni* DNA that can be detected by the assay is sufficient to detect, by visual inspection alone, the presence of the parasite 7 days after exposure of the snails to 8 miracidia, and in pooled samples.

By the use of the optimized LAMP assay, we detected three times more the infection by *S. mansoni* in snails when compared to parasitological examination using shell-crushing method, revealing six active transmission areas for schistosomiasis in both Mucuri and Jequitinhonha Valleys. Molecular methods also allowed the mapping of potential transmission foci through the identification of *B. glabrata* in much of the surveyed area, as demonstrated in the maps generated by this study.

5 Conclusion

Parasitological methods based on the detection of *S. mansoni* larval forms in *Biomphalaria* snails are limited and are affected by the variation in disease prevalence in different regions, such that false negative results may often be obtained when these methods are applied. The optimization of a LAMP assay provides a sensitive, specific, rapid, and precise diagnostic alternative, with a performance as good as other molecular approaches evaluated in this study. However, as an isothermal method, LAMP is easier to perform directly in the field or in low-infrastructure laboratories. Considering the challenges to controlling schistosomiasis, or even to interrupt its transmission in endemic areas, mapping and monitoring transmission foci with higher accuracy will improve decision-making processes enabling more appropriate allocation of public funding and resources aimed at the elimination of schistosomiasis as a public health problem.

Abbreviations

LAMP: loop-mediated isothermal amplification; MG: Minas Gerais; DNA: deoxyribonucleic acid; PCR-RFLP: polymerase chain reaction restriction fragment length polymorphism; PCR: polymerase chain reaction; LS-PCR: low stringency polymerase chain reaction; qPCR: real-time quantitative polymerase chain reaction; gDNA: genomic deoxyribonucleic acid; ITS: internal transcribed spacer; rDNA: ribosomal deoxyribonucleic acid; mtDNA: mitochondrial deoxyribonucleic acid; Fiocruz: Oswaldo Cruz Foundation; Fiocruz-CMM: Medical Malacology Collection; GPS: global positioning system; LPMR: Lobato Paraense Mollusk Room; dpi: days post-infection; MV: Mucuri Valley; JV: Jequitinhonha Valley.

Declarations

- **Ethics approval and consent to participate:**

Not applicable

- **Consent for publication:**

Not applicable

- **Availability of data and materials:**

Not applicable

- **Competing interests:**

The authors declare that they have no competing interests.

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- **Authors' contributions:**

Conceptualization: RLC, OSC and SGM; Methodology: SGM and RLC; Validation: SGM and FGSN; Formal analysis: SGM, CTF, RLC and RGCS; Investigation: SGM and FGSN; Resources: RLC and OSC; Data curation: SGM, RLC and CTF; Writing - original draft: SGM; Writing – review & editing: RLC, CTF, OSC and RGCS; Visualization: SGM, FGSN, RGCS and CTF; Supervision: RLC and CTF; Project Administration: RLC and SGM; Funding acquisition: RLC and CTF. All authors read and approved the final manuscript.

