A field survey using LAMP assay for detection of *Schistosoma mansoni* in a low-transmission area of schistosomiasis in Umbuzeiro, Brazil: assessment in human and snail samples.

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Abstract

Background. In Brazil, schistosomiasis is a parasitic disease of public health relevance, mainly in poor areas where *Schistosoma mansoni* is the only human species encountered and *Biomphalaria straminea* is one of the intermediate host snails. A nested-PCR based on a specific mitochondrial *S. mansoni* minisatellite DNA region has been successfully developed and applied as a reference method in Brazil for *S. mansoni* detection, mainly in host snails for epidemiological studies. Nowadays, the amplification efficiency of LAMP is known to be higher than PCR. The present work aimed to assess the utility of our previously described SmMIT-LAMP assay for *S. mansoni* detection in human stool and snail samples in a low-transmission area of schistosomiasis in the municipality of Umbuzeiro, Paraíba State, Northeast Region of Brazil.

Methodology/Principal findings. A total of 427 human stool samples were collected during June-July 2016 in the municipality of Umbuzeiro and an overall prevalence of 3.04% (13/427) resulted positive by duplicate Kato-Katz thick smear. A total of 1175 snails identified as *Biomphalaria straminea* were collected from 14 breeding sites along the Paraíba riverbank and distributed in 46 pools. DNA from human stool samples and pooled snails was extracted using the phenol/chloroform method. When performing the SmMIT-LAMP assay a total of 49/162 (30.24%) stool samples resulted positive, including 12/13 (92.31%) previously resulting Kato-Katz positive and 37/149 (24.83%) previously Kato-Katz negative. By nested-PCR, only 1/46 pooled DNA snail samples was positive. By SmMIT-LAMP assay, the same sample also resulted positive and another one more was positive from a different breeding site. Data of human and snail surveys

were used to build risk maps of schistosomiasis incidence using kernel density analysis.

Conclusions/Significance. This is the first study in which a LAMP assay was evaluated in both human stool and snail samples from a low-transmission schistosomiasis-endemic area. Our SmMIT-LAMP proved to be much more efficient in detection of *S. mansoni* in comparison to the 'gold standard' Kato-Katz method in human stool samples and the reference molecular nested-PCR in snails. The SmMIT-LAMP has demonstrated to be a molecular useful tool to identify foci of transmission in order to build risk maps of schistosomiasis.

Introduction

Schistosomiasis has been a public health problem in Brazil for decades with 4-6 million people infected with *Schistosoma mansoni* and around 25 million at risk of infection [1]. Nineteen of the twenty-six federal states of Brazil are the most affected by the disease, especially in the northeastern region of the country. Special program for schistosomiasis control was implemented more than 40 years ago, decreasing prevalence, morbidity, and mortality over the past years [2]. Nevertheless, parasitological or immunological test are not effective for detecting *S. mansoni* infection in low prevalence areas and polymerase chain reaction (PCR)-based molecular diagnostic methods have been successfully developed and applied in endemic areas of schistosomiasis in Brazil [3-7]. However, they are not still widely used in low-income countries due to the highly technical requirements and skilled personnel making them unviable for routine application in field conditions.

Snails of genus *Biomphalaria* are best known for their role as intermediate hosts of the S*chistosoma mansoni* which are able to produce hundreds or thousands of cercariae for months. Detection of cercarial shedding by infected snails after exposure of the specimens to light has been the most traditionally method used to detect active sites for snail-to-human transmission [8]. This technique has several disadvantages: non-shedding of snail during the prepatent period, lack of experienced personnel for identification acute infection, and difficulty in differentiating the morphology of the cercariae between trematodes species. To avoid these limitations, the detection of *S. mansoni* DNA in snail has been a good option offering greater sensitivity than classical methods with the advantage of detecting parasite of pooled snail samples. Therefore, several PCR-based assays have been developed to detect snails infected with *S. mansoni* [9, 10]. One of the most used has been a nested-PCR for monitoring *S. mansoni*-infected *Biomphalaria* spp. and has been approved as reference test to identify active foci of schistosomiasis transmission in Brazil [11]. However, PCR-based techniques are difficult to be applied in endemic areas of schistosomiasis because of the highly techniques requirements and skilled personnel is required.

