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Field evaluation of a loop-mediated isothermal amplification (LAMP) platform for the detection of *Schistosoma japonicum* infection in *Oncomelania hupemsis* snails

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Abstract: *Schistosoma* infection in snails can be monitored by microscopy or indirectly by sentinel mice. As both these approaches sometimes miss infections, more sensitive tests are needed, particularly in low-level transmission settings. In this study, the loop-mediated isothermal amplification (LAMP) technique, designed to detect a specific 28S ribosomal *S. japonicum* gene with high sensitivity, was compared to microscopy using snail samples from 51 areas endemic for schistosomiasis in five Chinese provinces. The results were compared with those by polymerase chain reaction (PCR) adding DNA sequencing as a reference when needed. The testing of pooled snail samples showed that a dilution factor of 1/50, i.e. one infected snail plus 49 non-infected ones, would still result in a positive reaction after the recommended number of amplification cycles. Testing a total of 232 pooled samples, emanating from 4,006 snail specimens, with the LAMP assay showed a 6.5% rate of infection, while traditional microscopy found only 0.04% positive samples in the same materials. Parallel PCR analysis confirmed the diagnostic accuracy of the LAMP assay, with DNA sequencing even giving LAMP a slight lead. Microscopy and the LAMP test were carried out at local schistosomiasis-control stations demonstrating that the potential of the latter assay to serve as a point-of-care (POC) test with results available within 60-90 minutes, while the more complicated PCR test had to be carried out at the National Institute of Parasitic Diseases (NIPD) in Shanghai, China. In conclusion, LAMP was found to be clearly superior to microscopy and as good as, or better, than PCR. Application of LAMP testing would be useful for surveillance and risk prediction as it requires less time than other techniques and can be used under field conditions, which improves and accelerates schistosomiasis control.

Key words: *Schistosoma japonicum*; *Oncomelania hupensis*; Snail; 28S ribosomal DNA; PCR; Loop-mediated isothermal amplification (LAMP); Pooled samples; China

1. Introduction

Schistosomiasis, one of the neglected tropical diseases (NTDs), is a worldwide public health problem caused by one of six species of the *Schistosoma* parasite that affects >200 million people in Africa, South America, and Southeast Asia [1, 2]. In China, The Philippines and three small pockets on the Indonesian island Sulawesi, the disease is a zoonosis caused by *Schistosoma japonicum* [1-3]. The intermediate snail host required, *Oncomelania hupensis* in China, is distributed all over central and south China, including the country's endemic areas that range from the Yangtze River Valley and the southern plains to the mountainous regions of Sichuan and Yunnan Provinces [4]. In 2004, a new integrated control strategy was introduced [5, 6] involving reduction of infection sources by fencing off transmission sites, replacement of water buffaloes (an important reservoir) with tractors for agricultural work as well as improved sanitation with access to clean water and latrines. These approaches have markedly reduced the infection rate, both in humans, domestic animals and the intermediate snail host [7]. Indeed, the changes accomplished are so profound that it makes difficult to monitor the remaining transmission sites by only testing humans and domestic animals [8]. On the other hand, snail diagnosis by microscopy or the use of the sentinel mice approach [9] are not only labour-intensive and time-consuming, but also not sufficiently sensitive. To sustain the success achieved with regard to schistosomiasis elimination in China, highly sensitive snail-monitoring systems capable of assessing residual transmission in real time are now needed [10].

Due to its high sensitivity, molecular diagnosis has emerged as a promising approach for the detection of suspected, low-level presence of pathogens [11-13]. However, resources essential for molecular diagnostics, such as bio-safety cabinets, stable supply of electricity and well-experienced technicians, are rare in peripheral laboratories in developing countries, limiting application of sophisticated technology. Hamburger and colleagues investigated the use of the loop-mediated isothermal amplification (LAMP) technology, which employs a polymerase that amplifies the target DNA gene sequence with high specificity and rapidity under isothermal conditions [14], for the detection of infections due to *S. mansoni* and *S. haematobium* showing excellent results, not only confirming that the LAMP technique works in the laboratory, but also in the field in Africa [15], later confirmed in Brazil by Gandasegui

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et al. [16] and Kumagagi et al. [17] in China, where latter research group developed a diagnostic platform based on a target 28S ribosomal DNA (rDNA) specific for *S. japonicum* (not reacting with *S. mansoni*) and showed that snails experimentally infected with only one miracidium could be detected less than 24 hours after infection.

