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Development of a PCR Assay for Diagnosing Trematode (*Opisthorchis* and *Haplorchis*) Infections in Human Stools

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Abstract. We developed a combined conventional polymerase chain reaction (PCR) and real-time PCR (qPCR)-based assay for detecting and discriminating between *Opisthorchis viverrini* and *Haplorchis taichui* parasite infections. The first PCR amplifies the mitochondrial cytochrome c oxidase subunit I (*COI*) genes of parasites, and differential diagnosis is achieved by performing qPCR with specific primers and SYBR Green I. The detection limit of the assay was found to be 2.0×10^2 plasmid copies in a test in which a stool sample was spiked with a single egg, which is equivalent to 5 eggs per gram (EPG). The testing of 34 clinical stool samples that had been demonstrated to contain "*Opisthorchis*-like" eggs by microscopy showed that the novel assay exhibited a sensitivity of 100% for "*Opisthorchis*-like" parasitic infections, and 71% and 91% of these samples were found to be infected with *O. viverrini* and *H. taichui*, respectively. A further four parasitic infections were diagnosed in the 16 negative samples, and the microscopic findings of these samples were confirmed to be false negatives by sequencing analysis. The assay also displayed high specificity during the testing of 10 samples containing other common parasites. The fact that our qPCR SYBR Green I–based assay detected submicroscopic traces of parasitic DNA and was able to differentiate between parasites that produce eggs with similar morphologies indicates that it has a good potential for development of diagnostic application to use in areas where multiple parasites coexist.

INTRODUCTION

Two parasites *Opisthorchis viverrini* (Opisthorchiidae family) and *Haplorchis taichui* (Heterophyidae family) are known to cause food-borne trematode diseases. The life cycles of both parasites involve the intermediate hosts, snails and fish, with humans being the definitive host. They cause infections in human through eating raw fresh water fish. These parasite infections have a major public health issue in the Lao People's Democratic Republic (Lao PDR) and Thailand. *Opisthorchis viverrini* infection is known to associate with cholangiocarcinoma (CCA),[1] whereas *H. taichui* infection is considered to be relatively innocuous, it remains a concern because it caused severe pathological changes in tissue.[2]

The identification of two trematodes in Opisthorchiidae and Heterophyidae families, as well as in Lecithodendriidae and Echinostomatidae families is still based on microscopic observation of their egg from fecal samples. This can be a challenge as they have similar egg morphology and requires more experienced microscopist to make any distinction. Therefore, there was a coexisting of two parasites with the eggs of similar morphology, they often described as "Opisthorchis-like" eggs in the clinical laboratory of Lao.[3,4] The adult worms recovered from purged stools are still required for making definitive species identification.[5–7] A novel method to detect and discriminate between a carcinogenic fluke and non-carcinogenic fluke may be useful to provide accurate information of the disease and help put in place public health interventions that will prevent CCA in the region.

Several different PCR-based techniques for discriminating between *O. viverrini* and *H. taichui* have been developed. These tests used DNA from adult worms, metacercariae, or human stool specimens and exhibited varying degrees of success. Some critical issues that affect such assays are low DNA concentrations, PCR inhibitors, and a lack of specificity during the initial egg identification. Previous comparative studies have detected discrepancies between the *O. viverrini* egg detection results obtained by microscopy and PCR.[8] The latter study suggested that PCR is not able to detect *O. viverrini*, probably due to incorrect identification during the microscopic examinations of fecal eggs. The presence of DNA polymerase inhibitors in stool extracts has also been reported to be a problem, and some studies have tried to improve PCR outcomes by cleaning up stool specimens during the DNA extraction process to remove inhibitors.[9–12]

Other PCR-based techniques involving the purification of the PCR template, sequencing analysis, temperature optimization, or the use of restriction enzymes to cut the PCR products post-amplification have also been tested.[13–15] Recently, multiplex PCR, loop-mediated isothermal amplification, qPCR-based fluorescence resonance energy transfer, and high-resolution melting analysis have been successfully used to detect *O. viverrini* and discriminate it from *Clonorchis sinensis*.[16–19] However, there have not been any reports about PCR assays for discriminating between *O. viverrini* and *H. taichui*.

SYBR Green I, a dye that is used in qPCR, is a widely accepted and cheap molecular detection tool.[20] T_m peak values, which are dependent on the GC/AT ratios and lengths of the nucleotide sequences of amplified PCR products, are used to identify specific DNA molecules during fluorescence melting curve analysis.[21] Some researchers have reported that T_m peak values can be used to distinguish between different parasite[22] and bacterial[23] species. This technique is particularly useful in instances where the most widely used qPCR probes are not able to amplify mutated DNA.[24]

In this study, we developed a protocol involving a combination of conventional PCR and qPCR SYBR Green I–based

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melting curve analysis for discriminating between *O. viverrini* and *H. taichui* parasite infections in human stool specimens, which were examined and confirmed for the presence and absence of "*Opisthorchis*-like" eggs by routine microscopic technique.

