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DNA DETECTION FROM *Schistosoma mansoni* BY LOOP-MEDIATED ISOTHERMAL AMPLIFICATION: IDENTIFICATION OF INFECTED MOLLUSCS IN THE PREPATENT PERIOD

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Abstract: Schistosomiasis is a neglected tropical disease, which infects about 240 million individuals worldwide. It is a parasitic infection transmitted by water, whose intermediate host is a mollusc of the genus: *Biomphalaria*. The aim of this study was to standardize the detection of DNA from *Schistosoma mansoni* by means of the loop-mediated isothermal amplification (LAMP) technique in samples of primary sporocysts, obtained from the laboratory cycle of *Biomphalaria glabrata*, strain Ourinhos, infected by *S. mansoni*, strain BH, at the Laboratory of Immunopathology of Schistosomiasis (LIM-06), Faculty of Medicine, ''Universidade de São Paulo" (FMUSP), São Paulo, Brazil. **Results**: We advanced in the elaboration of the LAMP technique protocol. The identification of molluscs infected by *S.mansoni* in the pre-patent period may contribute to surveillance actions.

Keywords*: Schistosoma mansoni; Biomphalaria glabrata; Loop-mediated-Isothermal Amplification.*

INTRODUCTION

Schistosomiasis is a parasitic disease caused by a Trematode of the genus: *Schistosoma*. In Brazil only the species: *mansoni* has medical and epidemiological importance. According to the World Health Organization (WHO) it is considered one of the 20 Neglected Tropical Diseases (NTDs), which affects about 240 million individuals worldwide and more than 779 million risk contracting it (Crego et al., 2021).

In Brazil, approximately 25 million individuals live in areas where they are at risk of contracting the disease. This endemic affects 19 federative units, considered endemic areas to be a continuous strip of land that covers the states of Rio Grande do Norte towards the South (Brasil, 2014).

In the state of São Paulo, areas of autochthonous transmission of schistosomiasis are located in: Vale do Ribeira, Vale do Paraíba, Litoral Norte, Baixada Santista, Grande Campinas and some municipalities in the Metropolitan Region of São Paulo, including the capital (Secretary of State for Health, 2009). In these areas the intermediate host, snail of the species: *Biomphalaria tenagophila* (*B. tenagophila),* is predominant (Carvalho et al., 2008). The only exception is related to cases originating from natural breeding grounds for molluscs of the species: *Biomphalaria glabrata* (*B. glabrata*), restricted to water collections in the middle section of the Paranapanema River. In a study carried out by Palasio et al for the identification and distribution of mollusc species present in the State of São Paulo, the presence of *B. glabrata* in the Christoni creek, in the municipality of Ourinhos (Palasio et al., 2019).

Taking into account the potential of *B. glabrata* to adapt to the lifecycle of the *Schistosoma*, it has become important to improve the diagnosis of infection caused by *Schistosoma* in the intermediate host. Detection based on primary sporocysts that develop in molluscs during the pre-patent infection phase may contribute to transmission control, especially in areas of low endemicity, as is the case of the municipality of Ourinhos. (Palasio et al., 2019).

Molecular biology techniques represent an important tool for the diagnosis of several pathogens, due to their high sensitivity and ability to differentiate species of the same genus. PCR (polymerase chain reaction) "*Polymerase Chain Reaction*") identified: *S. mansoni* in samples of human feces and urine and in infected snails (Steinauer; Blouin; Criscione, 2010). In addition, genetic markers such as microsatellites and mitochondrial DNA have been used as a tool to investigate

population migration frequencies (Durand; Sire; Théron, 2000).

Although conventional PCR and real-time PCR (qPCR) have great specificity, they are complex, requiring more time for a diagnosis, in addition to requiring laboratories with more resources (Oliveira, 2016).

