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Epidemiology of *Opisthorchis viverrini* in an endemic area of Thailand, an integrative approach

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Summary

An integrated epidemiological study of *Opisthorchis viverrini* consisting of risk factors analysis and parasitation prevalence determination in humans, as well as the assessment the roles of host reservoirs (dogs and cats) and intermediate hosts (cyprinid fish) was carried out in the vicinity of Huay Luang dam, Udon Thani province, in the north east of Thailand. The survey was conducted from June 2014 to July 2016 in three sub-districts. Fecal samples were collected from 5,347 participants in 22 villages and the overall prevalence of *O. viverrini* was found to be 31.5 %. Risk factors for liver fluke infection were determined from questionnaires analysis which sought demographic data and information about the eating behaviors of the participants. The behavior of raw fish consumption correlated significantly with parasitation in the studied areas. The risk factors for *O. viverrini* infection in humans were associated significantly with age, education, and habitation within a two kilometer range from nearby water and food sources. Questionnaires showed that the drug treatments were not always appropriate. The stools of domestic dogs (n = 468) and cats (n = 262) collected for parasitological analysis and results showed that the zoonotic role of dogs should not be underestimated as has been done previously/in past. The incidence of *O. viverrini* metacercariae in four cyprinid fishes *Barbonymus gonionotus* (n = 124), *Cyclocheilichthys repasson* (n = 843), *Hampala dispar* (n = 276) and *Henicorhynchus siamensis* n = 946), were determined and overall values ranged from 2.4 % to 23.1 %. There was a seasonal variation in metacercariae intensity which ranged from 1 – 125 metacercariae per fish. The study concluded that transmission ecology should be taken into consideration in developing of control strategies against opisthorchiasis.

Keywords: cholangiocarcinoma; *Opisthorchis viverrini*; epidemiology; reservoirs; intermediate host

Introduction

Chronic infection caused by the Southeast Asian liver fluke (*Opisthorchis viverrini*) is a critical risk factor for the development of the bile duct cancer cholangiocarcinoma (CCA), what is a major health concern in South-East Asia (Thailand, Laos People's Democratic Republic, Cambodia and central Vietnam) (Sripa *et al.*, 2010; Aunpromma *et al*., 2012; Miyamoto *et al*., 2014; Kaewpitoon *et al*., 2015; Khuntikeo *et al*., 2016). In Thailand at least 6 million people are estimated to be currently infected (Andrews *et al*., 2008). *Opisthorchis viverrini* infection in humans and in dog and cat host reservoirs (Sithithaworn & Haswell-Elkins, 2003) occurs via the consumption of a raw or uncooked fish which contains infective metacercariae (Sripa *et al*., 2007; Donthaisong *et al*., 2014; Madsen *et al*., 2015; Onsurathum *et al*., 2016) In addition, fermented fish also contain high concentrations of nitrosamines that are

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known to be potent human carcinogens (Thamavit *et al*., 1993). Once metacercariae are ingested they migrate to the intrahepatic bile duct via the common bile duct and once the fluke becomes gravid (in about 30 days) the eggs are excreted by feces (Harinasuta & Harinasuta, 1984). Species diversity and the abundance of cyprinid fish, which are available all year round, are involved in the life cycle across north east Thailand and facilitate the maintenance of life cycle of this trematode. A study across 20 provinces in the north east of Thailand showed that 13 of them are tested positive for the occurrence to this parasite (Pinlaor *et al*., 2013). Despite detected overall decline in the prevalence of this helminthiasis in Thailand (from 14.7 % to 8.7 %, 1980 – 1981, Preuksaraj *et al*., 1982) the last national survey performed in 2009 (Sithithaworn *et al*., 2012), in the provinces of the north east of Thailand, detected the prevalence still as high as 50 % in numerous areas (Sithithaworn *et al*., 2012). Several reasons can at least partially explain the difficulties associated with reduction of opistorchiasis prevalence. Primarily, it is well rooted tradition of uncooked or undercooked fish (Cyprinidae) consumption (Prasongwatana *et al*, 2013). Another strongly held belief in the north east Thailand culture that contributes to the maintenance of high prevalence of this trematode is that drinking of strong alcohol can kill the worms (Grundy-Warr *et al*., 2012).

