Rapid Molecular Detection of Opisthorchis viverrini in Human Fecal Samples by Real-Time Polymerase Chain Reaction

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Abstract. Real-time fluorescence resonance energy transfer (FRET) polymerase chain reaction (PCR) supplemented with melting curve analysis is a highly sensitive and fast method offering a high throughput. We report the development of a real-time FRET PCR for molecular detection of Opisthorchis viverrini in human fecal samples. The diagnostic sensitivity, specificity, accuracy, and positive and negative predictive values of this method were 97.5%, 100%, 98.9%, 100%, and 98.2%, respectively. The sensitivity was not significantly different from that of the quantified formalin-ethyl acetate concentration technique, the gold standard (P > 0.05). The procedure has potential for diagnosis of human opisthorchiasis in disease-endemic areas, for large epidemiologic investigations involving at risk populations, and monitoring eradication programs of the liver fluke, which causes hepatobiliary diseases and induces cholangiocarcinoma.

INTRODUCTION

Human opisthorchiasis caused by the liver fluke Opisthorchis viverrini is a disease endemic in Southeast Asian countries including the Lao People’s Democratic Republic, Cambodia, Vietnam, and Thailand.1 The estimated prevalence ranges from 9.4% to 79% and approximately 9 million persons are infected.2,3 In developed countries, the disease has become more prevalent with the influx of immigrants from Asia.4–6 Humans are infected by eating raw or semi-cooked cyprinoid fishes harboring the O. viverrini infective stage called metacercariae. General symptoms include dyspepsia, fatigue, and upper quadrant abdominal pain. Gall bladder enlargement can be demonstrated by ultrasonography.7 In severely or chronically infected patients cholangitis, biliary calculi, fibrosis of the periportal system, obstructive jaundice, cholecystitis, and even bile duct cancer can occur.8–11 The classic method for the diagnosis of human opisthorchiasis is by microscopic examination of fecal samples. However, much experience is needed to differentiate O. viverrini eggs from lecithodendriid and heterophyid parasites, which have similar egg features. Therefore, the sensitivity of microscopic examination is dependent on the skill and training of the personnel. Furthermore, this method is time-consuming. To overcome the constraints of the microscopic method, various sensitive and specific molecular methods such as classic polymerase chain reaction (cPCR)–based techniques capable of DNA amplification from helminthic eggs in stool samples have been reported,12–16 but these methods still lack the desired reliability.

Recently, a real-time based PCR method proved to be sensitive and specific for detection of O. viverrini DNA in intermediate hosts and liver tissues.17–19 The effectual real-time PCR has increasingly superseded the cPCR because of its greatly improved molecular detection and its capability to distinguish microorganisms belonging to the same genus. It is not only accurate, rapid, and can express the quantity of specific DNA in samples,20 but it also discriminates species or strains of several pathogenic agents by melting curve analysis. In addition, this method offers a high throughput, is done in a closed system, which removes the potential risk associated with cross-over contamination because it does not require agarose gel electrophoresis for identification of the amplicons, and has a wide dynamic ability.

Because this method has not been applied for the detection of O. viverrini in fecal specimens, our study was designed to use a real-time fluorescence resonance energy transfer (FRET) PCR, another assay arrangement of the real-time based PCR, combined with melting curve analysis for the detection of parasite DNA in human stool samples. The aim of our study was to develop a rapid, sensitive, and specific method and to improve its efficacy for the diagnosis of human opisthorchiasis.

MATERIALS AND METHODS

Fecal specimens and parasitologic method. Sixty-five fecal specimens were collected from patients of various ages and sex who visited the Srinagarind Hospital, Faculty of Medicine, Khon Kaen University, Thailand. The specimens were examined for parasites by the quantitative formalin ethyl acetate concentration technique (QFECT).21 The total number of O. viverrini eggs was given as eggs per gram (EPG) of feces (range = 17–8,820 EPG, geometric mean = 124.4 EPG). Of these 65 samples, 40 contained eggs of O. viverrini, 5 contained eggs of Taenia spp., 10 contained eggs of minute intestinal flukes, and 10 contained larvae of Strongyloides stercoralis. Thirty negative fecal samples were obtained as controls from apparently healthy adults whose stool examination gave no evidence of any intestinal parasitic infection and whose history did not include eating raw or semi-cooked foods. The specimens were labeled blindly and used for extraction of DNA.