The Loop Mediated Isothermal Amplification (LAMP) technique *"Loop-mediated Ishotermal Amplification")* was first introduced by Notomi et al. in 2000 as an alternative to other PCR-based techniques (Diego et al., 2021). The LAMP assay takes place under isothermal conditions (60-65°C) and is a method based on DNA synthesis using a DNA polymerase capable of strand displacement. It can be performed using two to three pairs of primers. Internal primers are called *forwar dinner primer* (FIP) and *backwar dinner primer* (BIP). External primers are designated as F3 and B3. The primers are used simultaneously to initiate DNA synthesis, generating a DNA stem loop for subsequent LAMP cycling, in which the target will be recognized by four sequences. Therefore, the target selectivity is expected to be greater than that obtained in PCR. The mechanism and steps of the reaction are illustrated in figure 1. (Notomi et al., 2000).

It is a simpler technique to perform, has a low cost, does not require complex equipment and presents numerous approaches to detect the result, even with the naked eye (Diego et al., 2021). The LAMP technique facilitates the examination of grouped molluscs, making it possible to monitor larger areas and areas of low transmission. (Gandasegui, 2016).

Table 1 presents a synthesis of the works carried out using molecular markers with the objective of improving the diagnosis of species of *Schistosoma mansoni* and *Schistosoma haematobium* in definitive and intermediate hosts.

File/ Year	Theme	Technique (marker used)	Goal
Espírito-Santo MCC et al., 2012	Two sequential PCR amplifications for detection of *Schistosomamansoni* in stool samples with low parasite load	cPCR; Nested PCR; TaqMan® Real-Time PCR (qPCR)	Diagnostic enhancement
Vicente-Crego B et al., 2021	Application of a genus-specific LAMP assay for Schistosome Species to detect Schistosoma hybrids haematobium x Schistosoma bovis	LAMP	Diagnostic enhancement
Gomes et al., 2009	Identification of eggs *Schistosoma* spp. in human stool sample	Conventional PCR	Diagnostic enhancement
Le; Blair and McManus, 2000	Genome project: *Schistosoma*	Mitochondrial DNA sequencing	Mitochondrial DNA genotyping of *Schistosoma* spp.
Soto-Fernández P et al., 2020	Molecular Markers for Detecting Schistosoma Speciesby Loop-Mediated Isothermal Amplification	LAMP	Diagnosis and surveillance of schistosomal infections.

Table 1 – List of works involving the investigation of the genome of Schistosoma spp. and discovery of diagnostic methods in human biological material.

Source: Personal archive (2022).