To our knowledge, an integrated study consisting of parasite prevalence and risk factors analysis in humans as well as the assessment the role of intermediate (cyprinid fish) and host reservoirs (dogs and cats) has never been carried out. Despite antiparasitic treatments being frequently used, which in principle can be highly effective, the prevalence and reinfection rates for this liver fluke remains still high (Saengsawang *et al*., 2016). To improve this situation a better understanding of the epidemiology of this foodborne parasite and the human factors relevant to its treatment are required to examine.

The present study aims to fill this gap by determining the distribution (prevalence and intensity) across several vertebrates that are essential to the life cycle of this trematode. We have examined areas which are endemic (the north east of Thailand) and analysed the risks of the population inhabiting this territories.

Materials and Methods

Study area and fecal sample collection

The survey was conducted in the vicinity of Huay Luang dam, the largest reservoir in the upper part of the north east of Thailand between June 2014 and July 2016. Three sub-districts (Nam Phon, Nong Bua Ban and Khok Sa-ard) of Udon Thani province, where

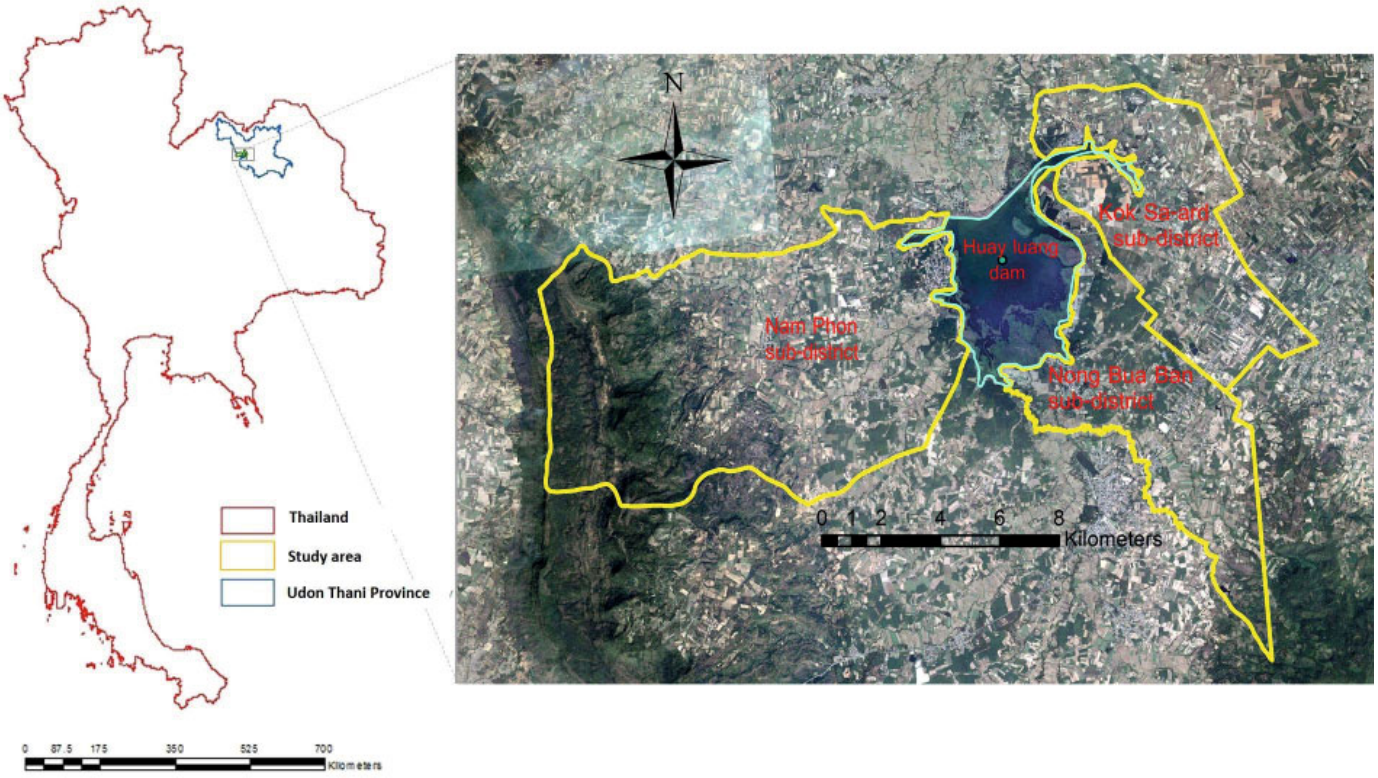

Fig. 1. Map of the surveyed area.

at least 30,000 people reside in an area of 247 km^2, were included in this study (Fig. 1). Fecal samples from 22 villages were collected from 5,347 participants and fixed in 10 % formalin before being transported to a laboratory at the Udon Thani Rajabhat University. Official letters explaining the aim of the program, the development of strategies for opisthorchiasis control were sent to the heads of villages, community leaders, school principals, the children's parents and to the individual households. Visits were made to each village to explain the benefits of the survey as well as the National Opisthorchiasis Control Programme. The study protocol was approved by the Human Ethics Committee of the Ministry of Public Health.

Risk factors for the liver fluke infection were evaluated by using questionnaires to collect demographic data such as age, sex, occupation (agriculture or public servant), monthly income (less than or greater than 10,000 bahts) and smoking habit (yes/no) together with the information about previous parasitic treatments and eating behaviours.

