Parasitol Res (2011) 108:1193–1198
DOI 10.1007/s00436-010-2162-x

ORIGINAL PAPER

Sensitive and rapid detection of *Paragonimus westermani* infection in humans and animals by loop-mediated isothermal amplification (LAMP)

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Received: 16 September 2010 / Accepted: 10 November 2010 / Published online: 25 November 2010

Abstract In the present study, a loop-mediated isothermal amplification (LAMP) assay was developed and validated for the detection of *Paragonimus westermani* adults, metacercariae, and eggs in human and animal samples. The LAMP amplification can be finished in 45 min under isothermal condition at 60°C by employing a set of four species-specific primer mixtures and the results can be checked by naked-eye visualization. No amplification products were detected with deoxyribunucleic acid (DNA) of related trematode species including *Fasciola hepatica*, *Fasciola gigantica*, *Clonorchis sinensis*, *Opisthorchis viverrini*, *Schistosoma mansoni*, and *Schistosoma japonicum*. The method was further validated by examining *P. westermani* DNA in intermediate hosts including freshwater crabs and crayfish, as well as in sputum and pleural fluid samples from patients of paragonimiasis. These results indicated that the LAMP assay was highly specific, sensitive, and rapid, and it was approximately 100 times more sensitive than conventional specific PCR. The LAMP assay established in this study provides a rapid and sensitive tool for the detection of *P. westermani* DNA in freshwater crabs, crayfish, sputum, and pleural fluid samples, which has important implications for effective control of human paragonimiasis.

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Introduction

Human paragonimiasis is caused by trematodes of the genus *Paragonimus* which parasitize in the lungs of humans. The disease is mainly endemic in China, Korea, Japan, and some other Asian countries, where people have the habit of eating raw or undercooked freshwater crabs or crayfish which may be infected with infective *Paragonimus metacercariae* (Kim et al. 2009). Now, paragonimiasis is having a wilder distribution due to the movement of the world population and the pursuit of eating exotic and delicate foods (Liu et al. 2008; Lane et al. 2009; Sohn et al. 2009; Ikehara et al. 2010). Paragonimiasis is considered an important food-borne parasitic disease in China (Liu et al. 2008; Zhou et al. 2008).

At present, approximately 28 species of *Paragonimus* genus are recognized: two species in Africa, five species in the Americas, and 21 species in Asia (Miyazaki 1982). Among them, *Paragonimus westermani* has long been considered to be the most important causative agent of paragonimiasis in Asia (Miyazaki 1978). *P. westermani* causes pulmonary, neurologic, and abdominal diseases by infecting lungs, brains, spinal cords, and other organs in humans and animals including dogs, tigers, cats, pigs, cattle, mink, and feral carnivores.

Various techniques have been established for the diagnosis of human paragonimiasis, including parasitological and immunological methods. These methods are conventionally performed by the detection of *P. westermani* eggs in human sputum or pleural fluid. However, it remains difficult to examine the infection intensity of *P. westermani* in crabs or crayfishes. It is also difficult to determine metacercariae and eggs of *P. westermani* to species level, since they are very similar to that of other digeneans morphologically. Furthermore, freshwater crabs or crayfish with only a few metacercariae harbored cannot be reliably recognized as infected using microscopic examination (Devi et al. 2010). Detection of eggs in early or late infection stage or extrapulmonary infection stage is difficult, because eggs rarely exist in these stages. Immunodiagnostic methods have proven sensitive for early diagnosis of human paragonimiasis. Although they are useful for the screening of patients in endemic areas, they are not suitable for the survey of intermediate hosts or sputum and pleural fluid samples (Lee et al. 2006, 2007; Na et al. 2006; Zhao et al. 2007; Kirino et al. 2009).

Recently, polymerase chain reaction (PCR) assays have been described for the detection of *P. westermani* in infected freshwater crabs or crayfishes and have significantly increased our ability in the detection of *P. westermani* infection (Sugiyama et al. 2002; Li et al 2005; Tandon et al. 2007; Doanh et al. 2007, 2009; Devi et al. 2010). However, highly precision thermocyclers are needed in PCR techniques, which prevent the widespread use of these approaches in field conditions.