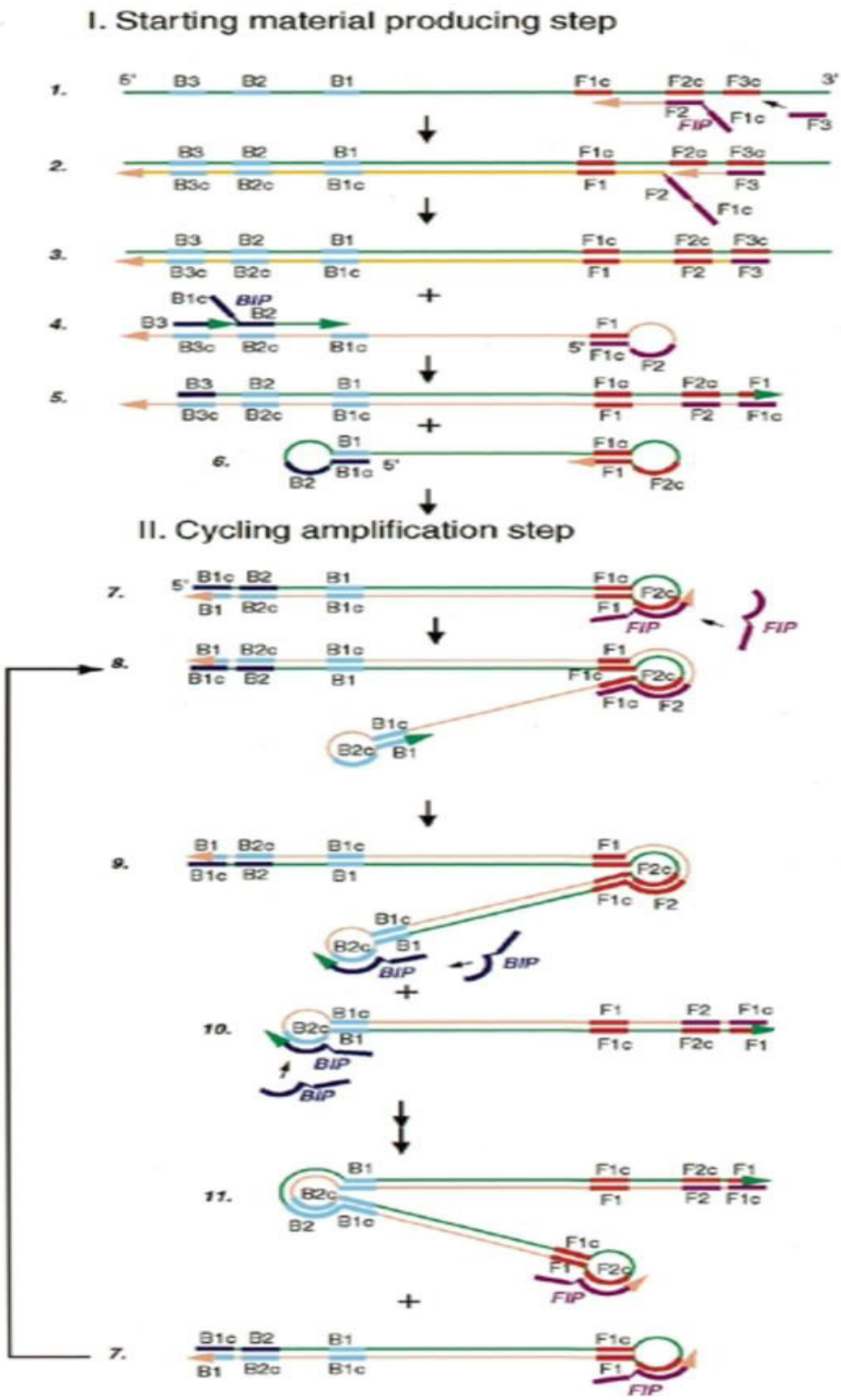

Figure 1: Steps of the LAMP reaction. Process starting from FIP primer. Source: Notomi et al., 2000.

JUSTIFICATION

In the municipality of Ourinhos, SP, Brazil, from 2008 to 2010, 16 autochthonous cases of schistosomiasis mansoni were registered, according to the Center for Epidemiological Surveillance (CVE). It is a region with large population migration processes.

The presence of *B. glabrata*, intermediate host most susceptible to infection caused by *S. mansoni,* the geographic and environmental conditions, makes the region an area with greater potential for transmission of this helminth and, consequent increase in endemicity.

It is known that the detection of positive molluscs is a difficult task in regions of medium and high endemicity. The difficulty increases even more in situations of low endemicity, which make the search for foci of infection much more difficult, since a low parasite load does not prevent human infection (Carvalho; Coelho; Lenzi, 2008).

Given these factors, this study aimed to improve the diagnosis in the intermediate host using the Loop-mediated Isothermal Amplification (LAMP) technique in the pre-patent phase of the infection, seeking to contribute to the surveillance and control of schistosomiasis.

GENERAL GOAL

The aim of this study was to standardize the detection of DNA from *Schistosoma mansoni* by means of the Loop Mediated Isothermal Amplification (LAMP) technique in samples of primary sporocysts, obtained from the laboratory cycle of *Biomphalaria glabrata*, Ourinhos strain, infected by *S. mansoni*, strain BH, at the Schistosomiasis Immunopathology Laboratory (LIM-06).

SPECIFIC GOAL

a) To standardize the detection of adult *S. mansoni* worms obtained in the routine laboratory cycle;

b) To standardize the detection of DNA extracted from primary sporocysts of *S. mansoni* by LAMP reaction in *B. glabrata* (BH and Ourinhos lineage) experimentally infected;

c) To compare the sensitivity of the LAMP reaction with TaqMan qPCR (real-time PCR) for detection of DNA extracted from primary sporocysts of *S. mansoni* em *B. glabrata.*

METHODOLOGY

ETHICAL ASPECTS

This research project (1684/2021) was submitted and approved by the Ethics and Research Committee of the Faculdade de Medicina da "Universidade de São Paulo" and by the Ethics and Research Committee involving Animals, of the Institute of Tropical Medicine, according to the norms, which comply with the Laws (6.638/79 and 9605/98), Decree 24.645/34, the Ethical Principles in Animal Experimentation (COBEA) (Geneva, 1985) and other instructions regulating animal research.

