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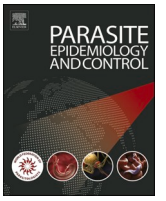

Hight prevalent of *Opisthorchis viverrini* infection and coincident *Haplorchis* metacercariae in cyprinid fishes in upper northeastern region of Thailand

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ABSTRACT

Cyprinid fishes are commonly acted as second intermediate hosts of *Opisthorchis viverrini* and *Haplorchis* spp. This research investigated the prevalence and intensity of both parasites in cyprinid species. This process is regularly used as a guideline for disease surveillance and as a preventive measure in the Upper Northeastern region of Thailand. Fish samples were collected from both lentic and lotic water sources between November 2020 and October 2021, and classified them by their species. Acid pepsin-HCL solution was used to digest the individual samples and create a build-up of precipitate in order to view metacercariae using a stereomicroscope. A total of 3583 fish were caught and classified into 23 species. Out of these fish, 5.49% of them were found *O. viverrini*, 26.54% *Haplorchis taichui*, 10.04% *Haplorchis pumilio*, and 9.29% *Haplorchoides* sp. Ten species of fish were observed to have been infected with *O. viverrini*. Amongst the collected fish, *M. obtusirostris* had the highest prevalence of infection at 52.94%, while *B. gonionotus* had only 1.71%. The prevalence of *O. viverrini* infection was highest at 27.41% in Udon Thani Province. No prevalence of *O. viverrini* infection was observed in Bueng Kan Province. High prevalence of *O. viverrini* infection in cyprinid fishes was found in both lentic and lotic water sources in almost all provinces in the region. The prevalence of infection and intensity depended on the fish species and fish habitats.

1. Introduction

The disease causes by liver fluke (*Opisthorchis viverrini*) infection has been and continued to be one of the public health challenges in Southeast Asia. It has been reported that more than 10 million people are infected with this liver fluke, including Thais, Laotians, Vietnamese and Cambodians. In Thailand, the disease is most widespread in Northeastern Thailand (Andrewa et al., 2008; Sripa et al., 2015; Sripa and Echaubard, 2017). The disease is caused by the local traditional food consuming of raw cyprinid fishes that carry the infective larvae, metacercariae, which infests the human bile ducts. Liver fluke can live inside a human bile ducts for 20 years or until the host dies. Chronic infection can lead to Cholangiocarcinoma, which is one of the most common causes of death of people in the endemic areas (Sripa et al., 2007). According to Sithithaworn et al. (2012), the current prevalence of opisthorchiasis and clonorchiasis

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in people in Mekong River Basin is 60.8%, 29.5%, 24.7%, 20.0%, 13.8%, 8.8%, and 5.2% in the provinces of Nakhon Phanom, Mukdahan, Nong Bua Lamphu, Sakon Nakhon, Udon Thani, Nong Khai, and Loei, respectively. All these provinces are located in the Upper Northeastern Thailand, which is considered to be a highly endemic area. This study investigated cyprinid fishes, which are the main source of protein for people in Northeastern Thailand. People or animals consuming raw cyprinid fishes carrying the infective larvae, metacercariae then can get the disease (Boonchot and Wongsawad, 2005). Species of cyprinid fishes most commonly found infective larvae are *Cirrhinus jullieni, Cyclocheilichthys apogon, C. armatus, C. reason, Hampala dispa, Puntius gonionotus, P. leiacanthus, P. orphoides, P. partipentazona, Mystacoleucus atridoralia* and *Anematichthys repasson* (Sithithaworn et al., 1997; Waikagul, 1998; Pinlaor et al., 2013; Laoprom et al., 2021). It was also found that different fish were affected by different types of liver fluke. People in the endemic areas consuming raw fish such as *Koi Pla, Laab Pla, Pla Som, Pla Jom,* and *Pla Ra* can get the infection (Pumidonming et al., 2018).