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Figures

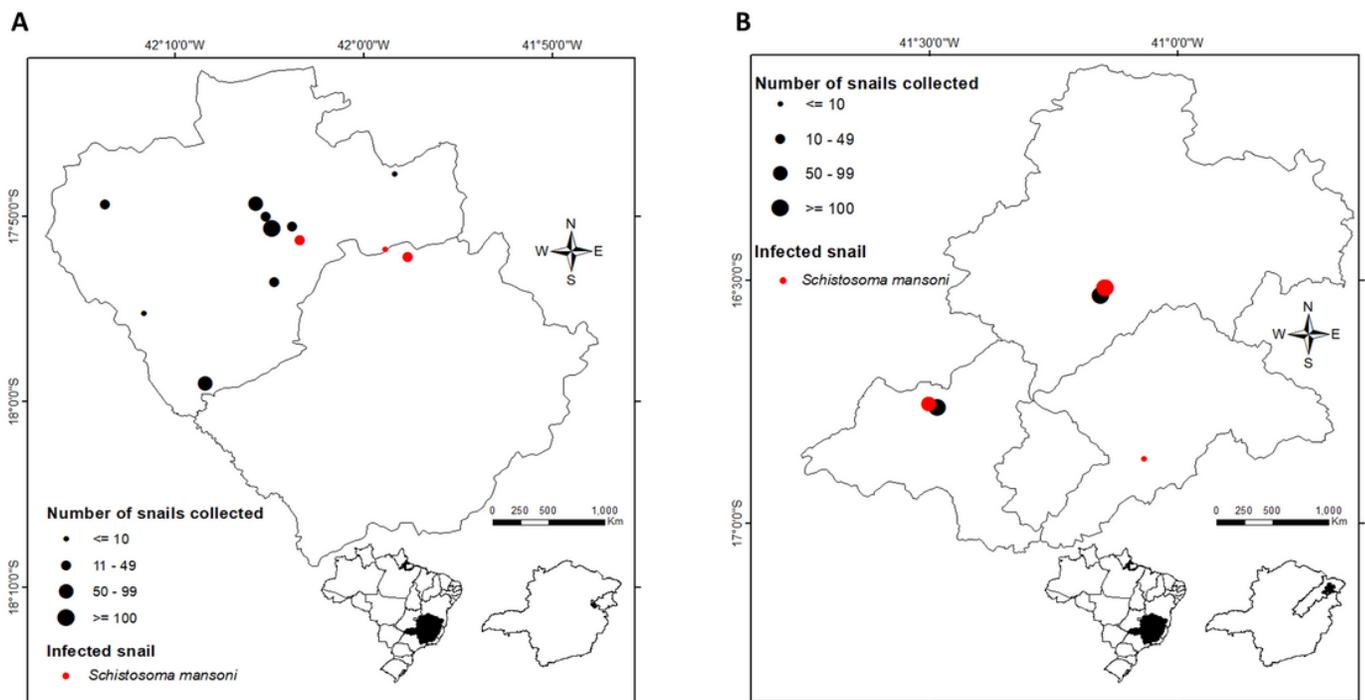


Figure 1

Study areas maps. The dots represent the collection sites and the population density of snails in the (A) Mucuri Valley region and (B) Jequitinhonha Valley region. Red dots represent the location where *S. mansoni* infection was detected in snails after the use of molecular methods. Note: The designations employed and the presentation of the material on this map do not imply the expression of any opinion whatsoever on the part of Research Square concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. This map has been provided by the authors.

