

# Development of Mitochondrial Loop-Mediated Isothermal Amplification for Detection of the Small Liver Fluke *Opisthorchis viverrini* (Opisthorchiidae; Trematoda; Platyhelminthes)

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Mitochondrial DNA sequences offer major advantages over the more usual nuclear targets for loop-mediated isothermal amplification approaches (mito-LAMP) because multiple copies occur in every cell. Four LAMP primers [F3, FIP (F1c+F2), BIP (B1c+B2), and B3] were designed based on the mitochondrial *nad1* sequence of *Opisthorchis viverrini* and used for a highly specific assay (mito-OvLAMP) to distinguish DNA of *O. viverrini* from that of another opisthorchiid (*Clonorchis sinensis*) and other trematodes (*Haplorchis pumilio*, *Haplorchis taichui*, *Fasciola hepatica*, and *Fasciola gigantica*). Conventional PCR was applied using F3/B3 primer pairs to verify the specificity of the primers for *O. viverrini* DNA templates. All LAMP-positive samples could be detected with the naked eye in sunlight, by gel electrophoresis (stained with ethidium bromide), and by addition of SYBR green I to the product in sunlight or under UV light. Only DNA from *O. viverrini* yielded amplification products by LAMP (and by PCR verification), and the LAMP limit of detection was as little as 100 fg ( $10^{-4}$  ng DNA), indicating that this assay is 10 to 100 times more sensitive than PCR. Field testing was done using representative egg and metacercarial samples collected from localities where the fluke is endemic. With the advantages of simplicity, rapidity, sensitivity, and cost effectiveness, mito-Ov-LAMP is a good tool for molecular detection and epidemiology studies in regions or countries where *O. viverrini* is endemic, which can lead to more effective control of opisthorchiasis and trematodiasis.

Opisthorchiasis caused by the fish-borne zoonotic liver fluke *Opisthorchis viverrini* Poirier, 1886 (Southeast Asian liver fluke), is a major disease in Thailand, Laos, Cambodia, and southern Vietnam (7, 13, 23, 24, 26, 27). This species can induce cholangitis and cholangiocarcinoma, the latter being the most severe consequence of chronic opisthorchiasis (22, 24, 26). The consumption of raw or improperly cooked freshwater fish containing infective metacercariae of *O. viverrini* remains a traditional habit in Vietnam, Thailand, and Laos, and it can lead to opisthorchiasis and its potentially fatal sequelae (5, 21, 24). Typically, infections by *O. viverrini* go unnoticed until severe symptoms and/or pathological lesions of the hepatic and biliary system are detected in patients admitted to medical stations and hospitals (3, 25, 26).

Recently, a simple, cost-effective, easily performed technique, known as loop-mediated isothermal amplification (LAMP) (15, 17), was developed that permits the rapid and highly specific amplification of a trace amount of DNA under isothermal conditions (normally at 63°C). The LAMP reaction is highly specific due to its use of four LAMP primers [namely, F3, FIP (F1c+F2), BIP (B1c+B2), and B3] binding to six independent regions in the target sequence (15, 19). Successful LAMP depends on the appropriate selection of the target gene, the optimal design of turn-back primers (10), and the generation of visible turbidity derived from the production of magnesium pyrophosphate (14). More importantly, the LAMP reaction is performed in inexpensive equipment such as a regular water bath or a heating block, and its products are easily observed with the naked eye or visualized by the addition of fluorescent dyes such as SYBR green I, with or without using a UV lamp (15). LAMP has been successfully developed for rapid detec-

tion of a range of zoonotic platyhelminths, such as *Fasciola hepatica* and *Fasciola gigantica* (1), *Clonorchis sinensis* (4), *Schistosoma japonicum* (29), *Taenia* spp. (16), *Paragonimus westermani* (6), and, recently, *O. viverrini* (2). For these LAMP reactions, nuclear genes were mainly chosen as the target regions.

Greater sensitivity will be possible if the target nucleotide sequence is present in high copy numbers and is highly specific and widely conserved within a particular pathogen species or group (10, 15, 19). For this reason, mitochondrial sequences have major advantages over the more usual nuclear targets. The animal mitochondrial genome is a double-stranded DNA circle (normally 13.5 to 20 kb) containing 36 or 37 genes (2 rRNA, 22 tRNA, and 12 or 13 protein-coding genes) (8, 11). Each cell contains many mitochondria, providing many copies of any mitochondrial DNA (mtDNA) target region. Furthermore, the circular mitochondrial DNA molecules are relatively resistant to degradation. Complete or near-complete mtDNA genomes for various zoonotic parasitic pathogens of public health concern are becoming available in the GOBASE database (<http://gobase.bcm.umontreal.ca/>) (18), including many helminths obtained by us and our collaborators (8, 11). Additionally, updated information is available from our own growing collecting of mitochondrial sequences from