Loop-mediated isothermal amplification (LAMP) technology was discovered seventeen-years ago [12], being a powerful tool to apply as point-of-care testing in resource-poor settings [13]. LAMP assay has been developed for molecular detection and diagnostics of several Neglected Tropical Diseases (NTDs) and applied mainly in NTDs produced by protozoa as human African trypanosomiasis and leishmaniasis [14, 15]. Additionally, LAMP assays have already been successfully described for detecting NTDs produced by helminth parasites, including filariasis, soil-transmitted helminthiases and foodborne trematodiases [16-21]. Recently, several molecular monitoring LAMP-based assays have been also developed for the detection of schistosomes [22-26]. In a previous work, a 620 bp sequence corresponding to a mitochondrial *S. mansoni* minisatellite DNA region was selected as a target for designing a LAMP-based method to detect *S. mansoni* DNA. This technique, called SmMIT-LAMP, was developed by our research group allowed us to detect *S. mansoni* DNA testing stool samples from infected mice [27].

Thus, with the aim to apply SmMIT-LAMP as a cost-effective molecular tool for the detection of *S. mansoni* in field applicable conditions, in this study we assess SmMIT-LAMP in human and snail samples collected in an endemic area of Brazil. Moreover, the results obtained by Kato-Katz analysis of human stool samples and nested-PCR performed in snails will be compared with the SmMIT-LAMP assay. It is the first time that a LAMP-based method is used to identify transmission foci and to evaluate the epidemiological risk of acquiring schistosomiasis.

Methods

Ethic statement

The study was approved by the Aggeu Magalhães Research Center Ethics Committee (protocol approval no. CAAE 56338916.6.0000.5190). Participation in the study was voluntary and prior parental consent. Participants were given detailed explanations about the aims, procedures and possible benefits of the study. Written informed consent was obtained from all subjects prior to the collection of biological samples for parasitological and molecular evaluation. Parents or guardians of children who participated in the study provided written informed consent on the child's behalf. All participants with confirmed infection received appropriate treatment. All samples were coded and treated anonymously.

Study area, population and mapping

The study was conducted during June and July 2016 in the municipality of Umbuzeiro, located in the Agreste region of Paraíba State in the Northeast Region of Brazil (Fig 1). The municipality of Umbuzeiro covers an area of 181,327 km^2 and has a population of 9,300 inhabitants (51.28 inhabitants/km^2) with 3,986 and 5,314 people living in urban and rural areas, respectively, at 2010 census [28]. This location was chosen for the study because it is a known rural area with a low-endemicity of schistosomiasis and there were no records of mass treatment of the

population within the last ten years. Moreover, this municipality is crossed by the Paraíba River, the only hydrographic basin in the region and the population has work and leisure activities centered on the river.

The locality was georeferenced by means of the global positioning system (GPS) technology, using a GPS receiver (Garmin, model eTrex) with minimum accuracy of 10 meters, configured in the Universal Transverse Mercator (UTM) projection Datum SIRGAS 2000. Using the TrackMaker Pro software, the GPS receiver data was transferred to a computer, making it possible to save files (map, case distribution, breeding sites and foci) in format that were used in the spatial data analysis, which was done by means of the ArcGis software and 10.1. The shapefiles of Brazil, Paraíba and Umbuzeiro were obtained at both open access websites IBGE (http://mapas.ibge.gov.br/bases-e-referenciais/bases-cartograficas/malhas-digitais.html) and the Digital Elevation Model satellite images from Geo Catálogo MMA (http://geocatalogo.mma.gov.br/index.jsp).

Collection of samples.

Human stool sampling and parasitological tests. A total of 427 participants from 127 households were included in the study. The average household size was 3.36 people per household. Participants, including 199 males (46.60%) and 228 females (53.39%) with a median age of 29.81 (range 1-91; SD: 20.83) were registered and recruited from door to door for the parasitological survey. Each participant was given a parasitological flask for stool collection. Samples were collected on a second visit the following morning. A single stool sample was individually obtained from each participant.

After collection, samples were transported to the Schistosomiasis Laboratory and Reference Service of CPqAM/Fiocruz for parasitological screening by Kato-Katz technique [29]. Duplicate Kato-Katz thick smear slides were prepared from each stool sample for the detection of *S. mansoni* eggs by well-trained technicians. After preparation of slides, the remaining samples were kept at -20ºC until further DNA extraction for molecular analysis as described below.

Snail sampling and processing. To determine the snail collection locations a survey along the Paraíba riverbank was carried out according to the presence of *Biomphalaria* snails and the use of the river for leisure, labor activities or crossing path as the main epidemiological criteria. *Biomphalaria* snails were *in situ* sorted out based on shell characteristics. Up to 14 snail breeding sites were located and selected for the study. In each breeding site, snails were collected using scoops and tweezers for 15 min and placed into properly labeled moistened ventilated plastic tubs for later transportation to the Schistosomiasis Laboratory and Reference Service of CPqAM/Fiocruz.