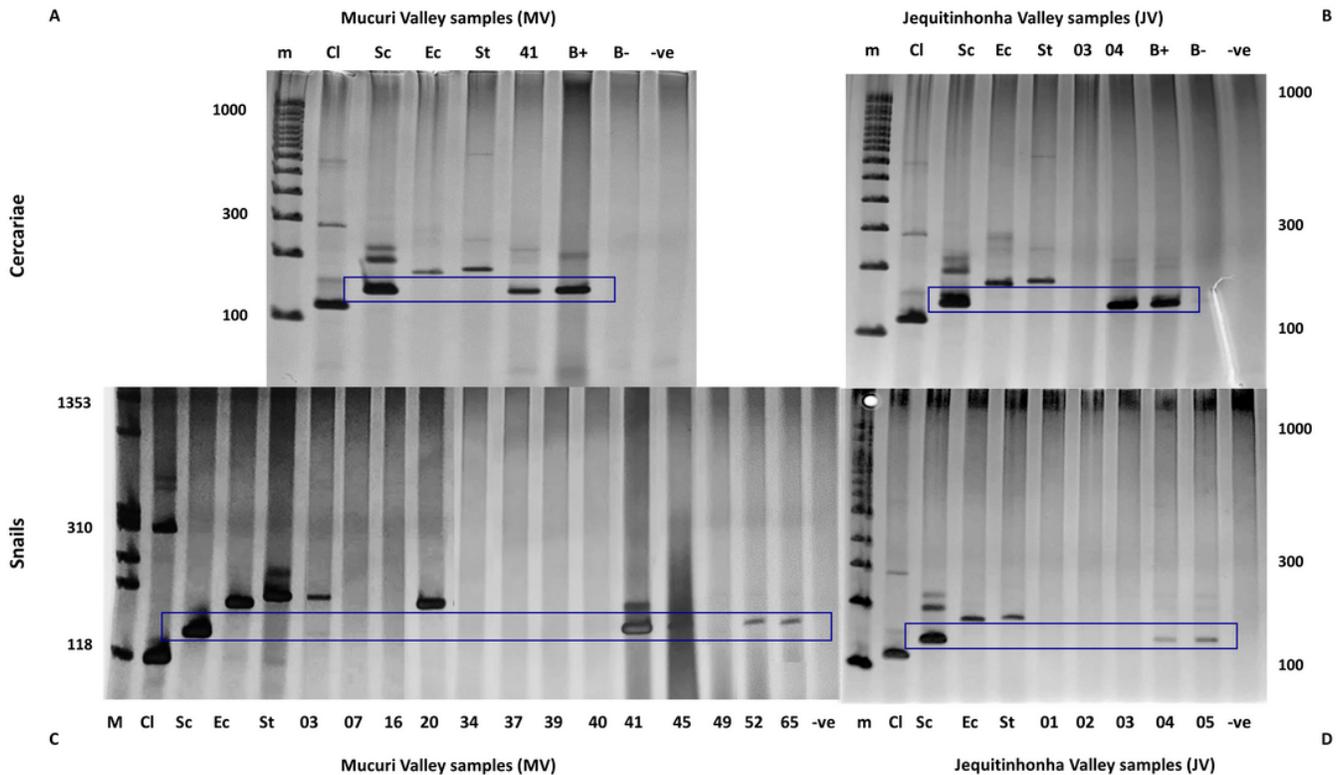


Figure 2

Multiplex PCR results. Identification of the cercariae isolated during the parasitological examination of snails collected in the (A) Mucuri and (B) Jequitinhonha Valleys. Schistosomatidae family DNA was detected in the collection sites MV 41 and JV 04. Investigation of the presence of Trematode infection in snails collected in the (C) Mucuri and (D) Jequitinhonha Valleys. Schistosomatidae family DNA was detected in the collection sites MV 41, MV 45, MV 52, MV 65, JV 04 and JV 05. A coinfection with the Strigeidae and Schistosomatidae families was detected in the collection site MV 03. Echinostomatidae family DNA was detected in the collection site MV 20. m, 100bp marker; M, Phi X 174 Hae III marker; Cl, Cercariae belonging to Clinostomidae family; Sc, Cercariae belonging to Schistosomatidae family; Ec, Cercariae belonging to Echinostomatidae family; St, Cercariae belonging to Strigeidae family; B+, *B. glabrata* infected with *S. mansoni*; B-, *B. glabrata* non-infected with *S. mansoni*; -ve, Negative control.