MATERIALS AND METHODS

Collection and preparation of stool samples. The Center of Malariology, Parasitology, and Entomology (CMPE), Vientiane City, Lao PDR, provided a pooled stool sample in April 2012. The pooled sample contained a mixture of purged stool specimens that had been found to contain "*Opisthorchis*-like" eggs. All of the samples were collected from individuals from central Khammouane Province, which is a region known for its relatively high incidence of parasite infections.[25]

A further 50 clinical stool specimens were obtained from patients at Mahosot Hospital, Vientiane, Lao PDR. Patients that were suspected of having parasitic infections at the outpatient department were asked to provide a stool sample of approximately 2 g in a wide-mouthed container, which was then subjected to a routine parasitic microscopic laboratory examination using direct fecal smear method. A maximum of three direct fecal smear samples were produced from each sample before a diagnostic result was obtained. The abovementioned clinical samples included samples that were collected from 34 patients that had been diagnosed with *O. viverrini* infections (*O. viverrini* is a term that used in local hospitals; however, in this study we will refer to these samples as containing "*Opisthorchis*-like" eggs). The remaining 16 fecal specimens were found to be "*Opisthorchis*-like" eggs parasite-negative during microscopic examinations. Although other coexisting parasitic eggs were found in some of these specimens such as *Taenia* spp., *Ascaris lumbricoides*, *Trichuris trichiura*, hookworms, *Enterobius vermicularis*, and *Strongyloides stercoralis* (Table 3).

All samples were first processed by making a 50 mL suspension containing approximately 2 g stool material in 0.85% saline solution. The suspension was strained through a mesh of 1 × 1 mm to remove any debris and then strained again with a mesh measuring 70 × 70 μm. Next, the strained filtrate was washed with 50 mL 0.85% saline solution and centrifuged for 5 minutes at 2,000 rpm before the supernatant was discarded. This washing process was performed twice before the sediment containing the parasitic eggs was stored at −30°C for further analysis.

Genomic DNA (gDNA) extraction. The gDNA of the parasitic eggs was extracted from the fecal pellets using a stool extraction kit (QIAGEN, Hilden, Germany) for DNA purification. The spin column silica membrane–based nucleic acid purification system together with the InhibitEX matrix tablet included in the kit for reducing stool inhibition were used according to the manufacturer's instructions. The DNA concentration was quantified by spectrophotometry.

Amplification of the target *COI* gene and PCR-inhibited test. Partial fragments of the target mitochondrial cytochrome c oxidase subunit I (*COI*) gene were amplified using a standard 50 μL PCR reaction mix containing Emerald Amp MAX PCR master mix (Takara Bio, Shiga, Japan) as a source of Taq DNA polymerase. The concentration of the gDNA was adjusted to 10 ng/μL with distilled water, and 10 μL of the mixture was used as a DNA template together with previously described primers (MCOI-A as a forward primer and MCOI-B as a reverse primer) (Table 1). These primers were used to amplify trematode parasite DNA in a phylogenetic study of food-borne trematodes and an intraspecies investigation of *O. viverrini* in Lao PDR and Thailand.[26,27] The PCR amplification conditions involved 40 cycles of 94°C for 10 seconds, 40°C for 30 seconds, and 72°C for 30 seconds with a final extension step of 15 minutes at 72°C.

All extracted gDNA samples (50) that were infected and negative of "*Opisthorchis*-like" eggs were subjected for PCR inhibitor test. The specific primer was used for screening human beta actin DNA detection.

Cloning and DNA sequencing of the *COI* genes from the pooled stool samples. The partial *COI* gene PCR products of the pooled sample were cloned with the TA PCR cloning kit using the pTAC-2 vector (size: 2,786 bp; BioDynamics Laboratory, Tokyo, Japan) to identify the different parasites in the pooled sample. After performing PCR and confirming the size of the target amplicon (444 bp) during electrophoresis on 1.5% agarose gel, the PCR products were ligated into the pTAC-2 vector as per the manufacturer's instructions. The vector was then transformed into *Escherichia coli* DH5α cells using the heat shock method, before the cells were spread onto a media plate containing selective antibiotics and X-Gal (Wako Pure Chemical Industries, Osaka, Japan). Colonies were selected based on the size of the target fragment during sequencing analysis using the BigDye® Terminator v3.1 cycle sequencing kit (Becton Dickinson Biosciences, Bedford, MA). The plasmid sequences were then compared with the sequences in GenBank database (National Institute for Biotechnology Information, Bethesda, MD) to determine the identity of the parasites in the pooled sample.