A number of specimens from each breeding site were selected at random for species confirmation using standard taxonomic identification keys [30]**.** In order to identify the snails breeding sites as potential transmission foci for schistosomiasis,

all the snails were exposed to artificial light to shed the cercariae in case they were infected [31]. Then, snails were divided into 46 batches containing a maximum number of 30 snails/pool for easy handling and processing maintaining their identification according to the different 14 breeding sites. Afterwards, snails were storage at -20ºC until further DNA extraction for molecular analysis.

DNA obtaining and molecular analysis.

DNA obtaining. Human stool samples (5-10 g/each) and pools of snails (4-30 speciments/batch) were used for DNA extraction using an adapted phenol/chloroform method [32]. Briefly, the feces or the whole snails -including the shell and the soft parts- were homogenized in 10 mL of lysis solution (10mM NaCl, 0.5% SDS, 25 mM EDTA, 10 mM Tris-HCl, pH 8.0). Following a brief centrifugation (5000 rpm), the supernatant was extracted with phenol/chloroform and precipitated with isopropanol. The pellet was resuspended in 1 mL of TE buffer. Then, 2 µL of DNA purified from stool or snail pooled samples were used for molecular analysis.

Two-step nested PCR for snails analysis. A nested PCR was performed using two pairs of primers in two sequential reactions as previously described by Melo et al., (2006) [11] with modifications. Briefly, 50 pmol of outer primers (Schfo19 and Unvfo2) were used in the first PCR, and 50 pmols of internal primers (Schfo17 and Schre16) were used in the second PCR. Two microlitres of the product of the first PCR was used as template for the second PCR. The mixtures for both PCR reactions were prepared containing 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTP 50 pmol of each primer and 2.5 units of Taq DNA polymerase (Amersham Pharmacia Biotech, USA). In the first reaction, program was run for 30 cycles, consisting of denaturation at 92 ºC for 30 s, annealing at 65 ºC for 1 min and extension at 72 ºC for 1 min. In the second PCR, program was the same, with the exception of annealing temperature at 58 ºC. Several positive (*S. mansoni* DNA) and negative (no template) controls were included in each PCR run. PCR products (5 µL) were detected in 2% agarose gels and photographed over an UV light system.

SmMIT-LAMP for human stool samples and snails analysis

All the human stool and pooled snails samples were tested using the reaction mixture and specific primer set for LAMP assay –SmMIT-LAMP- previously established by Fernández-Soto et al., (2014) [27]. The SmMIT-LAMP method amplifies a specific sequence corresponding to a mitochondrial *S. mansoni* minisatellite DNA region (GenBank Acc. No. L27240). Briefly, the reaction was carried out with a total of 25 µL reaction mixture containing 40 pmol of each FIP and BIP primers, 5 pmol of each F3 and B3 primers, 1.4 mM of each dNTP (Intron), 1x Isothermal Amplification Buffer -20 mM Tris-HCl (pH 8.8), 50 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Tween20- (New England Biolabs, UK), 1 M betaine (Sigma, USA), supplementary 6 mM of $MgSO_4$ (New England Biolabs, UK) and 8 U of

Bst 2.0 WarmStart DNA polymerase (New England Biolabs, UK) with 2 µL of template DNA. Reaction tubes were placed in an economic heating block at a constant temperature of 63ºC for 60-90 min and then heated at 80ºC for 5 min to stop the reaction. In all SmMIT-LAMP trials positive *(S. mansoni* DNA) and negative (water instead DNA) controls were always included.

The LAMP-positive results could be visually inspected by the naked eye by color change after adding 2 µL of 1:10 diluted 10,000x concentration fluorescent dye SYBR® Green I to the reaction tubes. Green fluorescence was clearly observed in successful LAMP reaction, whereas it remained original orange in the negative reaction. To avoid as much as possible the potential risk of cross-contamination with amplified products, all tubes were briefly centrifuged and carefully opened before adding the fluorescent dye.

Spatial data analysis

Data of human and snails surveys and results of parasitological and molecular analysis were used to build risk maps. Based on the number of snails collected in each station a thematic map demonstrating the abundance of snails in the breeding sites and foci of transmission was built. A kernel density analysis (kernel intensity estimator) was also performed to draw a risk map of schistosomiasis incidence according to the diagnostic methods used for detection. Kernel density estimation (KDE) is a statistical technique of interpolation, nonparametric method, which produces a continuous surface (cluster) of the density calculated at all locations for visual identification of hotspots without changing their local characteristics [33]. The parameters used to kernel analysis were the method data classification "equal interval" and bandwidth method defined using an adaptive beam as more appropriate for analyzing local studies [34]. The area unit was defined in m^2 and the kernel spatial resolution in 10 meters.

