PREVALENCE OF TREMATODE LARVAE IN INTERMEDIATE HOSTS: SNAILS AND FISH IN KO AE SUB-DISTRICT OF KHUEANG NAI, UBON RATCHATHANI PROVINCE, THAILAND

Jittiyawadee Sripa[1], Nadda Kiatsopit[2] and Supawadee Piratae[3]

[1]College of Medicine and Public Health, Ubon Ratchathani University, Ubon Ratchathani; [2]Department of Parasitology, Faculty of Medicine, Khon Kaen University, Khon Kaen; [3]Faculty of Veterinary Sciences, Mahasarakham University, Mahasarakham, Thailand

Abstract. Ko Ae Sub-district of Khueang Nai, Ubon Ratchathani Province, Thailand is located in an endemic area for *Opisthorchis viverrini* and other fish-borne zoonotic trematodes (FZT) infection. This study shows the status in Ko Ae Sub-district of FZT infection based on availability of intermediate hosts and necessary requirements for the transmission of FZT. A cross-sectional survey of intermediate hosts of FZT, including *Bithynia siamensis goniomphalos* and cyprinoid fish, was conducted from April 2013 to December 2014. Examination of 1,000 snails revealed 3.4% were infected with trematode cercariae, with a density of infection greater than 100 cercariae per infected snail. Six groups of morphologically-distinguishable trematode cercariae were identified, namely, cystophorous, echinostome, furcocercous, mutabile, parapleurolophocercous, and xiphidio, the latter being the most predominant type. Among 250 cyprinoid fish samples with metacercariae present at their caudal fins and examined for FZT by pepsin digestion, metacercariae of *Haplorchis taichui*, *H. pumilio*, and *Centrocestus formosanus* were found. Unidentified metacercariae collected from fish caudal fins were subsequently shown using a PCR-based assay to be *C. formosanus*. No infection by *O. viverrini* in the intermediate hosts, *Bithynia siamensis goniomphalos* and cyprinoid fish was evident. The study provides new information regarding trematode larvae infection in the primary and secondary intermediate hosts of FZT in this area of Thailand.

Keywords: *Bithynia siamensis goniomphalos*, cyprinoid fish, fish-borne zoonotic trematode, prevalence, rural Thailand

INTRODUCTION

Northeast Thailand is an endemic area for *Opisthorchis viverrini* and other fish-borne zoonotic trematodes (FZT) infection both in habitants and domestic animals. Several factors, including geographical location, optimal climate, poor sanitation, and availability of intermediate hosts are causes of high prevalence and transmission (Sithithaworn *et al*, 2012).

Owing to the consumption of raw fish, poor sanitation, and a wide distribu-

Correspondence: Jittiyawadee Sripa, College of Medicine and Public Health, Ubon Ratchathani University, Ubon Ratchathani 34190, Thailand. Tel: +66 (0) 45 353900 ext 5859; Fax: +66 (0) 45 353901
E-mail: jittiyawadee.s@ubu.ac.th

tion of freshwater snails, *B. s. goniomphalos* and cyprinoid fish in this part of Thailand are key factors, which enhance transmission of *O. viverrini* and other FZT (Pinlaor *et al,* 2013; Wang *et al,* 2015). Surveys conducted in endemic areas for *O. viverrini* and other FZT to clarify the status of trematode larvae infection in intermediate hosts detected various species of trematode larvae, including non-opisthorchiid and lecithodendrid, in *B. s. goniomphalos* and cyprinoid fish collected from several provinces in northeast Thailand (Pinlaor *et al,* 2013; Namsanor *et al,* 2015). In addition, co-infection with *O. viverrini* was reported (Wongsawad *et al,* 2012). Several surveys reported virgulate cercariae as the common infections in *B. s. goniomphalos* while the prevalence of snails infected with opisthorchiid cercariae is usually relatively low (Sri-Aroon *et al,* 2005; Kiatsopit *et al,* 2015; Namsanor *et al,* 2015).

In the case of trematode metacercariae, a high prevalence of opisthorchiid metacercariae was usually found in wild fish caught in the endemic area (Pinlaor *et al,* 2013). This finding is at odds contrasted with results from fish from aquaculture farms, which showed a high prevalence of other types of FZT metacercariae, such as *Centrocestus formosanus* and *Haplorchis taichui* (Pitaksakulrat *et al,* 2013). Thus, the prevalence of FZT infections can vary in different endemic areas. Monitoring risk areas for FZT infections by means of determining the prevalence of FZT larvae in intermediate hosts becomes of importance.