Received 2 November 2011 Returned for modification 6 December 2011

Accepted 27 January 2012

Published ahead of print 8 February 2012

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doi:10.1128/JCM.06277-11

**TABLE 1** List of samples and organisms providing *nad1* sequences for designing primers and DNA template used in the assessment of the specificity and sensitivity of LAMP

Species	Sample	Accession no. <sup>a</sup>	Origin	Purpose	
				Primer design	Specificity/sensitivity
<i>O. viverrini</i>	OvBD1	EU443831	Binh Dinh, Vietnam	✓	✓
	OvBD	DQ882172	Binh Dinh, Vietnam	✓	✓
	OvDL3	DQ882174	Dac Lak, Vietnam	✓	✓
	OvPY3	EU443833	Phu Yen, Vietnam	✓	
	OvQN	EU443832	Quang Nam, Vietnam	✓	
	OvHU		Hue City, Vietnam	✓	
	OvL	DQ882175	Vientiane, Laos	✓	✓
	OvKK		Khon Kaen, Thailand	✓	✓
	OvKO		Khon Kaen, Thailand	✓	✓
	OvMH		Thailand	✓	
	OvVT1	GQ401025	Vientiane, Laos	✓	
	OvCP1	GQ401064	Champasak, Laos	✓	
	OvKM7	GQ401046	Khammouane, Laos	✓	
	OvSV12	GQ401060	Savannakhet, Laos	✓	
	OvKD1	GQ401082	Kandal, Cambodia	✓	
	OvSK1	GQ401096	Sakao, Thailand	✓	
	OvKS	EU022343	Kalasin, Thailand	✓	
	OvLP	EU022346	Lampang, Thailand	✓	
	OvSK	EU022348	Sakon Nakhon, Thailand	✓	
	OvMS	EU022350	Maharakham, Thailand	✓	
<i>C. sinensis</i>	CsAmur-RU	FJ381664	Amur River, Russia	✓	
<i>O. felinus</i>	OfUT-RU	EU921260	Novosibirsk, Russia	✓	
<i>F. gigantica</i>	FgIN-IN		Indonesia	✓	
	FgNB-VN	EU260072	Ninh Binh, Vietnam	✓	✓
<i>F. hepatica</i>	FhAU-AU	AF216697	Geelong, Australia	✓	✓
<i>C. sinensis</i>	CsND-VN		Nam Dinh, Vietnam	✓	✓
<i>F. buski</i>	FbN-VN		Nghe An, Vietnam	✓	✓
<i>H. pumilio</i>	HpuD2-VN		Nghe An, Vietnam		✓
<i>H. taichui</i>	HtaTL-TH		Bangkok, Thailand		✓

<sup>a</sup> Samples with no accession number were obtained in this study.

*O. viverrini* (T. H. Le et al., unpublished data). Using these resources, we have developed a range of mtDNA-based LAMP assays (i.e., mito-LAMP) for detection of common helminthic zoonoses of public medical and veterinary importance. The mito-LAMP strategy could also be developed for detection of agents of other neglected tropical diseases. Here, we chose a mitochondrial protein-coding gene, *nad1*, as a LAMP target for *O. viverrini*. This gene encodes one of the subunits of nicotinamide dehydrogenase. Many *nad1* sequences are available from *O. viverrini* strains of different geographic origins and from other species of trematode, including some closely related to *O. viverrini*. This makes it possible to design primers for *O. viverrini*-specific mito-LAMP (mito-OvLAMP).

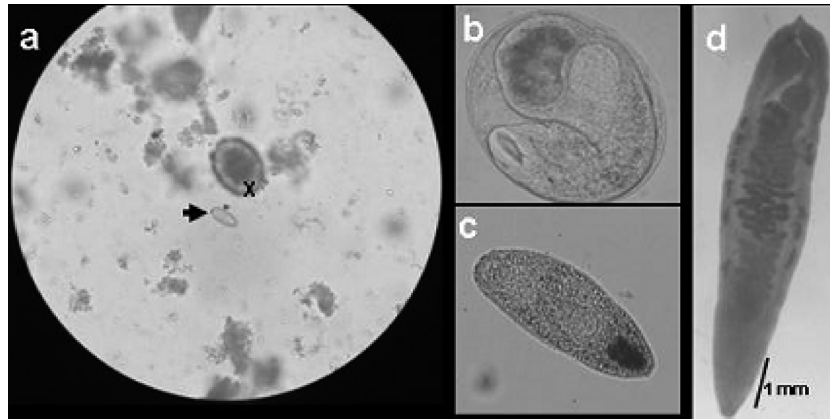
In this paper, we present the development of a mito-LAMP strategy for *O. viverrini* targeting *nad1* and of experimental assays for evaluation of sensitivity and specificity in order to provide a method for rapid detection of any life stage of this zoonotic trematode in any country where the organism is endemic.

