Detection of Early and Single Infections of *Schistosoma japonicum* in the Intermediate Host Snail, *Oncomelania hupensis*, by PCR and Loop-Mediated Isothermal Amplification (LAMP) Assay

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Abstract. Polymerase chain reaction (PCR) with the specific primer set amplifying 28S ribosomal DNA (rDNA) of *Schistosoma japonicum* was able to detect genomic DNA of *S. japonicum*, but not *S. mansoni*, at 100 fg. This procedure enabled us to detect the DNA from a single miracidium and a snail infected with one miracidium at just 1 day after infection. We compared these results with those from loop-mediated isothermal amplification (LAMP) targeting 28S rDNA and found similar results. The LAMP could amplify the specific DNA from a group of 100 normal snails mixed with one infected snail. A PCR screening of infected snails from endemic regions in Anhui Province revealed schistosomal DNA even in snails found negative by microscopy. PCR and LAMP show promise for monitoring the early infection rate in snails, and they may be useful for predicting the risk of infection in the endemic places.

INTRODUCTION

Schistosomiasis japonica is a relatively neglected tropical disease, and it is a chronic zoonotic parasitic disease in China, the Philippines, and small pockets of Indonesia.¹ In China, the causative organism, *Schistosoma japonicum*, and its intermediate snail host, *Oncomelania hupensis*, are distributed along the Yangtze River valley and recently, in the hilly and mountainous regions of Sichuan Province.² Since the mid-1950s, the People's Republic of China has markedly decreased the prevalence of schistosomiasis through mass-chemotherapeutic treatment and the control of the intermediate snails.^{3,4} However, a complete eradication of this disease is difficult in endemic areas. The estimated prevalence in the provinces of Hunan, Hubei, Jiangxi, Anhui, Yunnan, Sichuan, and Jiangsu was 4.2%, 3.8%, 3.1%, 2.2%, 1.7%, 0.9%, and 0.3%, respectively, in 2004.⁵ A total of 564, 207, 83, and 57 acute cases of *S. japonicum* infection were reported nationwide in 2005, 2006, 2007, and 2008, respectively.⁶ These findings suggest that control measures must be improved among at-risk populations, especially in lake and marshland regions. A new integrated strategy was tested for the control of schistosomiasis in China.^{7,8} It involved the reduction of infectious sources by the replacement of water buffaloes with tractors for agricultural work, improved access to clean water and general sanitation, better livestock management through fencing to isolate schistosomal egg sites, and better feces management using newly constructed latrines on-shore. These strategies markedly reduced the infection rate in both humans and intermediate snails in the pilot areas. Remarkably, the prevalence of infected snails reportedly decreased to almost 0% in some areas.⁸ To maintain these successes, it may be useful to use new snail-monitoring systems in such areas.

Molecular tools such as conventional polymerase chain reaction (PCR) and improved DNA amplification methods have been shown capable of detecting schistosome DNA in a variety of samples. A highly repetitive, 121-base pair (bp) sequence has been used to detect DNA from *S. mansoni* and *S. haematobium* in stool, serum, urine, and plankton samples.^{9–13} Because no similar repetitive sequence has been found in the *S. japonicum* genome, the repetitive non-long terminal repeat (LTR) retrotransposon SjR2¹⁴ was used for DNA detection as a target sequence.¹⁵ In an experimental rabbit model, the SjR2 sequence was detected in serum (1 week after infection) and stool samples using a PCR assay, and the 230-bp band of SjR2 was absent at 10 weeks after treatment with praziquantel,¹⁶ and real-time PCR was applied to the detection of SjR2 gene from cercaria in an environmental water sample.¹⁷ Alternatively, real-time PCR was also applied to the detection of a mitochondrial nicotinamide adenine dinucleotide (NADH) dehydrogenase I gene at low intensity in an infected pig model.¹⁸ Another highly repeated sequence, 28S ribosomal DNA (rDNA), was used for multiplex PCR to detect a distinct *Schistosoma* sp. from human urine samples.¹⁹

