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Rapid detection of *Opisthorchis viverrini* copro-DNA using loop-mediated isothermal amplification (LAMP)

Yuji Arimatsu^a, Sasithorn Kaewkes^{b,c}, Thewarach Laha^b, Sung-Jong Hong^d, and Banchob Sripa^{a,c}

^aTropical Disease Research Laboratory, Division of Experimental Pathology, Department of Pathology, Faculty of Medicine, Khon Kaen University, Khon Kaen 40002, Thailand ^bDepartment of Parasitology, Faculty of Medicine, Khon Kaen University, Khon Kaen 40002, Thailand ^cLiver Fluke and Cholangiocarcinoma Research Center, Khon Kaen University, Khon Kaen 40002, Thailand ^dDepartment of Medical Environmental Biology, College of Medicine, Chung-Ang University, Dongjak-gu, Seoul, 156-756, Republic of Korea

Abstract

Opisthorchis viverrini and other foodborne trematode infections are major health problem in Thailand, the Lao People's Democratic Republic, Vietnam and Cambodia. Differential diagnosis of *O. viverrini* based on the microscopic observation of parasite eggs is difficult in areas where *Clonorchis sinensis* and minute intestinal flukes coexist. We therefore established a rapid, sensitive and specific method for detecting *O. viverrini* infection from the stool samples using the loop-mediated isothermal amplification (LAMP) method. A total of five primers from seven regions were designed to target the internal transcribed spacer 1 (ITS1) in ribosomal DNA for specific amplification. Hydroxy naphthol blue (HNB) was more effective to detect the LAMP product compared to the Real-time LAMP and turbidity assay for its simple and distinct detection. The LAMP assay specifically amplified *O. viverrini* ITS1 but not *C. sinensis* and minute intestinal flukes with the limit of detection around 10^{-3} ng DNA/ μ L. The sensitivity of the LAMP was 100% compare to egg positive samples. While all microscopically positive samples were positive by LAMP, additionally 5 of 13 (38.5%) microscopically negative samples were also LAMP positive. The technique has great potential for differential diagnosis in endemic areas with mixed *O. viverrini* and intestinal fluke infections. As is is an easy and simple method, the LAMP is potentially applicable for point-of-care diagnosis.

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*Corresponding author: Banchob Sripa, Tropical Disease Research Laboratory, Departments of Pathology, Faculty of Medicine, Khon Kaen University, Khon Kaen 40002, Thailand. Tel: +66-43363113, Fax: +66-43-202024, banchob@kku.ac.th.

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Keywords

Loop-Mediated Isothermal Amplification; LAMP; *Opisthorchis viverrini*; Stool examination; Internal transcribed spacer 1; Internal transcribed spacer 2

1. Introduction

Liver fluke infection caused by *Opisthorchis viverrini* is a major public health problem in Thailand, the Lao People's Democratic Republic, Cambodia and southern Vietnam with an estimate of over 8-10 million people are infected [1]. Recent surveys have revealed a high prevalence of liver fluke infection with up to 70% in certain villages along the Lawa Lake, Khon Kaen Province, Thailand [2]. Moreover, the infection is also prevalent in cats and dogs, the reservoir hosts (35-36.4% and 0.3-3.8%, respectively) [3, 4] and Cyprinid fish intermediate hosts (up to 95%) [5]. Diagnosis of *O. viverrini* infection in humans is usually done by microscopic observation of parasite eggs collected from feces. However, the eggs of *O. viverrini* are morphologically similar to those of *Clonorchis sinensis* and minute intestinal flukes, such as, *Centrocestus caninus* and *Haplorchis taichui*. The diagnoses of *O. viverrini* and *C. sinensis* are particularly important because both parasite infections are risk factors for developing cholangiocarcinoma [10, 11]. A rapid, accurate and specific stool analysis is required, since these liver and intestinal flukes coexist in Southeast Asian countries [7-9].

Diagnosis of *O. viverrini* infection using PCR has been reported for specific diagnosis of opisthorchiasis [6]. Internal transcribed spacers (ITS1 and ITS2) have frequently been used as a molecular marker to differentiate closely related species because a high level of interspecific sequence divergence is common. ITS1 is specific for the discrimination of parasite species, but is less sensitive for diagnosis (i.e. 76.2%), whereas ITS2 is less specific but highly sensitive (i.e. 95.2%) [12]. A decade ago, a loop-mediated isothermal amplification (LAMP) method which amplifies DNA with high specificity, efficiency and rapidity was developed [13]. The LAMP assay does not require a thermocycler and allows visual detection of DNA amplification by turbidity. It has been applied successfully to detect pathogenic microorganisms, including parasites, such as, *C. sinensis* [14], *Schistosoma japonicum* [15], *Paragonimus westermani* [16], *Fasciola hepatica* and *Fasciola gigantica* [17]. The objective of the present study was to establish a LAMP-based approach for the sensitive and rapid diagnosis of *O. viverrini* in stool samples.

