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Identification of fish-borne trematode DNA in cyprinoid fish using molecular detection

Wu Zhiliang[1], Ratchadawan Aukkanimart[2,3,4,5], Thidarut Boonmars[3,4,5,*], Viengxay Vanisaveth[6], Aung Phyo Wai[2], Amkha Sanephonasa[6], Sakhone Laymanivong[7]

[1] Department of Parasitology, Faculty of Medicine, Gifu University,Gifu, Japan
[2] Department of Parasitology, Faculty of Medicine, Khon Kaen University,Khon Kaen 40002, Thailand.
[3] Neglected, zoonosis, and vector-borne disease group, Faculty of Medicine, Khon Kaen University, Khon Kaen 40002, Thailand
[4] Liver Fluke and Cholangiocarcinoma Research Center, Khon Kaen University, Khon Kaen 40002, Thailand.
[5] Rajamangala University of Technology Isan Sakornnakhon Campus, Sakornnakhon 47150 Thailand
[6] Deputy chief Laboratory of Centre of Malariology, Parasitology, and Entomology, Ministry of Health, Lao PDR
[7] Faculty of Medicine Technology, University of Health Science, Lao PDR
*Corresponding author: bthida@kku.ac.th

Abstract

Human liver and minute intestinal flukes are highly prevalent in Southeast Asia. In cases of mixed infections found in a single cyprinoid fish, the metacercariae are necessary in order to identify the morphology. This requires the fish to be digested, which is a process comprising of many steps while taking an entire day to finish. It also necessitates experienced laboratory technicians to identify them under a microscope. As a result, this study attempted to use PCR assays targeting the ITS2 regions to identify and differentiate among species. The length of the PCR amplicons of Opisthorchis viverrini Centrocestus sp and Haplorchis taichui were 380 bp and 530 bp, respectively, for ITS2. Moreover, using the ITS2 primers for preliminary analysis, we were able to detect at least 0.5 metacercariae in 40 mg of fish. This suggests that molecular detection of fish-borne trematode DNA in fish may be useful in identifying fish for quarantine.

Keywords: PCR, DNA, metacercaria, fish, trematodes

1. Introduction

Fish-borne trematode infections have led to trematodes becoming a major public health problem worldwide. With over 40 million people infected, these infections are among the most prevalent neglected tropical diseases. Most of these infections are in Southeast Asia [1], especially northeast Thailand 75% trematode infected people are infected by liver fluke. *Opisthorchis viverrini* and intestinal flukes such as *Haplorchis taichui* and *Centrocestus* sp. are widespread in humans and infections are generally caused by ingestion of raw or undercooked cyprinoid fish, which is popular in this region [2, 3, 4 & 5]. Metacercariae (flukes at the infective stage) are in the meat, gills, fins, head and scales of cyprinoid fish [6 & 7]. In our field study, we observed fish with a clear oval of color at the scale surface visible to the naked eye (submitted elsewhere). We found trematode metacercariae in the young cyprinoid fish, which had been obtained from fish farms and natural reservoirs. This study is the first to report on fish-borne trematode (FBT) (*O. viverrini*, *H. taichui* and *Centrocestus*) DNA detection in cyprinoid fish using ITS2 primers, a process that may be useful in identifying fish for quarantine.

2. Materials and Methods

2.1. Cyprinoids

The cyprinoids used in this study were obtained from fish farms in Khon Kaen and Maha Sarakham provinces (Figure 1) and a local market which captures fish from freshwater reservoirs in Khon Kaen province. All cyprinoid fish were divided into 2 groups: uninfected and infected, by visual detection of metacercariae at the scales and DNA detection using PCR.

2.2. Visual metacercaria detection

Fish from both natural freshwater reservoirs and farms were placed on a table in the sunlight. Fish scales which displayed small oval capsule-like shapes were collected and examined for metacercariae under a microscope (Figure 2). Fish without visible metacercariae on the scales were used for DNA extraction and detection of FBT DNA by ITS2 primers using PCR.

Figure 1 Cyprinoid fish used in this study

Figure 2 Infected scales of a cyprinoid fish (A) as observed by the naked eye sunlight (B). Arrows indicate metacercariae in gray spots.