Primer design and qPCR. The plasmid sequences of the partial *COI* gene PCR products of *O. viverrini* and *H. taichui* were used to design the primers for use in the qPCR. The sequences were intraspecies aligned with sequences from the GenBank database and then interspecies aligned with

TABLE 1
Primers for PCR and real-time PCR SYBR Green I–based assays

No.	Primer name	Direction	Nucleotide sequence (5′–3′)	Length (bp)	Product size (bp)
1	MCOI-A	F	TTT TTT GGG CAT CCT GAG GTT TA	23	444
2	MCOI-B	R	TAA AGA AAG AAC ATA ATG AAA ATG AGC	27	
3	PKZ1613F	F	CTT CTT TGG TTA TGC GGG TTT AGT TC	26	218
4	PKZ1613R	R	TTA TCA GAT CCC AAA ACA GGC CC	23	
5	QV301F	F	GTC ATA TCT GTA CGA CGT TCA CTG GT	26	155
6	QV301R	R	GCT AAA AAA CAC AGC GGT GCC	21	

F = forward; R = reverse; PCR = polymerase chain reaction.

the sequences of the clones in the pooled sample. Primers were then designed based on the conserved regions for each species, as shown in Table 1.

The qPCR was performed using a reaction mixture containing 10 µL of 0.2 µM each forward and reverse primer, 5 µL of 2× SsoAdvanced universal SYBR Green supermix Taq (as a source of Taq DNA polymerase; Bio-Rad Laboratories, Hercules, CA), and 1 µL of the prepared plasmid DNA or a 100 times diluted solution of the *COI* gene PCR product of one of the clinical stool samples as a DNA template. The qPCR was carried out using the Rotor-Gene Q rPCR system (QIAGEN) at an initial temperature of 95°C for 30 seconds, followed by 40 cycles of 15 seconds at 95°C and 30 seconds at 60°C.

Sensitivity and specificity of the primers. To determine the sensitivity and the limit of detection, 1.6×10^7 copies of the *COI* gene plasmids of the two parasites were subjected to 5-fold serial dilution, and then the diluted samples were subjected to qPCR using the conditions that were described. Amplification curve and T_m peak value analyses were used to calculate the correlation coefficient and consensus peak values of the *O. viverrini* and *H. taichui* primers. Moreover, *O. viverrini* and *H. tachui* gDNA from egg was used to evaluate the detection limit of the assay. The egg gDNA was extracted under two different conditions, i.e., it was 1) directly extracted from 1, 2, or 4 egg(s) and 2) extracted from 200-mg stool specimens (which had been demonstrated to be parasite-negative by microscopic methods and our present protocol) that had been spiked with 1, 2, or 4 egg(s). Ten nanogram of the gDNA that was directly extracted from the eggs was subjected to 10-fold serial dilution. The DNA concentration of the resultant solution was quantified by spectrophotometry, and 1 µL was used as a DNA template. As for the gDNA extracted from the egg-spiked stool specimens, it was subjected to the same process as the directly extracted gDNA and was then used in the combined PCR assay to determine the detection limit of the assay.

The analytical specificity of each primer was evaluated by using gDNA between two species *O. viverrini* and *H. taichui*, as well as of other parasites such as *Haplorchis pumilio*, *Echinostoma malayanum*, *S. stercoralis*, *A. lumbricoides*, *Ancylostoma duodenale*, *Trichuris trichura*, *Taenia saginata*, and *Schistosoma mansoni*. The reaction was run using the individual templates for each parasite or a mixture of the templates for all parasites under the combined PCR conditions described above.

The clinical stool specimens (50), which were infected and negative for "*Opisthorchis*-like" eggs, were also tested using the specific primers for *O. viverrini* and *H. taichui* under the combined PCR conditions mentioned above. The positive *O. viverrini* and/or *H. taichui* sample qPCR products were sequenced to confirm specific primer of amplification.

Ethics statement. This study was approved by the National Ethics Committee for Health Research, Ministry of Health, Lao PDR (Reference No. 276/NECHR), and also by the institutional review board of Kansai Medical University (Reference No. 0758). Written consent was obtained from each participant.

RESULTS

Cloning and sequencing analysis of the *COI* genes in the pooled sample. We cloned the products detected during the PCR analysis of the pooled sample and obtained 21 *COI* gene sequences. When the sequences were compared with the sequences in GenBank database, it was found that five sequences matched the *O. viverrini* sequence (JF739555), eight matched the *H. taichui* sequence (KF214770), and three matched the *T. saginata* sequence (AB821273). Five other clones did not match any known sequence in the GenBank database. The confirmed cloned nucleic acid sequences of *O. viverrini* and *H. taichui* were subjected to multiple sequence alignment.

Furthermore, comparisons were performed among the sequences from the pooled stool samples, the clones of *O. viverrini* and *H. taichui COI* gene sequences, and the sequences in GenBank (JF739555 and KF214770), and cross-species identity values were calculated. The *O. viverrini COI* gene clone sequences were highly homologous with the *O. viverrini* GenBank sequence (JF739555), and The *H. taichui COI* gene clone sequences were highly homologous with the *H. taichui* sequence in GenBank database (KF214770) (both cross-species homology values were 99.50%). These findings suggested that the pooled sample contained a mixture of eggs from *O. viverrini* and *H. taichui*, and that the cloned sequences could be used to design specific primers that would not cross-react with each other. In addition, plasmids containing these sequences could be used as positive or negative controls for each parasite.