2. Materials and methods

2.1. Parasites and stool specimens

Opisthorchis viverrini metacercariae were obtained from naturally infected Cyprinid fish captured from a freshwater reservoir in Khon Kaen Province, Thailand. The fish were digested by pepsin-HCl. After several washings with normal saline, the metacercariae were collected and identified under a dissecting microscope. Viable metacercariae were used to infect hamsters (*Mesocricetus auratus*), which were maintained at the animal facility of the Khon Kaen University Faculty of Medicine. After 2-3 months of infection, adult *O. viverrini* flukes were recovered from their bile ducts. Stool specimens were collected from

school children who live near the Lawa Lake in Khon Kaen Province, Thailand. The specimens were processed by standard formalin-ethyl acetate concentration technique and examined for parasites' eggs under a microscope. The number of *O. viverrini* eggs observed was given as eggs per gram (EPG) of feces [18]. *Clonorchis sinensis* adult worms were prepared in Korea using similar procedures as those for *O. viverrini*. Cercariae of *O. viverrini*, *Centrocestus caninus* and *Haplorchis taichui* were obtained from *Bithynia* snails by standard shedding techniques.

2.2. DNA extraction

Genomic DNA was extracted from adult *O. viverrini* worms using the PureGene Core kit A (QIAGEN) and dissolved in a 50 µl elution buffer. The DNA was used for analyzing the specificity and sensitivity of the LAMP assay. Stool samples microscopically positive for *O. viverrini* eggs were subjected to DNA extraction (n= 37). In addition, 13 stool samples negative for *O. viverrini* eggs by microscopy were also randomly selected and subjected to DNA extraction. Briefly, the stool samples (200 mg) were suspended in 1.4 ml ATL tissue lysis buffer and subjected to 5 cycles of freezing in liquid nitrogen followed by thawing them at 98–100 °C. DNA was then isolated from the supernatant using the QIAamp DNA Mini Stool Kit according to manufacturer's instruction with slight modifications. Final elution of DNA was made in 50 µl of elution buffer instead of 200 µl as recommended by the manufacturer. Control DNA from *C. sinensis*, *C. caninus* and *H. taichui* was extracted by the same method as described for *O. viverrini* adults.

2.3. LAMP assay

As shown in Fig. 1A, LAMP primers for the amplification of six distinct regions of the target DNA were designed from the *O. viverrini* ITS1 region (GenBank accession no. EU038151) using PrimerExplorer V4 software (<http://primerexplorer.jp/e>). The reaction mixture comprised of Tris-HCl (20 mM, pH 8.8), KCl (10 mM), MgSO₄ (8 mM), (NH₄)₂SO₄ (10 mM), Tween 20 (0.1%), Betaine (1.6 M), deoxynucleotide triphosphates (1.4 mM each), primers FIP and BIP (1.6 µM each), primers F3 and B3 (0.2 µM each), primer LB (0.8µM) (Fig. 1B), Bst DNA polymerase (8 U; New England BioLabs), and template DNA (2 µL). Double-distilled water was finally used to fill the system to 25 µL. No template DNA was added in the negative control reaction. The mixture was incubated at 65 °C in a heat block for 60 min and then heated at 80 °C for 5 min to terminate the reaction.

2.4. Detection of LAMP product

Three different reaction mixtures were prepared for detection of LAMP product. The first LAMP assay contained 1:3000 diluted SYBR green I (Invitrogen) and detected by real time monitoring using Mx3005P QPCR System (STRATAGENE, USA) [19]. The second reaction contained 120 µM hydroxy naphthol blue (HNB), which changes the color of solution from violet to sky blue when the DNA is amplified [20]. The third assay was inspected by turbidity of magnesium pyrophosphate, a by-product of the amplification reaction. For distinct detection of the formed magnesium pyrophosphate, the tubes were centrifuged for 10 seconds to conform a pellet at the bottom of the tube. The amplified product was also visualized using 10 µL of 1:100 diluted SYBR green I (Invitrogen) and

adding 1 μ L of each LAMP product. The reaction tubes were placed on a light box and an UV box irradiated at 365 nm and images were photographed.