To extract DNA, 100 mg of feces were washed with 200 μL of normal saline solution by vortexing and centrifugation at 8,000 × g for 5 minutes. The supernatant was discarded and the fecal pellet was frozen at −20°C. The frozen feces were homogenized with disposable polypropylene pestles (Belco Glass Inc., Vineland, NJ) and extracted using the QIAamp® DNA stool mini kit (Qiagen, Hilden, Germany). One microliter of eluted DNA solution was used for the real-time FRET PCR.

This study was reviewed and approved by the Human Ethics Committee of Khon Kaen University (no. HE511023). Informed consent was obtained from all human adult participants and from parents or legal guardians of minors.

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**Real-time FRET PCR.** For amplification and quantification, the LightCycler PCR and detection system (LightCycler 2.0; Roche Applied Science, Mannheim, Germany) was used. The real-time FRET PCR was performed in glass capillaries using the specific primer pair OV-F (5′-CAG TGA GTG TCT ATT GCC TAA-3′) and OV-R (5′-GTA CAT CTC TAA AAG GTT GCT-3′) (Proligo, Singapore) and a pair of adjacent oligoprobes. One probe was labeled at the 5′ end with the LightCycler Red 640 fluorophore (5′-Red 640-AGA AGG GCG AAA CCG GTC GTG G-Phosphate-3′) (OVLC640 probe) and the other was labeled at the 3′ end with 530 fluorescein (5′-GGG ACT CCT ACC TGA TAG CCC-Flou 530-3′) (OVFL530 probe) (Tib Molbiol, Berlin, Germany). Probes and primers were designed to bind to the pOV-A6 specific DNA probe sequence (GenBank accession no. S80278). Regarding the specificity of the probes and primers, no fluorescence signal appeared when purified DNA from Centrorurus spp., Haplorchis taichui, Fasciola gigantica, Echinostoma malayanum, Paragonimus heterotremus, Haplorchoides spp., Stellantchasmus spp., or animal schistosomes was tested.

The reaction mixture (20 μL) consisted of LightCycler FastStart DNA Master HybProbe (Roche Applied Science), 2 mmol/L MgCl₂, 0.3 μmol/L OV-F primer, 0.3 μmol/L OV-R primer, 0.2 μmol/L OVLC640 probe, and 0.2 μmol/L OVFL530 probe, respectively. Samples were processed by accomplishing 45 cycles of repeated denaturation (10 seconds at 95°C), annealing (30 seconds at 50°C), and extension (10 seconds at 72°C). The temperature transition rate was 20°C/second. A melting curve was constructed after amplification by heating the product at 20°C/second to 95°C, cooling it to 40°C, maintaining it at this temperature for 30 seconds, and then heating it slowly at 0.1°C/second to 75°C. The fluorescence intensity change was evaluated throughout the slow heating phase. Each running experiment had at least one negative control consisting of 5 μL of distilled water. A positive DNA control plasmid was created by cloning a PCR product of the pOV-A6 specific DNA probe sequence into the pCR4-TOPO vector (Invitrogen, Carlsbad, CA). The real-time FRET PCR results were calculated from duplicate experiments.

The melting temperatures and melting peaks were obtained as described. Melting curves were conducted to show the hybridization of probes and specific amplified products, which were validated by agarose gel electrophoresis. The PCR products were sequenced in both directions to confirm that the amplification products were O. viverrini by using the DYEnamic ET Dye terminator cycle sequencing Kit (Amersham Biosciences, Piscataway, NJ) and a MegaBACE DNA analysis system (Amersham Biosciences).

**Standardization of the real-time FRET PCR.** To assess the detection limit of the real-time FRET PCR, 100-mg aliquots of individual negative fecal control samples were separately inoculated with a serial dilution (2 × 10⁰ to 2 × 10⁶) of O. viverrini-positive control plasmid or of O. viverrini genomic DNA (3 × 10⁻⁴ to 3 ng) or of 1, 2, 4, 8, 16, 32, and 64 O. viverrini eggs, respectively. The O. viverrini eggs were recovered as described. The study samples were further processed for genomic DNA extraction and the resultant DNA was used in the real-time FRET PCR assay.