Primers and qPCR SYBR Green I–based assay. The primers used to amplify the *O. viverrini COI* gene were as follows: forward, QV301F and reverse, QV301R. Similarly, the primers used to amplify the *H. taichui COI* gene were as follows: forward, PKZ1613F and reverse, PKZ1613R (Table 1).

The sensitivity analysis of 5-fold serial dilutions of the two parasites' *COI* gene products produced parallel amplification curves for *O. viverrini* and *H. taichui*, as shown in Figure 1A and C, respectively. The detection limit of the assay was determined after 30 cycles and was calculated to be 2.0×10^2 copies for both parasites using standard curves that exhibited strong linear relationships. The correlation coefficients for *O. viverrini* ($R^2 \geq 0.9984$; Figure 1B) and *H. taichui* ($R^2 \geq 0.9958$; Figure 1D) indicated that the fluorescence levels observed during the PCR were a good indicator of the relative concentrations of the parasites' *COI* gene plasmids. During the melting curve analysis, consensus peaks for each dilution were observed (Figure 1E), and the mean T_m peak values (±SD) for *O. viverrini* and *H. taichui* were 83.21 (±0.05) and 82.14 (±0.08), respectively (Table 2). Regarding the sensitivity tests performed using 10-fold serial dilutions of 10 ng pure *O. viverrini* and *H. tachui* gDNA (from 1, 2, or 4 egg[s]), the qPCR assay exhibited a detection limit of 1×10^4, which was equivalent to 1 pg gDNA. As for the results obtained using the egg-spiked (1, 2, or 4 egg[s]) samples (200 mg) of uninfected human feces, the combined PCR assay was able to detect the presence of a single egg, which was equivalent to a detection limit of 5 eggs per gram (EPG).

The specificity of the primers for *O. viverrini* and *H. taichui* was assessed using the amplification curves and T_m peak values for the *O. viverrini*, *H. taichui* template DNA or an artificial mixture for templates. The findings suggested that there was no cross-amplification because the specific primers for *O. viverrini* did not amplify the *H. taichui COI* gene and vice versa. In addition, neither the *O. viverrini*-specific nor