Loop-mediated isothermal amplification (LAMP) is a simple, sensitive, and rapid DNA detection method.²⁰ The LAMP reaction requires only a single enzyme, *Bst* DNA polymerase, that can synthesize a new strand of DNA while simultaneously displacing the former complementary strand, thereby enabling DNA amplification at a single temperature. The LAMP reaction can be achieved using four primers (FIP, BIP, F3, and B3), two of which (F3 and B3) contribute to the formation of a stem-loop structure, whereas the other two (FIP and BIP), designed complementary to the inner sequence of the stem-loop structure, are used for amplification of the target sequence. This provides a higher specificity to the reaction than conventional PCR methods.²⁰ The LAMP assay has been widely applied for diagnosis and detection against several infectious diseases, including *Plasmodium*,²¹ *Trypanosoma*,²² *Leishmania*,²³ and *Taenia*.²⁴ In the present application, LAMP targeting to SjR2 for detecting the DNA from *S. japonicum* was also reported.²⁵

In the present study, we evaluated the performance of the PCR method by comparing SjR2 and 28S rDNA from

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S. japonicum. Next, we detected the schistosomal DNA from experimentally infected snails at 1 day after infection and detected schistosomal DNA from wild snails collected from endemic areas of Anhui Province in China. We also applied a LAMP assay to detect infected snails on-site in endemic local areas. Finally, we developed a simple, rapid, and safe screening method for determining the infection rate of snails in endemic areas after implementation of the above-described integrated strategy and detected infections using the LAMP assay with DNA extracted from a large number of snails.

MATERIALS AND METHODS

Parasites and snails. *S. japonicum* was maintained using ICR mice as a final host and *O. hupensis nosophora* from a non-endemic area (Yamanashi strain) as an intermediate host. The livers from infected mice were digested with 1 mg/mL collagenase and 0.5 mg/mL actinase, and then, purified eggs were put into water to hatch the miracidia. The collected miracidia were experimentally infected to each snail in a 96-well plate. Wild snails from endemic areas in China (*O. hupensis hupensis*) were collected from three places in Anhui Province as follows: (1) Shankou-city (30.52° N, 116.93° E) in marshland regions of Anqing county, (2) Shun'an town (30.56° N, 117.54° E) in the sand regions of the Yangtze River in Tongling county, and (3) Guanghui City (30.56° N, 117.45° E) in the marshland regions of the Yangtze River in Tongling county. Figure 1 presents detailed locations about each area. The snails were picked up in Anqing in March 2007 and in Tongling in September 2007. The collected snails were crushed and checked for infection under microscopy before preparation for DNA extraction.

DNA extraction. To detect schistosomal DNA by PCR and LAMP assay, we applied the DNA extraction method using heated NaOH.²⁶ Briefly, the counted miracidium was put into a 200- μ L volume of 50 mM NaOH and heated at 95°C for 30 minutes. After centrifugation, the 50- μ L supernatant was recovered and then mixed to an equal volume of 1 M Tris-HCl (pH 8.0). This solution was directly used as a template (1 μ L) for the PCR and LAMP methods. For direct extraction from a single infected snail (non-endemic area), each snail was also put into a distinct tube, and 200 μ L of 50 mM NaOH solution

was added to the tube. After crushing the snail with tweezers, the DNA was extracted using the above procedures. A large-scale DNA extraction from different numbers (100, 50, 25, 10, 5, and 1) of snails from non-endemic area was also performed with 10 mL of 50 mM NaOH in a 50-mL tube that was heated at 95°C for 60 minutes. After neutralization with 1 M Tris-HCl (pH 8.0), 1 μ L of the solutions was directly used as a template. Genomic DNA of *S. japonicum* was purified from adult worms using the Get-pure DNA Kit (Dojindo, Kumamoto, Japan), and the concentration of DNA was measured with a spectrometer.