Although extensive studies were undertaken in many endemic areas of Northeast Thailand, some areas have been neglected. For instance, Ubon Ratchathani Province located in the lower part of the northeast, was shown to be endemic for FZT infection due to a high prevalence of opisthorchiasis and incidence of cholangiocarcinoma (Tungtrongchitr *et al,* 2007; Manwong *et al,* 2013), and while a high number of infected patients were identified in many districts of the province, including Khueang Nai District, there is a lack of data on factors responsible for transmission of FZT in the area.

This study investigated the status of FZT infection in intermediate hosts, including *B. s. goniomphalos* and cyprinoid fish, in the Ko Ae Sub-district by means a cross-sectional survey. It is anticipated that the epidemiological evidence provided by this study will provide important information regarding FZT transmission and be the first step in an estimation of the transmission capacity of FZT in this area of Thailand.

MATERIALS AND METHODS

Location of collection sites

Ko Ae Sub-district of Khueang Nai, Ubon Ratchatthani Province was chosen as the study area of FZT transmission. Ten locations in rice fields, canals, and ponds used by humans as sources of food and water were chosen as sampling sites for *B. s. goniomphalos*. The locations were as follows: 15º 22′ 02.81″N, 104º 36′ 50.50″E; 15º 21′ 59.56″N, 104º 36′ 42.65″E; 15º 21′ 51.47″N, 104º 36′ 57.84″E; 15º 21′ 48.69″N, 104º 36′ 22.56″E; 15º 21′ 36.88″N, 104º 36′ 37.47″E; 15º 21′ 39.30″N, 104º 37′ 34.49″E; 15º 21′ 29.32″N, 104º 36′ 50.83″E; 15º 22′ 00.62″N, 104º 36′ 50.23″E; 15º 21′ 34.00″N, 104º 37′ 10.60″E; and 15º 21′ 25.91″N, 104º 37′ 29.28″E. Sample collection was carried out from April 2013 to December 2014. Freshwater snails of species *B. s. goniomphalos* were collected every two months by two trained persons using counts per minute of the time sampling method (Olivier and Schneiderman, 1956). Snails

were collected by hand scooping every 10 minutes for 1 hour at each sampling site. Snails were cleaned and washed with chlorine-free tap water to remove mud and plant materials, dried and kept in a container protected from light before being taken to the College of Medicine and Public Health, Ubon Ratchathani University for investigation.

Screening snails for cercariae

Infection of trematode cercariae in snails was examined using a shedding method (Kaewkes *et al*, 2012a). Each snail was placed in a small cup (3 cm in diameter and 2.5 cm in high) containing chlorine-free tap water, which was covered with a perforated lid and kept in the dark overnight. At 08:00 AM each snail was exposed to a strong artificial illumination for 5 hours to stimulate cercariae shedding and individually examined for trematode infection under a stereo microscope. Morphology of cercariae stained with 1% iodine was recorded using a digital camera fitted to the light microscope for morphological characterization (Frandsen and Christensen, 1984). Cercariae were identified at the family level and, in some cases, at genus level. The number of positive snails was recorded and percent infected snails calculated.

Cyprinoid fish collection

Cyprinoid fish were randomly collected from natural ponds and canals in the rice fields of Ko Ae Sub-district from April 2013 to December 2014. All fish samples were transferred on ice to the Tropical Disease Research Laboratory, Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand for trematode metacercariae screening at the caudal fin under a light microscope and subsequently for pepsin digestion (Srisawangwong *et al*, 1997). A total of 250 cyprinoid fish with metacercariae at their caudal fins were identified for species and measured for body length, width and weight. The fish were grouped according to species before being subjected to enzyme digestion. Trematode metacercariae were identified under a stereo microscope based on morphological criteria (Thu *et al*, 2007). Infection intensity was calculated by counting the number of metacercariae per fish species.

