Development of a PCR assay and pyrosequencing for identification of important human fish-borne trematodes and its potential use for detection in fecal specimens

Chairat Tantrawatpan1,2, Pewpan M Intapan1,3, Tongjit Thanchomnang1,4, Oranuch Sanpool1,3, Penchom Janwan1,3, Viraphong Lulitanond1,5, Lakkhana Sadaow1,3 and Wanchai Maleewong1,3*

Abstract

**Background:** Small liver and minute intestinal flukes are highly prevalent in Southeast Asia. Definitive diagnosis of parasite infection is usually achieved parasitologically by finding the fluke eggs in feces. However, their eggs are difficult to differentiate morphologically in fecal samples, even for experienced technicians. The present study developed a PCR assay coupled with DNA pyrosequencing for identification of the fish-borne trematodes (FBT), *Opisthorchis viverrini*, *Clonorchis sinensis*, *Haplorchis taichui*, *H. pumilio* and *Stellantchasmus falcatus*, and to evaluate potential detection in fecal specimens, and identification and differentiation of cercarial and metacercarial stages.

**Methods:** Primers targeting the partial 28S large subunit ribosomal RNA gene were designed and about 46–47 nucleotides were selected as the target region for species identification by a PCR assay coupled with a pyrosequencing technique.

**Results:** The nucleotide variations at 24 positions, which is sufficient for the identification of the five species of FBT were selected. The method could identify *O. viverrini* and *C. sinensis* eggs in feces, cercarial and metacercarial stages of *O. viverrini*, and metacercarial stage of *H. pumilio* and *H. taichui*. The detection limit was as little as a single *O. viverrini* or *C. sinensis* egg artificially inoculated in 100 mg of non-infected fecal sample (equivalent to 10 eggs per gram), indicating highly sensitivity. The method was found to be superior to the traditional microscopy method and was more rapid than Sanger DNA sequencing.

**Conclusions:** DNA pyrosequencing-based identification is a valuable tool for differentiating *O. viverrini* and other *Opisthorchis*-like eggs, and can be applied to epidemiological studies and for molecular taxonomic investigation of FBT in endemic areas.

**Keywords:** Pyrosequencing, Identification, *Opisthorchis viverrini*, *Clonorchis sinensis*, *Haplorchis taichui*, *Haplorchis pumilio*, *Stellantchasmus falcatus*

* Correspondence: wanch_ma@kku.ac.th
Background

Human fishborne trematode (FBT) infections caused by liver and intestinal trematodes are especially problematic in Asian countries including Vietnam, Lao PDR, Cambodia and Thailand, for example, *Opisthorchis viverrini*, *Clonorchis sinensis*, *Haplorchis taichui*, *Haplorchis pumilio*, *Stellantchasmus falcatus* [1]. According to the World Health and the Food and Agriculture Organization of the United Nations, more than 40 million people were infected with FBT in 2002 [2]. *Opisthorchis viverrini* has been reported frequently in several countries such as Thailand, Lao PDR, South Vietnam and Cambodia [3]. *Clonorchis sinensis*, which is endemic in the People’s Republic of China, Korea, Taiwan and North Vietnam [4-6] has been reported in Thailand in one study [7]. More than 200 million people are at risk of contracting clonorchiasis, whereas 15–20 million are infected and 1.5–2 million present symptoms and complications [6]. Infection by *O. viverrini* has a major public health impact with chronic infections associated with the development cancer of the bile duct (cholangiocarcinoma) and the liver (hepatocarcinoma) in humans [8]. Since endemic areas of *C. sinensis* and *O. viverrini* are closely next to each other [1,5] with the report of co-endemic areas in Thailand [7], and since the number of travelers visiting endemic areas of those parasites has been expanding, both flukes may overlap in Southeast Asia.

The heterophyids *H. taichui* and less frequently *H. pumilio* are the most common minute intestinal flukes. *Haplorchis taichui*, *H. pumilio* as well as *S. falcatus* are prevalent and cause infections frequently in Thailand [9] and Vietnam [10]. Mixed infections of *O. viverrini* with other minute intestinal flukes of the Heterophyidae and Lecithodendriidae families have also been reported [11-16].