POPULATION OF ADULT MOLLUSCS AND WORMS *S.MANSONI*

The experimental cycle of *S. mansoni,* BH/IMT lineage is maintained in the Immunopathology of Schistosomiasis laboratory, Laboratório de Investigação Médica-06 (LIM-06) of the Faculdade de Medicina da: ‘’Universidade de São Paulo”, where the study will be carried out.

A new experimental cycle of *S. mansoni*, designated as Ourinhos lineage was developed in 2016, from samples of snails:*B. glabrata*, originating from the Christoni neighborhood, in the municipality of

Ourinhos, Vale do Paranapanema, São Paulo, Brazil, collected between the months of December 2015 and March 2016 by the Superintendence for Control of Endemic Diseases (SUCEN) following established instructions (Brasil, 2008). The current study population involves molluscs and adult forms of *S.mansoni*, related to these two cycles.

STUDY PROJECT

It is, therefore, an experimental study that was carried out on DNA samples extracted from molluscs of the species: *B. glabrata* originating from the Christoni district, in the municipality of Ourinhos, Vale do Paranapanema, SP, Brazil, compared with that extracted from the adult worm of *S. mansoni* and *B.glabrata/BH*.

The new creation of molluscs:*B.glabrata*, received the name of strain:Ourinhos (*B.glabrata*/Ourinhos).

Thus, three phases of the study were carried out from the establishment of the experimental cycle, according to the schedule below (Figure 2).

In order to evaluate the sensitivity and specificity of the LAMP technique, the genetic material of three groups was analyzed:

- Group I- Molluscs: *B. glabrata/BH, not infected*
- Group II- Molluscs: *B. glabrata/* Ourinhos infected by *S.mansoni*;
- Group III - S. mansoni adult worms infected by *S.mansoni*.

Group I - six DNA samples extracted from molluscs strain BH/IMT negative for *S.mansoni* infection (negative control); Group II - ten samples of DNA extracted from molluscs strain Ourinhos infected with 30 miracidia, after 72 hours; Group III – six DNA samples extracted from adult worms of