Hence, a study of different species of cyprinid fishes infected with liver fluke (*O. viverrini*) and knowledge about the percentage of infection can be used as a guideline for disease surveillance and as a preventive measure in the endemic areas of both lentic and lotic water sources in the Upper Northeastern region of Thailand.

2. Material and methods

2.1. Ethic approval

The study protocols and design were approved by the Institutional Animal Care and Use Committee of Rajamangala University of Technology Isan, based on the Ethics of Animal Experimentation of National Council Research of Thailand (Approval ID proposal 5/2565).

2.2. Study areas and sample collection

The samples (cyprinid fishes) were collected from lentic water sources such as a pond, or a dam reservoir in the upper Northeastern region of Thailand, or from lotic water sources such as Maekhong River, Songkhram River, or Loei River. The samples were collected between November 2020 and October 2021. The area of study included the provinces of Nakhon Phanom, Mukdahan, Loei, Sakon Nakhon, Nong Bua Lamphu, Nong Khai, Udon Thani and Bueng Kan (Fig. 1). Five districts were chosen within each province for sample collection. One kilogram of fish was collected by fishermen from 40 stations each and the collection was included fish ports and markets. The samples were stored in an ice box. The samples were classified in Department of Fisheries Laboratory, Faculty of Natural Resources, Rajamangala University of Technology Isan, Sakon Nakhon Campus.

2.3. Classifying fish types and collecting metacercariae from cyprinid fishes

The fish samples were taken out of the ice box and cleaned. Then, the samples were classified by species according to Taki (1974); Rainboth (1996); and FishBase[1] guidelines. Metacercariae was then collected by putting individual fish into a blender and blended it then adding a 0.25% pepsin-1.5% HCl solution with a ratio of 1:1 to digest the samples. The samples were then put inside a shaking water bath, at the temperature of 37 degrees Celsius for one hour. The samples were subsequently sieved using 1200, 300, 250, 106 μm mesh, respectively. Then, 0.85% NaCl solution was used to wash out the precipitates that could pass through the 106 μm mesh. The sediments were precipitated in a sedimentation jar by adding 0.85% NaCl and leaving it for 5–10 min.

The precipitates were filtered again and a stereomicroscope used to view metacercariae. A compound microscope to was used to identify the types of worms based on its morphology, such as the size of the cyst, suckers, and excretory bladder (Vajrasthira et al., 1961; Scholz et al., 1990). The total number of fish found with metacercariae was recorded.

2.4. Data analysis

The total number of fish found with *O. viverrini* was analyzed, and the prevalence of overall fish infected with *O. viverrini*, the prevalence of each type of fish infected with *O. viverrini*, and the prevalence of fish infected with *O. viverrini* in each province in the Upper Northeastern Thailand were calculated. The evaluation of infection rate of *O. viverrini* in Cyprinid fish was calculated using:

$$\text{Prevalence of infection} = \text{number of infected fish (for each type of worm) x } 100/\text{Total of examined Fish.}$$

2.5. Statistical analysis

Data of the intensity and prevalence of metacercaria infection were presented in mean ± SD. Statistical analysis was used one-way ANOVA in SPSS 11.0 for window, SPSS Inc., Chicago, IL, USA. A *P*-value less than 0.05 was considered statistically significant.