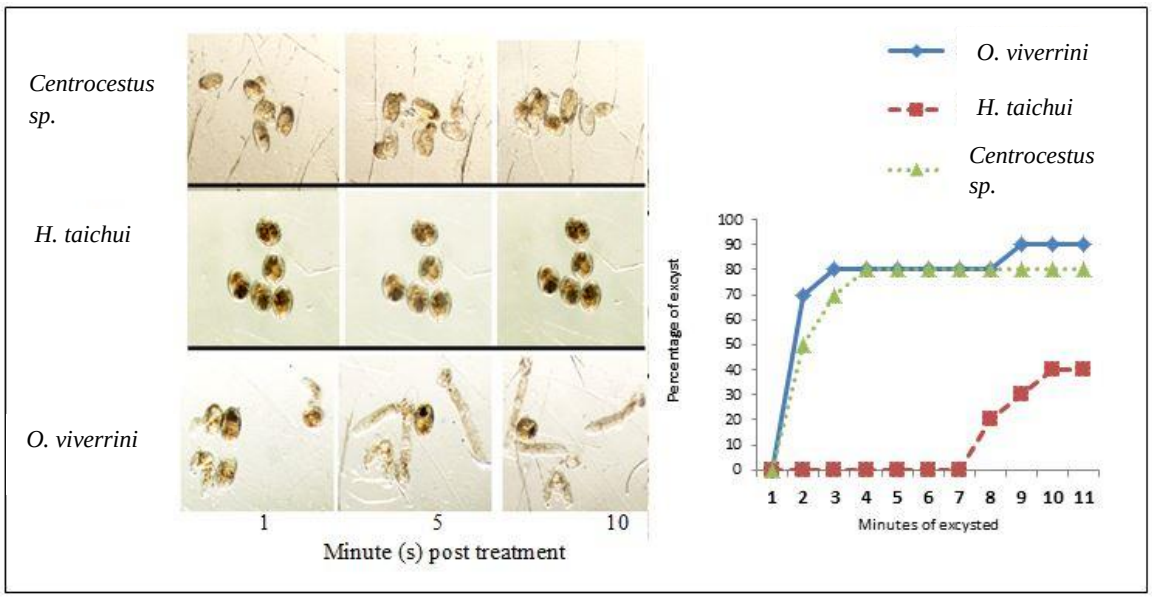

Figure 3 Metacercariae excystation in KOH pH 9 at various time points

2.3. Collection of metacercariae

Metacercariae of *O. viverrini*, *H. taichui* and *Centrocestus* sp. were obtained from naturally-infected cyprinoid fish captured from a freshwater reservoir in an endemic area of Khon Kaen Province in Northeast Thailand. Fish were minced and digested with pepsin-HCl, filtered and then washed with normal saline until clear. Metacercariae of fish-borne trematode (*O. viverrini*, *H. taichui* and *Centrocestus* sp.) were identified based on the metacercaria key under a dissecting light microscope. The optimum conditions for metacercaria excystation were created using KOH solution at pH 9. Approximately 80% of Metacercariae of *O. viverrini*, and *Centrocestus* sp. excysted within the 3 minutes and approximately 20-40% of *H. taichui* metacercariae had excysted at 7 minutes (Figure 3).

2.4. Adult worm preparation

To confirm the results of metacercaria identification, mice were infected with *H. taichui* or *Centrocestus* sp. metacercariae and 10 days thereafter, were euthanized for collection of the adult flukes. In the case of *O. viverrini* metacercariae, hamsters euthanized and the adult worms were collected 30 days after infection**.** The adult worms of each type were used as a positive control for morphological identification and DNA extraction.

2.5. DNA extraction

Fifty metacercariae or a single adult worm each of *O. viverrini*, *H. taichui* and *Centrocestus* sp. or fish were used for DNA extraction. Fifty metacercariae or adult worms were ground and incubated in 60 ^{0}C in proteinase K - TE buffer for 1 hr, protein precipitated using phenol and chloroform, and DNA precipitated using 0.01 v/v sodium acetate and 2.5v/v absolute ethanol. The DNA pellet was dissolved and kept at -20 ^{o} C until used.
For DNA extraction from individual metacercariae, each metacercaria from each trematode was broken using a pin under microscopy and then immediately used for a DNA template. DNA was then amplified directly using conventional PCR.