Identification of unidentified metacercariae and metacercariae in fish caudal fins by quantitative (q)PCR

A pool of unidentified metacercariae collected from 20 cyprinoid fish caudal fins of a particular species were subjected to genomic DNA extraction using a phenol/chloroform method (Pitaksakulrat *et al*, 2013). Genomic DNA of unidentified metacercariae was used as template for assay by qPCR. PCR primers specific for *O. viverrini* were OV-F (5′-CAGTGAGTGTCTATTGGCTAA-3′) and OV-R (5′-GTACTACTCATAAGGTTGCGT-3′), generating an amplicon of 162 bp (Sermswan *et al*, 1991). Primers for *H. taichui* were LC1 (5′-CGAGTATCGATGAAGAACGCAGC-3′) and HT4sp (5′-GTGCACAAAGAATTGCATGG 3′) (amplicon of 558 bp), and for *C. formosanus* LC1 and Centsp (5′-CCAATGCCGAGATCACAGACAAG-3′) (amplicon of 367 bp) (Pitaksakulrat *et al*, 2013). PCR mixture of 20 μl contained 1 μl of genomic DNA (1:10 dilution of test, and of *H. taichui*, and *C. formosanus* as internal positive controls), 10 μl of 2X Maxima SYBR Green/ROX qPCR master mix (Thermoscientific, Rockford, IL), 0.4 μl (10 pmol) of each primer, and 7.2 μl of distilled H_2O. Thermocycling was performed in a LightCycler480 II® instrument (Roche, Basel, Switzerland) as follows: 95ºC for 30 seconds; followed by

40 cycles of 95ºC for 5 seconds and 60ºC (for *O. viverrini*) or 55ºC (for *H. taichui* and *C. formosanus*) for 20 seconds. In order to verify specificity of PCR amplification, a melting curve was constructed using the following program: 95ºC for 30 seconds, 65ºC for 15 seconds, followed by a continuous increase to 95ºC. The melting temperature (Tm) of each amplicon was analysed using a LightCycler480 gene scanning software (Roche, Basel, Switzerland). Genomic DNA of *O. viverrini*, *H. taichui*, and *C. formosanus* metacercariae were included as positive controls. Deionized water was included as a negative control. The generation of amplicons were confirmed by agarose gel-electrophoresis. Each assay was conducted in triplicate. Result is considered negative when c_T is > 33.

RESULTS

Identification of trematode cercariae

A total of 1,000 *B. s. goniomphalos* samples were collected from rice fields, canals, and ponds throughout Ko Ae Subdistrict. Of these, 34 snails (3.4%) were infected with cercariae, 41% by xiphidio cercariae (large, medium, small, and black virgulate types), 26% by furcocercous cercariae, 12% by parapleurolophocercous cercariae, 9% by cystophorous cercariae, 9% by echinostome cercariae, and 3% by mutabile cecariae. All infected snails had an intensity of cercaria infection of > 100 cercariae per snail but no snail was infected by *O. viverrini* cercariae (Fig 1).

Identification of trematode metacercariae

A total of 250 fish samples consisting of 10 species of cyprinoid fish (Table 1) were found to have metacercariae at their caudal fins. All fish samples were subjected to pepsin digestion and then identification of trematode metacercariae. Similar species of trematode metacercariae were found in different species of fish. The most common trematode metacercariae found in this area were *C. formosanus*, *H. pumilio*, and *H. taichui* with prevalence of 25%, 9% and 1%, respectively. The most common fish species in this area were *Puntioplites falcifer*, *Cyclocheilichthys enoplos*, *C. apogon*, and *Hampala dispar* and were infected by FZT, including *C. formosanus*, *H. pumilio*, and *H. taichui* with different metacercaria burdens, with *H. dispar* being the highest. However, 64% of the metacercariae could not be identified by their morphology (Fig 2).

Identification of metacercariae by qPCR

Positive results by qPCR were obtained only from using primer pair specific for *C. formosanus* in both unidentified metacercariae and those from three fish caudal fins. Tm of *C. formosanus* amplicon was 79.1- 80.4ºC. Gel-electrophoresis of *C. formosanus* amplicon revealed the expected size of 367 bp (Fig 3).