[1] http://www.fishbase.org/home.htm

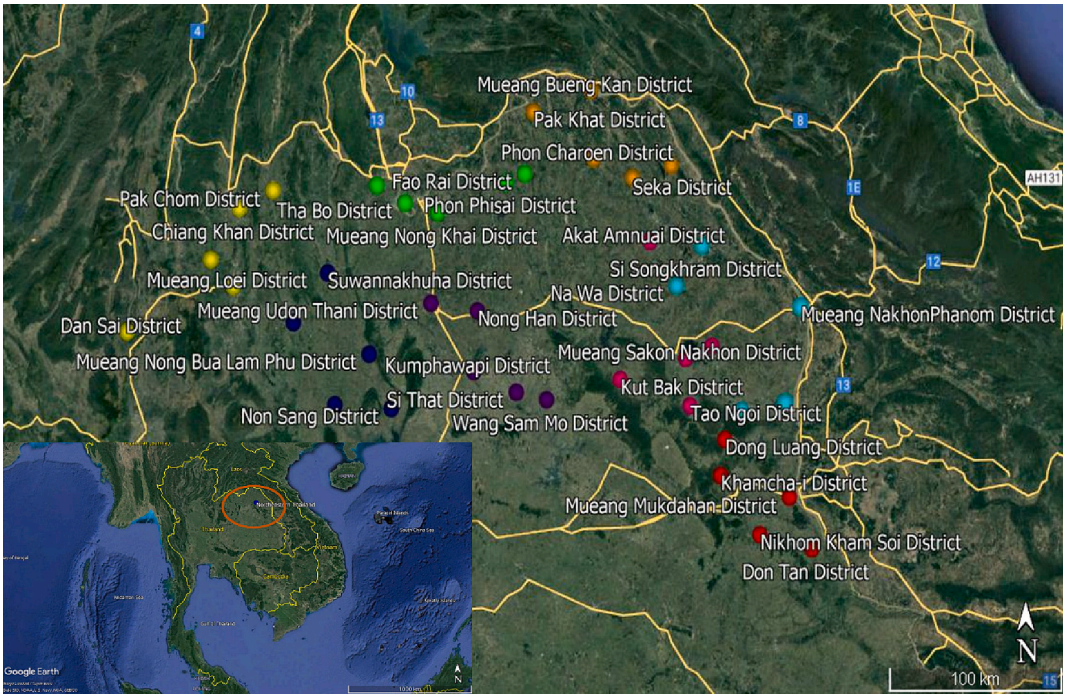

Fig. 1. Sample collection locations in eight provinces in the Upper Northeastern region of Thailand: Loei (yellow dots), Nong Khai (green dots), Nong Bua Lamphu (Blue dots), Udon Thani (purple dots), Mukdahan (red dots), Sakon Nakhon (pink dots), Nakhon Phanom (Light blue dots), and Bueng Kan (orange dots). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3. Results

3.1. Types cyprinid fishes found of O. viverrini metacercariae in upper northeastern Thailand

This study investigated *O. viverrini* metacercariae in cyprinid fishes in Upper Northeastern Thailand. A total of 3583 fish were collected and classified into 23 species including, *Henicorhynchus siamensis, Puntioplites waandersi, Hampala dispar, Cyclocheilichthys repasson, Osteochilus hasselti, Paralaubuca typus, Paralaubuca riveroi, Barbonymus gonionotus, Barbodes altus, Puntius brevis, Labiobarbus siamensis, Morulous chrysophykadian, Probarbus jullieni, Osteochilus melanopleura, Rasbora daniconius, Clupeichthys aesarnensis, Mystacoleucus obtusirostris, Barbodes schwanefeldi, Amblyrhynchichthys truncates, mystacoleucus marginatus, Cyclocheilichthys enoplos, Hypsibarbus wetmorei* and *Tor douronensis* (Table 1). It was found that 1841 fish tested positive for metacercariae, overall prevalence of 51.38%. Metacercariae larvae is classified into 4 types or into 2 groups, which are *Haplorchis taichui, Haplorchis pumilio, Haplorchides* sp. and liver fluke of *Opisthorchis viverrrini* metacercariae (Fig. 2). It was found that 197 fish were infected with *O. viverrrini* (5.49%), 951 fish were infected with *H. taichui* (26.54%), 360 fish were infected with *H. pumilio* (10.04%) and 333 fish were infected with *Haplorchides* sp. (9.29%) (Table 1). In addition, the most fish were infected with *C. aesarnensis* (88.23%), followed by *M. marginatus* (73.33%), *P. jullieni* (71.42%), *H. wetmorei* (71.42%) and *A. truncates* (44.44%) (Table 1).