A novel nucleic acid amplification method named loop-mediated isothermal amplification (LAMP) was developed by Notomi et al. (2000). This assay can amplify target deoxyribunucleic acid (DNA) to a quantity as high as 10^9 copies in less than 1 h under isothermal conditions, and no thermocycler is needed (Notomi et al. 2000; Nagamine et al. 2002). Further, the amplification product can be visually detected with the addition of fluorescent dyes such as SYBR Green I (Poon et al. 2006). Four LAMP primers, which are designed to recognize six distinct regions on the target gene, assure the specific amplification of the target DNA.

LAMP assays have been developed successfully for the detection of many viral, bacterial, and fungal diseases (Maruyama et al. 2003; Endo et al. 2004; Okafuji et al. 2005). Recently, this method has also been developed successfully for the diagnosis of parasitic infections such as malaria, trypanosomiasis, theileriosis, babesiosis, schistosomiasis, and clonorchiasis (Kuboki et al. 2003; Poon et al. 2006; Alhassan et al. 2007; Iseki et al. 2007; Thekisoe et al. 2007; Xu et al. 2009; Cai et al. 2010).

The objective of the present study was to develop a simple and cost-effective LAMP assay for rapid detection of *P. westermani* DNA in humans and animal samples based on its second internal transcribed spacers (ITS-2) of nuclear ribosomal DNA (rDNA). Data on the sensitivity and specificity of the method were reported, and the applicability of the LAMP assay for the detection of adult, metacercariae, and egg stages of *P. westermani* was demonstrated.

Materials and methods

Parasite samples

Adult worms of *P. westermani* were collected from dogs experimentally infected in our laboratory. *P. westermani* metacercariae were collected from freshwater crabs and crayfish muscles from Fujian and Guangdong provinces, China. *P. westermani* eggs were collected from sputum and pleural fluid samples from patients who have been diagnosed and identified by morphology (Table 1). Several trematodes infecting humans, namely, *Fasciola hepatica*, *Fasciola gigantica*, *Clonorchis sinensis*, *Opisthorchis viverrini*, *Schistosoma mansoni*, and *Schistosoma japonicum*, were included as 'heterologous control samples' for assessing the specificity of the LAMP assay. All parasite materials were preserved in 70% ethanol and kept at −20°C until the extraction of genomic DNA.

Table 1 Samples of *Paragonimus westermani* from China used in the present study

Sample codes	Location	Stage
Crabs 1–20	Fujian Province	Metacercariae
Crayfishes 1–15	Guangdong Province	Metacercariae
Sputum and pleural fluid from patients 1–17	Guangdong Province	Egg
Adult worm	Our laboratory	Adult

Thirty-five freshwater crab and crayfish muscle samples with *P. westermani* metacercariae infected (infection intensity ranged from 1 to 8 metacercariae per gram of muscle), as well as sputum and pleural fluid samples from patients who have been diagnosed and identified by morphology, were used. Furthermore, two crab and two crayfish samples without *P. westermani* metacercariae, as well as three sputum samples from healthy persons confirmed by microscopic examination, were used as control. All these materials were kept at −86°C until the extraction of genomic DNA.

DNA preparation

Total genomic DNA from *P. westermani* adults, metacercariaes, eggs, noninfected freshwater crab and crayfish muscles, sputum, and pleural fluid samples from healthy persons, as well as the heterologous control samples, were extracted, respectively, by SDS/proteinase K treatment. Then the DNA samples were column-purified (Wizard® SV Genomic DNA Purification System, Promega; Zhao et al. 2009; Ai et al. 2010) and eluted into 60 μl H_2O, respectively, according to the manufacturer's recommendations. The integrity of all the DNA samples were validated by successful amplification of the mitochondrial cytochrome c oxidase subunit 1 (*cox*1) using primers and protocols described previously (Bowles et al. 1992; data not shown).

LAMP assay

The successful amplification of LAMP assay depends on the specificities of primers designed, and a set of four specific primers (B3, F3, BIP, and FIP) that recognize a total of six distinct sequences (B1, B2c, B3, F1c, F2, and F3) of the target DNA region are required. The ITS-2 sequence of *P. westermani* rDNA was retrieved from GenBank (AF159604) and was used for the design of species-specific primers with Primer Explorer V4 software (http://primerexplorer.jp/e; Table 2). The primers were designed and selected based on the criteria described by Notomi et al. (2000).