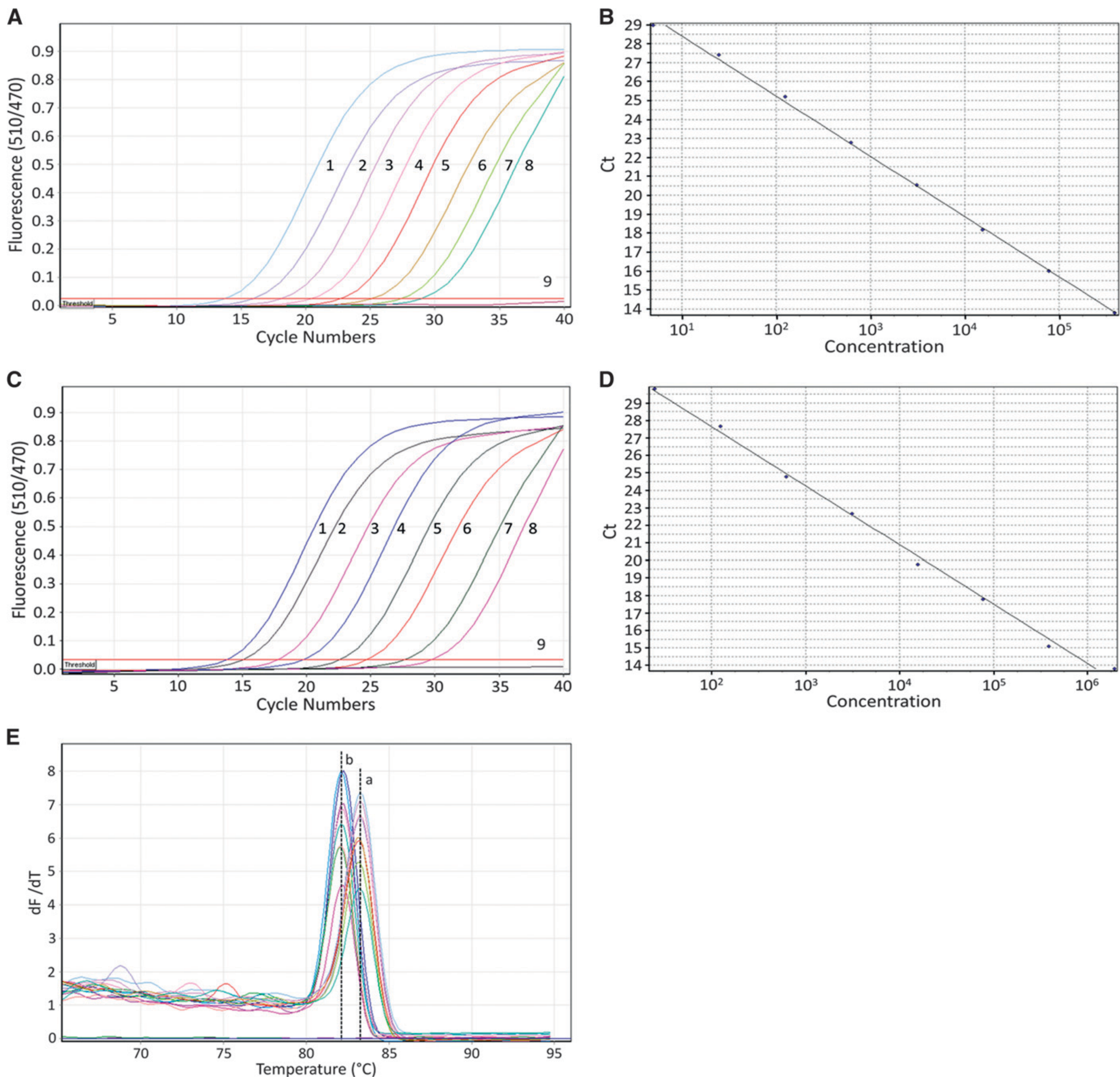

FIGURE 1. Analytical sensitivity of the specific primers for *Opisthorchis viverrini* and *Haplorchis taichui* during the real-time polymerase chain reaction SYBR Green I–based assay. (**A**) and (**C**) show the amplification curves obtained using 5-fold serial dilutions of the COI plasmids for *O. viverrini* and *H. taichui*, respectively, whereas (**B**) and (**D**) show the corresponding correlation coefficients for the relationship between the threshold cycle and the relative concentrations of the plasmids. The templates (1–8) were diluted from 1.6×10^7 copies, and the minimum detection limit was found to be 2.0×10^2 copies. The negative control (9) (no DNA template) was not amplified. (**E**) Melting curve analysis showing consensus T_m peak values of 83.21 (±0.05) for *O. viverrini* and 82.14 (±0.08) for *H. Taichui* (see the vertical [a] and [b] dashed lines, respectively).

the *H. taichui*-specific primers amplified *COI* gene DNA of *H. pumilio*, *E. malayanum*, *S. stercoralis*, *A. lumbricoides*, *A. duodenale*, *T. trichura*, *T. saginata*, and *S. mansoni* or a mixture of the templates and the negative control (no DNA template). These results indicate that the primers could be used to validate the detection of *O. viverrini* and *H. taichui* in clinical stool samples.

Evaluation of the clinical stool samples. qPCR was used to evaluate the partial parasitic *COI* gene PCR products obtained from human fecal specimens. The results for the 50 clinical stool samples that were examined with the newly designed primers for each species are shown in Table 3. The T_m peak values of the *O. viverrini* and *H. taichui* amplification curves were similar to those of the known *COI* gene plasmids, as shown in Table 2. Of the 34 stool specimens that were shown to contain "*Opisthorchis*-like" eggs by routine microscopy, *O. viverrini* was detected in 24 specimens (71%), and Figure 2A shows a representative amplification curve and the corresponding T_m peak value for these specimens. On the other hand, *H. taichui*

TABLE 2
The mean T_m peak value (±SD) from the melting curve analysis of *Opisthorchis viverrini* and *Haplorchis taichui COI* gene plasmid and fecal *COI* gene DNA

Primer	*COI* gene plasmid	Fecal *COI* gene DNA
QV301F-R (*O. viverrini*)	83.21 (±0.05)	83.11 (±0.12)
PKZ1613F-R (*H. taichui*)	82.14 (±0.08)	82.09 (±0.06)

COI = cytochrome c oxidase subunit I

was detected in 31 specimens (91%) (Figure 2B), and 21 (62%) specimens were found to have been coinfected with both parasites (Figure 2C). In the other 16 specimens that were found to be negative for "*Opisthorchis*-like" eggs during the microscopic examinations, *O. viverrini* was detected in one sample, and *H. taichui* was detected in four samples. The clear amplification curves and corresponding T_m peak values obtained for these samples are shown in Figure 2D. These results clearly demonstrated the capability of our combined PCR assay to detect and differentiate between *O. viverrini* and *H. taichui* at the submicroscopic level.