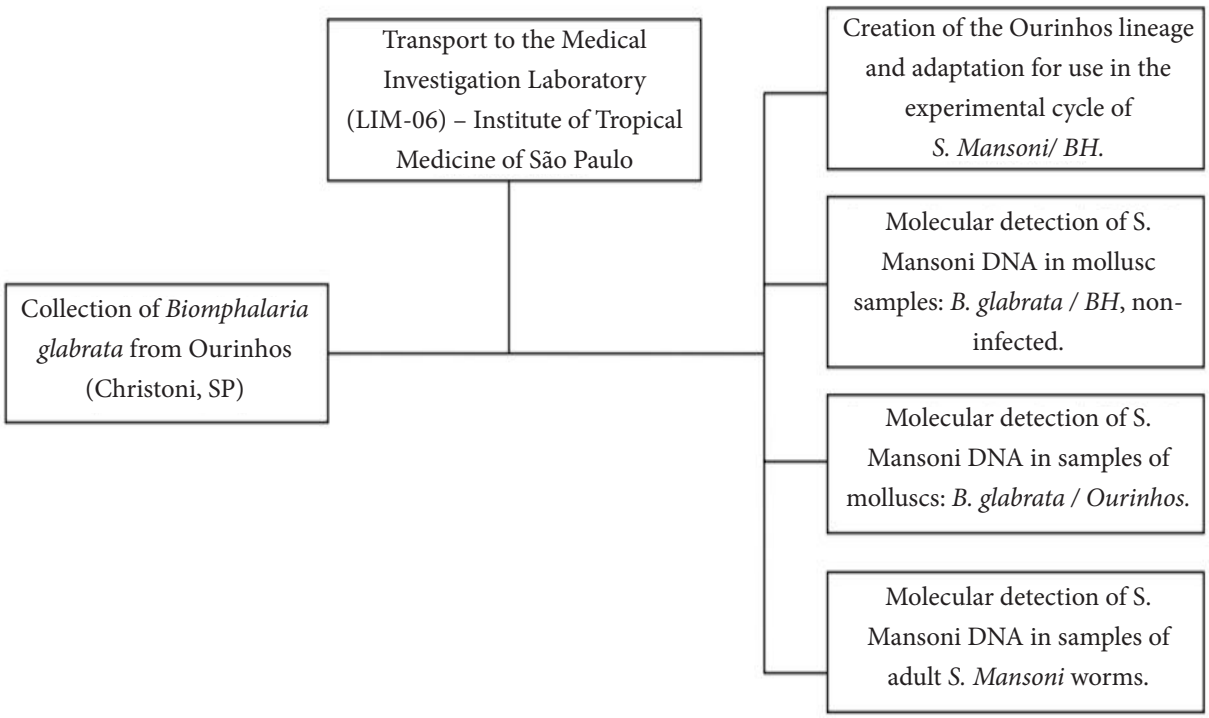

Figure 2: Study design for evaluation of snails: B. glabrata of Ourinhos experimentally infected with miracidia of S. mansoni, lineage BH. Source: Personal archive (2022).

S.mansoni. A sample of water was added to the groups to control contamination of the reaction.

PRIMERS SELECTION

The set of primers selected for detection of S. mansoni by LAMP have already been described in the literature. The outer (F3 and B3) and inner (FIB and BIP) primers were designed based on the SM1-7 repeated tandem of *S. mansoni* for every 121 base pairs. Figure 3 shows the primers used in this assay and figure 4 their location within the target sequence.

LAMP REACTION AND PRODUCT DETECTION

Reactions were carried out in 0.2 mL tubes. The reaction mix contained: 25X primer solution containing 40 ul FIP, 40 ul BIP, 5 ul F3 and 5 ul B3; 10x isothermal amplification buffer; MgSO4 (100 mM); dNTP mixture (10 mM); 8,000 U/ml of Bst 2.0 WarmsStar enzyme (*New EnglandBiolabs*); 10.5 ul of nuclease-free water. Samples of adult worms and *B.glabrata,* uninfected samples were tested with a volume of 10ng and 20ng, according to the DNA concentration quantified by the equipment: *Qubit*. The samples from Ourinhos were tested with a volume of 10ng. The reaction was incubated at 65°C for 60 minutes and then heated to 80°C for 20 minutes to stop the reaction.

The reaction result was visually inspected by colorimetric change by adding 2ul (diluted 1:10) of SYBR Green I fluorescent dye (ThermoFisher) to the reaction tubes. The color change to yellow was observed in

S. mansoni (Sm1-7)	F3	GAT CTG AAT CCG ACC AAC CG
	B3	AAC GCC CAC GCT CTC GCA
	FIP: F1c + F2	AAATCCGTCCAGTGGTTT **TTTT** GAAAATCGTTGT ATCTCCG
	BIP: B1c + B2	CCGAAACCACTGGACGGA **TTTT** TATTTTTAATCT AAAACAAAC ATC

Figure 3: F3= direct external primer; B3= outer primer backwards; FIB= direct internal primer composed of primers F1c and F2; BIP= reverse internal primer composed of primers B1c and B2. Source: ABBASI et al., 2010.