## MATERIALS AND METHODS

**Parasite samples.** Samples of *O. viverrini* from Vietnam were collected from humans in the central part of the country (Binh Dinh, Phu Yen, Dac Lak, Quang Nam, and Thua Thien-Hue provinces) (7, 13), and adults and eggs of Thai origin (Khon Kaen province) were provided by Paiboon Sithithaworn of Khon Kaen University. Other samples used as controls for specificity were collected by us, including a related small liver fluke (*Clonorchis sinensis*), large liver flukes (*Fasciola hepatica* and *F. gigantica*), a giant intestinal fluke (*Fasciolopsis buski*), and minute intestinal flukes (*Haplorchis pumilio* and *H. taichui*) (Table 1).

Stool specimens were collected from men over 45 years old who lived in an area where *O. viverrini* is endemic (a village in Phu Yen province of central Vietnam). Stools from individual donors were screened for parasite eggs using the Kato-Katz method (9); the eggs were collected for microscopic examination and identification (Fig. 1), and stool samples were subjected to DNA extraction.

Metacercariae and/or metacercarial cysts of any trematode species present were pooled from muscle samples of freshwater fish (*Carassius auratus gibelio*), a silver carp weighing up to 30 or 40 g and commonly



**FIG 1** Photomicrographs of samples collected from fish and people living in the village where *O. viverrini* is endemic used in this study. (a) Egg of *O. viverrini* (arrow) and an egg of *Ascaris lumbricoides* (X) (magnification,  $\times 400$ ); (b and c) metacercarial cyst and a metacercaria from fish (magnification,  $\times 100$ ); (d) unstained adult *O. viverrini* worm from a patient (magnification,  $\times 40$ ).

eaten by habitual consumers of raw fish. These were sampled randomly from ponds. A single fish in this area commonly contains metacercariae of a number of trematode species. Entire fish or large portions of fish muscle were digested using 2% pepsin at pH 2. The digested material was passed through a 1-mm-mesh sieve and allowed to settle until the sediment was clearly visible. Several rounds of washing with physiological saline (i.e., 0.85% [wt/vol] NaCl), settling, and decanting the supernatant followed until metacercarial cysts could be seen in the sediment. All samples (adult worms, pooled trematode eggs from stools, and metacercariae and metacercarial cysts from fish) were stored in 70% ethanol and kept at  $-20^{\circ}\text{C}$  prior to DNA extraction.

**Genomic DNA preparation.** Genomic DNA was extracted from individual *O. viverrini* metacercariae and metacercarial cysts and from adult *O. viverrini* and worms of other species using a commercial QIAamp DNA extraction kit (Qiagen Inc.) as described previously (13). Additionally, for stools known to contain trematode eggs, an AccuPrep stool DNA extraction kit (Bioneer, Daejeon, South Korea) was used. Briefly, after the addition of 400  $\mu\text{l}$  SL buffer to 100 mg stool, the tube was vortexed for 30 s and then incubated at  $60^{\circ}\text{C}$  for 10 min. Following centrifugation at 12,000 rpm for 5 min, the supernatant was transferred to a new tube, and 400  $\mu\text{l}$  binding buffer added to this tube, which was incubated again at  $60^{\circ}\text{C}$  for 10 min. After the addition of 100  $\mu\text{l}$  isopropanol, the tube was vortexed for 5 s and briefly centrifuged. The supernatant liquid was transferred into the binding column fitted onto a collection tube, and the combination was then centrifuged at 8,000 rpm for 1 min. The column was fitted to a new tube, 500  $\mu\text{l}$  washing buffer 1 was added, and the tube was centrifuged again at 8,000 rpm for 1 min. This step was repeated using washing buffer 2, followed by centrifugation at 13,000 rpm for 1 min. Finally, the column was fitted onto a collection tube, 100  $\mu\text{l}$  elution buffer was added, and after standing at room temperature for 5 min, the combination was centrifuged at 8,000 rpm for 1 min. The eluted genomic DNA from stool samples was stored at  $-20^{\circ}\text{C}$  until use.

The identity of each trematode DNA sample was confirmed by species-specific PCR as previously described for *C. sinensis* (13), for heterophyids (28), and for *Fasciola* spp. (12). DNA concentration was estimated using a GBC UV/visible 911A spectrophotometer (GBC Scientific Equipment Pty. Ltd., Australia). Each sample was diluted to 100 ng/ $\mu\text{l}$ , and 1  $\mu\text{l}$  was used as the template in a normal LAMP or PCR in a 25- $\mu\text{l}$  volume.