We describe here the application of LAMP using pooled snail DNA samples based on a protocol of visual LAMP detection developed by Tomita et al. [18], where the amplification pyrophosphate ion by-product combines with a divalent metal ion to form an insoluble salt. Adding calcein, a bivalent fluorescein/manganese complex, to the reaction solution results in strong fluorescent signal from positive reactions enabling visual discrimination by the naked eye without specialized equipment. We compared the results of LAMP with that of atest founded on the polymerase chain reaction (PCR) with DNA sequencing applied as arbiter.

The purpose of the present study was not only to confirm the sensitivity of the LAMP method when used for the detection of *S. japonicum* DNA in infected snails in known endemic settings, but also to investigate and validate its application under field conditions soon after snail collection. An added aim was to find out if the procedure could be speeded up by testing pooled DNA samples in place of testing the snails one by one.

2. Materials and Methods

Our study describes and evaluates the first version of a LAMP-based platform, which integrates different kinds of data enabling translational research that is expected to lead to improved surveillance of schistosomiasis transmission. *O. hupensis* snails were collected during the spring over a period of three years (2013-2015) from all endemic regions in China covering five provinces [4]. The LAMP approach used is shown schematically in the form of a flow chart (Figure 1). As we were interested to see if the field work could be accelerated by testing samples consisting of pooled snails, DNA from various numbers of snails were investigated together before the main study was undertaken.

Fig 1 about here

2.1. Snail collection, microscopy and DNA extraction

Oncomelania snails were collected from areas with known human schistosomiasis infection rates varying between 0.9% and 2.8% [19]. We used 232 pooled samples (resulting

from 4006 single snails) coming from 51 villages in 15 counties in Hunan, Hubei, Jiangxi, Anhui and Yunnan provinces. The snails were firstly examined for infection with *S. japonicum* by traditional microscopy methods, in which snails were crushed by pressure between clean glass plates and scrutinized, one by one, then checked whether cercariae and sporocysts presented inside snail under the microscope at low magnification (generally 10 ×) after the pieces of shell had been removed with a clean needle. The remained snail bodies were used for test by either conventional PCR or LAMP assay.

After microscopy, the snail bodies were pooled in clean 2.0 ml tubes (not exceeding 50 snails in each), adding 50-200 μL of Tris-EDTA (TE) buffer (Takara Biotech, Dalian, China) and the tissue collections homogenized. TE lysis liquid (consisting of Tris to stabilize the pH near 8.0 and ethylenediaminetetraacetic acid (EDTA) to chelate cations like Mg^{2+} was added to the snail homogenates and the tubes kept in a water bath at 56°C for 2-3 hours, then stored at -20 °C and until use. This TE buffer was used to solubilize DNA and also protect it from degradation.

2 .2. PCR assay.

A conventional PCR assay (Takara Biotech, Dalian, China) was carried out in parallel with the LAMP assay (see below) and used as reference standard. Paired primer strands, i.e. 5′-GGTTTGACTATTATTGTTGAGC-3′ and 5′-CTCACCTTAGTTCGGACTGA-3′,. targeting the *S. japoniucm* 28S (Sj28S) rDNA was used. The ingredients were 1 μL of each primer, 3 μL DNA template, 0.2 mM deoxynucleotide (dNTP) solution, 1.25 units of a highly thermostable DNA polymerase from the thermophilic bacterium *Thermus aquaticus* (Taq DNA) and 2.5 μL 10× buffer (pH 8.8 Tris-HCL, KCL and $MgCL_2$). The initial activation (cycle 1) was set as 3 min at 94°C followed by 33 30-sec cycles at 94°C, 56°C for 50 sec and 72°C for 1 min. The final extension step was carried out for 7 min at 72°C. The amplified yield products were visualized by ethidium bromide staining after gel electrophoresis on 1.5% agarose and photographed.

2.3. DNA sequencing

In order to make sure that the PCR product exactly matched to the target sequence, the 330 bp band was separated by gel electrophoresis, cloned into the Pmd19-T vector (Takara Biotech, Dalian, China), transferred into *E. coli* cells, strain *DH5α*. and then cultured in Luria-Bertani (LB) medium with ampicillin (100 μg/ml) at 37°C in a 5.0 ml tube. After 12-16 hours

of culture, plasmid DNA was extracted from the bacterial colonies using a DNA extraction kit. The purified plasmid DNA was sent to a commercial company (Sangon Biotech, Co. Ltd., Shanghai, China) for sequence analysis confirming the sample to be a complete match to Sj28S rDNA when compared with the National Center for Biotechnology Information (NCBI) database using the basic local alignment search tool (BLAST), a program that compares sequences obtained to sequence databases and calculates the statistical significance of matches.