Diagnosis of FBT infection in humans is usually done by microscopic observation of parasite eggs in feces. However, it is difficult to differentiate *O. viverrini* and *C. sinensis* eggs as well as to discriminate opisthorchid eggs from lecithodendrid and heterophyid eggs i.e., *H. taichui*, *H. pumilio*, *S. falcatus* and *Centrocestus caninus* because of their morphological similarity [17]. To overcome the pitfalls of traditional microscopic methods, various sensitive and specific molecular methods have been developed to discriminate between eggs of different species of FBT. However, the rapid, concurrent and high throughput identification of five species of FBT, *O. viverrini*, *C. sinensis*, *H. taichui*, *H. pumilio* and *S. falcatus* is still lacking.

Recently, DNA pyrosequencing of the PCR amplicons, the direct sequencing by the synthesis of short nucleotide fragments has been successfully applied in a variety of cases, including genotyping and species level identification of protozoan parasites [18-21], trematodes [22] and nematodes [23].

In the present study, we report the molecular identification of life cycle stages, of *O. viverrini* and *C. sinensis*, *H. taichui*, *H. pumilio* and *S. falcatus* by using the PCR assay and a high-throughput sequence analysis, pyrosequencing technique of the amplicons based on hypervariable regions within 28S rRNA genes.

Methods

Parasite and sample collections

Adult worms of *O. viverrini* (Khon Kaen strain, Northeast Thailand) and *C. sinensis* (Thai Binh strain, Vietnam; the generous gift from Institute of Ecology and Biological Resources, Vietnam Academy of Science and Technology, Hanoi, Vietnam) were obtained from experimentally infected hamsters and naturally infected cats, respectively, and used for genomic DNA extraction of positive control DNAs. Metacercariae of *H. taichui*, *H. pumilio* and *Stellantchasmus falcatus* were collected from naturally infected cyprinid fishes from fresh water reservoir after pepsin-HCl digestion. Human stool specimens infected with *O. viverrini* were collected from left-over specimens from patients who visited Srinagarind Hospital, Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand, and cat stool specimens infected with *C. sinensis* were obtained from Thai Binh Province, Vietnam. Cercariae of *O. viverrini* were obtained from experimentally infested *Bithynia siamensis* goniomphalos snails by light shedding technique. All of cercariae, metacercaria, eggs and adults of each specimen were morphological identified by microscopy and PCR/Sanger DNA sequencing. This study was approved by the Khon Kaen University Ethics Committee for Human Research (reference number HE541243).

Primer design

The large subunit of ribosomal RNA (28S rRNA) genes of *O. viverrini* (GenBank:HM004188), *C. sinensis* (GenBank:JF823989), *H. taichui* (GenBank:HM004187), *H. pumilio* (GenBank:HM004191), and *S. falcatus* (GenBank:KF241630) available in GenBank were selected to find suitable regions for discrimination of these five parasites. After alignment of the 28S rRNA genes of those 5 species, we selected about 46–47 bp fragments in 28S rRNA genes as the suitable target region used for identification and differential detection by pyrosequencing. At this region, 24 nucleotide variations within 47 nucleotides are assumed to be sufficient for the identification of five parasites. Therefore, the conserved PCR primers (OVPyro28S_F; 5′-TTTGCTGGTCTGGTCGGGTAGGC-3′ and biotinylated OVPyro28S_R; biotin-5′-CCGCGTCCTCGGACATAC-3′) and sequencing primer (OVPyro28S_S; 5′-TCCGATGAGCAGTA-3′) were designed by using pyrosequencing assay design software (PyroMark Q96 ID software version...
genes were sequenced in both directions. For confirmation and validation of sequence in-combiant plasmids were propagated in \textit{Escherichia coli} JM109. The nucleotide sequences of the inserted products were obtained from conventional PCRs using according to the manufacturer’s protocols. Genomic DNA was eluted in 50 μl of distilled water. Two hundred milligrams of frozen feces was homogenized and extracted using the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s protocol. DNA extracted from feces was eluted in 100 μl of distilled water.