**Data analysis.** The diagnostic sensitivity, specificity, accuracy, and predictive values were calculated and expressed using a standard method. The sensitivity of two different methods was compared by using McNemar’s χ² test.

**RESULTS**

Regarding the standardization of the real-time FRET PCR, the detection limit in fecal specimens was approximately 2 × 10⁶ copies of positive control plasmid (Figure 1), which is equivalent to 3 × 10⁻² ng of O. viverrini genomic DNA when using 35 cycle numbers as the cut-off between positivity and negativity. To estimate the capability of O. viverrini egg detection, as little as one egg could be detected in 100 mg of negative control sample.

The DNA extracted from the fecal specimens that contained O. viverrini eggs and other parasite material and the negative control samples were separately examined by applying the real-time FRET PCR assay merged with a melting curve analysis of the specific hybridization probes and PCR products. The real-time FRET PCR yielded positive results with 39 (cycle number range = 26.5–34.8, mean ± SD = 31.8 ± 2.6, median = 31.7) of the 40 O. viverrini egg-positive stool samples by QFECT (Table 1). All stool samples with other parasite material and the 30 negative control samples were negative. The melting curve analyses for the O. viverrini DNA are shown in Figure 2. The mean ± SD melting temperature value of the O. viverrini DNA in the fecal sample was 66.3 ± 0.2.

Regarding validation of real-time FRET PCR amplifications, the positive control plasmids (Figure 3, lane 2) and O. viverrini egg-positive stool samples (Figure 3, lanes 3 and 4) showed a 162-basepair product, whereas genomic DNA from the negative control samples (Figure 3, lane 5). Taenia spp. egg–positive samples (Figure 3, lane 6), S. stercoralis larvae–positive samples (Figure 3, lane 7), and minute intestinal fluke egg–positive samples (Figure 3, lane 8) were not amplified. The 162-basepair amplified product was sequenced and the resulting data were ascertained with pOV-A6–specific DNA sequence (Genbank accession no. S80278).

![Amplification Curves](image-url)

**Figure 1.** Amplification plot of fluorescence (y-axis) versus cycle numbers (x-axis) showing the analytical sensitivity of the real-time polymerase chain reaction for detecting Opisthorchis viverrini plasmid DNA mixed with normal fecal sample. A. O. viverrini plasmid (2 × 10⁶ copies/sample); B. O. viverrini plasmid (2 × 10⁵ copies/sample); C. O. viverrini (plasmid 2 × 10⁶ copies/sample); D. O. viverrini (plasmid 2 × 10⁵ copies/sample); E. O. viverrini plasmid (2 × 10⁶ copies/sample); F. O. viverrini plasmid (2 × 10⁵ copies/sample); G. O. viverrini (plasmid 2 × 10⁶ copies/sample); H. O. viverrini plasmid (2 × 10⁵ copies/sample); I. O. viverrini plasmid (2 × 10⁴ copies/sample); J. distilled water.
REAL-TIME PCR FOR THE DETECTION OF O. VIVERRINI

Table 1

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<th>Relationship between the diagnoses of <em>Opisthorchis viverrini</em> infection by real-time PCR and QFECT methods in fecal samples*</th>
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<td>Real-time FRET PCR test result</td>
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*PCR = polymerase chain reaction; FRET = fluorescence resonance energy transfer; QFECT = quantitative formalin ethyl acetate concentration technique. The sensitivity of the two methods was not significantly different (P > 0.05) (Table 1). The rates of real-time PCR positivity within different *O. viverrini* intensity groups are shown in Table 2. The real-time FRET PCR assay yielded positive results in 100% of the fecal samples with > 99 EPG and in 94.4% of fecal samples with 1–99 EPG. The lowest egg count detected with the real-time FRET PCR was 17 EPG. However, one fecal sample with 18 EPG showed a negative result.

DISCUSSION

Detection of *O. viverrini*-specific sequences by cPCR for the diagnosis of human opisthorchiasis in fecal samples has yielded a varying sensitivity,13-16 which shows the need for a more rapid, sensitive, and specific method. We used OV-F and OV-R primers for diagnosis of *O. viverrini* infection in human fecal specimens by cPCR. Results also showed lower sensitivity (65%) than that of the QFECT (P < 0.05). Another potential pitfall was the need to analyze the results by agarose gel electrophoresis, which requires a large amount of time, has a limited throughput, and has the tendency to carry-over contamination, and show as illusory outcomes.