**Design of LAMP primers for *O. viverrini*.** LAMP primers specific for *O. viverrini* were designed following inspection of the multiple alignment of 27 *nad1* sequences, including 20 of *O. viverrini* (Table 1). Four LAMP primers, F3, FIP(F1c+F2), BIP(B1c+B2), and B3 (Table 2), were designed based on regions conserved in *O. viverrini* but differing in other species of trematode (Fig. 2). PrimerExplorer V4 software (<http://primerexplorer.jp/elamp4.0.0/index.html>)

was used to determine the primer sequences. The F1c and F2 sequences of the forward inner primer (FIP) as well as the B1c and B2 sequences of the reverse inner primer (BIP) are joined by TTTT spacers.

**PCR assays using outer primers F3 and B3 to confirm specificity and sensitivity.** Conventional PCR using the external primers F3 and B3 was performed on each DNA template of *O. viverrini* and on a range of other trematodes (listed in Table 1), to test specificity of the primers. An amplicon of 216 bp was expected for *O. viverrini*. PCR was conducted in a 25- $\mu\text{l}$  reaction volume containing 12.5  $\mu\text{l}$  PCR master mix (Fermentas Inc.), 2  $\mu\text{l}$  (5 pmol/ $\mu\text{l}$ ) each primers F3 and B3, 1  $\mu\text{l}$  DNA template (100 ng/ $\mu\text{l}$ ), and 7.5  $\mu\text{l}$  water. A negative (no DNA) control was included. The reaction conditions were an initial step at  $94^{\circ}\text{C}$  for 3 min, then 30 cycles of  $94^{\circ}\text{C}$  for 30 s,  $50^{\circ}\text{C}$  for 30 s, and  $72^{\circ}\text{C}$  for 1 min, followed by a final extension at  $72^{\circ}\text{C}$  for 5 min.

The sensitivity of the PCR was also assayed to establish the detection limit of *O. viverrini* DNA. A serial dilution of genomic DNA of a Vietnamese sample (OvBD1) was used. The quantities of template used were 10, 1,  $10^{-1}$ ,  $10^{-2}$ ,  $10^{-3}$ ,  $10^{-4}$ , and  $10^{-5}$  ng DNA. The assay was performed with 1  $\mu\text{l}$  of the diluted template in each case. A negative (no DNA) control was included. The PCR products (10  $\mu\text{l}$  of each) were examined on a 1% agarose gel, stained with ethidium bromide, and visualized under UV light (Wealtec).

**LAMP performance and assays.** The LAMP reaction was performed in a total volume of 25  $\mu\text{l}$  containing 12.5  $\mu\text{l}$  of  $2\times$  LAMP reaction buffer [40 mM Tris-HCl (pH 8.8), 20 mM KCl, 16 mM  $\text{MgSO}_4$ , 20 mM  $(\text{NH}_4)_2\text{SO}_4$ , 0.2% Tween 20, 1.6 M betaine, 2.8 mM each deoxynucleo-

**TABLE 2** Sequence of primers for LAMP reaction

Primer <sup>a</sup>	Sequence (5'–3')	Length (bp)
F3	GTGTTATGTCGTGTGACTG	19
B3	CCAACCCAAATAACAACATCC	21
FIP(F1c+F2)	ACTAGCTCCCTCTCCGCTTC <i>TTTTT</i> ATGCTTTGTGAGTG TAATCGT	46
BIP(B1c+B2)	ACGTTCCATTTACTTGTCTT TTTGCTTTTGAACCTGAAC CTAACCAGGAA	49

<sup>a</sup> F3, forward outer primer; FIP, forward inner primer (comprising F1c and F2 sequences with a TTTT link [italics] added between them); BIP, reverse inner primer (comprising B1c and B2 sequences, with the TTTT link); and B3, reverse outer primer.