Positive control plasmids containing 119 bp PCR products of \textit{O. viverrini} and \textit{C. sinensis}, and 118 bp PCR products of \textit{H. taichui}, \textit{H. pumilio} and \textit{S. falcatus} 28S rRNA genes were constructed by cloning these PCR products into the pGEM®-T Easy vector (Promega, WI) according to the manufacturer’s instructions. The PCR products were obtained from conventional PCRs using the OVPyro28S\textsubscript{F} and OVPyro28S\textsubscript{R} primers. The recombinant plasmids were propagated in \textit{Escherichia coli} JM109. For confirmation and validation of sequence information, the nucleotide sequences of the inserted genes were sequenced in both directions.

**Polymerase chain reaction (PCR) and pyrosequencing assays**

Conventional PCR was performed with OVPyro28S\textsubscript{F} and biotinylated OVPyro28S\textsubscript{R} primers which were used for amplification region of 28S rRNA gene. The PCR reaction was carried out in a 25 μl final reaction volume, which composed of 1× PCR buffer (Invitrogen, Carlsbad, CA) with 0.2 mM each of dNTPs, 1.5 mM MgSO\textsubscript{4}, 0.2 μM each of OVPyro28S\textsubscript{F} primer and biotinylated OVPyro28S\textsubscript{R} primer, 0.625 U of Platinum Taq DNA polymerase high fidelity (Invitrogen) and 2 μl of the DNA sample in a GeneAmp PCR system 9700 thermal cycler (Applied Biosystems, Singapore). The amplification procedure was as follows: 5 minutes at 94°C for initial denaturation followed by 35 cycles of denaturation for 30 seconds at 94°C, annealing for 30 seconds at 60°C, and extension for 30 seconds at 72°C, followed by a final extension for 7 minutes at 72°C. Two microliters of the PCR products was used to detect and verify the molecular size of PCR products on a 1% agarose gel. The specific amplification products that contained biotinylated primer were then prepared before performing pyrosequencing. To check the reliability of pyrosequencing data, all PCR products of each sample that was amplified with OVPyro28S\textsubscript{F} and non-biotinylated OVPyro28S\textsubscript{R} primers similarly with the above condition, was then subjected to sequencing by the Sanger method and compared with the pyrosequencing data. The pyrosequencing assays were performed by using the PyroMark™ Q96 ID instrument (Biotage, Uppsala, Sweden) according to the manufacturer’s protocols. The procedure was similar as previously described [20] with the exception that 0.4 μM OVPyro28S\textsubscript{S} specific sequencing primer was used before performing pyrosequencing reaction.

**Analytical sensitivity and specificity**

To determine sensitivity of egg detection in feces, 2 μl of DNA extracted from 100 mg of non-infected hamster and cat feces obtained from the Northeast Laboratory Animal Center, Khon Kaen University which were artificially mixed with 1, 2, 5, 10 and 30 eggs of \textit{O. viverrini} and \textit{C. sinensis} were also examined, respectively. The spiked experiments were repeated in triplicate. For the evaluation of analytical specificity, 1 μl (3 ng) of control genomic DNAs from other organisms were analyzed. The panel of other organisms was \textit{Necator americanus}, \textit{Strongyloides stercoralis}, \textit{Ascaris lumbricoides}, \textit{Trichuris trichiura}, \textit{Capillaria philippinensis}, \textit{Taenia spp.}, \textit{Schistosoma mekongi}, \textit{S. japonicum}, \textit{Echinostoma malayanum}, \textit{Fasciola gigantica}, \textit{Paragonimus heterotremus}, \textit{Isospora belli}, \textit{Trichostrongylus spp.} and \textit{Giardia lamblia}. Genomic DNAs of human leukocytes as well as DNA from non-infected fishes and snails tissues, and DNA from non-infected hamster and cat feces were also included. To compare efficacies of microscopic examination, Sanger DNA sequencing and pyrosequencing assays, identification and diagnosis of \textit{O. viverrini} infected-human (\(n = 5\)) and \textit{C. sinensis} infected-cat (\(n = 5\)) feces, individual opisthorchid-like cercaria (\(n = 4\)) from infected snails and individual