The evaluation of the infective stage of *O. viverrini* in cyprinid fishes revealed 10 different types of metacercariae in the sample fish including *H. siamensis*, *P. waandersi*, *H. dispar*, *C. repasson*, *O. hasselti*, *B. gonionotus*, *B. altus*, *L. siamensis, O. melanopleura* and *M. obtusirostris* (Fig. 3). The results showed that fish infected with *O. viverrini* metacercariae the most was *M. obtusirostris* at 52.94% ($n = 17$), followed by *O. melanopleura* at 43.47% ($n = 23$), *H. siamensis* at 13.21% ($n = 401$), *B. altus* at 11.14% ($n = 305$), *C. repasson* at 6.09% ($n = 476$), *O. hasselti* at 5.06% ($n = 395$), *L. siamensis* at 5.04% ($n = 218$), *H. dispar* at 3.58% ($n = 391$), *P. waandersi* at 1.89% ($n = 528$), and *B. gonionotus* at 1.71% ($n = 407$) (Table 1).

3.2. Percentage of fish which tested positive for O. viverrini for each province in upper northeastern Thailand

The percentage of the fish which tested positive for liver fluke at metacercariae stage was the highest in Udon Thani, accounting for 27.41%, of all districts including Mueang Udon Thani District, Kumphawapi District, Wang Sam Mho District, Sri That District, and Nong Han District. The types of fish were *H. siamensis, O. hasselti, H. dispar, P. waandersi* and *B. altus.* The second highest percentage of the which fish tested positive for *O. viverrine* metacercariae was found in Sakon Nakhon, accounting for 17.77%. The types of fish were *H. siamensis and C. repasson.* In Nong Bua Lamphu, the fish which tested positive for *O. viverrine* metacercariae were *H. siamensis, L. siamensis, O. hasselti* and *B. gonionotus* (17.26%). In Nakon Phanom, the fish which tested positive for *O. viverrine* metacercariae were *B. altus* and *O. melanopleura* (14.72%). In Loei, the fish which tested positive for *O. viverrine* metacercariae were *C. repasson, H.*

Table 1
Prevalence of *Opisthorchis viverrini* and *Haplorchis* sp. in Upper Northeastern Thailand.