Statistical methods

Statistical analyses were performed using GraphPad Prism software package (version 6, GraphPad Software, Inc., San Diego, CA, USA; https://www.graphpad.com). Comparison of LAMP results with those obtained by microscopy were analyzed by McNemar's test for matched pairs. Comparisons were considered significant at a p-value < 0.05. The diagnostic sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were calculated for the SmMIT-LAMP and the Kato-Katz method using the MedCalc statistical program version 15.2.2 (MedCalc Software, Ostende, Belgium) according to the software instruction manual (www.medcalc.org).

Results

Parasitological analysis by Kato-Katz

A total of 13/427 (3.04%) human stool samples were positive by duplicate Kato-Katz thick smear, including samples obtained from 5 males and 8 females (median age 45; range 14-90; SD 22.76). In all Kato-Katz positive slides, the *S. mansoni* eggs count was very low, as well as the number of egg per gram of feces (EPG) with an average from 12 to 180. Up to 4/13 positive slides were negative at least in one of the two analyses (S1 Table).

Spatial distribution of parasitological positive cases is represented in Fig 1A. All Kato-Katz positive cases were detected in a zone located at the northeast of the study area (Fig 1B). In that zone, a total of 162 samples had been previously collected in the parasitological survey, counting the 13 Kato-Katz positive samples obtained and 149 Kato-Katz negative samples. These 162 samples were further subjected to molecular analysis by LAMP assay as described below.

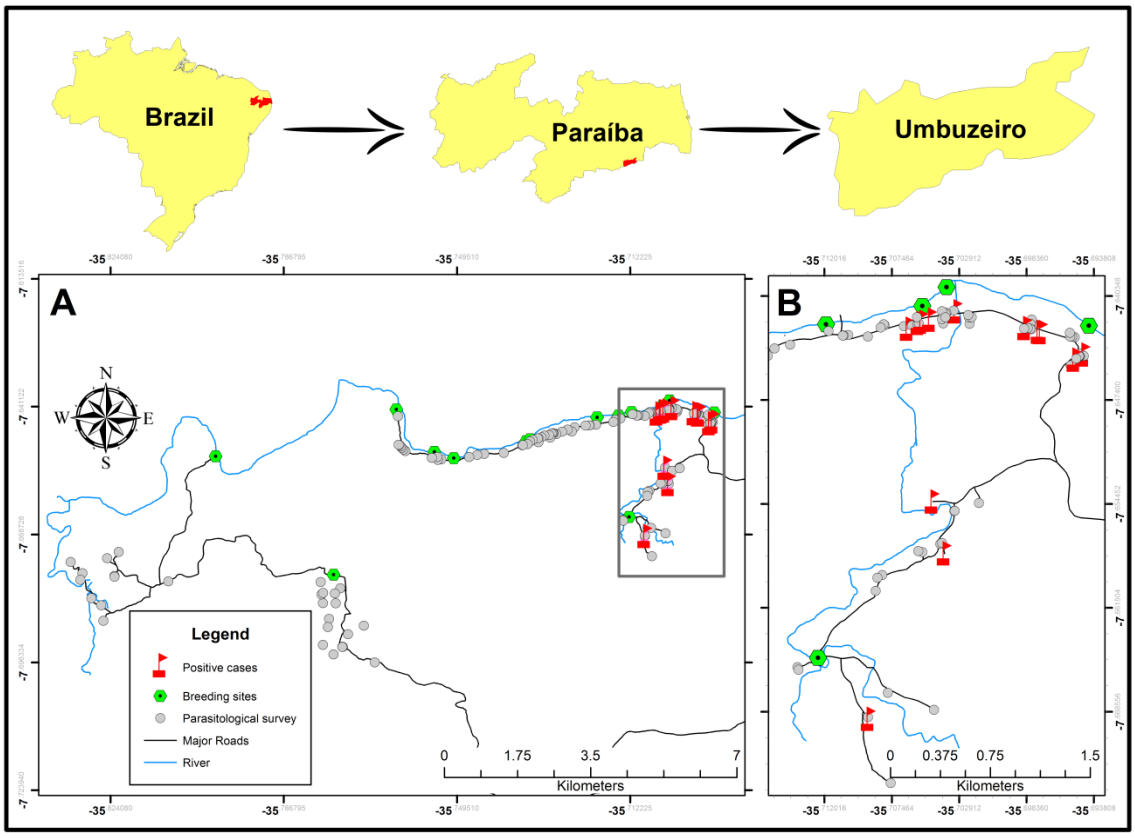

Fig 1. Map of the study area in the municipality of Umbuzeiro, located in the Agreste region of Paraíba State in the Northeast Region of Brazil. (A) Distribution of households included in parasitological survey (grey points); Kato-Katz positive samples (red flags) and breeding sites of *Biomphalaria straminea* (green points). (B) Enlargement of the area of the study where Kato-Katz positive results were obtained.