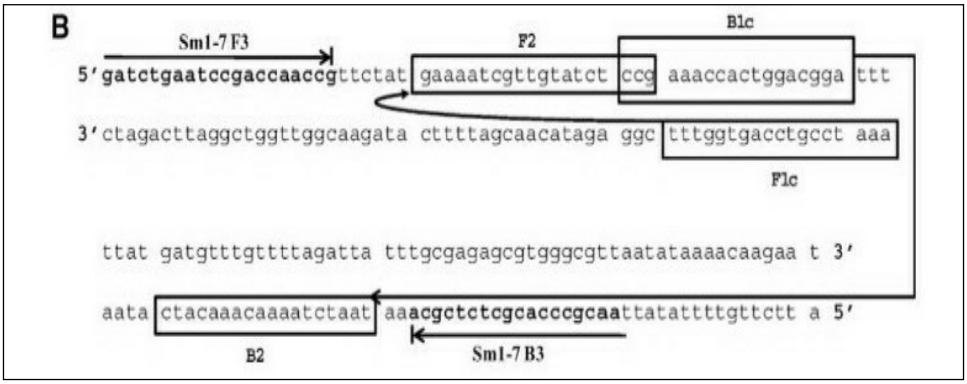

Figure 4: Localization of Isothermal Amplification Mediated by Forward (F3) and Backward (B3) Loop, and internal primers within the respective Sm1-7 S sequence. mansoni. Fonte: ABBASI et al., 2010.

positive reactions, while the orange color was maintained in negative reactions. In addition, the products were monitored by 1.5% agarose gel electrophoresis and visualized under UV light.

RESULTS

LAMP REACTION IN SAMPLES OF ADULT S.MANSONI WORMS AND UNINFECTED MOLLUSCS

To evaluate the sensitivity and specificity of the technique, the LAMP reaction was performed using samples of adult *S. mansoni* worms (Group III) and uninfected B. glabrata/ IMT molluscs (Group I) as a negative control.

The result indicated detection of fluorescence with the naked eye in the positive samples (Group III), verified by the change to fluorescent green color, generated by the dye SYBR Green I. These samples were also exposed to ultraviolet light, which intensified the positivity of the reaction observed by eye. naked (Figure 5). In the negative samples (Group I) there was no color change, remaining in orange.

The third way to identify the LAMP reaction in this study was agarose gel electrophoresis, which resulted in bands. Agarose gel electrophoresis carried out with samples from Group I (non-infected snails of the BH/IMT lineage) did not show positivity, causing the absence of ladder-like bands (Figures 6 and 7).

LAMP TEST ON SAMPLES OF LINEAGE MOLLUSCS: OURINHOS

The LAMP assay using samples of molluscs strain Ourinhos infected by *S.mansoni* (Group II), resulted in positivity only when exposed to ultraviolet light, due

Figure 5: Samples exposed to ultra-violet light. A: Group I in orange (non-infected BH/IMT molluscs strain BH/IMT). B: Group III in green (infected adult worms). Both groups contained a water sample (highlighted in red) for contamination control. Source: Personal archive (2022).