2.4. LAMP assay

We performed the LAMP assay targeting the Sj28S gene with a reaction mixture of 7.5 µL nuclease-free water, 2×12.5 µL reaction buffer, 1.0 µL primer mixture (25×), 1.0 µL (8 µ/µL) Bst, a *Bacillus stearothermophilus* DNA polymerase homologue used for DNA strand displacement, and 1.0 µL calcein. We used 0.2 ml reaction tubes dispensing 23 µL of the reaction mixture together with 2.0 µL of the sample to be tested into each tube. In addition, 23 µL reaction mixture was added to Sj28S rDNA target in one tube (positive control) and to nuclease-free water in the other (negative control). All reaction tubes were incubated at 65°C for 60～90 min, followed by inactivation of the enzyme by incubation of the tubes at 85 °C for 5 min and then observing the tubes for colour shift (fluorescence) using daylight illumination from the lateral side.

2.4.1. Testing samples from different parasite stages

To confirm whether DNA from a single miracidium could be detected by the LAMP assay using the Sj28S rDNA primers, we collected DNA using the extraction and purification kit from one miracidium and performed the test with 5 independent samples. Further, we carried out infection experiments with the intermediate snail host followed by collection of the DNA from a mother sporocyst, a daughter sporocyst as well as mature cercariae subjecting the samples for testing using the LAMP procedure. *Trichobilharzia* cercariae were used as specificity control

2.4.2. Use of pooled snails samples

To evaluate the detection capability limit of the LAMP method before the main study, we performed a preliminary test, in which one schistosome-positive snail was mixed with different numbers of schistosome-negative snails in the following ratios: 1/5, 1/10, 1/20, 1/50, 1/100 and 1/200.

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For practical reasons, each snail pool in the main study came from the same area (though separate from one pool and another). Depending on availability, the number of snails in the pools could not be standardized but we made sure that it was always kept at ≤50. The end result of this exercise was 232 pools made out of a total of 4,006 collected snails. Depending on how many snails were pooled, the pools were kept in 2.0-ml or 50-ml centrifuge tubes together with 400 µL or 2 ml, respectively, of a lysis buffer from a DNeasy blood and tissue kit, (Qiagen, Valencia, CA, USA).

2.5. Validation

To assess the quality of LAMP assay, a panel of nucleic acid extracts and test kits were dispatched to 28 separate testing health agency laboratories in the endemic areas at the provincial and county levels where laboratory personnels were well trained to operate the LAMP assay. Each nucleic acid sample was thawed, divided into 100 µL aliquots, coded and refrozen. The next day, all samples were packaged and sent by overnight shipment to the same laboratories that were all blinded to the assigned individual identification numbers. Each laboratory tested each sample 5 times using a standard LAMP protocol provided by National Institute of Parasitic Diseases (NIPD) at China Center for Disease Control (CDC) based in Shanghai. The outcomes were scored according to a 0-100 scale, where 5 equal results were given a 100 score, 4 times a 80 score and so on.

3. Results

The preliminary test of snail pools in different ratios showed that large-scale testing could be carried out based on 50 snails without risk of missing a positive result.

Figure 2 about here

The ready-to-use LAMP test kit evaluated here, which amplifies the Sj28S gene target, demonstrated excellent diagnostic accuracy in comparison to the reference PCR, and a diagnostic sensitivity significantly superior to that of microscopy (Table 2). In the analysis of the 232 pooled samples tested, 217 were negative and 14 positive by both assays, while the remaining single sample was negative by PCR but positive by DNA sequence analysis, which showed that LAMP had a higher sensitivity than PCR. The results further demonstrated that schistosome DNA included in a single miracidium is sufficient to be amplified by the LAMP

assay using the 28S rDNA primers, and not only that but the LAMP technology is also capable of detecting a schistosome infection in the sporocyst stages as well as mature cercariae ready for release (Figure 3). The *Trichobilharzia* cercariae used to test the specificity were negative.