2.6. Polymerase chain reaction for detection of FBT metacercaria DNA

A total of 10 µl of master mix composed of 1 µl of DNA, 1 µl of 0.1mM dNTPs, 1 µl of 2.5 mM $MgCl_2$, 1 µl of 1xPCR buffer , 1 µM of forward primer, 1 µM of reverse primer , 0.02 unit/ µl RBC Taq Polymerase 0.04 µl, and 4 µl of deionized water. PCR analysis was performed using a PCR instrument (C1000^{TM} Thermalcycler, BIO-RAD).

2.7. Primers for polymerase chain reaction

Opisthorchis viverrini, *Haplorchis taichui* and *Centrocestus* sp**.** primers were ITS2-F: 5_-CTT GAACGC ACA TTG CGG CCA TGG G-3 and ITS2-R: 5_-GCG GGT AAT CACGTC TGA GCC GAG G-3, of which the product lengths were 380 bp, 530 bp, and 380 respectively (Sato *et al*, 2009).

3. Results

3.1. Detection of FBT DNA from metacerceria and adult flukes using ITS II primers

DNA of *O. viverrini*, *H. taichui*, and *Centrocestus* sp**.** collected from metacercaria and adult worms were used for testing the primers. The results agreed with a previous report, which found the single bands of *O. viverrini* and *Centrocestus* sp. at 380 and *H. taichui* at 530 bp. Moreover, our study found the amplicon of *Centrocestus* sp. at 380 bp, which was also observed in *O. viverrini* (Figure 4).

3.2. Detection of FBT DNA in fish using ITS II primers

O. viverrini, *H. taichui* and *Centrocestus* sp**.** DNA were each mixed with fish DNA and the ITS II primers were used in a PCR reaction. The result found the single bands of *O. viverrini* and *Centrocestus* sp at 380 bp and *H. taichui* at 530 bp, which was similar to the positive control (Figure 5). However only *O. viverrini* DNA was mixed with uninfected fish DNA at various concentrations (1:70, 1:140, 1:210, 1:240, and 1:350 v/v). The results showed that the single band of OV DNA (380 bp) was observable a concentration of at least 0.015 µg or about ½ of a metacercaria 30 mg of fish, or at a concentration of 25.5 µg / µl, as shown in Figure 6.