**Figure 1 Multiple Alignment of the 28 large subunit of ribosomal RNA from 5 fishborne trematodes.** Alignment of (285 RNA) genes of Opisthorchis viverrini (HM004188), Clonorchis sinensis (JF839989), Haplorchis taichui (HM004187), H. pumilio (HM004191), and Stellantchasmus falcatus (KF241630) available in GenBank were selected to find a suitable region for discrimination these five species. The solid arrows indicate the position of OVPyro28S\textsubscript{F} (forward primer) and biotinylated OVPyro28S\textsubscript{R} (reverse primer) for template amplification. A dash arrow indicates OVPyro28S\textsubscript{S} (sequencing primer), and a rectangular box shows the position of the 47 nucleotides as the target region used for species level identification. Dots indicate identical nucleotides between lines. Dash indicates gap between nucleotides.
opisthorchiid-like (n = 3) or Haplorchis-like (n = 3) metacercaria from infected fishes were performed.

Results

Multiple alignment of 28S rRNA sequences and pyrosequencing assays

Multiple alignment of the 28S rRNA genes from O. viverrini (GenBank:HM004188), C. sinensis (GenBank:JF823989), H. taichui (GenBank:HM004187), H. pumilio (GenBank:HM004191), and S. falcatus (GenBank:KF241630) available in GenBank showed that 47 nucleotides (for O. viverrini and C. sinensis) and 46 nucleotides (for H. taichui, H. pumilio, and S. falcatus) after the 3′ end of sequencing primer as the target region was sufficient for identification of the five target FBT by pyrosequencing technique (Figure 1 and Table 1). The nucleotide sequence profiles at the target regions of each flukes were demonstrated as the pyrogram results (Figure 2). Within 46–47 nucleotides, 24 positions were nucleotide variations, which was useful in classifying and discriminating the sequenced FBT species (Table 1). Among 5 species, O. viverrini could be discriminated from the others by seven nucleotide positions (T/C13G, C15T, C26T, A27G, A32C/-, C33G/T, and C39T) and C. sinensis distinct from the others by three nucleotide positions (C32A/-, G33C/T, and T35C) (Table 1 and Figure 2). By using this region as the marker, H. taichui and H. pumilio separated from O. viverrini, C. sinensis and S. falcatus by 3 nucleotide positions (T5G, T28C and T43C), while H. taichui and H. pumilio can be differentiated each other by using 5 nucleotide positions (C3T, A9G, C21T, T23C, and G45A). Similarly, S. falcatus have two unique nucleotides at the position number 37 (T) and 41 (T), which were used for identification from the others. All samples of O. viverrini, C. sinensis, H. taichui and H. pumilio and S. falcatus included genomic DNAs and positive control plasmids exhibited the same sequence except the T → C substitution at the position no. 13 of O. viverrini was found (Figure 3). The sequences of five trematodes by pyrosequencing showed the same sequences with Sanger sequencing results (Additional file 1: Figure S1).

Specificity and sensitivity analysis

The specificity of the PCR primer set was analyzed and the results showed that no amplification product was detected with DNAs of other parasites (see Methods) or genomic DNAs from human leukocytes or uninfected cat and hamster feces or snail and fish tissues (Figure 4). Furthermore, pyrosequencing result of other organisms and negative control showed no pyrogram (Additional file 2: Figure S2). The method could detect as little as either a single egg of O. viverrini or a single egg of C. sinensis artificially inoculated in 100 mg of non-infected hamster and cat feces, respectively.

Detection efficacy of pyrosequencing compared with microscopic examination and Sanger sequencing

For evaluation of the detection efficacy of the applications, DNAs extracted from the fecal specimen of five human opisthorchiasis patients and two opisthorchiid-like infected metacercariae from infected fishes as well as four unidentified cercariae from infected snails by microscopic examination were used. The results showed that the samples, which present the O. viverrini sequences pattern were similar to the O. viverrini positive control plasmid (Table 2). Moreover, DNAs extracted from five C. sinensis infected cat feces which have been previously identified by microscopic examination and Sanger DNA sequencing, could be confirmed as the C. sinensis with the same sequence by pyrosequencing (Table 2). Additionally, three Haplorchis-like metacercariae which was previously identified by microscopic examination similarly showed the Sanger DNA sequencing and pyrosequencing results with two H. pumilio and one H. taichui (Table 2). Interestingly, one opisthorchiid-like metacercaria which was first identified by microscopical examination exhibited the H. pumilio pyrogram (Table 2).