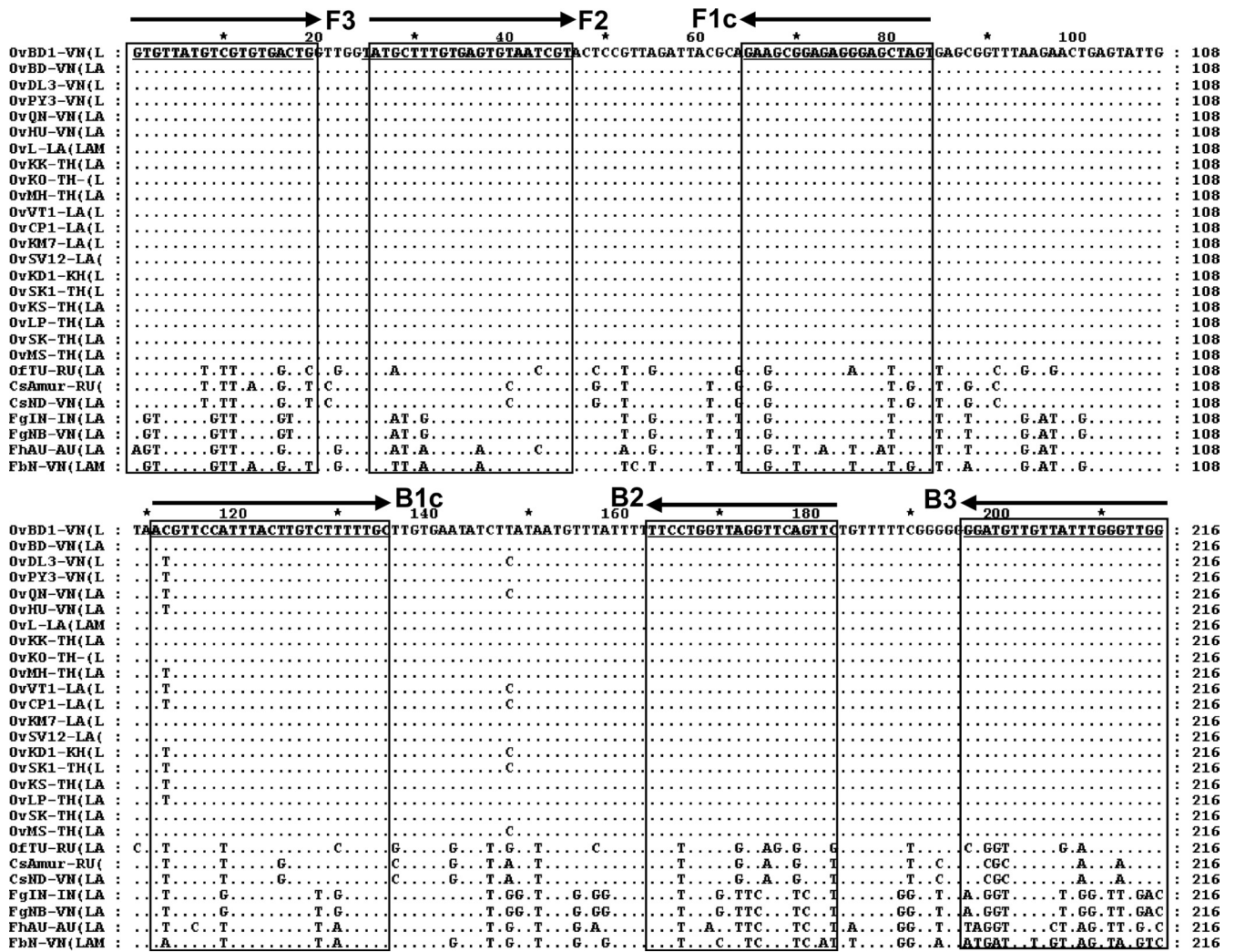


FIG 2 Comparative alignment of 27 *nad1* sequences from *O. viverrini* and related trematodes for demonstration of variability between species in regions chosen for the primers for mito-OvLAMP (see Table 1 for a list of samples and strains from which *nad1* sequences were obtained). The top sequence is the corresponding OvBD1 *nad1* sequence (Vietnam); dots in the sequences below indicate identity. The horizontal arrows indicate the directions (sense and antisense) of the LAMP primers (underlined), including F3 (forward outer primer), F2 and F1c (forward inner primers); B1c and B2 (reverse inner primers), and B3 (reverse outer primer). Regions used for OvLAMP primers are highly conserved among *O. viverrini* samples but differ in other trematodes.

side triphosphate], 2.5  $\mu$ l 10 $\times$  *Bsm* DNA polymerase buffer, 1  $\mu$ l of primer mix (20 pmol FIP, 20 pmol BIP, 2.5 pmol F3 primers, and 2.5 pmol B3 primers), 1  $\mu$ l of target genomic DNA, and 7  $\mu$ l of diethyl pyrocarbonate (DEPC)-treated water. First, the mixture was heated at 95 $^{\circ}$ C for 3 min and then chilled on ice. Next, 1  $\mu$ l (8 units) of the *Bsm* DNA polymerase large fragment (Fermentas Inc.) was added. The mixture was incubated at 63 $^{\circ}$ C for 70 min in a water bath and then heated to 80 $^{\circ}$ C for 5 min to terminate the reaction. The LAMP products were inspected to determine positivity by the naked eye (i) directly in sunlight, (ii) after the addition of SYBR green I (Invitrogen Inc.) in sunlight, (iii) under UV light after the addition of SYBR green I, and (iv) by electrophoresis in 1.5% agarose gel with ethidium bromide staining under UV light (Wealtec).