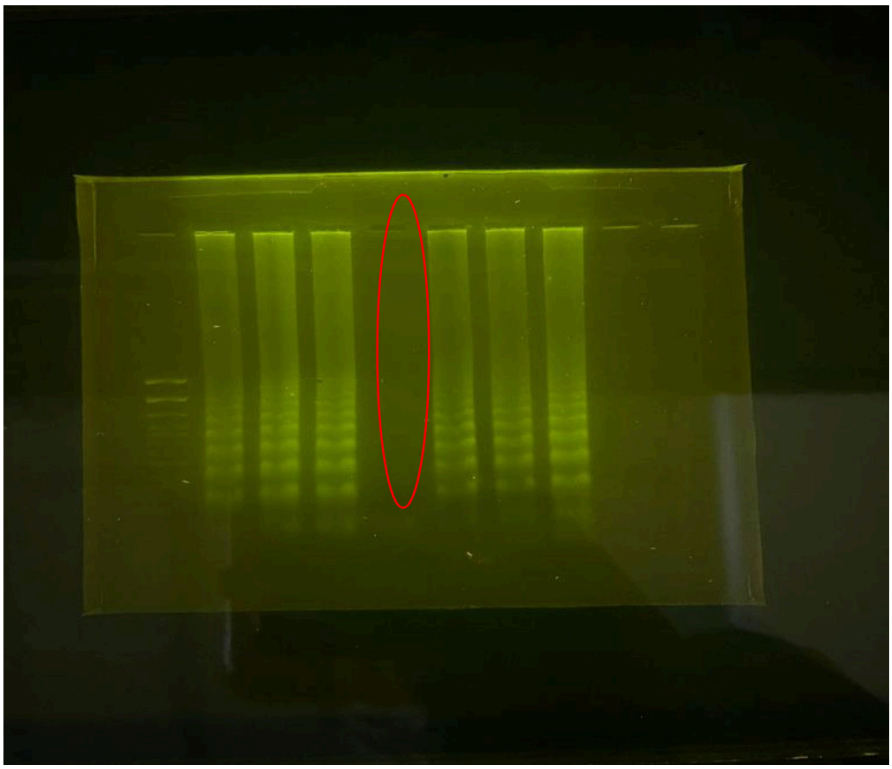

Figure 6: Agarose gel electrophoresis performed with samples of adult worms infected by S.mansoni (Group III) and a sample containing water (negative control) in red. Note the positivity of the reaction with the presence of ladder-like bands. Source: Personal archive (2022).

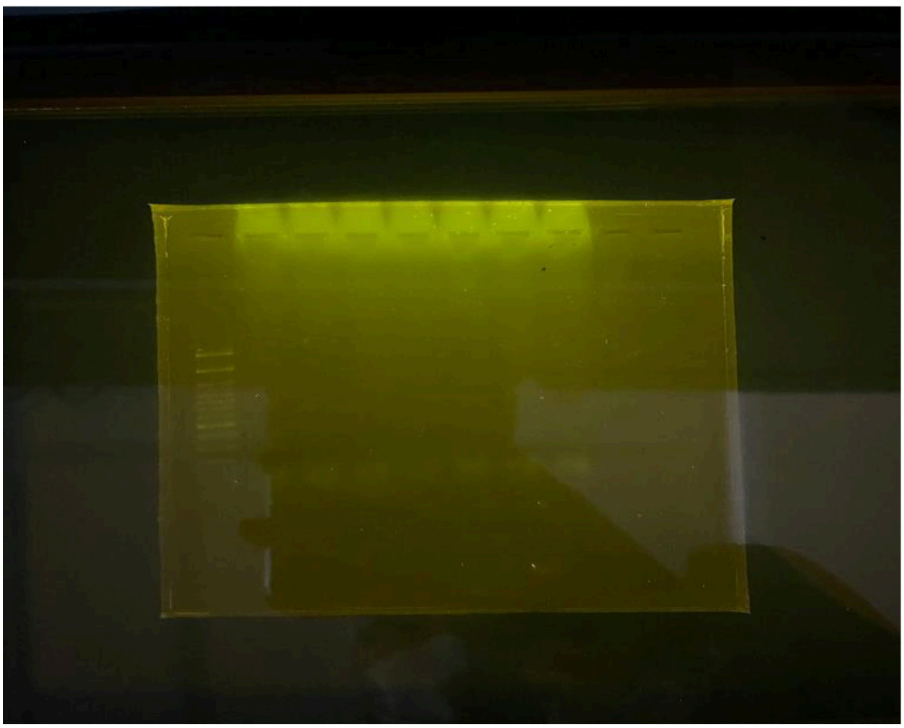

Figure 7: Agarose gel electrophoresis performed with samples from Group I (non-infected snails BH/IMT lineage) and a sample containing water. Source: Personal archive (2022).

Figure 8: A: Positive samples of Ourinhos that changed to green color after addition of SYBR Green I dye. B: A positive sample of adult worm that changed to green color is highlighted in red and a water sample is highlighted in orange. Source: Personal archive (2022).

to the color change from orange to green, as shown in Figure 8. amplicons by agarose gel electrophoresis was not possible in this assay.

DISCUSSION

Schistosomiasis is an endemic parasitic disease in 74 tropical and subtropical countries (World Health Organization). Among the different species of Schistosomas, in Brazil only the mansoni species has medical and epidemiological importance. Palasio et al, in their study on the diversity of Biomphalaria in risk areas, pointed to the presence of B. glabrata in the Christoni creek, in the municipality of Ourinhos. Taking into account the potential of B. glabrata as an intermediate host for adaptation to the life cycle of Schistosoma, early diagnosis of the infection has become important, which could facilitate the development of actions that interrupt the cycle, before human infection (Abbasi et al., 2021).