Table 1 Regions of the 28S large subunit of ribosomal RNA genes (28S rRNA) and nucleotide patterns used to differentiate Opisthorchis viverrini, Clonorchis sinensis, Haplorchis taichui, H. pumilio, and Stellantchasmus falcatus by the pyrosequencing technique

| Types (Accession No.) | Nucleotide positions after the 3′ end of sequencing primer |
|-----------------------|----------------------------------------------------------|
|                       | 3   | 5   | 7   | 9   | 13  | 15  | 16  | 18  | 19  | 21  | 23  | 26  | 27  | 28  | 29  | 32  | 33  | 35  | 36  | 37  | 39  | 41  | 43  | 45  |
| O. viverrini (HM004188) | C   | G   | T   | G   | C   | T   | T   | T   | C   | G   | C   | T   | C   | A   | C   | A   | C   | C   | C   | C   | C   | C   | G   |
| C. sinensis (JF823989)   | G   | T   | G   | C   | G   | C   | T   | T   | T   | T   | G   | C   | C   | A   | G   | G   | G   | C   | T   | C   | C   | G   |
| H. taichui (HM004187)    | C   | T   | G   | C   | G   | T   | T   | T   | C   | C   | G   | A   | A   | C   | T   | T   | T   | G   | G   | G   | G   | G   | G   |
| H. pumilio (HM004191)    | T   | T   | G   | C   | G   | T   | C   | C   | A   | G   | C   | T   | T   | T   | G   | G   | G   | G   | G   | G   | G   | G   | G   |
| S. falcatus (KF241630)   | T   | G   | G   | G   | T   | C   | T   | C   | G   | C   | T   | C   | A   | T   | T   | A   | T   | G   | C   | T   | C   | C   | G   |

*Nucleotide for identification of O. viverrini; †Nucleotide for identification of C. sinensis; ‡Nucleotide for identification of H. taichui and H. pumilio; §Nucleotide positions used for discrimination between H. taichui and H. pumilio; ‖Nucleotide for identification of S. falcatus.
Discussion
The FBT, including liver and intestinal flukes, are recognized as an important group of emerging and re-emerging human pathogens especially to populations living in low- and middle-income countries [24]. Two species of liver flukes, *O. viverrini* and *C. sinensis*, and more than 50 species of intestinal flukes, including *Haplorchis* spp., *S. falcatus*, and *Centrocestus* spp. are important representative examples of the FBT in Southeast Asia [24]. Differential detection of the FBT eggs in fecal specimens is
difficult and frequently results in misidentification due to similar morphology [17] especially in co-infection areas [12,25,26].

Many molecular techniques have been developed for the detection of a range of FBT, i.e., multiplex PCR and real-time PCR for identification of *C. sinensis* and *O. viverrini* [27,28], random amplified polymorphic DNA-PCR method for differential detection of *O. viverrini* and *H. taichui* [29], PCR targeting ITS regions for *O. viverrini*, *C. sinensis*, *H. pumilio* and *H. taichui* [30], PCR-restriction fragment length polymorphism (RFLP) for detection of *O. viverrini*, *C. sinensis*, and *H. taichui* [31] and for *H. taichui*, *H. pumilio*, *H. yokogawai*, Procerovum varium, *S. falcatus*, and *Centrocestus formosanus* [32]. Our study could identify life cycle stages of *O. viverrini*, eggs in infected feces, metacercariae from infected fishes, and cercarial stages from infected snails; *C. sinensis* eggs in infected feces as well as *H. pumilio*, *H. taichui* and *S. falcatus* metacercariae from infected fishes at the species level by a pyrosequencing approach. Because this primer set was designed based on the highly conserved regions of the 28S rRNA gene from 5 FBT examined herein, it could have major applications in the identification and differentiation of other FBT.

In the present study, our developed pyrosequencing technique is a rapid and high throughput method, which can obtain results from 96 samples within 1 hour after PCR amplification. The reasonable cost estimate for pyrosequencing for a 96 sample analysis was $220 ($4.58 per sample). This was an approximate estimation and would vary depending upon the commercial source of reagents used. To check the reliability of pyrosequencing data, PCR amplicons were sequenced by the Sanger method and compared with the pyrosequencing data. Both sets of sequence data were completely identical with each other at the 46–47 nucleotides of target.