DISCUSSION

The results of this survey confirmed Ko Ae Sub-district of Khueang Nai, Ubon Ratchatthani province located in the lower part of Northeastern Thailand as an endemic region for FZT infection. FZT was shown to maintain a complete life cycle in this area. The contamination of feces in natural water reservoirs before distribution to paddy rice fields was considered to be the origin of FZT transmission (Kaewkes *et al*, 2012b).

FZT larvae found in snails and fish from Ko Ae Sub-district included *H. taichui*, *H. pumilio* and *C. formosanus* but not *O. viverrini*. These flukes are readily transmitted to habitants due to reports of the local inhabitants behavior of eating raw fish (Suwannahitatorn *et al*, 2013).

Table 1
Prevalence of fish-borne trematode metacercariae in cyprinoid fish from Ko Ae Sub-district, Khueang Nai District, Ubon Ratchathani Province, Thailand.

Fish species	Number of fish examined	Width		Length		Number of metacercariae recovered per host (*N*)[a]	Number of metacercariae classified according to morphology (% prevalence)[b]				Total number of metacercariae (%)[c]
		Min (cm)	Max (cm)	Min (cm)	Max (cm)		*H. taichui*	*H. pumilio*	*C. formosanus*	Unidentified	
Puntioplites falcifer	91	4.5	9	11	21	0.37 (34)	-	10 (29)	9 (26)	15 (44)	34 (15)
Soldier river barb (*Cyclocheilichthys enoplos*)	63	3	7	14.5	24.5	1.59 (100)	2 (2)	8 (8)	26 (26)	64 (64)	100 (43)
Beardless barb (*Cyclocheilichthys apogon*)	55	4	5	14	15.5	0.69 (38)	1 (3)	1 (3)	15 (39)	21 (55)	38 (16)
Eye-spot barb, Spotted Hampala barb (*Hampala dispar*)	23	3	5	12	20	0.04 (23)	-	2 (9)	6 (26)	15 (65)	23 (10)
Epalzeorhynchos chrysophekadion	1	5.5		20		11 (11)	-	-	1 (9)	10 (91)	11 (5)
Java barb, Silver barb (*Barbonymus gonionotus*)	7	2	3	7.5	9.5	1.86 (13)	-	-	1 (8)	12 (92)	13 (6)
Puntius brevis	7	2	3	10	12.5	0.43 (3)	-	-	-	3 (100)	3 (1)
Red tailed tinfoil (*Barbonymus altus*)	1	4.5		13		6 (6)	-	-	-	6 (100)	6 (3)
Siamese mud carp (*Henicorhynchus simensis*)	2	2.3	2.8	10.5	12.5	1.5 (3)	-	-	-	3 (100)	3 (1)
Total	250	-	-	-	-	0.92 (231)	3 (1)	21 (9)	58 (25)	149 (64)	231

[a]Total number of recovered metacercariae. [b]No *O. viverrini* detected. [c]% of total recovered metacercariae.