SmMIT-LAMP analysis of human stool samples

The SmMIT-LAMP results obtained after testing the stool samples in comparison to Kato-Katz results are showed in Fig 2. When performing the SmMIT-LAMP assay a total of 49/162 (30.24%) stool samples resulted positive, including up to 12 of the 13 (92.31%) previously resulting Kato-Katz positive and, additionally, 37/149 (24.83%) previously Kato-Katz negative.

MacNemar's test showed a statistical significant relation between LAMP results and microscopy-detected *S. haematobium* infections (p-value<0.0001). Considering the microscopy findings by Kato-Katz as the reference standard, the following diagnostic parameters were calculated for the SmMIT-LAMP in this study: 92.86% sensitivity (95% CI: 66013% -99.82%); 80.11% specificity (95% CI: 73.64% - 85.59%); 26.00 % positive predicted value (95% CI: 20.28% -32.67%) and 99.33% negative predicted value (95% CI: 95.75% -99.90%).

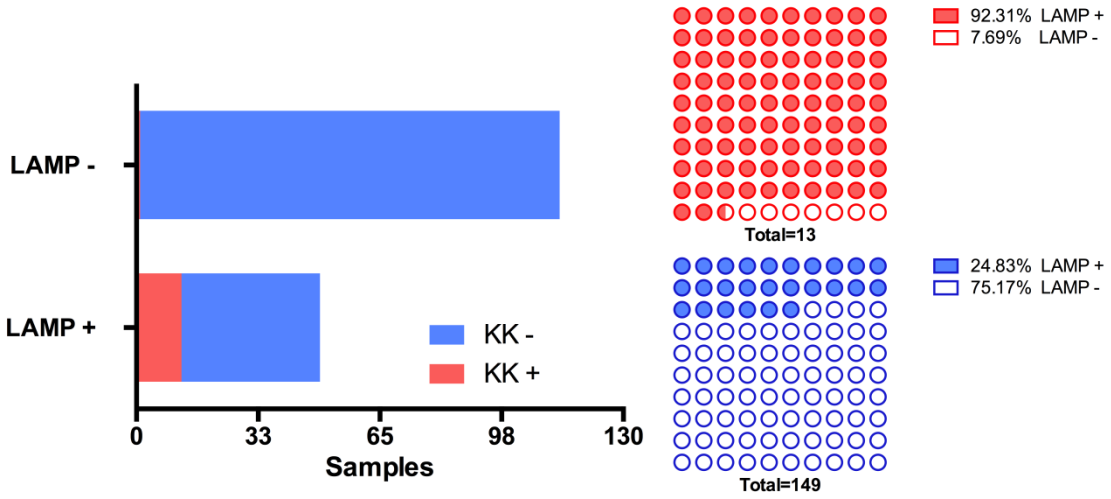

Fig 2. Results obtained by LAMP assay in comparison to Kato-Katz. Blue and red color shows negative (KK-) and positive (KK+) samples by Kato-Katz. Graphics bars show the number of LAMP positive (LAMP+) and negative (LAMP-) samples. Circles show the percentage of samples LAMP positive and negative in comparison to Kato-Katz positive and negative samples.

Snail identification, cercarial shedding, nested PCR and SmMIT-LAMP analysis.

A total of 1175 snails were collected with an average number of specimens per breeding site of 83.92 (range 4-370; SD: 109.44). All snails were identified as *Biomphalaria straminea*. None of the snails examined by exposure to artificial light for cercariae to emerge was identify as infected.

When testing by nested-PCR the 46 pooled DNA snails samples, only one pool resulted positive. By SmMIT-LAMP assay, the same pooled snails sample also resulted positive and another pool was positive from a different breeding site.

Geographical distribution of breeding sites where pooled snails samples resulted positive by molecular assays is shown in Fig 3. In these two points the abundance of snails was the largest of the survey. Additionally, the two pooled snail positive samples were located in the same area where the highest number of microscopy-positive results were previously detected. According to this, two potential foci of schistosomiasis transmission were identified in the study area.