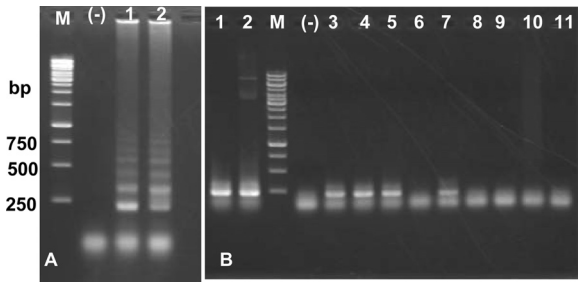
The specificity of LAMP was examined using 1  $\mu$ l of genomic DNA (100 ng/ $\mu$ l) in each case, from six samples of *O. viverrini* (Vietnam, Laos, Thailand origin); one sample of *C. sinensis* (Vietnam); three fasciolids, *F. hepatica* (Australia), *F. gigantica* (Vietnam), and *Fasciolopsis buski* (Vietnam); and two heterophyids, *H. pumilio* (Vietnam) and *H. taichui* (Thailand) (Table 1). A negative control (no DNA) was included.

The sensitivity of the mito-OvLAMP was assayed with the 10-fold

dilution series of DNA as described above (i.e., 10, 1, 10 $^{-1}$ , 10 $^{-2}$ , 10 $^{-3}$ , 10 $^{-4}$ , and 10 $^{-5}$  ng). The assay was performed with 1  $\mu$ l of template in each case. A negative control (no DNA) was included. The LAMP products were subjected to electrophoresis on a 1.5% agarose gel stained with ethidium bromide, followed by visualization after addition of SYBR green I by the naked eye in sunlight and under UV in a Dolphin apparatus (Wealtec).

## RESULTS

**LAMP and PCR performance for confirmation of the *O. viverrini* template.** To confirm the identity of DNA from *O. viverrini*, two DNA templates (OvBD1, from Binh Dinh, Vietnam, and OvKK, from Khon Kaen, Thailand) were used for a LAMP and a PCR experiment. The temperature for LAMP was set to 63 $^{\circ}$ C for 70 min (1 to 2 $^{\circ}$ C lower or higher than 63 $^{\circ}$ C was tested with less positive results [data not shown]). Each LAMP reaction yielded a typical ladder-like pattern on an agarose gel stained with ethidium bromide, while the negative control did not (Fig. 3A). When SYBR

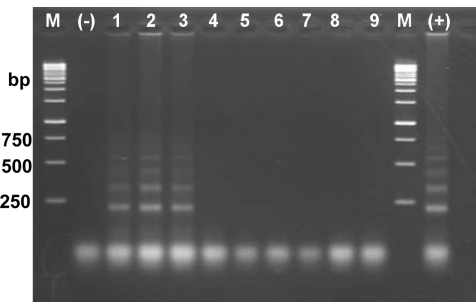


**FIG 3** Confirmation of the identity of *O. viverrini* genomic DNA (OvBD1, Vietnam; OvKK, Thailand) by loop-mediated isothermal amplification (LAMP) and PCR. A total of 100 ng of template was used in each case. (A) Products of mito-OvLAMP on 1.5% agarose stained with ethidium bromide. Lanes: M, 1-kb ladder marker (numbers on the left are sizes in base pairs); (-), negative control (no DNA); 1 and 2, LAMP products from templates of OvBD1 and OvKK, respectively. (B) Electrophoresis of PCR products of 216 bp (using primers F3 and B3) on 1% agarose. Lanes: M, 1-kb ladder marker; (-), negative control (no DNA) for templates, see Table 1); 1, OvBD1 (*O. viverrini*); 2, OvKK (*O. viverrini*); 3, OvDL3 (*O. viverrini*); 4, OvPY3 (*O. viverrini*); 5, OvQN (*O. viverrini*); 6, CsND (*Clonorchis sinensis*); 7, OvL (*O. viverrini*); 8, FhAU (*Fasciola hepatica*); 9, FgNB (*F. gigantica*); 10, HpuD2 (*Haplorchis pumilio*); 11, HtaTL (*H. taichui*).