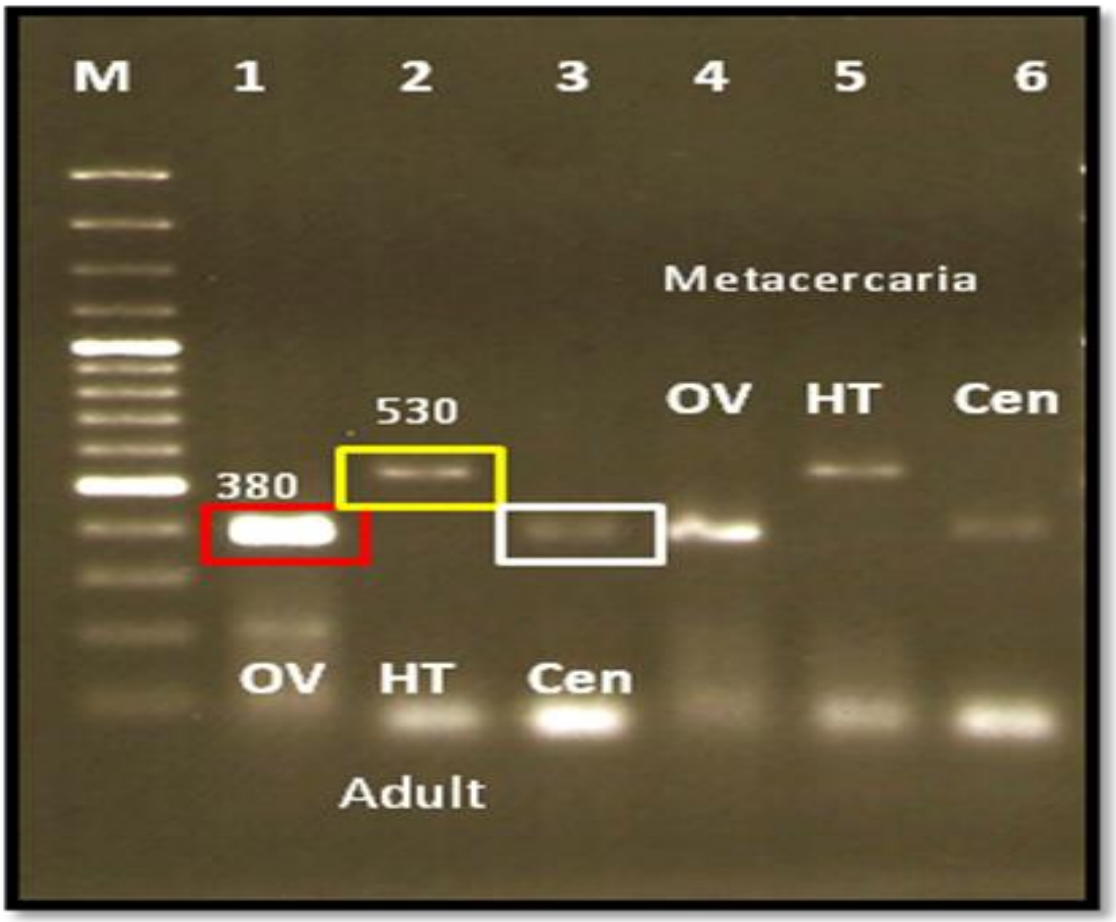

Figure 4 Metacercaria and adult DNA detection using ITS2 primers. OV, *Opisthorchis viverrini* DNA; HT, *Haplochis taichui* DNA; Cen, *Centrocestrus* sp. DNA

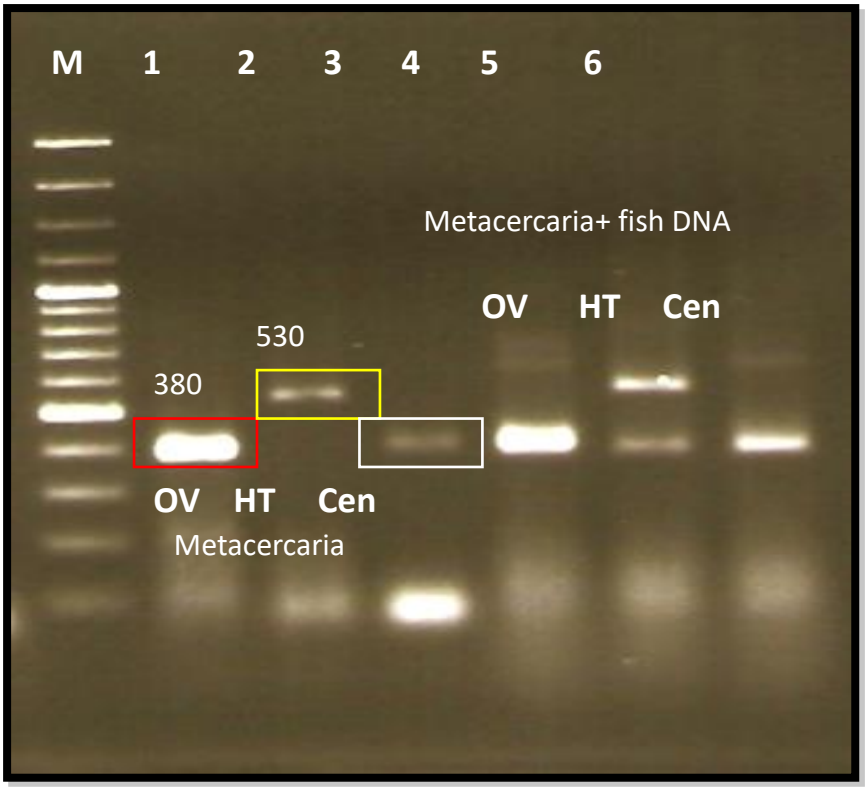

Figure 5 Fish-borne trematode DNA detection using ITS2 primers. OV, *Opisthorchis viverrini* DNA; HT, *Haplochis taichui* DNA; Cen, *Centrocestrus* sp. DNA