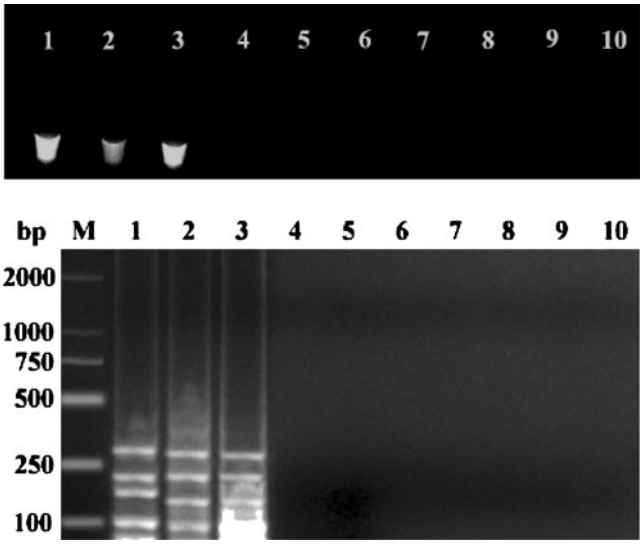

Fig. 1 Specificity assessment of the LAMP assay for detection of *Paragonimus westermani*. *Upper*: Visual examination of LAMP products. *Bottom*: Agarose gel electrophoresis of amplified products. *Lanes 1–9* represent *P. westermani* adult, *P. westermani* metacercariae, *P. westermani* eggs, *Fasciola hepatica*, *Fasciola gigantica*, *Clonorchis sinensis*, *Opisthorchis viverrini*, *Schistosoma mansoni*, and *Schistosoma japonicum*, respectively. *Lane 10* represents no-DNA control. *M* represents a DNA size marker (ordinate values in bp)

The LAMP assay was carried out in a 25-μl reaction mixture containing 10×*Bst*-DNA polymerase buffer (2.5 mM each), betaine (10 μM each), deoxynucleotide triphosphates (2.5 mM each), $MgSO_4$ (10 mM each), FIP and BIP (1.6 mM each), loop-F and loop-B (0.8 μM each), F3 and B3 primers (0.2 mM each), *Bst* DNA polymerase (8 U, New England BioLabs), ddH_2O, and template DNA (1 μl). The template DNA was omitted in the reaction of negative control. The mixtures were incubated at 60°C and then heated at 80°C for 10 min to terminate the reaction. Because of the high sensitivity of LAMP reaction, controls (positive and negative) were included in each run, and all precautions to prevent cross-contamination were considered such as minimizing manipulation of the reaction tubes, performing the steps of LAMP assay in different rooms, and adding sterile paraffin wax to the mix to prevent contamination.

Table 2 LAMP primers for the amplification of the second internal transcribed spacer (ITS-2) of *Paragonimus westermani* rDNA

Primer	Length	Sequence (5′–3′)
F3	18	CAAAAAGTCGCGGCTTGG
B3	18	CACGCGCAACATGAACCA
FIP (F1c+F2)	41	GGTGCGCGCGAGTATGTTAGG-CGTGATCTCCCCAATCTGGT
BIP (B1c+B2)	40	CCTTGACGGGGATGTGGCAA-CAGATGAAGACAGGACAGCG
Loop F	19	AACGCCATAGATCTGGCAC
Loop B	18	CGGAATCGTGGCTCAGTA