2.5. PCR assay

Conventional PCR was performed with the outer primers F3 and B3 which were used for amplification of *O. viverrini* ITS1 region in the LAMP assay. The PCR reaction was carried out in a 25 μ L system with 10 $\times$ PCR buffer (2.5 μ L), 0.2 mM of each dNTPs, 0.4 μ M of each B3 and F3 primers, and 1.25 U of i-Taq DNA polymerase (Intron biotechnology), and 2 μ L of DNA sample in a thermocycler (Applied Biosystems) under the following conditions: after an initial denaturation at 95 $^{\circ}$ C for 7 min, then 35 cycles of denaturation (30 s at 95 $^{\circ}$ C), annealing (30 s at 60 $^{\circ}$ C), and extension (30 s at 72 $^{\circ}$ C), and a final extension for 7 min at 72 $^{\circ}$ C. Five microliters of PCR products (213 bp) were examined on a 1% agarose gel and stained with ethidium bromide (EtBr) and photographed.

2.6. PCR-RFLP assay

PCR was also performed by using ITS2-specific primers [7] shown in Fig. 1B. The estimated size of the amplified products is 375 bp, 381 bp and 526 bp for *O. viverrini*, *C. sinensis* and *H. taichui*, respectively. The PCR conditions were the same as described above. To differentiate *O. viverrini* from *C. sinensis*, amplified ITS2 products were digested with *Acu*I. The PCR amplicon of *O. viverrini* does not possess a restriction site for *Acu*I and remains uncut (375 bp), whereas that of *C. sinensis* has a single *Acu*I site and gives rise to two bands at 286 bp and 95 bp. Two μ L of PCR product were digested with 2.5 units of the restriction endonuclease *Acu*I (New England Biolabs) at 37 $^{\circ}$ C for 3 hours in a volume of 10 μ L.

3. Results

3.1. Sensitivity of LAMP

The concentration of the genomic DNA of *O. viverrini* adult worm was measured by spectrophotometer and diluted with 10 mM Tris HCl (pH 8.8) to a final concentration of 10 ng/ μ L. Subsequently, a 10-fold serial dilution was prepared ranging from 1×10^1 to 1×10^{-5} ng/ μ L and each of the diluted DNAs was used as a template for LAMP and conventional PCR. After amplifications, the products were visually detected on a 1% agarose gel and the sensitivity of the two assays was compared. The results showed the detection limit of the LAMP assay was 10^{-3} ng/ μ L. Similar sensitivity was observed by conventional PCR, using both LAMP outer primers (F3, B3), and ITS2 primers (RTFlukeFa, RTFlukeRa). However the intensity of the amplified product was clearly different between the two methods, indicating that LAMP assay gives distinct results more than the conventional PCR (Suppl. 1).

The Real-time LAMP assay using SYBR green I as an intercalator was successful to detect the LAMP products by Mx3005P QPCR System (Fig. 2). The fluorescence intensity rose during the LAMP reaction using DNA sample ranging from 1×10^1 to 1×10^{-3} ng/ μ L within 40 minutes. The same result was obtained using the HNB as a metal ion indicator, which changes the color of solution after DNA amplification. The LAMP product detecting by

turbidity, spin down pellet and SYBR green I as a DNA dye solution (Suppl. 2), also showed similar results.

3.2. Specificity of LAMP

Specificity of the LAMP was done by using genomic DNA of *O. viverrini* (adult worms, metacercariae and cercariae), *C. sinensis* adult worms and minute intestinal flukes (MIF), namely, *C. caninus* and *H. taichui*. The concentration of the genomic DNA of each sample was measured by spectrophotometer and diluted with 10 mM Tris-HCl (pH 8.8) to a final concentration of 1.0 ng/μL and applied to the LAMP and PCR assays. The DNA samples were confirmed by PCR-RFLP assay. The results showed that the LAMP could differentiate *O. viverrini* from *C. sinensis*, *H. taichui* and *C. caninus*, indicating the reaction is specific (Fig. 3).

3.3. Stool sample analysis

Conventional PCR and LAMP analysis were compared by using stool samples (Table 1). With the PCR method, the results showed 9 positive (18.0%) and 41 negative (82.0%) out of 50 specimens. With reference to microscopic examination, the sensitivity of the PCR was 9 in 37 egg-positive stool specimens (24.3%). Using the same DNA samples, 37 out of the 37 samples (100.0%) showed positive results with the LAMP system. Overall, the percentage of *O. viverrini* positive by LAMP was higher than that of microscopic examination in the 50 stool samples examined. Of the samples negative for *O. viverrini* eggs (n=13), 5 (38.5%) were positive by LAMP. Using the microscopic examination as a gold standard, the diagnostic sensitivity and specificity of LAMP assay were calculated at 100% and 61.5%, respectively. The positive rates of LAMP assay were consistently higher than those of conventional PCR within different *O. viverrini* intensity groups (Table 2).