The stools of domestic dogs and cats were collected by confining them to the house of their owners. This particular approach allows performing the correlation between fecal sample results with the data obtained from the human occupants living in the same house. The samples were fixed in 10 % formalin and then transported to a laboratory at Udon Thani Rajabhat University for parasitological analysis. The Animal Ethics Committee of the Udonthani Rajabhat University approved this part of the study.

Modified formalin ethyl-acetate concentration technique

The quantitative formalin ethyl acetate concentration technique with slight modification was performed. One gram of feces was fixed with 10 % formalin and filtered through 2 layers of gauze after which the suspension was centrifuged at 2,500 rpm for 3 minutes (EBA20S, Hettich, Singapore). The pellet was then re-suspended in 10 ml of 10 % formalin and 3 ml of ethyl-acetate and centrifuged at 2,500 rpm for a another 3 minutes. Lipid floating on the supernatant was removed after separating it by ringing with an applicator stick from the walls of the tube. One ml of 10 % formalin was then added to the sediment and the number of eggs per gram of liver fluke infected feces was assessed individually by two experienced researchers.

Isolation of metacercariae from cyprinid fishes

Cyprinid fish traditionally caught by local people living around the Huay Luang dam were examined for the metacercariae within 24 hours after the collections. The sampling was conducted on monthly basis from February 2014 to January 2016 and the isolation of *O. viverrini* metacercariae was achieved by pepsin digestion (Pinalor *et al.*, 2013). Each whole fish was identified at the species level, weighed individually and minced by electric blender before being immersed in an aqueous solution containing 0.25 % pepsin, 1.5 % HCl and 0.85 % NaCl. The mixture was then incubated at 37 °C for 2 hours before the digested fish were filtered using 4 consecutive metal sieves (1100; 350; 250 and 140 μm

Table 1. Characteristics and prevalence of *O. viverrini* in humans.

Characteristics	Stools (n)	Positive (n)	Prevalence (%)	Average of EPG (range)
Gender				
Male	2,565	742	28.9	305.1 (12 – 3,594)*
Female	2,782	946	34.0	43.2 (7 – 160)
Age				
<25	825	61	7.4	33.6 (7 – 176)
25 - 35	1,074	385	35.8	63.1 (36 – 296)
36 – 45	1755	512	29.1	113.7 (33 – 3,594)
46 – 55	1,149	531	46.2	231.8 (24 – 1,686)*
>55	544	199	36.5	23.5 (11 – 464)
Sub-district				
Nam Phon	2,762	1,374	49.7	162.8 (7 – 3,594)*
Nong Bua Ban	1,518	221	14.5	145.3 (16 – 254)
Khok Sa-ard	1,067	93	8.7	145.3 (27 – 653)
Total	5,347	1,688	31.5	74.2 (7 – 3,594)

* – significance $p < 0.05$

Table 2. Prevalence and intensity of *O. viverrini* in dog, cat and human hosts.