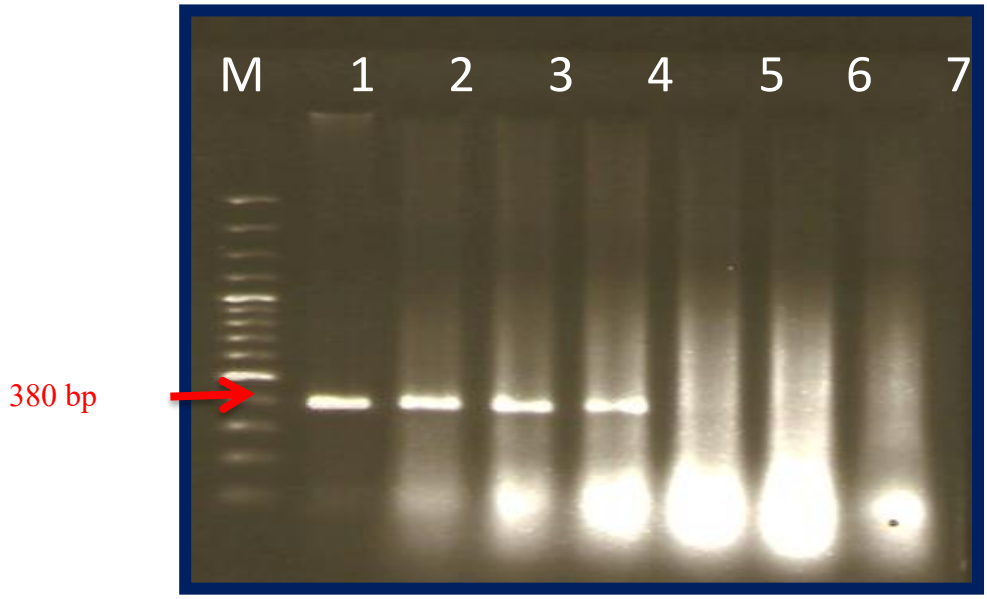

Figure 6 The quantity of *Opisthorchis viverrini* (OV) DNA detection in fish DNA using conventional PCR
Lane1= Positive control (adult OV DNA)
Lane2= OV Metacercaria DNA + fish DNA (1:70)
Lane3= OV Metacercaria DNA + fish DNA (1:140)
Lane4= OV Metacercaria DNA + fish DNA (1:210)
Lane5= OV Metacercaria DNA + fish DNA (1:280)
Lane6= OV Metacercaria DNA + fish DNA (1:350)
Lane7= Negative control (fish DNA)
fish DNA conc. 8.5 µg/ µl
meta-OV DNA 0.06 µg/ µl

4. Discussion and conclusion

FBT are medically important around the world. This is especially true of *C. sinensis* - which can be found in Korean, Chinese and Vietnamese populations who eat raw cyprinoid fish - and *O. viverrini*, which can be found in Thai, Laos and Cambodian populations. Generally, detection of eggs in feces is the most common method for diagnosing infection. The collection of cyprinoid fish and observation and identification of metacercariae under a microscope are necessary to detect infection by fish-borne trematodes in fish. Our present results have shown that fish-borne trematodes could detected simply by using visual detection and molecular examination. Visual detection of FBT metacecariae is convenient in that there is no equipment necessary and no additional costs involved. However, this method is limited in that it cannot detect light infections or metacercaria-infected muscle. Therefore, the detection of FBT DNA in cyprinoid fish by ITS2 primers using PCR demonstrated in this study may be more useful for fish quarantine in fish farms or exports. All FBT DNA was able to be detected using these primers even through the product sizes were quite similar. The PCR technique is a common and acceptable technique for detecting DNA and has many applications [8], [9], [10], [11] & [12]. ITS1 and ITS2 were chosen based on the mitochondria DNA [13] that can be used to distinguish between *O. viverrini* and *H. taichui* [9] & [14]. However, our present study shows that these primers could not be used to distinguish between *O. viverrini* and *Centrocestus* sp, as shown in Figures 4 and 5. However, this present study suggests that visual detection is useful for early detection and can be employed by consumers of cyprinoids. Furthermore, molecular detection trematode DNA in fish is useful in identifying fish for quarantine or in fish farms to control and reduce fish-borne trematode infection.

5. Acknowledgements

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