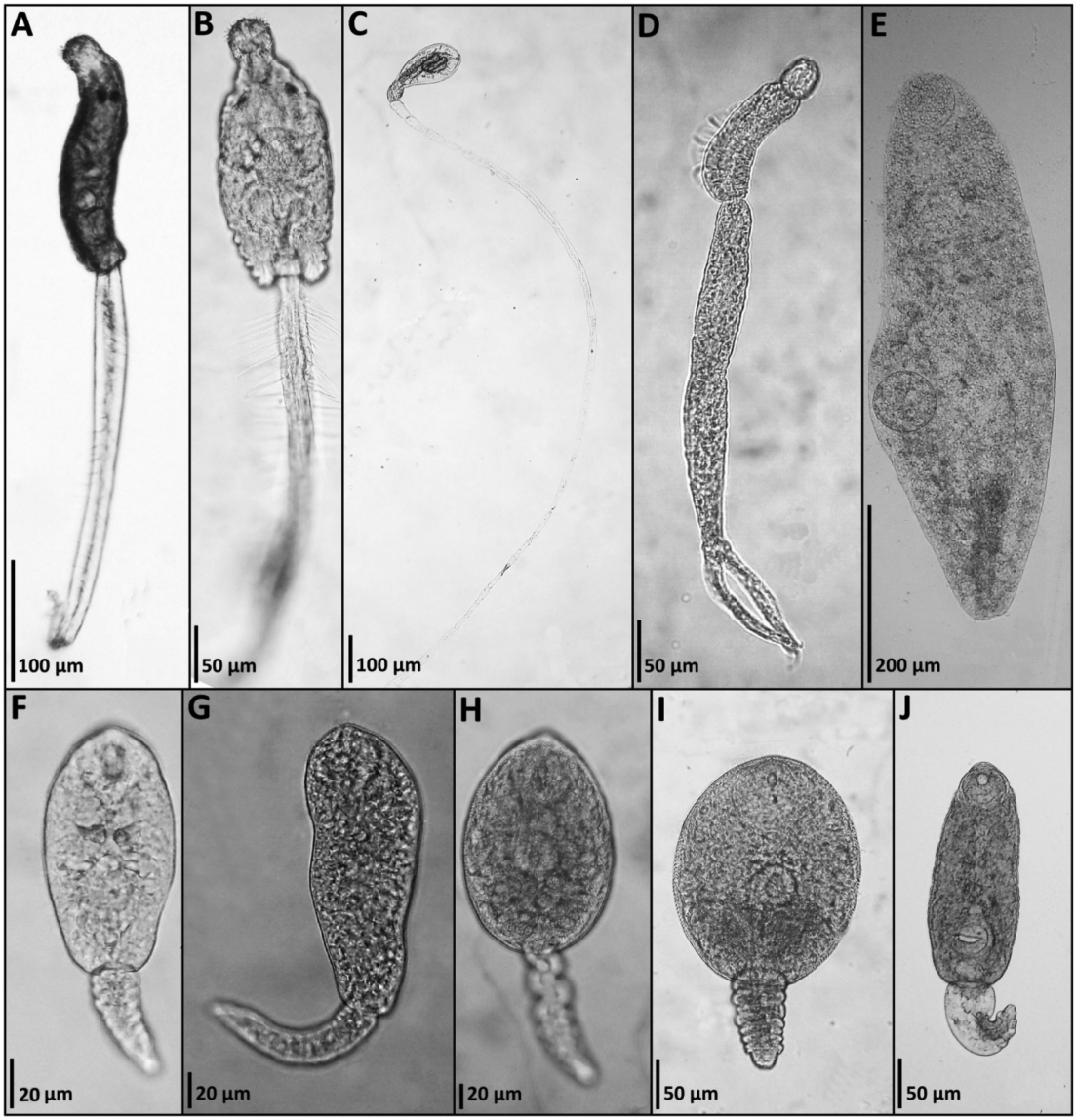

Fig 1–Cercariae from snail *B. s. goniomphalos* collected from Ko Ae Sub-district, Khueang Nai District, Ubon Ratchathani Province, Thailand. (A and B), parapleurolophocercous cercaria. (C) cystophorous cercaria. (D) furcocercous cercaria. (E) mutabile cercaria. (F-I) xiphidiocercaria, virgulate types. (J) small echinostome cercaria.

Although these flukes do not cause serious symptoms, *H. taichui* has been identified as a pathogenic agent in cases of irritable bowel syndrome (Sukontason *et al*, 2005; Watthanakulpanich *et al*, 2010). In addition, presence of the zoonotic trematode, *C. formosanus*, is becoming an important public health issue (Hung *et al*, 2013). Mixed infections by *C. formosanus* with *O. viverrini* and *H. taichui* were reported in people who frequently consume uncooked freshwater fish from endemic areas (Chai