4. Discussion

Our established LAMP is a rapid method, which can obtain results within 40 min, using a heat box or a water bath to maintain the temperature at 65 °C. The performances of LAMP assays using Real-time LAMP, HNB, and turbidity were compared. All methods show clear differences between the negative control and positive samples. Regarding the sensitivities of the three different LAMP methods, similar results were obtained among them. The LAMP products detected by HNB were used throughout because of simplicity, distinct results, and since other detection methods may cause cross contamination through opening the caps of the tubes with amplified DNA. The limit of detection of the LAMP method was 10⁻³ ng DNA/μL.

The PCR technique has been used to detect *O. viverrini* infection in fish, snails and experimentally infected hamsters [21, 22]. However, few of these studies have been employed for the detection of *O. viverrini* DNA in human stool specimens and they have shown varying sensitivity [23, 24]. Therefore, the sensitivity of detection needs to be improved when stool samples are used in the test. The sensitivity of PCR detection depends largely on the quality of extracted DNA, since several inhibitors in stools are known to inhibit Taq DNA polymerase [25]. It is also known that stools may contain high

concentrations of polyphenols, tannins, polysaccharides, terpenes, and resins which derive from food that limit DNA extraction success [26]. Modified QIAamp DNA Mini Stool Kit DNA extraction procedure using hexadecyltrimethylammonium bromide (CTAB) is useful to remove these compounds and successfully extract DNA from stool of primates [27] and also *O. viverrini* DNA from eggs in stool samples [28]. However, this study used only the QIAamp stool kit, therefore contaminants which may inhibit the polymerase reaction seem to remain in our stool DNA samples. Interestingly, our LAMP assay showed higher sensitivity than conventional PCR when used the same DNA samples of egg-positive cases. However, LAMP assay showed low sensitivity in stool sample without repeated freeze-thaw step during DNA extraction (data not shown). The results indicate that LAMP amplification is resistant to the contaminants included in the extracted DNA from the stool sample. However, radical physical crush of the *O. viverrini* eggs is necessary to expose miracidium to reagents during DNA extraction. The *Bst* DNA polymerase used in LAMP amplification is more resistant to inhibitors than other DNA polymerases [29], and also this DNA polymerase is active at relatively high temperatures (65 °C), which helps the reduction of nonspecific priming. Therefore, LAMP technique is effective for amplifying DNA from the stool samples, which possibly contain inhibitors of DNA polymerase.

In this study 5 false positive reactions occurred in the LAMP assay to detect copro-DNA. There is a possibility that microscopists may miss parasite eggs in stool samples due to light infections. The second possibility may be due to the quantity of stool sample examined. For microscopic analyses, 2 drops of egg sediments are generally examined while DNA is extracted from 100 mg of feces for LAMP assay. The other possibility may be the cross reaction between our LAMP primers with DNA from other organisms that are not included in our study. We did not use the stool samples of healthy people in nonendemic areas as negative control, but our established LAMP had no cross reaction with common minute intestinal flukes (*C. caninus* and *H. taichui*) and *C. sinensis*. The LAMP method is simple having only one step amplification, unlike other PCR techniques that require restriction endonuclease for differential diagnosis of these parasites [7,12]. Therefore, it is feasible to use for differential diagnosis of *O. viverrini* in areas with high prevalence of minute intestinal flukes in Thailand [8,9], or even in *C. sinensis* endemic areas in Vietnam [1].

In conclusion, LAMP assay has inherent characteristics that make it advantageous as a diagnostic test which can assist in bringing point-of-care diagnosis to patients. Since DNA amplification and reading of results require minimum equipment, the technique has great potential for use in the endemic areas of mixed *O. viverrini* and other foodborne trematode infections. Future studies will be aimed at the application of this LAMP procedure for routine diagnosis of opisthorchiasis with further validation of DNA extraction method and improvement of sensitivity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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A

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1 AACCTGCGGAAGGATCATTACAGTACACAAAGCCCAACACCTCAGTTAATCTGAGCATTGCGACGGGT
-----18S rRNA----><--ITS1--