![Figure 3](https://www.parasitesandvectors.com/content/7/1/88)

**Figure 3** Single nucleotide polymorphisms of *Opisthorchis viverrini*. Theoretical pyrogram patterns (top of each panel) and a representative raw data (bottom of each panel) of *Opisthorchis viverrini* from pyrosequencing. The areas show the pyrograms for the species differentiation in this cluster. Single nucleotide polymorphisms, exhibited in the light gray in the program were analyzed to discriminate thymine (A) and cytosine (B) of *O. viverrini*. 
region. Furthermore, the primer set used in this present study showed specificity with the 5’ FBT and no amplification product was found with control genomic DNAs.

There are several advantages of the pyrosequencing technique compared with Sanger sequencing; (i) pyrosequencing determines the exact sequence thereby providing the same accuracy as conventional sequencing methods, (ii) the technique dispenses with the need for labeled nucleotides, labeled primers and electrophoresis, (iii) large number of samples can be analysed in a short time, the work included analyses of test samples at the specified rate of 96 samples within 1 hour and (iv) the reagent costs are considerably lower for sequencing short stretches of DNA compared to currently available methods [33]. These are the reasons why this method could be the best-alternative way for molecular identification. However, there are some limitations; (i) pyrosequencing is easily capable of detecting PCR fragments that are 25–50 bp in length while longer fragments may pose a problem and (ii) the target sequence contained up to four homopolymers was limited for software interpretation. However, this problem can be clarified by manual interpretation.

Our pyrosequencing assay showed high sensitivity with the limit of detection of a single egg of *O. viverrini* and *C. sinensis* in feces, equivalent to 10 EPG. This result is quite similar with previous reports for the egg detection limits of either *O. viverrini* or *C. sinensis* single egg by PCR in fecal samples [31,34] as well as can be detected with ranges of 5–10 EPG in fecal samples by a singleplex Taqman probe-based real-time PCR assay for *C. sinensis* eggs [35,36], by a singleplex FRET-probe-based real-time PCR assay for *O. viverrini* eggs [37] and by a duplex real-time FRET PCR assay for *C. sinensis* eggs [28]. Variations in the detection limits noted in various reports were possibly due to inhibitors remaining in the fecal samples. Interestingly, we found nucleotide variation within *O. viverrini* at the position no. 13 (T → C substitution), indicating that this method can be implied for opisthorchiid flukes genotyping, similar to a previous report of the genotyping of *Blastocystis* [19].

**Conclusions**

In conclusion, the pyrosequencing technique can be a practical alternative method used in a high-throughput format for molecular detection of 5 species of FBT in Southeast Asia. This rapid, accurate and cost-effective
assay can be applied to identify several life cycle stages including eggs, cercariae, and metacercariae. However, this developed technique should be evaluated using larger sample sizes and more varied isolated samples or different parasite strains before practical use in clinical and field samples. Therefore, the method can have important implications for molecular genotyping and epidemiological studies of all five FBT in endemic countries.

**Additional files**

**Additional file 1: Figure S1.** Sanger sequencing results. Nucleotide sequences of 28S ribosomal RNA of Opisthochis viverrini, Clonorchis sinensis, Haplorchis taichu, H. pumilio, and Stellantchasmus falcatus by Sanger method. Highlighted nucleotides indicate sequencing primer position.

**Additional file 2: Figure S2.** Pyrosequencing results of other organisms and negative control. Representative pyrosequencing results of other organisms such as Necator americanus (a), Strongyloides stercoralis (b), Ascaris lumbricoides (c), Trichuris trichiura (d) and negative control (e) showed no pyrogram.

**Competing interests**

The authors declare that they have no competing interests.

**Authors’ contributions**

CT, PMI, VL and WM took part in the conception, planned and designed the study. CT, PMI and LS performed laboratory work and collected field samples. Therefore, the method can have important implications for molecular genotyping and epidemiological studies of all five FBT in endemic countries.