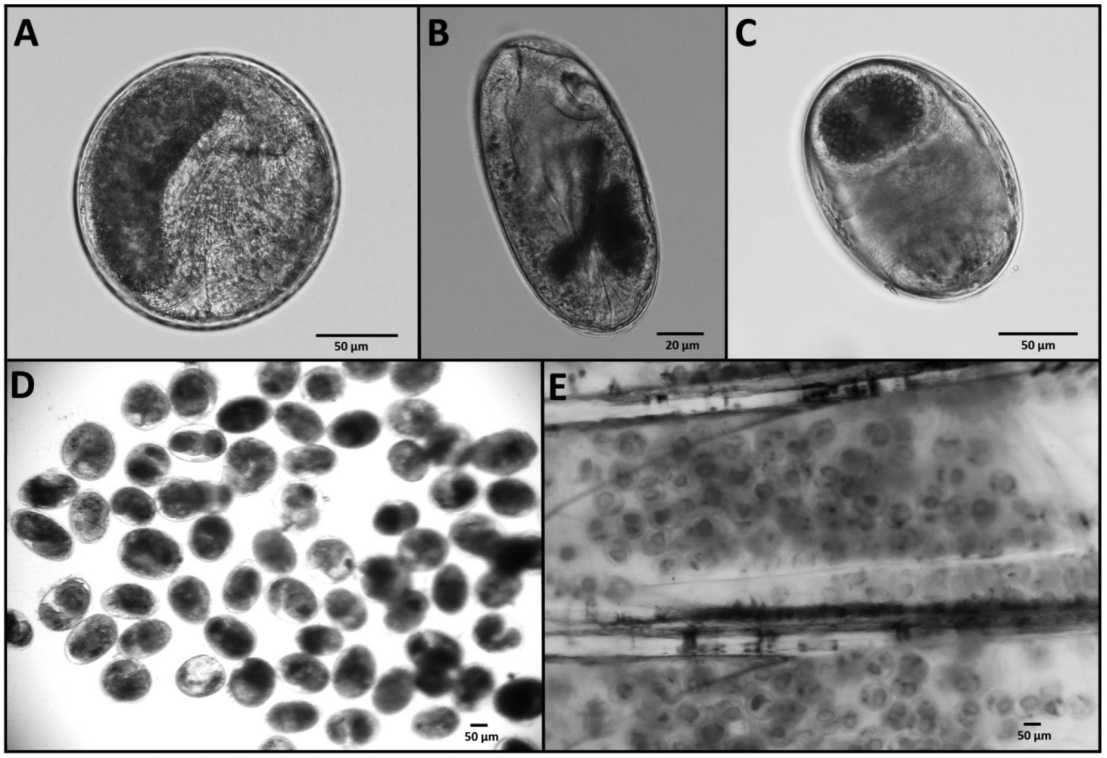

Fig 2–Trematode metacercariae infecting cyprinoid fish caudal fins from Ko Ae Sub-district, Khueang Nai District, Ubon Ratchathani Province, Thailand. (A) *H. taichui*. (B) *C. formosanus*. (C) *H. pumilio*. (D) unidentified metacercariae. (E) cyprinoid fish infected caudal fin.

et al, 2013). The discovery of infection and transmission of *H. taichui* and *C. formosanus* in intermediate hosts in this region should raise concern, and monitoring of infection in habitants and animals needs to be considered.

Several species of trematode cercariae reported in other parts of Thailand were also found in this survey. Echinostome cercariae, commonly infecting *Lymnea* spp (Joe *et al*, 1973), were found in *B. s. goniomphalos* from Ko Ae Sub-district. Low host specificity of *Echinostome* spp and high susceptibility to parasitic infection of *B. s. goniomphalos* may increase the infection rate of *Echinostome* spp in these snails. However, there is no infection of echinostome metacercariae in cyprinoid fish collected from the same area. Poor diagnostic characteristics and/or difficulties in identification may have been the cause of the loss of identification of certain echinostome metacercariae (Kostadinova *et al*, 2003). Use of RAPD-PCR has improved detection of echinostome metacercariae in intermediate hosts from Ko Ae Sub-district (Noikong and Wongsawad, 2014). Moreover, low host specificity of echinostome cercariae may increase the number of secondary intermediate hosts. Additional intermediate hosts, such as gastropods, snails, crustaceans, fish, and amphibians, should be collected from this area and examined for infection by *Echinostome* spp (Toledo and Fried, 2005). Ready extensive spread of *Echinostome* spp