DISCUSSION

One of the main problems with PCR-based assays for examining stool specimens is that it is difficult to detect specific DNA molecules using such methods because the inhibitors present in mixtures of stool samples can interfere with the PCR reaction.[28,29] Such inhibitors can inhibit any of the steps from DNA extraction to DNA extension during the PCR. These inhibitory effects might affect the DNA amplification efficiency of PCR; i.e., reduce the amount of the PCR product obtained, and hence, make the PCR products difficult to visualize during agarose gel electrophoresis. Lovis and others tried to develop a PCR-based assay involving nested PCR, two newly designed primer sets (outer and inner primers) for detecting the mitochondrial DNA of *O. viverrini*, and another two primer sets for detecting the mitochondrial DNA of *H. taichui*. They found that they were able to increase the sensitivity and the efficiency of the PCR assay from 81.3% to 93.7% by cleaning up and increasing the copy number of the templates. In addition, these methods performed at least as well and sometimes better than microscopic methods, such

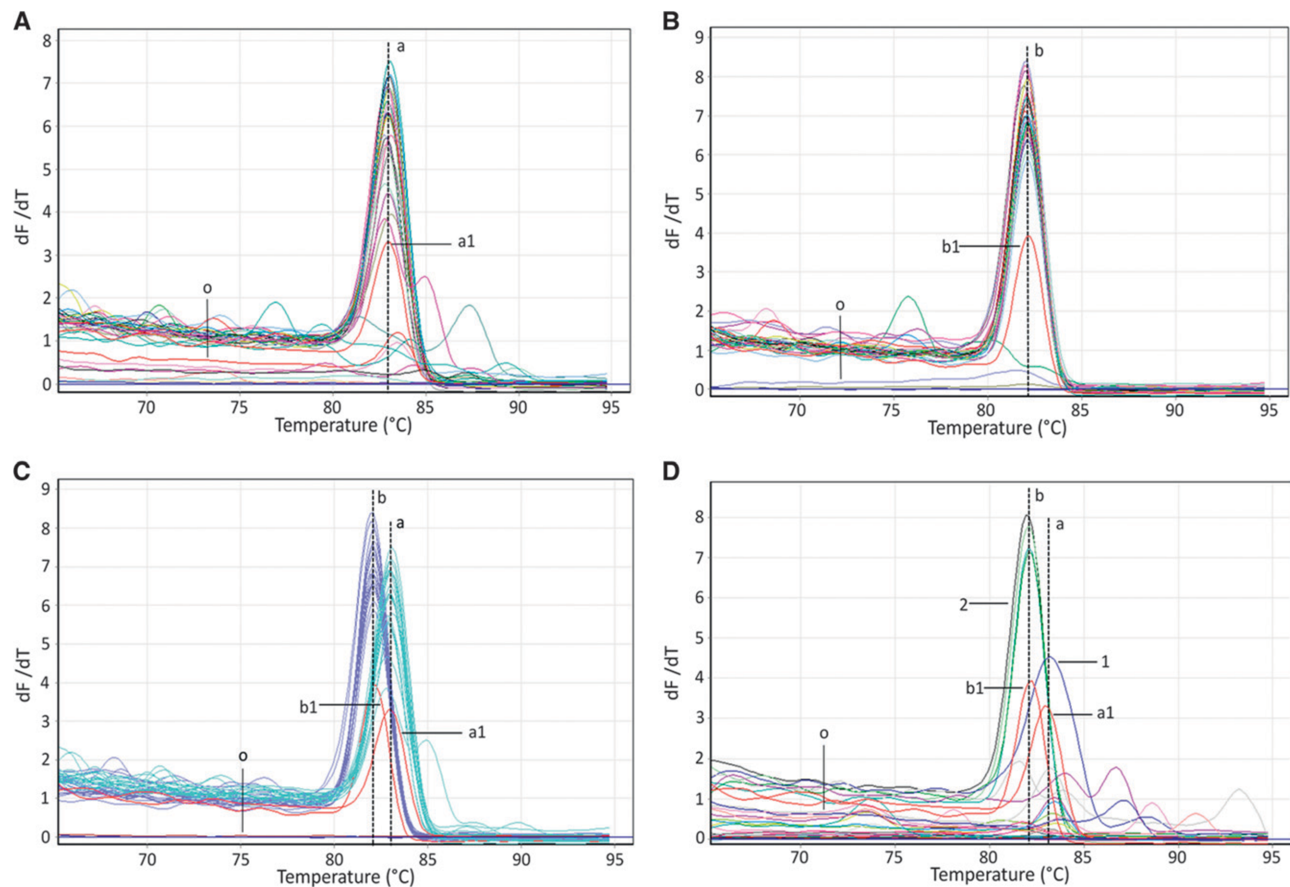

FIGURE 2. Testing of 50 clinical stool specimens using the combined polymerase chain reaction assay. The vertical (a) and (b) dashed lines show the consensus T_m peak values used to identify *Opisthorchis viverrini* (83.11 [±0.12]) and *Haplorchis taichui* (82.09 [±0.06]). (a1) and (b1) represent the detection limits of the *O. viverrini* and *H. taichui COI* gene plasmids, respectively. The letter O indicates the other templates that were not amplified by the two specific primers, including the negative control (no DNA template). In total, (**A**) 24 consensus T_m peaks were identified as *O. viverrini*, (**B**) 31 consensus T_m peaks were identified as *H. taichui*, and (**C**) 21 consensus *O. viverrini* T_m peaks and 21 consensus *H. taichui* T_m peaks were detected in the coinfected specimens. Furthermore, (**D**) 16 consensus T_m peaks were amplified by the *O. viverrini*– or *H. taichui*–specific primers in samples that were found to be negative for "*Opisthorchis*-like" eggs during microscopic examinations. Figures 2D-1 and 2D-2 show the amplification curves and T_m peak values for the samples in which *O. viverrini* (sample no. 35 in Table 3) and *H. taichui* (sample nos. 35, 36, 37, and 40 in Table 3) were detected, respectively.