71 CGTCATGCCCCGTTGGTCTTGCAGCATTGTCTGCCTAGGGCGGAGCGATTCCAGTTCCGTCATCTGTCTTG

141 CAGCATTGTCTGCCTAGGGCGGAGCGATCCTAGTTCCGTCATGTATAACCTACATGTATAACCATACATG

211 CTGCAGCGTTGTCTGCCTACGGTGGAGCGTTCCTAGTTCTACCAATTCCTGGCTATACCTGGCACATGTA

281 CTCAGTATGTGTGCCTACGTACAGTCGCGTTTCGGCAGGGTGCCTACCCGTCGTATGCTCTCGGTATGCT

351 CGCTTCCGTTGGTGGCCAGTCCATATTGGGGGTGACGGGATGTGCTGTGTCAGCATGGACAGTGTAGGCTT

421 AATGAGTGGGCTTGATGTGTGTGTTTCGAGCTACGGCTCACCACCGCCCTGATGTTGTGTTTCAAAC
-----F3----->-----F2----->

491 TGTTTTTACTGTGTTAAAGTGTCTCAGGCTGGCCTGGCTGGCTTGTGTTTCACTGCCCCGACATGCACCCGGT
<-----Flc----->-----B1c----->

561 GTTCTACACTGGACTGCATGTGCAGTCGCCCGGCGGTGCCTTATCCCGGGTACGACTGATAACGCCTCGG
-----LB----->-----B2----->-----B3--

631 TCATTGGGTTACCAATTGATCGAGATGAGTACAACCTCTGAGCGGTGGATCACTCGGCTCGTGTG
-----
--ITS1--><-----5.8S rRNA-----

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B

	Primers	Length	Sequence (5'-3')
	F3	18	TTCGAGCTACGGCTCACC
	B3	20	CCAAATGACCGAGGCGTTAT
LAMP	FIP (Flc+F2)	41	CAGGCCAGCCTGAGACACTTTACACCGCCCTGATGTTGTTG
(ITS1)	BIP (B1c+B2)	38	TTCCTGCCCCGACATGCACTCGTACCCGGGATAAGGC
	LB	20	CCGGTGTCTTACTGACTGGACT
PCR	RTFlukeFa	20	CTTGAACGCACATTGCGGCC
(ITS2)	RTFlukeRa	20	CACGTTTGAGCCGAGGTCAG

Fig. 1.

Opisthorchis viverrini ITS1 sequence and primer sequence (A) Name and location of each target sequence as a primer in the *O. viverrini* ITS1 gene. (B) Names and sequences of LAMP and PCR primers used in this study.

Fig. 2.

Real-time monitoring of LAMP reaction. The generated fluorescence intensity of DNA binding SYBR Green I was monitored at every 1 minute. Template DNAs were prepared from 10^1 to 10^{-5} ng/ μ L. Distilled water (D.W.) was used as a negative control.

Fig. 3. Specificity of LAMP and PCR-RFLP assays for *Opisthorchis viverrini* and minute intestinal flukes. Sample numbers are as follows: 1, negative control; 2, *O. viverrini* adults; 3, *O. viverrini* metacercariae; 4, *O. viverrini* cercariae; 5, *C. sinensis* adults; 6, *C. caninus* metacercariae; 7, *H. taichui* adults. (A) Visual examination of LAMP products by HNB. +, positive reaction; -, negative reaction. (B) PCR-RFLP assay. +AcuI, digestion with AcuI endonuclease; -AcuI, without digestion.

Table 1

Diagnostic abilities of PCR and LAMP to detect *Opisthorchis viverrini* copro-DNA from stool samples.

		Microscopic stool examination ^a		
		Egg-positive samples (N=37)	Egg-negative samples (N=13)	Total (N=50)
PCR	No. positive	9 (24.3) ^b	0 (0)	9 (18.0)
	No. negative	28 (75.7)	13 (100)	41 (82.0)
LAMP	No. positive	37 (100)	5 (38.5)	42 (84.0)
	No. negative	0 (0)	8 (61.5)	8 (16.0)

^a Stool samples confirmed by microscopic examination were used as standard to evaluate the diagnostic abilities of PCR and LAMP.

^b Percentages are shown in parentheses.

Table 2

Diagnosis of *Opisthorchis viverrini* infection by PCR and LAMP in different intensity groups based on microscopic analyses.

Intensity (egg/g)	No. of samples	PCR positive (%)	LAMP Positive (%)
0	13	0 (0)	5 (38)
1-49	12	0 (0)	12 (100)
50-99	8	2 (25)	8 (100)
100-499	14	5 (36)	14 (100)
>500	3	2 (67)	3 (100)