Host	Stools (n)	Positive (n)	Prevalence (%)	Average EPG (range in each village)
Dogs	468	81	17.3	78.1 (21 – 169)
Cats	262	64	24.4*	145.3 (36 – 296)*
Humans	5,347	1,688	31.5**	74.2 (7 – 3,594)*
Total/Average	6,077	1,833	30.1	82.1 (7 – 3,594)

* – significant $p < 0.05$; ** – significant higher than $p < 0.05$

apertures). Filtered fish pellets were sedimented in 0.85 % NaCl in a sediment jar several times until the supernatant was clear (the details of the technique are given in (Pinlaor et al., 2013)). Metacercariae of *O. viverrini* in the sediment were identified and counted using a stereo-microscope (Motic® DMW-143, USA).

Statistical analyses

Data are presented as average infection percentages. One-way ANOVA was used to compare the infection intensity (eggs per gram – EPG) in feces and compared with infective rates of metacercariae between different species of fish. Student's t-test and chi-squared tests were used for parametric and non-parametric analysis, respectively. Pearson's correlation coefficient was used to analyse correlations between parametric data and regression analysis was performed if the data exhibited significant correlations. Odds ratios (OR) were calculated to determine the association between cross sectional data to predict possible risk factors and analysed for all other predictive variables on the basis of logistic regression analysis. Statistical analyses were performed using the SPSS version 11.5 and p values less than 0.05 were considered statistically significant.

Post-treatment

Humans as well as the host reservoirs (dogs and cats) which were found to be parasitized with *O. viverrini* were cured with praziquantel. This treatment was part of the active prevention and control program implementation performed by the Faculty of Science at the Udonthani Rajabhat University.

Results

Prevalence and intensity of O. viverrini in human and host reservoirs

A total of 5,347 participants (2,565 males and 2,782 females) representing 40 % of the population older than 20 years old were screened (range: 20 – 82 years old, see Table 1). The overall prevalence of *O. viverrini* was 31.5 % (1,688 infected). However, the egg counts were statistically significant between sexes (305.1 EPG in males versus 43.2 EPG in females). As can be seen in Table 1, an age-dependence was found for *Opisthorchis* infection where infection peaked in the 46 – 55 years age range. The highest prevalence (49.7 %) and intensity (162.8, range 7 – 3,594 EPG) was found in the Nam Phon sub-district within the Nong Wua Sor district ($p < 0.05$).

The prevalence of *O. viverrini* infection in domestic dogs (n = 468) and cats (n = 262) is presented in Table 2. The rates of liver fluke infection in cats (24.4 %) were significantly higher than in dogs (17.3 %), while the highest prevalence was found in humans (31.5 %; $p < 0.05$). The average EPG in feces collected from dogs was 78.1 (range; 21 – 169) meanwhile the cats EPG was 145.3 (range; 36 – 296), and in humans the average EPG was 74.2 (range; 7 – 3,594). Data, confirmed significantly different infection rates in humans and cats when compared to dogs ($p < 0.05$).

Risk factors for O. viverrini infection

A logistic regression analysis and the odds ratios were performed and calculated from 1,634 questionnaires where 31.5 % of participants provided also fecal samples for screening (Table 3). The habit of eating a raw fish ($p = 0.04$; OR = 3.1) was found to be important and statistically significant in the areas studied. The univariate model for risk factors in *O. viverrini* human infection were significantly linked with gender ($p = 0.067$; OR = 1.6); age ($p \leq 0.035$; OR = 2.7); education ($p \leq 0.000$; OR = 12.6), the residence within a 2 kilometers range of nearby water ($p \leq 0.000$; OR = 2.2) and food selection factors ($p = 0.041$; OR = 2.0) were found important. Relating to the drug treatments, most of infected participants lack the knowledge of praziquantel use ($p \leq 0.000$; OR = 7.4) and had no history of parasitic treatment ($p \leq 0.037$; OR = 3.3). Despite the question not being included in the questionnaires, most of the interviewed participants were aware of albendazole treatment. No statistically significant results were found within the occupation ($p = 0.16$, OR = 1.1), smoking habit ($p = 0.51$, OR = 1.0) or monthly income ($p = 0.67$, OR = 1.2) categories.