Primer sets. To amplify the specific DNA of *S. japonicum*, the 28S rDNA gene (GenBank Accession No. Z46504) was selected as a target sequence. For the conventional PCR and LAMP methods, we designed specific primer sets (Table 1). As in the previous report, Sjr2 (GenBank Accession No. AF412221) primers were generated for conventional PCR¹⁶ and the LAMP assay²⁵ (Table 1). The LAMP primer sets were prepared to be high performance liquid chromatography (HPLC) purification grade.

PCR and LAMP assay. The PCR solution (20 μ L) was prepared with a standard procedure using Top polymerase (BIONEER, Daejeon, Korea). The reaction consisted of 35 cycles each at 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds. The PCR products were resolved by agarose gel electrophoresis and stained in ethidium bromide. The LAMP method was performed according to the manufacturer's instructions (Eiken Sci, Tokyo, Japan), except for use of the 20- μ L total reaction mixture. The LAMP reaction was performed at a constant 65°C. The amplification of the target gene was confirmed based on the turbidity of magnesium pyrophosphate and by gel electrophoresis.

RESULTS

Sensitivity and specificity of PCR and LAMP assay. To determine the sensitivity of the PCR and LAMP methods, we performed the reactions using *S. japonicum* genomic DNA from 10 pg to 10 fg, respectively, by serial dilution. As shown in Figure 2, PCR using specific primers amplified the band of 405 bp from 28S rDNA, and the PCR method was able to detect more than 100 fg of genomic DNA (Figure 2A). The LAMP assay had the same level of sensitivity as the conventional PCR assay (Figure 2B). Furthermore, both methods amplified only DNA from *S. japonicum* and none from *S. mansoni*. Thus, our methods distinguished the *S. japonicum* species from others. However, PCR using Sjr2 primers detected DNA at the level of 1 pg (Figure 2A), whereas LAMP did not detect the Sjr2 gene at all, contrary to a recent report²⁵ (data not shown). Taken together with these results, we performed the following experiments using 28S rDNA primers as the appropriate targeting genes because of higher sensitivity.

Detection of the schistosomal DNA from miracidia and infected snails. To confirm whether a single miracidium DNA could be detected by the PCR and LAMP assay using 28S rDNA primers, we extracted DNA using the heated NaOH method from one miracidium and performed both methods with 10 independent samples. The PCR and LAMP detected the DNA from one miracidium in all samples (Figure 3A and B), indicating that the total DNA included in a single miracidium was enough to be amplified by both the PCR and LAMP methods. Furthermore, we performed the infection experiment with the intermediate snail with a different number

FIGURE 1. Schema of the selective areas for snail sampling in Anhui Province of China. Three points located along the Yangtze River in Anhui Province are shown as closed circles, and the capital of each country is shown as an open circle. Shankou-city in Anqing county and Guanghui City in Tongling county were marshland regions, and Shun'an town in Tongling county was in the sand regions.

TABLE 1
Specific primer sets used in this study

PCR	Sj28S Forward primer; 5'-GGTTTGACTATTATTGTTGAGC-3' Reverse primer; 5'-TCTCACCTTAGTTCGGACTGA-3' SjR2 ¹⁶ Forward primer; 5'-TCTAATGCTATTGGTTTGAGT-3' Reverse primer; 5'-TTCCTTATTTTCACAAGGTGA-3'
LAMP	Sj28S F3 primer; 5'-GCTTTGTCCTTCGGGCGATTA-3' B3 primer; 5'-GGTTTCGTAACGCCCAATGA-3' FIP primer; 5'-ACGCAACTGCCAACGTGACATACTGGTCGGCTTGTTACTAGC-3' BIP primer; 5'-TGGTAGACGATCCACCTGACCCCTCGCGCACATGTTAAACTC-3' SjR2 ²⁵ F3 primer; 5'-GCCGGTTCCTTATTTTCACAAGG-3' B3 primer; 5'-CTAACATAATTTATCGCCTTGCG-3' FIP primer; 5'-CTACGACTCTAGAATCCCCTCCGCGAATGACTGTGCTTGGAATC-3' BIP primer; 5'-CCTACTTGATATAACGTTTCAACGTATTGGTTTGAGTTCACGAAACGT-3'