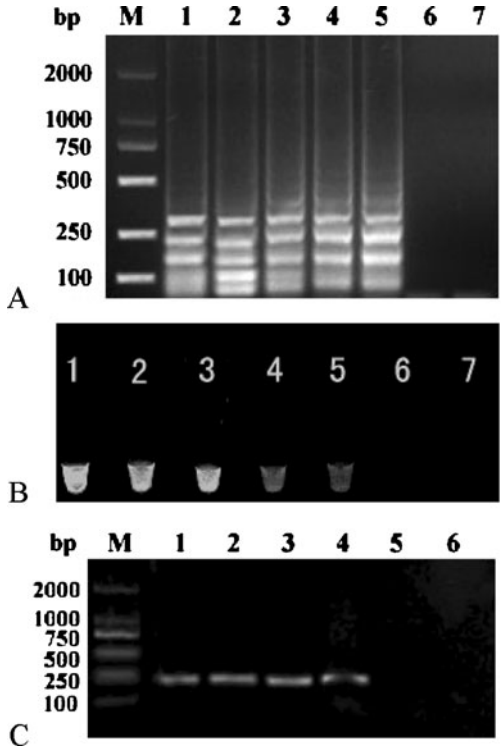

Fig. 2 Sensitivity assessment of the LAMP assay and comparison with conventional PCR for the detection of *Paragonimus westermani*. The conventional PCR was performed with primers F3 and B3. **A**, **B** Sensitivity of the LAMP assay. C Sensitivity of a conventional PCR. *Lanes 1–6* represent serial concentrations of *P. westermani* DNA in the range of 10^{-4}–10^{-9} ng/μl for **A** and **B**. *Lanes 1–5* represent serial concentrations of *P. westermani* DNA in the range of 10^{-3}–10^{-7} ng/μl for **C**. *Lane 7* in **A** and **B** and *Lane 6* in **C** represent no-DNA control. *M* represents a DNA size marker (ordinate values in bp)

Detection and confirmation of LAMP products

The LAMP amplification results were visually detected by adding 1 μl of 1:10 diluted 10,000×concentration fluorescent dye SYBR Green I (Invitrogen) to the reaction tube. The solution would turn to green if LAMP reaction was successful; otherwise, it would remain orange. The LAMP products were also monitored in a 2% agarose gel stained with ethidium bromide. The stained gel and the reaction tubes were then photographed using the ultraviolet (UV) image system (Gel documentation system, UVItec, UK).

Conventional PCR with the outer primers B3 and F3 of LAMP was performed. The PCR mix was a 25 μl system with 10×PCR buffer (2.5 μl), 0.2 mM of each dNTPs, 0.4 μM of each B3 and F3 primers, 1.25 U of Ex*Taq* polymerase (Takara), and 1 μl of DNA sample in a thermocycler (Biometra). The performing conditions were as follows: an initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation (30 s at 94°C), annealing (1 min at 55°C), and extension (30 s at 72°C), with a final extension for 5 min at 72°C. PCR products (5 μl) were examined in a 2% agarose gel, stained with SYBR green I and photographed.

Results and discussion

To determine the specificity of the LAMP assay for *P. westermani*, DNA samples from other trematode parasites including *F. hepatica*, *F. gigantica*, *C. sinensis*, *O. viverrini*, *S. mansoni*, and *S. japonicum* were used as control. It was found that target DNA was amplified only from DNA samples of *P. westermani* adults, metacercariae, and eggs, and no amplification of target DNA was found in the heterologous control samples (Fig. 1, upper). These products displayed typically ladder-like bands on agarose gels (Fig. 1, bottom). The specificity of the primers was also confirmed by BLAST search (http://www.ncbi.nlm.nih.gov/Blast) in the NCBI database (data no shown).

To determine the sensitivity of the LAMP assay for *P. westermani*, serial concentration of DNA samples were prepared. Briefly, the concentration of DNA samples of *P. westermani* metacercariae and eggs was measured three times by spectrophotometry to obtain an average concentration. Then they were diluted with 10 mM Tris–HCl (pH 8.8) to a final concentration of 100 ng/μl. Subsequently, tenfold serial dilutions ranging from 1×10^{-3} to 1×10^{-9} ng/μl were prepared and used as templates for LAMP or conventional

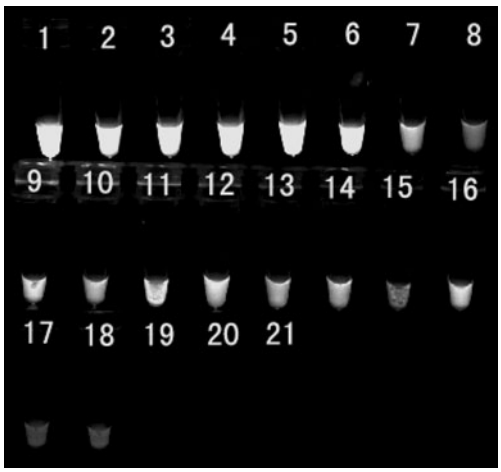

Fig. 3 LAMP amplification of the second internal transcribed spacer of *Paragonimus westermani* from freshwater crabs and crayfish samples infected with metacercariae, and sputum and pleural fluid samples from patients of paragonimosis. *Tube 1* represents *P. westermani* adult. *Tubes 2–4* represent freshwater crab samples infected with metacercariae of *P. westermani*. *Tubes 5–7* represent crayfish samples infected with metacercariae of *P. westermani*. *Tubes 8–13* represent sputum samples from paragonimosis patients. *Tubes 14–18* represent pleural fluid samples from paragonimosis patients. *Tube 19* represents a crab sample without metacercariae of *P. westermani*. *Tube 20* represents a sputum sample from healthy person. *Tube 21* represents no-DNA control