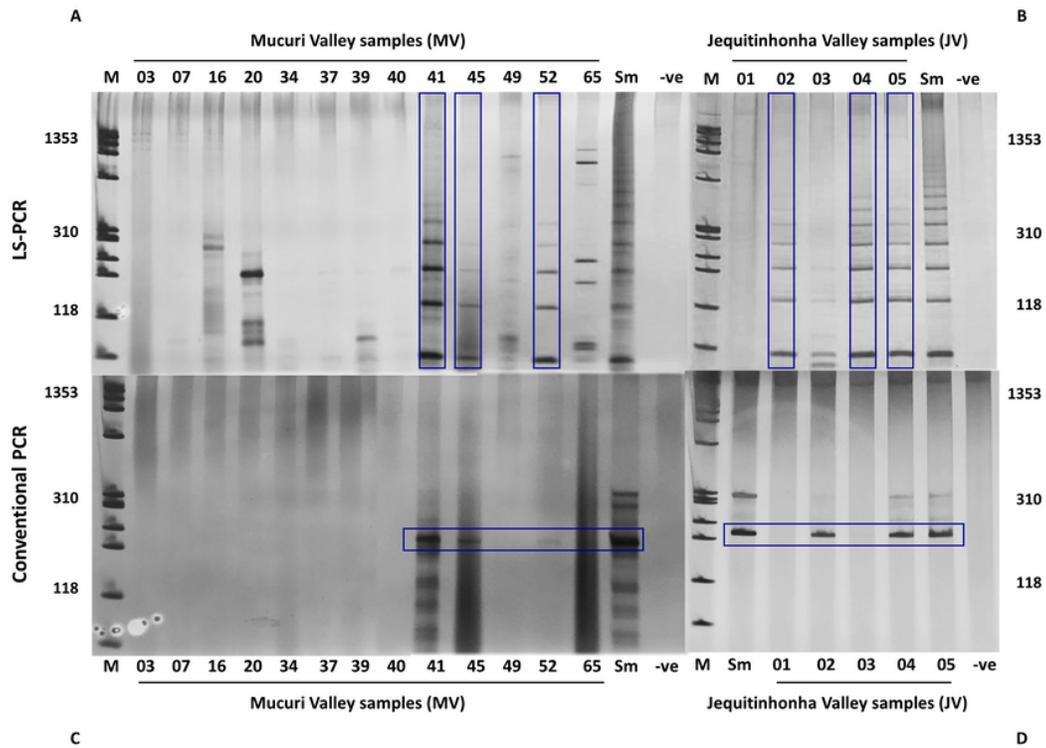


Figure 3

Detection by LS-PCR of *S. mansoni* infection in snail samples from (A) Mucuri and (B) Jequitinhonha Valleys, and by conventional PCR using snail samples from (C) Mucuri and (D) Jequitinhonha Valleys. Using both methods, infection was found in the collection sites MV 41, MV 45, MV 52, JV 02, JV 04 and JV 05. M, Phi X 174 Hae III marker; Sm, *S. mansoni*; -ve, Negative control.