of miracidia and extracted total DNA from each snail at 1 day after the infection. As a result, we found four positive samples out of a total of five samples infected with one miracidium, although all samples were positive in the five samples infected

with 5 or 10 miracidia, respectively (Figure 3C). We considered that one negative snail was not penetrated by a miracidium, because not all miracidia could enter the snail. These results showed that the PCR detected the schistosome-specific band

FIGURE 2. Sensitivity of the PCR and LAMP methods using genomic schistosomal DNA comparing 28S rDNA with SjR2 primers. (A) PCR was performed with different weights of genomic DNA, and the 28S rDNA primer set was able to detect 100 fg of DNA from *S. japonicum* but none from *S. mansoni*; the SjR2 primer set was able to detect just 1 pg of DNA. (B) The LAMP assay method showed the same sensitivity (100 fg) as the PCR method. Neither method reacted to DNA from *S. mansoni*, and no template (-) was the negative control.

FIGURE 3. Detection of a schistosome-specific band in genomic DNA extracted from naked miracidia and the experimentally infected snail by PCR and LAMP. (A) The DNA extracted from one miracidium was amplified by PCR. PCR detected the specific band (arrow) in each of 10 samples extracted distinctly from one miracidium but not the no-template sample (-). Genomic DNA (1 pg) of *S. japonicum* was used for the positive control. (B) The DNA extracted from one miracidium was amplified by LAMP assay. LAMP showed the positive results as the white turbidity of magnesium pyrophosphate in all 10 samples extracted distinctly from one miracidium (1–10) and *Sj* DNA (1 pg) as positive control (P) but not the no-template sample (N). (C) Each snail from the non-endemic area was experimentally infected with a different number of miracidia (0, 1, 5, and 10 miracidia/snail), and genomic DNA was extracted from each snail at 1 day after infection. The PCR method detected the schistosome-specific band in DNA from a snail infected with just one miracidium without amplifying DNA from non-infected snails. Each lane represents a distinct snail infected with the same number of miracidia.

TABLE 2
The comparison of detection rate between the PCR assay and microscopy method in wild snails from Anhui Province

	Shankou-city in Anqing		Shun'an town in Tongling		Guanghui city in Tongling	
	Microscopy positive	Microscopy negative	Microscopy positive	Microscopy negative	Microscopy positive	Microscopy negative
PCR positive	10	13	2	0	0	0
PCR negative	0	217	0	72	0	48
Positive rate of microscopic examination		4.2%		2.7%		0%
PCR positive rate		9.6%		2.7%		0%

in the DNA extracted from the infected snail with a single miracidium. Furthermore, using the same DNA prepared from the snails infected with a single miracidium of *S. japonicum*, the result of the LAMP method was consistent with that of the PCR method (data not shown). Thus, the PCR and LAMP methods have the high specificity and sensitivity and detect schistosomal DNA immediately after the infection to the snail host.

Detection of the schistosomal DNA in wild snails collected from endemic areas. To evaluate whether the PCR assay could detect schistosomal DNA from the infected snails in the endemic areas, we collected wild snails from three points, Shankou-city, Shun'an town, and Guanghui City of Anhui Province in China (Figure 1), in which the human infection rate is 4%, 0%, and 1.6%, respectively. As shown in Table 2 in snails collected from Shankou-city during the spring, the PCR method detected more positive snails than did the microscopy method with the observation of *S. japonicum* cercaria. Although all positive snails by microscopy were also positive by PCR, PCR also amplified the DNA of *S. japonicum* in the snails negative by microscopy. This indicates that PCR could detect the infection not only in the matured cercaria but also in the early sporocyst. However, in snails from Tongling collected in the autumn, PCR detected DNA only from the snails positive by microscopy.