Fish Types	Total fish samples	Prevalence	Prevalence each type of Trematodes			
			Haplorchis taichui	*Haplorchis pumilio*	*Haplorchoides* sp.	*Opisthorchis viverrini*
Henicorhynchus siamensis	401	207(51.62)	75(18.70)	48(11.97)	31(7.73)	53(13.21)
Puntioplites waandersi	528	252(47.72)	116(21.96)	60(11.36)	66(12.50)	10(1.89)
Hampala dispar	391	183(46.80)	110(28.13)	35(8.95)	24(6.13)	14(3.58)
Cyclocheilichthys repasson	476	216(45.37)	107(22.47)	38(7.98)	42(8.82)	29(6.09)
Osteochilus hasselti	395	194(49.11)	114(28.86)	41(10.37)	19(4.81)	20(5.06)
Paralaubuca typus	15	10(66.66)	6(40.00)	4(26.66)	0(0.00)	0(0.00)
Paralaubuca riveroi	69	42(60.86)	29(42.02)	7(10.14)	6(8.69)	0(0.00)
Barbonymus gonionotus	407	206(50.61)	135(33.16)	48(11.79)	16(3.93)	7(1.71)
Barbodes altus	305	171(56.06)	77(25.24)	35(11.47)	25(8.19)	34(11.14)
Puntius brevis	50	33(66.00)	14(28.00)	5(10.00)	14(18.00)	0(0.00)
Labiobarbus siamensis	218	115(52.75)	58(26.60)	17(7.79)	29(13.30)	11(5.04)
Morulous chrysophykadian	66	39(59.09)	24(36.36)	4(6.06)	11(16.66)	0(0.00)
Probarbus jullieni	14	10(71.42)	10(71.42)	0(0.00)	0(0.00)	0(0.00)
Osteochilus melanopleura	23	16(69.56)	3(13.04)	0(0.00)	3(13.04)	10(43.47)
Rasbora daniconius	58	42(72.41)	24(41.37)	5(8.62)	13(22.41)	0(0.00)
Clupeichthys aesarnensis	17	15(88.23)	15(88.23)	0(0.00)	0(0.00)	0(0.00)
Mystacoleucus obtusirostris	17	12(70.58)	0(0.00)	0(0.00)	3(17.64)	9(52.94)
Barbodes schwanefeldi	20	6(30.00)	4(20.00)	2(10.00)	0(0.00)	0(0.00)
Amblyrhynchichthys truncatus	9	4(44.44)	3(33.33)	0(0.00)	1(11.11)	0(0.00)
mystacoleucus marginatus	30	22(73.33)	9(30.00)	0(0.00)	13(43.33)	0(0.00)
Cyclocheilichthys enoplos	45	26(57.77)	12(26.66)	7(15.55)	7(15.55)	0(0.00)
Hypsibarbus wetmorei	21	15(71.42)	6(28.57)	0(0.00)	9(42.85)	0(0.00)
Tor douronensis	8	5(62.50)	0(0.00)	4(50.00)	1(12.50)	0(0.00)
Total of fish	**3583**	**1841(51.38)**	**951(26.54)**	**360(10.04)**	**333(9.29)**	**197(5.49)**

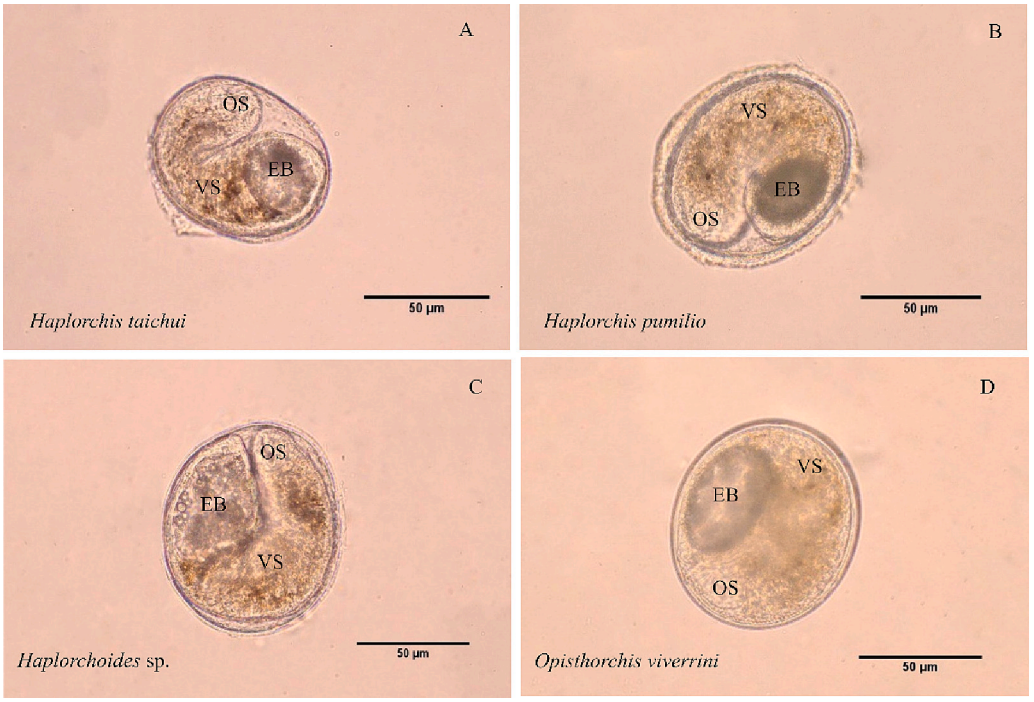

Fig. 2. Metacercariae found in cyprinid fishes in the upper northeastern Thailand: A = *Haplorchis taichui*, B = *Haplorchis pumilio*, C = *Haplorchoides* sp., D = *Opisthorchis viverrine*: OS = Oral sucker, VS = Ventral sucker, EB = Excretory bladder (All scale bar is 50 µm).