**Acknowledgments**

We thank Dr. Pham Ngoc Doanh from Institute of Ecology and Biological Resources, Vietnam Academy of Science and Technology, Hanoi, Vietnam for their assistance.

**Financial support**

This work was funded by grants from the TRF Senior Research Scholar Grant, Thailand Research Fund (WM, grant number RTA5880004), the National Science and Technology Development Agency (Discovery Based Development Grant); the Higher Education Research Promotion and National Research University Project of Thailand, Office of the Higher Education Commission and Khon Kaen University, Thailand.

**Author details**

1. Sripa B, Kawkes S, Intapan PM, Maleewong W, Brindley PJ: Food-borne trematodiases in Southeast Asia epidemiology, pathology, clinical manifestation and control. Adv Parasitol 2010, 72:305–350.

2. WHO: World Health Organization: Report Joint WHO/FAO Workshop on Food-Borne Trematode Infections in Asia. Workshop Report Series No. RS/2002/GE40 (VTN). Manila, Philippines: World Health Organization, 2002.

3. Sithithaworn P, Andrews RH, Nguyen VD, Wongsaaroj T, Sinoum M, Odermatt P, Nawa Y, Liang S, Brindley PJ, Sripa B: The current status of opisthorchiasis and clonorchiasis in the Mekong Basin. Parasitol Int 2012, 61:10–16.

4. Domy P, Praet N, Deckers N, Gabriel S: Emerging food-borne parasites. Vet Parasitol 2009, 163:196–206.

5. Keiser J, Utzinger J: Food-borne trematodiases. Clin Microbiol Rev 2009, 22:46–483.

6. Hong ST, Fang Y: Clonorchis sinensis and clonorchiasis, an update. Parasitol Int 2012, 61:17–24.

7. Traub RJ, Macaranas J, Munthign M, Leelayoova S, Cribb T, Murrell KD, Thompson RC: A new PCR-based approach indicates the range of Clonorchis sinensis now extends to Central Thailand. PLoS Negl Trop Dis 2009, 3:e367.

8. Fried B, Reddy A, Mayer D: Helminths in human carcinogenesis. Cancer Lett 2011, 305:239–249.

9. Waikagul J: Opisthorchis viverrini metacercaria in Thai freshwater fish. Southeast Asian J Trop Med Public Health 1998, 29:524–526.

10. Chai JF, De NV, Sohr WM: Foodborne trematode metacercariae in fish from northern Vietnam and their adults recovered from experimental hamsters. Korean J Parasitol 2012, 50:317–325.

11. Gboda M, Ditrich O, Scholz T, Vinessg T, Bouaphanh S: Current status of food-borne parasitic zooneses in Laos. Southeast Asian J Trop Med Public Health 1991, 22(Suppl):56–61.

12. Radomyos B, Wongsaaroj T, Wilaratanat P, Radomyos P, Praisevanich R, Meesomboon V, Jongkuansukkul P: Opisthorchiasis and intestinal fluke infections in northern Thailand. Southeast Asian J Trop Med Public Health 1998, 29:123–127.

13. Sukontason KL, Sukontason K, Piangjai S, Pungpak S, Radomyos P: Prevalence of Opisthorchis viverrini infection among villagers harboring Opisthorchis-like eggs. Southeast Asian J Trop Med Public Health 2001, 32(Suppl 2):229–236.

14. Chai JY, Park JH, Han ET, GuK SM, Shin EH, Lin A, Kim JL, Sohn WM, Yong TS, Eom KS, Min DY, Hwang EH, Phommasack B, Rim HJ: Mixed infections with Opisthorchis viverrini and intestinal flukes in residents of Vientiane Municipality and Saravane Province in Laos. J Helminthol 2005, 79:283–289.

15. Chai JF, Yong TS, Eom KS, Min DY, Jeon HK, Kim TY, Jung BK, Siabath L, Insisiengmay S, Phommasack B, Rim HJ: Hyperendemicity of Haplorchis taichui infection among riparian people in Saravane and Champasak province, Lao PDR. Korean J Parasitol 2013, 51:305–311.

16. Rim HJ, Sohn WM, Yong TS, Eom KS, Chai JY, Min DY, Lee SH, Hoang EH, Phommasack B, Insisiengmay S: Fishborne trematode metacercariae in Luang Prabang, Khammouane, and Saravane Province, Lao PDR. Korean J Parasitol 2013, 51:107–114.