Screening with large-scale DNA extraction from the infected snail by LAMP assay. The PCR method is difficult to use in the field in endemic areas because of the expense of the thermal cycler and the impracticality of performing gel electrophoresis and staining. To amplify the specific DNA without such problems, we applied the LAMP method, which can be performed at a constant temperature and the result can be determined without gel electrophoresis. The LAMP detected schistosomal DNA from a single miracidium of *S. japonicum* (Figure 2B) and the snail infected with a single miracidium (data not shown). Thus, the LAMP method should be useful for the detection of specific DNA in the field without the need for a thermal cycler or gel electrophoresis. We also screened the rate of infected snails in local areas using large-scale DNA extraction. Different numbers (99, 49, 24, and 4) of non-infected snails from non-endemic areas were prepared, and a single infected snail (1 day after infection with 10 miracidia) was mixed in each group. The snails were crushed together, genomic DNA was extracted in one tube, and each sample was assayed by the LAMP method. LAMP detected 28S rDNA of *S. japonicum* from all infected groups but not non-infected groups (Figure 4), indicating that it is useful for detecting schistosomal DNA from a large number of snails in the field in endemic areas.

DISCUSSION

Schistosomiasis-control activities in China since the mid-1950s have decreased the prevalence of human infection with

S. japonicum to less than 10%.^{27,28} Furthermore, a new integrated strategy was developed and proven effective in endemic areas.^{7,8} However, the complete eradication of schistosomiasis japonica and the prevention of its reemergence remain difficult. To monitor the infection rate and distribution of infected snails, we developed molecular detection tools based on the amplification of nucleic acid.

PCR targeting 28S rDNA amplified 100 fg of genomic DNA from only *S. japonicum* and none from *S. mansoni*. The ribosomal DNA was known to have a highly repetitive sequence in the genome,^{18,29,30} and each region has been shown to be useful for molecular diagnosis and identification of species

FIGURE 4. Detection of 28S rDNA from *S. japonicum* by LAMP assay in the total DNA from different numbers of non-infected snails artificially contaminated with a single infected snail. The snails infected with 10 miracidia were prepared and mixed with different numbers of snails (99 + 1, 49 + 1, 24 + 1, 9 + 1, 4 + 1, 0 + 1; normal + infected snails). Total DNA was extracted from each group and one non-infected snail (-), and the LAMP assay was performed. The 28S rDNA was amplified from all samples contaminated with the infected snail but not from non-infected snails by the LAMP assay. The results were confirmed based on the white precipitation (Upper) and gel electrophoresis (Lower).

in other infectious diseases.^{31,32} Our designed primer set was suitable for detection of the 28S ribosomal region from *S. japonicum*, and it performed better than had been previously reported, indicating that the sensitivity was 15 pg.¹⁹ Non-LTR retrotransposon, Sjr2, was also detected by PCR at a sensitivity of 1 pg, nearly coinciding with the previous result (0.8 pg).¹⁶ Our results indicated that the sensitivity of 28S rDNA is higher than that of Sjr2, because R2 sequences were specifically inserted into the 28S ribosomal region and the copy numbers of Sjr2 were restricted by that of the target 28S ribosomal DNA.³³

The LAMP assay is a rapid, specific, and convenient assay employing four primers and isothermal DNA polymerase, and this tool can be applied as a new molecular diagnosis in the field in endemic areas. Our results showed that the sensitivity of LAMP was the same as that of conventional PCR. In general, the LAMP method is more sensitive than the PCR method,^{20,22,34,35} although similar sensitivities between the two have also been reported.²⁴ This may reflect the fact that the sensitivity is dependent on the designed primers. However, the present study found that the sensitivity of PCR assay was sufficient to amplify the 28S ribosomal DNA from a single miracidium, and the results between the LAMP and PCR assays were completely consistent. Therefore, the LAMP assay seems capable of detecting a single miracidium rapidly and inexpensively in the field. Recently, Xu and others²⁵ investigated LAMP targeting Sjr2 and found that its sensitivity was 0.08 fg. We repeated their experiment using the reported primers,²⁵ but we found that the DNA from *S. japonicum* was not adequately amplified, because each sequence of the reported primers was not identical to the Sjr2 regions.