Figure 3 about here

The use of the inter-laboratory panel, including 28 laboratories from provincial and county level health agencies, demonstrated that the assays in the different laboratories agreed almost exactly with each other, only four laboratories had a slightly lower scores, one with score 80 in 2013, 2 with scores 60 and one with score 80 in 2011 and one with score 90 in 2015, respectively (Table1). Finally, as seen in the table, laboratories with lower scores than 100 in one year, did not have that in other years. The number of samples from 2013 was not sufficient to be tested in all laboratories.

Table 1 about here

Comparison between all tests carried out is shown in Table 2. The LAMP method detected more positive snails than did the microscopy method. Although all snails that were positive by microscopy were also positive by LAMP, the opposite was not true. The LAMP approach detected 7.5 times more infected snails than microscopy, while the PCR results were largely in agreement with those of the LAMP assay, except with respect to a single snail pool that was positive by LAMP and negative by PCR.

Table 2 about here

3. Discussion

Estimates of the prevalence of *S. japonicum* infection in its obligatory vector snail can be used as proxy for areas at risk for schistosomiasis. As pointed out by Hamburger et al. [13] and reiterated by Abbasi et al. [14], the snail infection rate provides a measure of the transmission from humans (and other definitive reservoir hosts) to the snail and can therefore serve as a marker of residual infection in an area. Although this is correct in principle, snail diagnosis not only depend on labour-intensive, tedious and time-consuming individual dissection of thousands of collected snails from the field [19], but the results are also not

sufficiently sensitive for Hamburger's statement to hold in practise. Again correct, but only until the advent of molecular diagnostics, which has revolutionized the testing for snail infected snails in the laboratory as well as in the field [13-17]. As the use of snail control as a complementary approach along with chemotherapy is now again found to be useful [20], snail diagnostics will have an increasingly important role to fill.

PCR-based assays have been around since the mid-1970s [11] but not used for schistosomiasis diagnosis until the end of the 1990s and the early 2000s, first for snails [13] and later for human infection [21-22]. However, the instability and variability inherent in enzymatic processes as well as the need for advanced equipment limit PCR applications in the field. In this regard, the LAMP approach is superior, as it can be adapted for application in field laboratories [23] by using ready-mixed reagents suitable for shipment at ambient temperature together with sample storage under minimal refrigeration [14-17].

The development of a molecular diagnostic platform with the desired simplicity and performance we had in mind, it was necessary to introduce an approach adaptable to county-level laboratories with limited resources. The manganese/calcein method of Tomita et al. [17] is an important contribution in this regard as it enables recognition of small quantities of DNA by means of a fluorescent signal emitted from the sample solution after amplification that was successfully used in China by Kumagagi et al. [18]. A dilution ratio of 1/50 was found to be useful (Figure 2), but if the number if infected snails decreases, as it is supposed to do with an elimination programme in force, the risk of mistakenly declare an area free of transmission increases if the snail pool contains too few snails. This could be counteracted by using a higher number of snails in the pool and increasing the time of amplification for the test but, on the other hand, the risk for false positives would then rise. This work shows that already a dilution factor of 1/50 accelerates the areal testing considerably as the snails tested can now come from a 50 times larger area than traditionally used, something that could be a practical value already now [24]. Importantly, we are already in a situation where More investigation might provide the needed room for improvement.

The strength of the present study is not only that it further improves the potential of the LAMP test that has already proved successful for snail diagnosis [17-18], but also that it targets a gene specific for *S. japonicum* and provides validation leading to the reliable use of the pooled-snail approach piloted by Hamburger et al. for *S. mansoni* [13] and Tong et al. for

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S. japonicum [25]. A further advantage is the understanding that the technique, although highly technical and sophisticated, can be read by the naked eye and is easy to learn and carry out as shown by the fact that it was successfully used for 232 samples at 28 different health agency laboratories in the rural areas. In addition, while sporocysts and germ balls are easily missed by traditional microscopic methods, the snails containing mother or daughter sporocysts were both positive in the day after infection. Therefore, the LAMP assay provides an appropriate method for the detection of pre-patent infections.

The LAMP test kit demonstrated excellent diagnostic accuracy in comparison with PCR at a level of sensitivity significantly superior to microscopy (Table 2). Indeed, the one sample reacting negatively by PCR but positively by LAMP, was also positive by DNA sequence analysis, which means that the LAMP adaption used here had in fact a slightly higher sensitivity than PCR. The risk of schistosomiasis still exists in China and snail control remains a significant challenge in the field. In the present study, the LAMP results indicated that Hunan, Hubei, Jiangxi, Anhui, and Yunnan Provinces contained infected snails, which is an indication of the risk of schistosomiasis transmission. The results can be used to guide further local investigations and snail control activities.