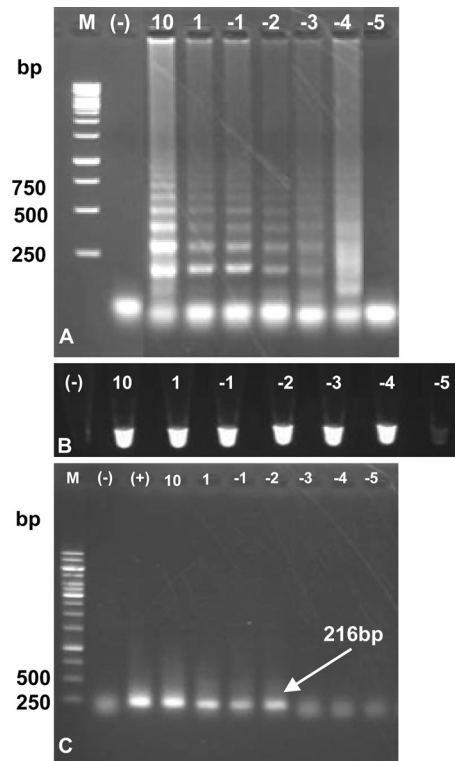
green I was added to the products, either under UV light or in sunlight, clearly positive results were seen with the naked eye. Both OvBD1 and OvKK and other *O. viverrini* templates generated the expected 216-bp PCR product, while samples from other trematodes yielded no amplicons (Fig. 3B).

**LAMP specificity for *O. viverrini*.** To determine the specificity of the mito-OvLAMP assay, genomic DNA samples from different life stages of *O. viverrini* and other trematodes, including *C. sinensis* (Vietnam isolate), *F. hepatica* (Australia), *F. gigantica* (Vietnam), *Fasciolopsis buski* (Vietnam), *H. pumilio* (Vietnam), and *H. taichui* (Thailand), were used. LAMP products were amplified only from DNA samples of *O. viverrini* eggs, adults, and metacercariae, and no amplification occurred with other trematode samples (Fig. 4).

**Analytical sensitivity of LAMP and PCR for *O. viverrini*.** Tenfold serial dilutions of *O. viverrini* genomic DNA of a Vietnamese isolate (OvBD1) were used to compare the sensitivity between the mito-OvLAMP and the PCR tests. Results indicated that



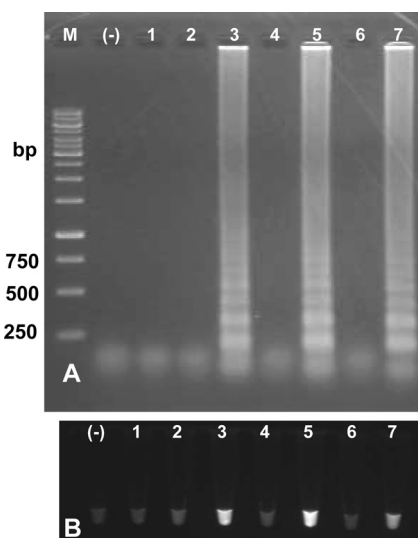
**FIG 4** Specificity assessment of the mito-OvLAMP assay by visualization on 1.5% agarose gel stained with ethidium bromide. Lanes: M, 1-kb ladder marker (numbers on the left are sizes in base pairs); (-), negative control (no DNA); (+), positive control (100 ng DNA template of *O. viverrini*); 1 through 9, LAMP reaction with DNA template (100 ng) from *O. viverrini* eggs, *O. viverrini* adult worm, *O. viverrini* single metacercaria, *C. sinensis* (CsND), *Fasciolopsis buski* (FbN), *F. hepatica* (FhAU), *F. gigantica* (FgNB), *H. pumilio* (HpuD2), and *H. taichui* (HtaTL).



**FIG 5** Sensitivity assessment of the LAMP assay for *O. viverrini* using serial dilutions of genomic DNA template and comparison with PCR (performed using outer primers F3 and B3). (A and B) Sensitivity analysis of the LAMP assay by visualization on 1.5% agarose stained with ethidium bromide (A) or under UV light after the addition of SYBR green I (B). (C) Sensitivity analysis of the conventional PCR revealed on 1% agarose (arrow indicates a 216-bp DNA fragment at the highest template dilution for PCR performance). Lanes: M, 1-kb ladder marker (numbers on the left are sizes in base pairs); (-), no-DNA template (water added) as a negative control; (+), 100 ng template (positive control), 10, 10 ng; 1, 1 ng; -1, 100 pg; -2, 10 pg; -3, 1 pg; -4, 100 fg; -5, 10 fg.

the detection limit for the LAMP reaction was between  $10^{-3}$  ng (1 pg) and  $10^{-4}$  ng (100 fg) of template (Fig. 5A and B), while it was  $10^{-2}$  ng (10 pg) for the conventional PCR assay (Fig. 5C). This suggested that the LAMP is 100 times more sensitive than the PCR assay.