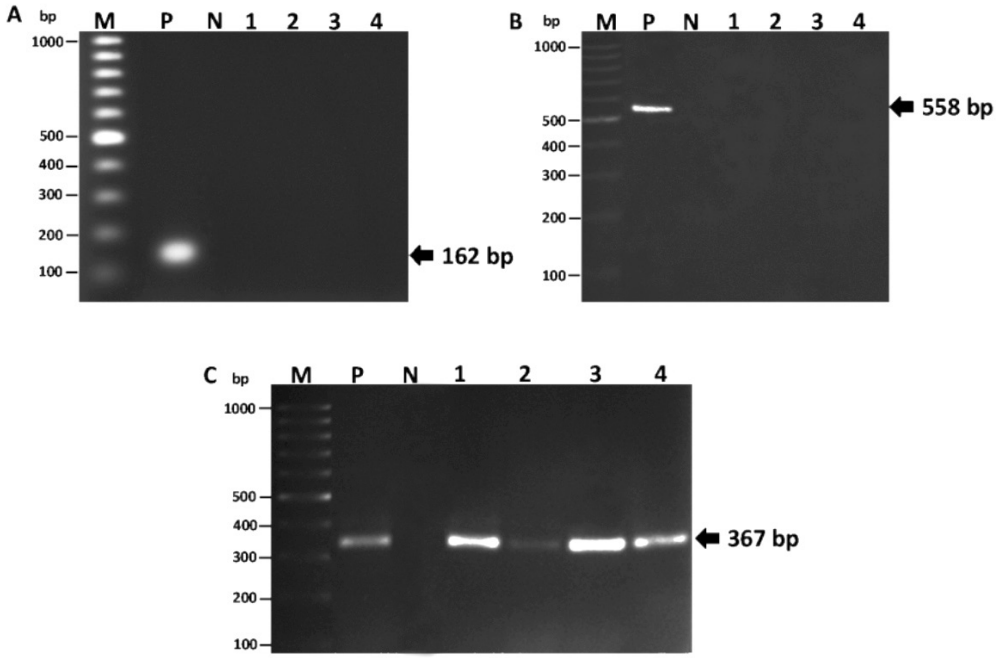

Fig 3–Amplicons of morphologically unidentified metacercariae analyzed by1.5% agarose gel-electrophoresis and visualized by ethidium bromide staining. Genomic DNA was PCR amplified using primers specific for (A) *O. viverrini*, (B) *H. taichui* and (C) *C. formosanus*. Lane M, DNA size markers; lane P, positive control of *O. viverrini* metacercarial DNA (panel A), *H. taichui* metacercarial DNA (panel B) and *C. formosanus* (panel C); lane N, negative control containing de-ionized water; lanes 1-4, unidentified metacercarial DNA from snail and cyprinoid fish infected caudal fin.

infection should be of concern as their low host specificity together with poor sanitation of inhabitants have been reported (Graczyk and Fried, 1998). Although mild symptoms are typical of *Echinostome* spp infection, the social and economic costs of morbidity from such infections need to be taken into consideration (Chai, 2009).

In this survey, infection of FZT cercariae including several animal trematode larvae was examined in *B. s. goniomphalos*, the specific intermediate host of *O. viverrini*. Several studies have reported infection of animal trematodes larvae, xiphidiocercariae, and furcocercous cercariae in diverse species of snails collected from various parts of Thailand (Mard-arhin *et al*, 2001; Dechruksa *et al*, 2007; Ukong *et al*, 2007; Chontananarth and Wongsawad, 2013). These animal trematodes are less of a medical concern for humans but are of significance in veterinary medicine (Frandsen *et al*, 1984).

This study investigated FZT metacercariae present at fish caudal fins using a screening method which reduces cost and time by means of enzyme digestion. However, this method is unable to identify all species of trematode metacercariae due to atypical morphology. SYBR green-based qPCR with specific primers was found to be successful in the identification of these previously unidentifiable metcercariae, allowing *C. formosanus* metacercariae to be

identified as being common in cyprinoid fish from this area. Although success in the use of conventional PCR in metacercaria identification were reported, SYBR green-based qPCR is still an attractive technique due to its sensitivity, specificity, and economy of time (Pitaksakulrat *et al*, 2013). However, although less sensitive and specific than hybridization-based qPCR, SYBR green-based qPCR is sufficient to identify trematode metacercarie (Fernandez *et al*, 2006).

In conclusion, the study shows that the status of FZT infection in freshwater snails and cyprinoid fish is important in monitoring human and veterinary health in Ko Ae Sub-district of Khueang Nai, Ubon Ratchatthani Province. There was no infection of liver fluke, *O. viverrini*, but data collected provided important information on the ecology and parasitic infestations associated with the health of local inhabitants and mammal animals. However, the information gained was dependent on a variety of existing factors and monitoring of the status of parasitic infection in known and other susceptible hosts in this area should be performed regularly.

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