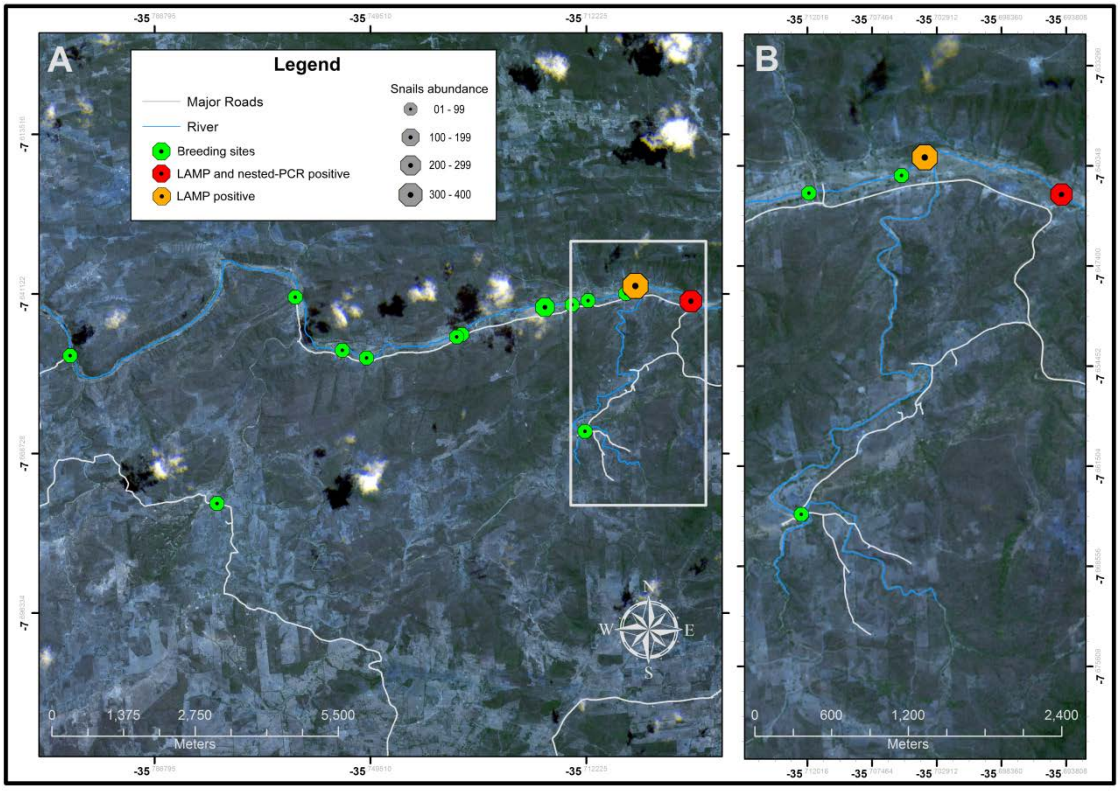

Fig 3. Geographical distribution of breeding sites showing positive pooled snails samples by molecular assays. (A) Breeding sites and identification of potential foci of transmission of *Schistosoma mansoni* using LAMP and nested-PCR (red point) and only LAMP assay (orange point). The abundance of snails is also indicated (grey points). (B) Enlargement of the area of the study where molecular assays positive results were obtained.

Risk of schistosomiasis infection

The distribution of both microscopy and SmMIT-LAMP positive cases by households included in the study and the risk maps of schistosomiasis infection generated with the kernel density method are shown in Fig 4. Only one positive result per household was detected when using the Kato-Katz technique (Fig 4A) whereas up to 5 positive results per household could be obtained when using the SmMIT-LAMP assay (Fig 4B). The potential risk of schistosomiasis transmission considering the results obtained when testing by Kato-Katz (Fig 4C) and SmMIT-LAMP (Fig 4D) is also represented. Two foci of schistosomiasis transmission were

located at the breeding sites where pooled snails samples resulted positive by molecular assays.