PCR. The LAMP products were visualized under UV light, and the fluorescent signals of the solutions were observed in the positive reactions without opening the tubes. Results showed that the detection limit of the LAMP assay was 10^{-8} ng/μl (Fig. 2A and B), while it was only 1×10^{-6} ng/μl for conventional PCR (Fig. 2C). Therefore, the sensitivity of the LAMP assay for the detection of *P. westermani* DNA was 100 times higher than that of the conventional PCR.

The applicability and practicality of the LAMP assay were evaluated for the detection of *P. westermani* metacercariae in freshwater crabs, crayfishes, or in sputum and pleural fluid samples of human paragonimiasis patients (Fig. 3). Totally, 35 muscle samples from freshwater crabs or crayfishes infected with *P. westermani* metacercariae, and 17 egg samples from sputum and pleural fluid of human paragonimiasis patients were used for positive evaluation. Two crab samples, two crayfish samples without metacercariae, and three sputum samples without eggs from healthy persons (confirmed by microscopic examination) were used as negative control. As expected, the 35 infected samples and the 17 sputum and pleural fluid samples produced positive results in the LAMP assay, even some samples with infection intensity as low as 1 metacercariae/g. However, no products were detected from the negative control samples of noninfected crabs or sputum samples from healthy persons (Fig. 3).

Freshwater crabs and crayfishes are the second intermediate hosts of *P. westermani*, and humans get infected with *P. westermani* metacercariae by ingesting raw or undercooked freshwater crabs or crayfishes. Hence, the accurate identification and detection of *P. westermani* metacercariae of *P. westermani* in freshwater crabs and crayfishes has important implications for the control and prevention of human paragonimiasis in endemic areas. Microscopic examination has still been the routine method for the detection of metacercariae in freshwater crabs, crayfishes, and sputum and pleural fluid samples from patients of paragonimiasis, but there is limitation of such method in that metacercariae are difficult to be detected and identified due to low intensity of infection. PCR techniques for the detection of *P. westermani* have been developed in recent years (Sugiyama et al. 2002; Li et al 2005; Tandon et al. 2007; Doanh et al. 2009; Devi et al. 2010). But complicated equipment and expert techniques are required, which makes the techniques not to be readily available in rural endemic regions. Moreover, the *Taq* DNA polymerase used in PCR assays is easily to be inhibited by biological substances.

The LAMP assay established in the present study is economic and easy to be performed, and it is more sensitive than conventional PCR in the detection of *P. westermani* infection. The designed loop primers allow the amplification to be finished in 45 min. Only a water bath or a heat block is required to carry out the amplification, which is readily convenient even in poorly equipped laboratories or in large-scale epidemiological studies in poor areas. The *Bst* DNA polymerase acts at a relatively high temperature, which helps to reduce nonspecific priming. Moreover, this DNA polymerase is also more resistant to inhibitors than *Taq* DNA polymerase (Poon et al. 2006). A large amount of white magnesium pyrophosphate precipitate will generate in positive samples, which allows the presence of *P. westermani* DNA to be easily identified by visual inspection, and the positive amplification can be viewed by adding fluorescent dyes such as SYBR Green I.

In conclusion, the results of the present study demonstrated that the established LAMP assay is a rapid and sensitive technique for detection of *P. westermani* metacercariae in freshwater crabs, crayfishes, or eggs in sputum and pleural fluid samples of human paragonimiasis patients, which provides a useful tool for the prevalence survey of *P. westermani* in freshwater crabs and crayfishes. Moreover, the technique has the potential to be applied in many clinical and epidemiological investigations of *P. westermani* infection in humans, which will contribute to the effective control of human paragonimiasis.

Acknowledgements This work is supported in part by the Program for National S&T Major Program (grant no. 2008ZX10004-011), National Key Technology R&D Program (grant no. 2008BAI56B03) and the State Key Laboratory of Veterinary Etiological Biology, Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences. The experiments comply with the current laws of the country in which the experiments were performed.

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