Table 3

Case data of samples showing presence and absence of "*Opisthorchis*-like" eggs by microscopy and *Opisthorchis viverrini* and *Haplorchis taichui* positive (+) and negative (–) samples by combined PCR

Sample no.	Sex	Age (years)	Direct fecal smear microscope–based technique: Parasite eggs identified	Combined PCR: *O. viverrini*	Combined PCR: *H. taichui*
Presence of "*Opisthorchis*-like" eggs and other parasitic eggs from 34 samples					
1	M	58	"*Opisthorchis*-like" egg, Hookworm	–	+
2	M	40	"*Opisthorchis*-like" egg	+	+
3	F	65	"*Opisthorchis*-like" egg, *Taenia* sp., *Blastocystis hominis*	+	+
4	M	17	"*Opisthorchis*-like" egg	+	+
5	M	23	"*Opisthorchis*-like" egg	+	+
6	F	34	"*Opisthorchis*-like" egg	+	–
7	M	67	"*Opisthorchis*-like" egg	+	+
8	F	44	"*Opisthorchis*-like" egg	+	+
9	F	58	"*Opisthorchis*-like" egg	–	+
10	F	59	"*Opisthorchis*-like" egg	+	+
11	M	38	"*Opisthorchis*-like" egg	–	+
12	F	70	"*Opisthorchis*-like" egg	–	+
13	M	22	"*Opisthorchis*-like" egg, *Trichuris trichura*	+	+
14	M	38	"*Opisthorchis*-like" egg	+	+
15	F	54	"*Opisthorchis*-like" egg	+	–
16	F	43	"*Opisthorchis*-like" egg, *B. hominis*, Hookworm, *Trichomonas hominis*	+	–
17	F	35	"*Opisthorchis*-like" egg	+	+
18	M	82	"*Opisthorchis*-like" egg	+	+
19	M	30	"*Opisthorchis*-like" egg	+	+
20	F	23	"*Opisthorchis*-like" egg	+	+
21	M	34	"*Opisthorchis*-like" egg	+	+
22	F	23	"*Opisthorchis*-like" egg, *T. trichura*	+	+
23	M	27	"*Opisthorchis*-like" egg	+	+
24	M	57	"*Opisthorchis*-like" egg	+	+
25	M	60	"*Opisthorchis*-like" egg	–	+
26	F	23	"*Opisthorchis*-like" egg	–	+
27	M	25	"*Opisthorchis*-like" egg	+	+
28	M	20	"*Opisthorchis*-like" egg	+	+
29	F	44	"*Opisthorchis*-like" egg	–	+
30	F	36	"*Opisthorchis*-like" egg	–	+
31	F	35	"*Opisthorchis*-like" egg	–	+
32	M	35	"*Opisthorchis*-like" egg	+	+
33	M	58	"*Opisthorchis*-like" egg	–	+
34	M	32	"*Opisthorchis*-like" egg	+	+
Absence of "*Opisthorchis*-like" eggs from 16 samples					
35	F	39	*Enterobius vermicularis*	+	+
36	M	33	*Ascaris lumbricoides*, Hookworm	–	+
37	F	26	*T. trichura*, Hookworm	–	+
38	F	52	*Strongyloides stercoralis*	–	–
39	M	57	*A. lumbricoides*	–	–
40	F	47	*A. lumbricoides*	–	+
41	F	22	*Trichuris trichura*	–	–
42	M	29	*Taenia* spp.	–	–
43	F	73	*S. stercoralis*	–	–
44	F	27	Hookworm	–	–
45	F	42	–	–	–
46	F	44	–	–	–
47	F	30	–	–	–
48	F	43	–	–	–
49	F	26	–	–	–
50	M	40	–	–	–

F = female; M = male; PCR = polymerase chain reaction.