4. Conclusion

The LAMP platform is an effective method for monitoring infected snails from field sites of endemicity areas despite expensive reagents and the risk of contamination that requires specific training of the staff in charge. We predict that the current test may replace microscopy for snail diagnosis because of greater accuracy and reduced delay in delivering results.

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Authors'contribbutions

LS and XZ conceived and designed the project, and supervised the study. QZ performed most of the experiments and analyzed the data and wrote the original draft. JX , ZL helped analysed the data, RB helped organize and write the article. TF, CL, and YJ helped with DNA extraction, PCR and LAMP

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experiments. SL and LY helped with snail samples collection in field. All authors reviewed and approved the manuscript.

Competing interests

The authors declare no competing financial interests.

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Table 1. Inter-laboratory comparison using LAMP for the detection of intermediate snail hosts infected by *S. japonicum*

Province	Laboratory	Score 2013	Score 2014	Score 2015
Hunan	IPD	100	100	100
	Hanshou	-	100	100
	Yuanjiang	-	100	100
	Yueyang	-	100	100
Hubei	CDC	100	100	100
	Gongan	-	100	100
	Hanchuan	-	100	100
	Jiangling	-	100	100
Anhui	IPD	100	100	100
	Wuhu	-	100	100
	Anqin	-	100	100
	Guichi	-	100	100
Jiangxi	IPD	100	100	100
	Poyang	-	60	100
	Duchang	-	60	100
Jiangsu	IPD	100	100	100
	Qixia	-	100	100
Sichuan	CDC	100	100	100
	Renshou	-	100	100
	Guanghan	-	100	100
Yunnan	CDC	100	100	100
	Dali	-	80	90
	Eryuan	-	100	100
Shanghai	CDC	100	100	100
Guangdong	CDC	100	100	100
Zhejiang	IPD	100	100	100
Guangxi	CDC	80	100	100
Chongqin	CDC	-	-	-

CDC = Center for Disease Control; IPD= Provincial Institute of Parasitic Diseases; Scores are the levels of agreement between 5 tests.

Table 2. Comparison between microscopy, LAMP and PCR in screening snail samples

Province	No. of counties included	No. of villages included	No. of snails tested	No. of pooled samples	Microscopy		LAMP		PCR	
					Pos.	%	Pos.	%	Pos.	%
Hubei	2	5	599	38	1	0.5	3	7. 9	3	7.9
Hunan	2	6	716	80	0	0	2	2.5	2	2.5
Jiangxi	6	25	1,183	34	0	0	5	14.7	4	11.8
Anhui	2	6	698	43	0	0	1	2.3	1	2.3
Yunnan	3	9	810	37	0	0	4	10.8	4	10.8
Total	**15**	**51**	**4,006**	**232**	**2**	**0.04**	**15**	**6.5**	**14**	**6.0**