By contrast, PCR, using 28S rDNA primer sets amplifying the *S. japonicum* DNA from the snails infected with a single miracidium at 1 day after infection, was able to detect a single individual of *S. japonicum* throughout the snail stages, whereas conventional microscopy can detect only mature cercaria of *S. japonicum*. Furthermore, PCR is useful for beginners without skill and knowledge, because this method can be specific to *S. japonicum* and can distinguish it from the other species. To evaluate whether our PCR method could be applied in endemic areas, we collected wild snails from endemic areas in Anhui province of China. After crushing the snails and checking for infection by microscopy, total DNA extracted from each snail underwent PCR using 28S rDNA primer sets. PCR amplified the product band from not only snails including the matured cercariae of *S. japonicum* but also snails where cercaria could not be seen by microscopy. This is further support for our hypothesis that PCR could detect the potential infection in the snails with early sporocysts. However, PCR never detected schistosomal DNA from cercaria-negative snails collected from Tongling in the autumn (September). It may be that the differences in the findings between the two areas reflect differences in the timing of new infections in snails as a function of the season and local factors. These areas were part of the marshlands of the Yangtze River where water levels fluctuate markedly because of rainfall and flooding. In autumn, domestic animals exit the marshlands because of the rising water level, which usually reaches the highest level of the year. Thus, transmission of miracidia to snails may be the most difficult in autumn, although domestic animals known to contain hosts of *S. japonicum* were found to be repeatedly infected throughout the year.^{36,37} These data suggested that the

PCR method has the potential to monitor the timing of the infection of snails in endemic areas.

Several previous reports have suggested that LAMP is useful for the detection of the infections in pathogen-carrying vectors.^{38,39} To evaluate the efficiency of the LAMP method for detecting infected snails, a large number of snails contaminated with a single infected snail were crushed together, and the total DNA was extracted in one tube. We then investigated whether LAMP could amplify the schistosomal DNA alone and found that LAMP could detect infection from a snail infected with *S. japonicum* in a group of 100 non-infected snails, indicating its use for detecting infection at a 1% infection rate. If snails (1,000–10,000 individuals) collected from several locations (e.g., 10–100 locations) were assayed, we expect that this method could precisely identify the infection rate in that area. The LAMP assay using 28S rDNA primers may be an effective tool, having the benefits of being rapid, easy, and inexpensive. Although the microscopy method is inexpensive, it is difficult to crush and observe a large number of snails. In particular, the novel LAMP method will make it possible to easily monitor very low infection rates of snails in endemic areas, where the new integrated strategy will be implemented.^{7,8}

In the present study, we evaluated PCR and LAMP assay targeting to 28S rDNA from *S. japonicum*. We found that PCR amplifying 28S rDNA could detect 100 fg of DNA from *S. japonicum* but none from *S. mansoni*. Furthermore, the PCR (and LAMP) method could detect the infection of *S. japonicum* in every stage inside the snail. In fact, PCR could detect potential infection from snails deemed negative for infection by microscopy that were sampled from wild snails collected from endemic areas. LAMP, which is rapid, easy, and safe to use in the field, was able to amplify the schistosomal DNA from a single infected snail in a total of 100 snails without marked inhibitions. PCR and LAMP targeting to 28S rDNA may be useful for monitoring the infection rate of snails in endemic areas and for confirming complete eradications against infected snails in the areas where the new integrated strategy is implemented.

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