**LAMP test with field samples collected from fish and humans.** The specific LAMP assay was used in attempts to detect *O. viverrini* metacercariae and cysts from silver carp and to detect eggs from feces of humans in an area where *O. viverrini* is endemic. Most of the inhabitants (mainly males) frequently eat raw fish in the traditional style (7, 13). Three pools of mixed metacercariae from fish muscle samples and four samples of mixed eggs collected from human feces (Fig. 1) were used for evaluation of mito-OvLAMP. One of three metacercarial samples and two of four fecal egg samples produced a positive LAMP reaction; these results were verified by visualization after addition of SYBR green I under UV light (Fig. 6). The positive result in the mito-OvLAMP assay was in agreement with the microscopic examinations of the *O. viverrini* eggs and metacercariae (Fig. 1) and with PCR using specific F3-B3 primer pairs (data not shown). Neither the number of metacercariae per fish nor egg counts (eggs per gram of feces) were calculated in this study.



**FIG 6** Results of specific LAMP assays for detection of *O. viverrini* using DNA material from mixed metacercariae extracted from freshwater fish and eggs from feces of humans. (A) Visualization on 1.5% agarose stained with ethidium bromide; (B) with SYBR green I under UV light. Lanes: M, 1-kb ladder marker (numbers on the left are sizes in base pairs); (-), LAMP negative control (no DNA template); 1 through 3, DNA of possibly mixed metacercariae from muscles of fish caught in ponds where *O. viverrini* is endemic; 4 through 7, LAMP products from DNA of eggs, possibly of more than one species, extracted from individual stool samples from humans (after raw fish consumption) in a village where *O. viverrini* is endemic.

## DISCUSSION

The problem of *O. viverrini*-contaminated fish and the increasing rate of cholangiocarcinoma in the community is a great concern. More effective methods to detect every life stage of this dangerous worm are required. Until now, a variety of microscopic, immunological, and PCR (and real-time PCR) approaches have been used to detect metacercariae in fish and to detect eggs and adult worms as well as antibodies in late-stage opisthorchiasis patients (24), including a LAMP assay using the internal transcribed spacer 1 (ITS-1) in the nuclear ribosomal gene cluster as a target (2). However, it would be more reasonable to use a mitochondrial target for a LAMP assay, due to its stability and the likely higher copy number for provision of DNA template. A typical animal cell is likely to contain hundreds of copies of the mitochondrial genome (20).

In this study, a mito-OvLAMP method targeting the *nad1* gene of *O. viverrini* was successfully developed. The selection of the mitochondrial *nad1* gene was appropriate because it contained highly conserved regions for all geographical strains of this species, forming an ideal target for the design of LAMP primers. This was specifically tested with a range of *O. viverrini* life stages, including eggs, metacercariae, and adults of different geographical origins. The mito-OvLAMP assay in this study is more sensitive than the conventional PCR, capable of producing LAMP ladder-like pattern products from as little as 100 fg of DNA template. The *Bsm* DNA polymerase large fragment worked well, generating a large amount of DNA products which could be visualized by adding fluorescent SYBR green I for inspection under UV light or with the naked eye. In this study, we have used four methods of determining positive reactions by visual inspection of the LAMP tubes: (i) with the naked eye in sunlight without any additions (turbidity

determined from the production of magnesium pyrophosphate); (ii) by gel electrophoresis (with ethidium bromide staining) to inspect the DNA generated; (iii) with the naked eye in sunlight after the addition of SYBR green I to the product; (iv) under UV light after the addition of SYBR green I to the product. The last three of these methods employ a sensitive fluorescent dye for detecting double-stranded DNA.

In fact, in our study, the first method (magnesium pyrophosphate turbidity) is not emphasized, since in sunlight it was difficult to determine sensitivity and variability between the tubes. Interestingly, all LAMP-positive reaction tubes examined under UV light after the addition of SYBR green I appear similar in intensity, although the amounts of product evident via LAMP on the agarose gel stained with ethidium bromide are clearly different. However, the distinction between the positive result at the lowest detection level and the negative at the next dilution is very sharp in sunlight or under UV light. We suggest that visualization using SYBR green I is most practical due to its ease of application in a basic laboratory.

In conclusion, the mito-LAMP method targeting mitochondrial *nad1*, developed in this study, demonstrated successful identification of any life stage of *O. viverrini* in diverse hosts in several areas where opisthorchiasis is endemic.

## ACKNOWLEDGMENTS

This work received financial support from the National Foundation for Science and Technology Development (NAFOSTED) in Vietnam (grant no. 106.16-2010.60) to Thanh Hoa Le. We express thanks to colleagues for their kind provision of materials used in this study and to international and national collaborators (provincial stations) for their assistance in the field and laboratory work. We extend our thanks to David Blair of School of Marine and Tropical Biology, James Cook University (Townsville, Australia), for the invaluable review of the manuscript.

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