as the Kato-Katz technique and formalin–ether concentration.[15] However, despite the advantages of the nested PCR technique, false-positive and false-negative diagnoses were detected when ribosomal DNA-, internal transcribed spacer 1(*ITS1*)-, and *ITS2* gene-based PCR assays were used to discriminate between *O. viverrini*, *C. sinensis*, *H. taichui*, and *H. pumilio* in an analysis of the same human stool specimens, and it was concluded that PCR cannot discriminate between these parasite species based on the size of the amplicon produced during agarose gel electrophoresis.[8] To discriminate between *O. viverrini* and *H. taichui*, Thaenkham and others[14] designed primers for amplifying the same *COI* gene region in adults, metacercariae, and eggs of both parasites. After PCR amplification at the optimal annealing

temperature, it was shown that these two parasite species cannot be identified based on the sizes of their amplicons on agarose gel electrophoresis. Thus, the amplicons were subjected to restriction enzyme digestion, and the genetic characteristics of *O. viverrini*, *C. sinensis*, and *H. taichui* were determined. However, if a weak amplicon is produced during PCR or a mutation is present in the target DNA sequence, this might reduce the sensitivity of the PCR restriction fragment length polymorphism technique.[30]

This study describes a protocol for diagnosing parasite infections based on the use of *COI* gene DNA derived from parasite eggs as a genetic marker, which attempted to jump over PCR inhibition in stool specimens as well. As started from the step of gDNA egg extraction from stool pellet by using a commercial stool DNA extraction kit, which included with InhibitEX matrix tablet, the InhibitEX matrix tablet helped to reduce PCR inhibitors and followed with screening of human beta actins to confirm PCR quality from the gDNA extraction process.

In this technique, a PCR involving a universal primer pair that has been demonstrated to be useful for amplifying the *COI* gene DNA of multiple parasites is used to increase the purity and enhance the amplification of the target region of the *COI* gene. In the latter stage, the PCR product was diluted with distilled water and it was enough for subjecting into qPCR without cleanup of PCR product, and then qPCR was performed with species-specific primers. The unique features of the amplification curves and T_m peak values of specific DNA molecules are used to identify the causative parasite. We initially used this assay to detect *O. viverrini* and *H. taichui COI* gene plasmids that had been produced by DNA cloning, and then it was used to validate clinical stool specimens. The stool specimens had been found to contain "*Opisthorchis*-like" eggs and other parasite eggs during microscopic examinations.

To the best of our knowledge, this is the first study to use fluorescent melting curve analysis to evaluate *COI* gene products to differentiate between two genospecies (*O. viverrini* and *H. taichui*). No cross-reactivity was detected when qPCR was performed using partial *COI* gene DNA for *O. viverrini*, *H. taichui*, or a mixture of other parasites template, and in positive cases, only one sharp T_m peak was detected. Furthermore, the differences between the T_m peak values of *O. viverrini* and *H. taichui* were greater than 1°C (Table 2). These findings suggest that our qPCR assay with species-specific primers is able to distinguish between *O. viverrini* and *H. taichui* with high specificity. In agreement with this, the assay was able to detect *O. viverrini* and *H. taichui* in stool samples that had been found to be "*Opisthorchis*-like" eggs-negative in microscopic examinations. Moreover, the assay was able to detect 1 pg of pure *O. viverrini* and *H. tachui* egg DNA (which was derived from a single egg) and 5 EPG in spiked egg stool specimens, which are similar to the findings obtained for other qPCR tests.[31] However, no cross-reactivity was detected during the testing of 10 clinical samples containing other parasites, which confirmed the specificity of the primers relative to the other parasites that commonly cause coinfections in Lao PDR[25] (Table 3).

The fact that our assay exhibited 100% sensitivity to parasitic infections (*O. viverrini*, 71%; *H. taichui*, 91%) when it was used to test samples that had been found to contain "*Opisthorchis*-like" eggs demonstrated that it is a good detection tool, while the fact that it detected parasites in 25% of the samples that had been found to be "*Opisthorchis*-like" eggs-negative during the microscopic examinations showed that it has a clear advantage over the current microscopic diagnostic techniques. Sequencing analysis confirmed that the four false-negative specimens included one coinfected specimen and three *H. taichui*–infected specimens (Table 3). Another reason for enhanced submicroscopic detection capability of our PCR assay could be, the use of an increased quantity of stool material (2 g) for detecting *O. viverrini* eggs.[32] These results suggest that our assay is highly sensitive and specific for detecting and differentiating between *O. viverrini* and *H. taichui*.

Inhabitants infested with *O. viverrini* and/or *H. taichui* parasites consumed raw fresh water fish cooked in traditional style, which were infected with parasitic metacercariae as single and/or mix. The prevalence rates were previously reported in Lao PDR community survey.[33,34] These studies used expelled adult worms to confirm the types of parasites present in each subject and showed that there is geographical variation in the prevalence of the two parasites and a need for local information about effective interventions and prioritization.[35] As molecular technology is becoming more affordable, it is hoped that the findings of this study will not only contribute to the development of better detection methods, but also aid research develop better diagnostic methods.

CONCLUSIONS

As the eggs of *O. viverrini* and *H. taichui* are very similar in terms of their size and morphology, it is difficult to distinguish between them during microscopic examinations of stool samples from infected patients. To differentiate between these two parasites, we designed new species-specific primers and used a combined PCR assay involving SYBR Green I and T_m peak value analysis, which exhibited high sensitivity and specificity.

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