siamensis and *M. obtusirostris* (11.68%). In Mukdahan, the fish which tested positive for *O. viverrine* metacercariae were *B. altus* and *O. hasselti* (7.61%). In Nong Khai, the fish which tested positive for *O. viverrine* metacercariae was *H. dispar* (3.55%). In Bueng Kan, no fish tested positive for *O. viverrini* metacercariae.

Over a one year period, the spread of *O. viverrini* metacercariae in cyprinid fishes was found in almost all provinces in the Upper Northeastern region of Thailand (except Bueng Kan) in both lentic and lotic water sources.

Fig. 3. Types of fish which tested positive for *O. viverrini.*

4. Discussion

This study investigated *O. viverrine* metacercariae in cyprinid fishes in eight provinces in the Upper Northeastern Thailand, including Nakon Phanom, Bueng Kan, Mukdahan, Loei, Sakon Nakhon, Nong Khai, Nong Bua Lamphu, and Udon Thani. We collected 3583 fish from both lentic and lotic water sources and classified them into 23 species metacercariae was found in 1841 fish. Many of the fish were the second intermediate host. Four types of flukes were found: minute intestinal flukes including *Haplorchis taichui*, *Haplorchis pumilio*, *Haplorchides* sp. and liver fluke including *Opisthorchis viverrini* metacercariae. This result is consistent with the report of infection in cyprinid fishes in Northeastern Thailand nearby Mekong Basin areas and a study on the prevalence of *O. viverrini* in Southeast Asia (Pinlaor et al., 2013; Prasongwatana et al., 2013; Dao et al., 2017; Myint et al., 2020; Laoprom et al., 2021) and minute intestinal flukes including *H. taichui, H. pumilio* and *Haplorchoides* sp. (Srisawangwong et al., 1997; Boonchot and Wongsawad, 2005; Myint et al., 2020). Liver flukes can cause diseases in human (Sithithaworn et al., 2012; Pinlaor et al., 2013) and animals such as dogs (0.37%, $n = 821$) and cats (35.51%, $n = 214$) (Aunpromma et al., 2012). Intestinal fluke can cause fewer diseases in humans compared to liver flukes or lung flukes (Sohn et al., 2014). In this study, the infection rate was the highest in *H. taichui* (26.54%) and lowest in *O. viverrini* (5.49%). This could be due to a lack of fish infected with liver flukes in the water sources from which the samples were obtained. This was consistent with the classification of metacercariae from cyprinid fishes studied in Khon Kaen where *H. taichui* metacercariae was found the most prevalent, followed by *O. viverrini* (Srisawangwong et al., 1997). In addition, Nithiuthai et al. (2002) reported the infection of metacercariae in cyprinid fishes in rivers in Nakon Ratchasima. They found metacercariae as high as 51.9% (*Haplochinae* spp. and *Heterophyidae* spp.). However, they did not find *O. viverrini.* This was different from the study of cyprinid fishes by Pinlaor et al. (2013) that found *O. viverrini* in Nakon Ratchasima, Northeastern Thailand.