In this project, the LAMP reaction was tested in samples of adult worms, infected and non-infected molluscs. Samples of adult worms were obtained from the experimental cycle of S. mansoni, strain BH/IMT maintained at the Laboratory of Immunopathology of Schistosomiasis, LIM-06. Samples of uninfected BH/IMT strain snails and infected Ourinhos strain snails were provided from previous projects.

As expected, samples from adult worms and molluscs strain BH/IMT tested positive for infection caused by S. mansoni, while samples from non-infected molluscs strain BH/IMT did not show positivity.

In agreement with the results obtained in this project, Abassi et al performed the LAMP reaction with the objective of detecting genetic material of S. mansoni in infected molluscs in the laboratory. The results were positive and infected molluscs were detected from one day after exposure to miracidia. When discussing the specificity of the technique, Abbasi et al. also emphasize the importance of primer design, as they are responsible for the specificity of the reaction and one of the challenges for the standardization of the LAMP assay.

This study experimentally evaluated the specificity and sensitivity of the LAMP reaction and, in addition, the specificity of the primers used was also evaluated when comparing the result obtained without samples of adult worms and in samples of uninfected BH/IMT lineage snails. The results showed that the LAMP reaction is a sensitive and fast technique, since in the samples of BH/IMT molluscs the genetic material of the molluscs was not detected.

The LAMP reaction has great advantages over other diagnostic methods, it is accessible, specific and fast, making it an attractive technique for use in endemic regions. In a study carried out by Diego et al, the LAMP assay was performed for different scenarios, for the diagnosis of schistosomiasis, for the detection of infected snails and for evaluating the effectiveness of the treatment, both in animal models and in humans. In both scenarios the LAMP reaction proved to be a highly sensitive technique. Especially in the LAMP reaction carried out to detect infected snails, the results obtained reveal that LAMP can be used as a fast, sensitive and inexpensive tool for the detection of infected snails compared to other molecular methods.

CONCLUSION

Diagnosis through the LAMP reaction could become a great ally for the detection of infected molluscs, making it possible to identify transmission foci and thus prevent the disease from reaching humans. The use of the LAMP technique can thus contribute to the use of field laboratories, often located in endemic areas. These were preliminary results of a standardization of the LAMP reaction, some points still need to be defined for standardization of the reaction in samples of primary sporocysts, however, with these results we can already point out a specificity and sensitivity of the LAMP reaction and its used reagents, mainly the primers.

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ATTACHMENT 1

Opinion referring to project 1684/2021 submitted and approved by the Ethics Committee on the Use of Animals of the Medicine Course of ‘’Universidade de São Paulo”.

Medicine course in ‘’Universidade de São Paulo”
Avenida Dr. Arnaldo, 455
Pacaembu, São Paulo - SP

ETHICS COMMITTEE FOR THE USE OF ANIMALS

We certify that the proposal entitled "**Sehistosoma mansoni DNA detection by loop-mediated isothermal amplification: identification of infected snails in early pre-latency**" registered under **number: 1684/2021** under the responsibility of **Maria Cristina Carvalho do Espírito Santo** and **Ana Beatriz Nascimento Costa**, submitted by the Department of Infectious and Parasitic Diseases, which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum vertebrata (except humans), for scientific research (or teaching) purposes is in agreement with the precepts of law, number 11,794, of October 2008, of decree number: 6,899 of July 15, 2009 and with the norms issued by the national council for the control of animal experimentation (CONCEA) and was approved by the COMISSAO DE ETICA NO USE OF ANIMALS (CEUA) of the Faculty of Medicine of USP on 07/20/2021.

Purpose	() Teaching (x) Scientific Research
Duration of authorization	Start: August 2, 2021 - End: August 2, 2022
Species/Strain/Breed	Molluscs
Number of animals	5
Weight/Age	Not applicable
Gender	Not applicable
Origin	Stream: Christoni-Ourinhos

CEUA FMUSP requests that at the end of the survey a report be sent with all activities.

CEUA – FMUSP, July 20, 2021

Doctor Eduardo Pompeu
(Coordinator)
Ethics Committee on the use of animals

Ethics Commission on the use of animals at FMUSP
E-mail: ceua@fm.usp.br