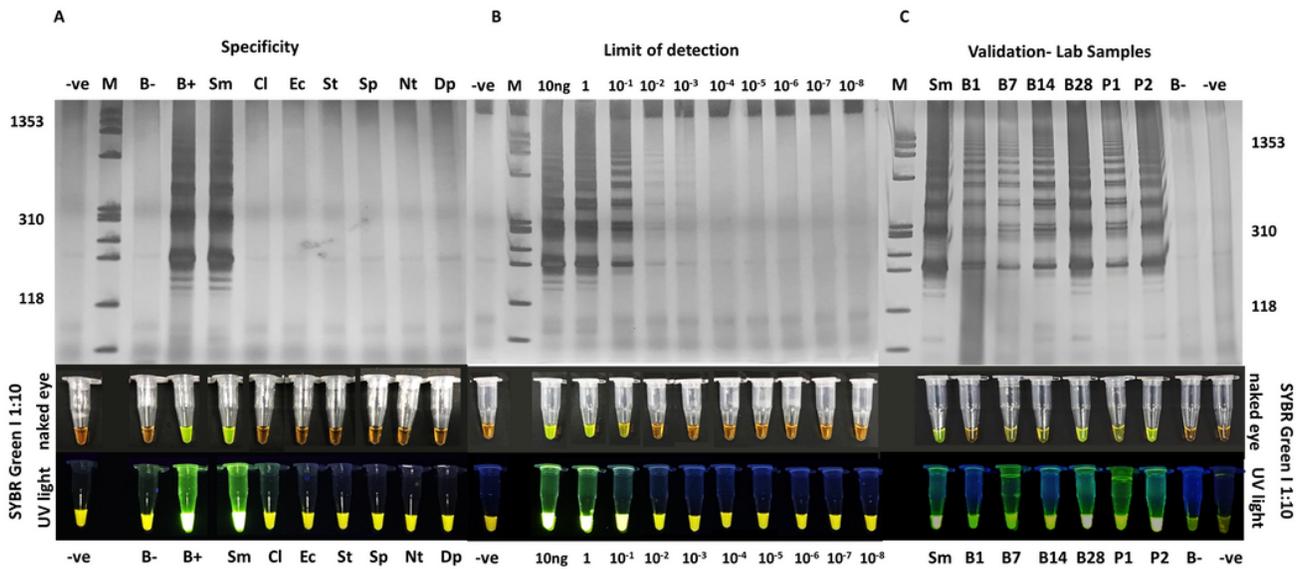


Figure 4

Optimization and validation of the LAMP assay. LAMP products were visualized either by silver-stained 6% polyacrylamide gels or visual inspection of reaction tubes using a 1:10 dilution of SYBR Green I by the naked eye (positive: yellow-green; negative: orange) or UV light exposure (positive: fluorescent; negative: non-fluorescent). (A) Specificity analysis of the optimized LAMP assay. No cross-reactivity was detected. (B) Analytical limit of detection. Optimized LAMP detects up to 10^{-1} ng of *S. mansoni* DNA. (C) Validation of the optimized LAMP assay using snails maintained in the laboratory in different stages of infection and pool conditions. *Schistosoma mansoni* DNA was detected in all conditions. M, Phi X 174 Hae III marker; B-, Negative snail; B+, Biomphalaria snail infected with *S. mansoni*; Sm, *S. mansoni*; Cl, Cercariae belonging to Clinostomidae family; Ec, Cercariae belonging to Echinostomatidae family; St, Cercariae belonging to Strigeidae family; Sp, Cercariae belonging to Spirorchiiidae family; Nt, Cercariae belonging to Notocotyliidae family; Dp, Cercariae belonging to Diplostomidae family; B1, Snail 1 day post-infection (dpi); B7, Snail 7 dpi; B14, Snail 14dpi; B28, Snail 28dpi; P1, Pool of 19 negative snails + 1 snail in pre-patent period of infection; P2, Pool of 19 negative snails +1 snail shedding cercariae; -ve, Negative control

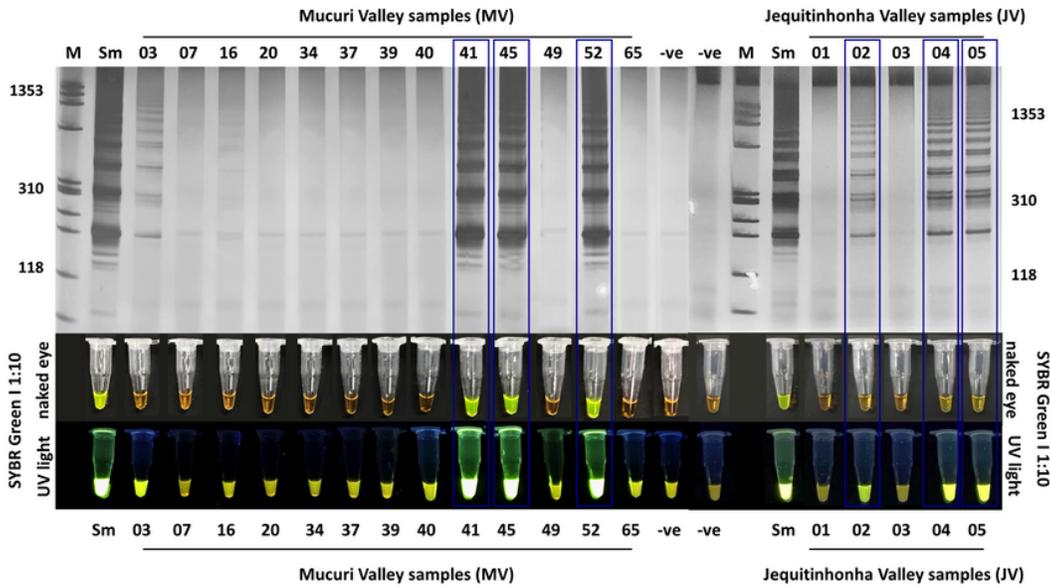


Figure 5

Mucuri and Jequitinhonha Valleys snails examined by LAMP assay to detect *S. mansoni* infection. LAMP products were visualized either using silver-stained 6% polyacrylamide gels or visual inspection of reaction tubes using a 1:10 dilution of SYBR Green I by the naked eye (positive: yellow-green; negative: orange) or UV light exposure (positive: fluorescent; negative: non-fluorescent). M, Phi X 174 Hae III marker; Sm, *S. mansoni*; -ve, Negative control

Supplementary Files

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