Our study found high prevalence of *O. viverrini* metacercariae in 10 different types of cyprinid fishes including *H. siamensis*, *P. waandersi*, *H. dispar*, *C. repasson*, *O. hasselti*, *B. gonionotus*, *B. altus*, *L. burmanicus*, *O. melanopleura* and *M. marginatus.* This is consistent with the study of Sithithaworn et al. (1997); Nithiuthai et al. (2002); Pinlaor et al. (2013); Laoprom et al. (2021). They found the infection of liver flukes including *O. hasselti*, *H. siamensis*, and *H. dispar* in cyprinid fishes in Northeastern Thailand. Our study found high prevalence of infection in cyprinid fishes living in public water bodies, wetlands, and flooded areas. The infection in each type of fish depended on the study areas where the samples were obtained from different types of fish were collected from different locations. In this study, the percentage of the fish infected with metacercariae of liver fluke was the highest in *M. obtusirostris* (52.94%). The percentage of the fish infected with liver flukes was the lowest in *B. gonionotus* (1.71%). This is different from the study of liver fluke infection in cyprinid fishes in Northeastern Thailand by Sithithaworn et al. (1997). They reported that the liver fluke infection varied with seasons. They found that the liver fluke infection was most prevalent in *P. leiacanthus* and *C. armatus* and lowest in *H. dispar.* A study conducted by Laoprom et al. (2021) revealed that the infection was highest in *H. dispar.* They reported that the severity of infection varied with seasons and types of fish. Touch et al. (2013) studied the liver fluke infection at metacercarial stage in cyprinid fishes in Southern Cambodia. They found that the percentage of liver fluke infection was 100% in *A. truncates.* Therefore, it is observed that liver fluke infection depends on locations, water sources, seasons, disease prevalence, fish types, and susceptibility of the fish. In addition, the snail hosts mediating the disease are members of the freshwater family Bithyniidae: *Bithynia siamensis siamensis* in central Thailand, *Bithynia funiculata* in northern Thailand, and *Bithynia siamensis goniomphalos* in northeast Thailand. (Brockelman et al., 1986; Burch and Upatham, 1989). The snail *Bithynia siamensis goniomphalos* is the intermediate host for the trematode *Opisthorchis viverrini* in

northeast Thailand (Wykoff et al., 1995; Brockelman et al., 1986; Burch and Upatham, 1989; Sri-Aroon et al., 2005).

In this study, liver fluke infection was found to be the most prevalent in Udon Thani (27.41%). Liver fluke was found in cyprinid fishes living in public water bodies in all studied districts. The disease was found in *H. siamensis, O. hasselti, H. dispar, P. waandersi,* and *B. altus.* This was consistent with the study on the effects of liver fluke infection on Cholangiocarcinoma by Sithithaworn et al. (2014). They found that the spread of liver fluke infection and the spread of Cholangiocarcinoma were most prevalent in Udon Thani. This study found no infection of *O. viverrini* metacercariae in cyprinid fishes in Bueng Kan (0.0%). This could have been because the water sources from which samples were collected had few fish infected with liver fluke at the infective stage. As a result, metacercariae was not found in the sample fish. This result was the same as the study conducted by Nithiuthai et al. (2002) which did not find metacercariae of liver fluke in cyprinid fishes in the water sources in Nakon Ratchasima. In addition, Sithithaworn et al. (2012) reported that the spread and prevalence of *O. viverrini* in Northeastern Thailand was the highest in Nakon Phanom, followed by Sisaket and was lowest in Nong Khai. This differed from the current study.

5. Conclusion

This study found liver fluke (*O. viverrini*) and minute intestinal flukes including *H. taichui*, *H. pumilio*, and *Haplorchides* sp. in cyprinid fishes. The spread of liver fluke at the metacercariae stage was discovered in ten different varieties of cyprinid fish over a one-year period. The spread of the disease was found in almost all investigated provinces in both lentic and lotic water sources. In Northeastern Thailand, water sources ware reservoirs for liver fluke diseases, liver diseases, and Cholangiocarcinoma. The infection rate and severity were different depending on the type of fish and their habitat.

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Declaration of competing interest

The authors declare that they have no competing interests.

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