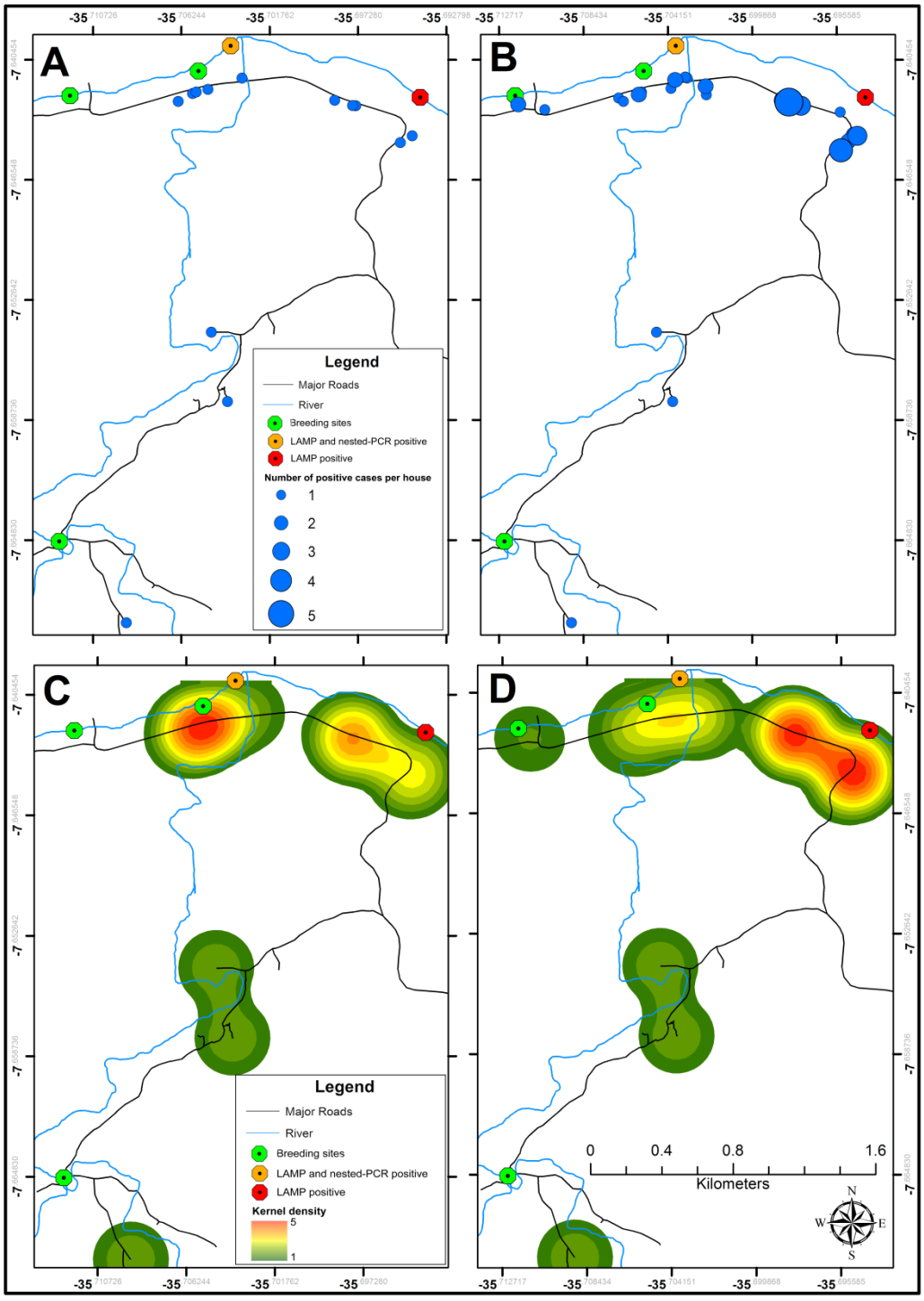

Fig 4. Risk maps of the schistosomiasis incidence according to Kato-Katz and SmMIT-LAMP results. (A) Distribution of cases by households including in the parasitological survey using Kato-Katz (B) Distribution of cases by household using LAMP assay; (C) Kernel risk map of the occurrence of cases by Kato-Katz method; (D) Kernel risk map of the occurrence of cases by the LAMP assay.

Discussion

Our study was conducted in a known low prevalence area of schistosomiasis in Brazil. Kato-Katz results obtained in the population survey corroborated previous results published from the study area [35]. Only 13 stool samples (3.04%) resulted microscopically positive including up to 10 samples with light infections (1-99 EPG) and, additionally, 4/13 positive cases presented absence of *S. mansoni* eggs at least in one of the two slides examined microscopically. These data are in line with the known low sensitivity of the Kato-Katz technique for diagnosing schistosomiasis in areas of low prevalence and parasite load [4, 36].