In this study, detection of *O. viverrini* eggs in fecal samples was performed by using a real-time FRET PCR with two primers and two individually fluorophore-labeled hybridization probes combined with a melting curve analysis. Probes and primers were based on a highly repetitive DNA sequence of the *O. viverrini* genome.22 This procedure could detect as little as one *O. viverrini* egg inoculated into a 100-mg fecal specimen, which is equivalent to 10 EPG. The high sensitivity of the method was not significantly different from that of the QFECT (P > 0.05), which indicates that this method can be used for diagnosis of human opisthorchiasis and for application with field samples, such as for determining the prevalence of *O. viverrini* in disease-endemic areas. Moreover, this technique has the advantage that it can diagnose infections with light intensities.

However, one fecal sample from a lightly infected person showed a negative result in duplicate experiments. This result may have been caused by the absence of *O. viverrini* eggs in the fecal sample used for the DNA extraction, which was caused by a non-homogeneous distribution of the helminthic eggs during preparation. It also could have been caused by technical problems during preparation such as unsuccessful breaking of the highly resistant *O. viverrini* eggs and poor release of DNA. This possibility could cause the sample to show a positive result by QFECT and a negative result by real-time FRET PCR. Regarding the specificity of the procedure, no fluorescence was visible and primers did not amplify the 162-basepair band when DNA extracted from other parasite-positive stools and negative control samples was tested, which indicated a specificity of 100%.

Table 2

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<th>Diagnosis of <em>Opisthorchis viverrini</em> infection by real-time FRET PCR in different intensity groups*</th>
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<td>No. of EPG by QFECT</td>
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*FRET = fluorescence resonance energy transfer; PCR = polymerase chain reaction; EPG = egg per gram (of feces); QFECT = quantitative formalin ethyl acetate concentration technique.

Figure 3. Ethidium bromide staining patterns of polymerase chain reaction (PCR) products after electrophoresis on a 2% agarose gel. **A** indicates the 162-basepair *Opisthorchis viverrini*-specific band. Lane 1, negative control containing no DNA; lane 2, PCR products obtained from positive control plasmid (106 copies/reaction); lanes 3 and 4, PCR products obtained from *O. viverrini* egg-positive fecal specimens; lane 5, PCR products from a negative control sample; lane 6, PCR products from *Taenia* egg-positive fecal specimen; lane 7, PCR products from *S. stercoralis* larvae-positive fecal specimen; lane 8, PCR products from minute intestinal fluke egg-positive specimen; lane M, DNA size markers (1 kb plus DNA ladder; Invitrogen, Carlsbad, CA).

Figure 2. Representative melting curve analysis of two fluorophore-labeled probes hybridized to the amplification products of the tandem repetitive sequence pOV-A6 DNA of *Opisthorchis viverrini*. The melting temperature (Tm) of the double-stranded fragment is visualized by plotting the negative derivative of the change in fluorescence divided by the change in temperature in relation to the temperature [- (d/dT) fluorescence (640/530)]. The turning point of the converted melting curve results in a peak and permits easy identification of the fragment specific Tm. Melting peaks of *O. viverrini* infected human fecal samples (A–E), the positive control plasmid (F), non-infected human fecal samples, *Taenia* spp.–infected, Strongyloides stercoralis–infected, and minute intestinal fluke–infected human fecal samples, and the negative control containing no DNA (G).
The real-time FRET PCR protocol offers an alternative to immunologic or molecular methods for detection of *O. viverrini* in fecal specimens. By virtue of this rapid method, the entire protocol of the real-time FRET PCR can be completed in less than one hour (after extraction of genomic DNA). The technique also eliminates the requirement for specifically trained personnel and laborious and time-consuming microscopic methods. A large number of specimens can be processed at the same time and only a small amount of fecal sample is necessary. The method is not influenced by the illusory bias inherent in microscopic examinations and avoids the misidentification of eggs of other digenean flukes. As reported, the diagnostic values were higher than those in previous reports.

In conclusion, we report a rapid, specific, and sensitive real-time FRET PCR for detection of *O. viverrini* in human stool samples. The method is an appropriate and powerful tool for diagnosis of human opisthorchiasis and for epidemiologic surveys of at risk populations and programs to eradicate liver flukes.

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