Figure 1 Flow chart of LAMP and PCR

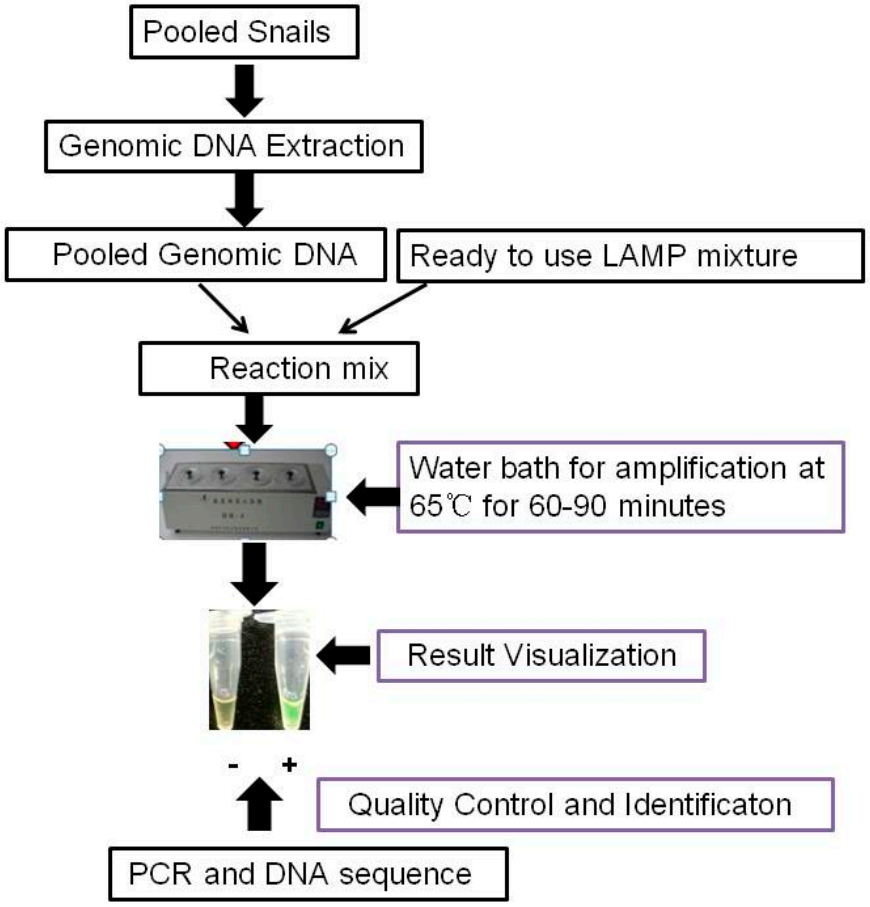

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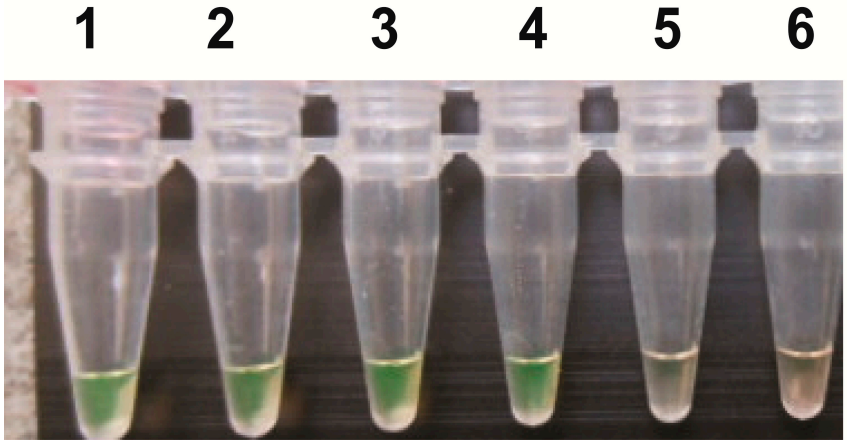
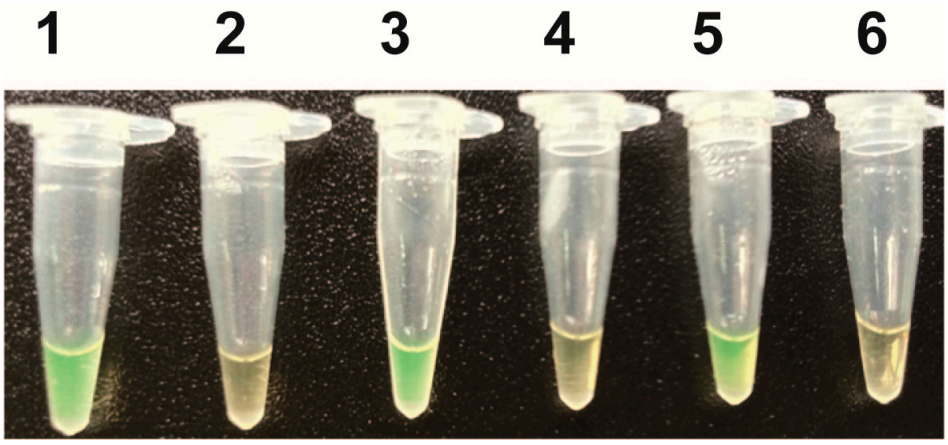
Figure 2. Investigation of LAMP diagnostic capability by testing serial dilutions of the Sj28S gene component



The following dilutions of infected and uninfected snails are shown: 1/4 (tube no. 1); 1/9 (tube no. 2); 1/19 (tube no. 3); 1/49 (tube no. 4); 1/99 (tube no. 5) and 1/199 (tube no. 6)

Figure 3. Detection of the Sj28S gene component in the different parasite developmental stages of in *Oncomelania hupensis* by the LAMP method



1. Mother sporocyst; 2. Negative control; 3. Daughter sporocyst; 4.Negative control; 5. Cercariae; 6. Specificity control (*Trichobilharzia* cercariae).