Molecular assays rise as a potential alternative to traditional parasitological methods in situations were highly sensitive diagnostic test are needed [37]. In this context, we tried to evaluate our SmMIT-LAMP assay in an area where the Kato-Katz previously showed a low sensitivity. When performing the SmMIT-LAMP a higher number of positive samples were detected with an overall prevalence of 30.24%. Moreover, of the Kato-Katz positive samples up to 92.31% were LAMP-positive, thus indicating a high sensitivity of the technique. Thus, our SmMIT-LAMP seems to be much more sensitive than microscopy eggs detection used commonly as the classical reference test for intestinal schistosomiasis. In addition, *S. mansoni* DNA for LAMP analysis was extracted using a cost-effective phenol/cloroform method without compromising the sensitivity of the technique. A number of LAMP assays have been previously reported for parasites detection with a minimal DNA extraction requirement, including schistosomes [38, 39]. Moreover, the SmMIT-LAMP results were easily visualized by color change by the naked eye. This is a great advantage for epidemiological surveys in low-income areas compared to other DNA-based molecular methods.

Biomphalaria straminea was the solely species identified as intermediate host in this studied area. This finding is according with a previous malacological survey in this region [40]. Among the three species of host snails for *S. mansoni* in Brazil, *B. straminea* is considered to be the most epidemiologically important species because of its wide geographic distribution [41]. Besides, *B. straminea* are naturally more resistant to *S. mansoni* infection than other snail species [42]. Thus, a number of epidemiological studies have demonstrated the utility of the nested-PCR method for *S. mansoni* detection in pooled *B. straminea* samples when the parasitological assays have not been effective [11, 40]. In our work, using this nested-PCR as reference one pooled sample of *B. straminea* was detected but, by contrast, no one was positive by classical cercarial shedding tests.

In order to test our SmMIT-LAMP in detection of *S. mansoni* in *B. straminea*, we analyzed the pooled snail samples to compare results with nested-PCR assay. The SmMIT-LAMP assay was originally designed on a sequence corresponding to a specific mitochondrial *S. mansoni* minisatellite DNA region [43]. This sequence was also previously used for designed a specific PCR-based method for detection of *S.*

mansoni with no cross-reaction with other Brazilian trematodes which have as intermediate hosts snails of genus *Biomphalaria* [10]. When applying the two molecular methods for snail samples screening two breeding sites were identified as active foci of schistosomiasis transmission including one detected by both nested-PCR and SmMIT-LAMP, and one more by SmMIT-LAMP. These two foci of transmission were located in the same zone of the study area where the Kato-Katz positive human cases were detected. In recent years, the large-scale screening by LAMP of pooled field-collected snails in transmission areas of schistosomiasis has been reported as a simple and efficient tool for snails surveillance, including *S. mansoni* in Brazil [44, 26]. In our study, the SmMIT-LAMP assay was applied for the first time to evaluate the *S. mansoni* infection not only in pooled field-collected snails but also in human stool samples.

Data obtained in SmMIT-LAMP and Kato-Katz tests were used to create Kernel density-based maps of risk of schistosomiasis. The Kernel density has previously been used to build maps of risk for several helminthiases, including schistosomiasis [45-47]. The risk areas obtained resulted to be closely located to the snail breeding sites identified as foci of schistosomiasis transmission by the SmMIT-LAMP and nested-PCR. Precisely, in those breeding sites the greatest contact between the population and the river was observed for work activities (extraction of sand from the river), domestic activities (washing clothes and dishes), and leisure activities (fishing and children's recreation). All these activities are known to be associated with transmission of schistosomiasis [48, 49]**.** In addition, this area is commonly used by the inhabitants as a route for crossing the river further increasing the risk of infection.

In summary, this is the first study in which a LAMP assay was evaluated in both human stool and snail samples from a low-transmission schistosomiasis-endemic area. Our SmMIT-LAMP proved to be much more efficient in detection of *S. mansoni* in comparison to the 'gold standard' Kato-Katz method in human stool samples and the reference molecular nested-PCR in snails. Moreover, SmMIT-LAMP has demonstrated to be a molecular useful tool to identify foci of transmission in order to build risk maps of schistosomiasis.

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S1 Table. Positive samples detected by duplicate Kato-Katz thick smear. The table shows the results obtained in the first (KKT-1) and the second (KKT-2) Kato-Katz technique and the mean of eggs per grams (EPG) of feces for each sample.

No. sample	KKT- 1	KKT-2	EPG
1	3	3	72
2	0	1	12
3	1	2	36
4	4	8	144
5	6	7	156
6	1	1	24
7	9	6	180
8	1	0	12
9	1	1	24
10	1	0	12
11	0	1	12
12	2	1	36
13	5	1	72