17. Kaewkes S, Ellins DB, Sithithaworn P, Haswell-Elkins MR: Comparative studies on the morphology of the eggs of Opisthorchis viverrini and lecithodendrid trematodes. Southeast Asian J Trop Med Public Health 1991, 22:623–630.

18. Sreekumar C, Hill DE, Miska KB, Via-VX MC, Yan L, Myers R, Dubey JP: Genotyping and detection of multiple infections of Taenia solium gondii using Pyrosequencing, Int J Parasitol 2005, 35:991–999.

19. Stenovold CR, Traub RJ, von Samson-Himmelstjerna G, Jessupgaard C, Nielsen HV, Thompson RC: Blastocystis: subtyping isolates using pyrosequencing technology. Exp Parasitol 2007, 116:111–119.

20. Stenovold CR, Lebbad M, Verweij JJ, Jessupgaard C, von Samson-Himmelstjerna G, Nielsen SS, Nielsen HV: Identification and delineation of members of the Entamoeba complex by pyrosequencing. Mol Cell Probes 2010, 24:403–406.

21. Lulitanond V, Intapan PM, Tantrawatpan C, Sankuntav N, Sanpoo O, Janwan P, Maleewong W: Molecular markers for detection and differentiation of Plasmodium falciparum and Plasmodium vivax in human blood samples by pyrosequencing. J Clin Microbiol 2012, 50:1465–1467.

22. Tantrawatpan C, Intapan PM, Janwan P, Sanpoo O, Lulitanond V, Srichantaratamee A, Ananam W, Maleewong W: Molecular identification of Paragonimus species by DNA pyrosequencing technology. Parasitol Int 2013, 62:341–345.

23. Sadaow L, Tantrawatpan C, Intapan PM, Lulitanond V, Boonmars T, Morakote N, Pozio E, Maleewong W: Molecular differentiation of Trichinella spiralis, T. pseudospiralis, T. papuae and T. zimbabwensis by pyrosequencing. J Helminthol 2013, 131:1–6.
24. Chai JY, Darwin Murrell K, Lymbery AJ: Fish-borne parasitic zoonoses: status and issues. Int J Parasitol 2005, 35:1233–1254.

25. Sayasone S, Vonghajack Y, Vanmany M, Rasphone O, Tesana S, Utzinger J, Akkhavong K, Odermatt P: Diversity of human intestinal helminthiasis in Lao PDR. Trans R Soc Trop Med Hyg 2009, 103:247–254.

26. De NV, Le TH: Human infections of fish-borne trematodes in Vietnam: prevalence and molecular specific identification at an endemic commune in Nam Dinh province. Exp Parasitol 2011, 129:355–361.

27. Le TH, Van De N, Blair D, Sithithaworn P, McManus DP: Clonorchis sinensis and Opisthorchis viverrini: development of a mitochondrial-based multiplex PCR for their identification and discrimination. Exp Parasitol 2006, 112:109–114.

28. Sanpool O, Intapan PM, Thanchomnang T, Janwan P, Lulitanond V, Doanh PN, Van Hien H, Dung Do T, Maleewong W, Nawa Y: Rapid detection and differentiation of Clonorchis sinensis and Opisthorchis viverrini eggs in human fecal samples using a duplex real-time fluorescence resonance energy transfer PCR and melting curve analysis. Parasitol Res 2012, 111:99–96.

29. Wongratanacheewin S, Pumidonming W, Sermswan RW, Maleewong W: Development of a PCR-based method for the detection of Opisthorchis viverrini in experimentally infected hamsters. Parasitology 2001, 122:175–180.

30. Rahman SM, Bae YM, Hong ST, Choi MH: Early detection and estimation of infection burden by real-time PCR in rats experimentally infected with Clonorchis sinensis. Parasitol Res 2011, 109:297–303.

31. Intapan PM, Thanchomnang T, Lulitanond V, Pongsakulchoti P, Maleewong W: Rapid molecular detection of Opisthorchis viverrini in human fecal samples by real-time polymerase chain reaction. Ann Trop Med Pub Health 2009, 81:917–920.