Prevalence and intensity of O. viverrini metacercaria in cyprinid fishes

Four cyprinid species, that are very common in catchments in the areas studied, were surveyed for *O. viverrin*i metacercariae: *Barbonymus gonionotus* (*Pla Ta-Pein* in Thai); *Cyclocheilichthys repasson* (*Pla Sai Ton); Hampala dispar* (*Pla Kasub Jud)* and

Table 3. Analysis of risk factors of *O. viverrini* infection in endemic areas.

Significant factors	questionnaires (n) (Total 1,634)	Infected n (%)	p value	Odd ratio (95% CI)
Gender				
Male	784	91 (11.6)	0.067	1.0
Female	850	123 (14.4)		1.6 (0.6-12.5)
Age				
> 50 years	532	51 (9.5)	0.035	1.0
< 50 years	1,102	163 (14.8)		2.7 (0.5-6.3)
Education				
2nd school and under	1,328	95 (7.1)	0.000	1.0
Higher than 2nd school	306	119 (38.8)		12.6 (3.2-18.0)
2 km close to water				
No	695	71 (10.2)	0.000	1.0
Yes	939	143 (15.2)		2.2 (0.5-6.6)
Life style and food selective vision				
by value of nutrition	612	47 (7.6)	0.041	1.0
by tradition	1,022	167 (16.3)		2.0 (0.7-8.1)
Knowledge of praziquantel treatment				
Yes	769	51 (6.6)	0.000	1.0
No	865	163 (18.8)		7.4 (1.2-14.3)
History of parasitic treatment				
Ever	738	61 (8.2)	0.037	1.0
Never	896	153 (17.0)		3.3 (0.6-13.6)
Eat raw fish in last 1 year				
Never	1,044	115 (11.0)	0.044	1.0
Often	590	99 (16.7)		3.1 (0.9-11.2)

Henicorhynchus siamensis (*Pla Kao Na*), (see details provided in Table 4). The highest infection prevalence among the four species of fish was found in *H. siamensis* (23.1 %, n = 946) and then with decreasing order in *C. repasson* (15.8 %, n = 843), *H. dispar* (5.4 %, n = 276) and *B. gonionotus* (2.4 %, n = 124). The infection intensity in each fish ranged from 1 – 125 metacercariae. Significantly higher numbers were found in *H. siamensis* and *C. repasson* when compared with *H. dispar* and *B. gonionotus* ($p < 0.05$).
Figure 2 shows that the seasonal average intensity of metacercariae infection during the January to April period was significantly higher in *H. siamensis* and *C. repasson* (infection range: 25 % – 33 %; intensity: 13 – 125 metacercariae/fish, $p < 0.05$), whereas decreased levels of infection were found during July to October period (range: 1 % – 8 %; intensity: 1 – 18 metacercariae/fish, $p < 0.05$).

Discussion

Our study suggests a well-established life cycle for *O. viverrini* in the Huay Luang dam area as it was simultaneously detected by high prevalence levels in humans, dogs, cats and cyprinid fish, each associated with considerable parasite loads.
In humans, a National survey of helminthiasis in Thailand in 2009 (Sithithaworn *et al*., 2012) showed that the north east of Thailand is an area in which *O. viverrini* is known to be endemic, where the prevalence across this region vary from 4.6 % to 60.8 %. The values reported in our recent study are higher (31.5 %) when compared with the average values found in Udon Thani Province (13.8 %) (Sithithaworn *et al*., 2012). In this study, at least 1,688 participants were infected suggesting the existence of a high risk

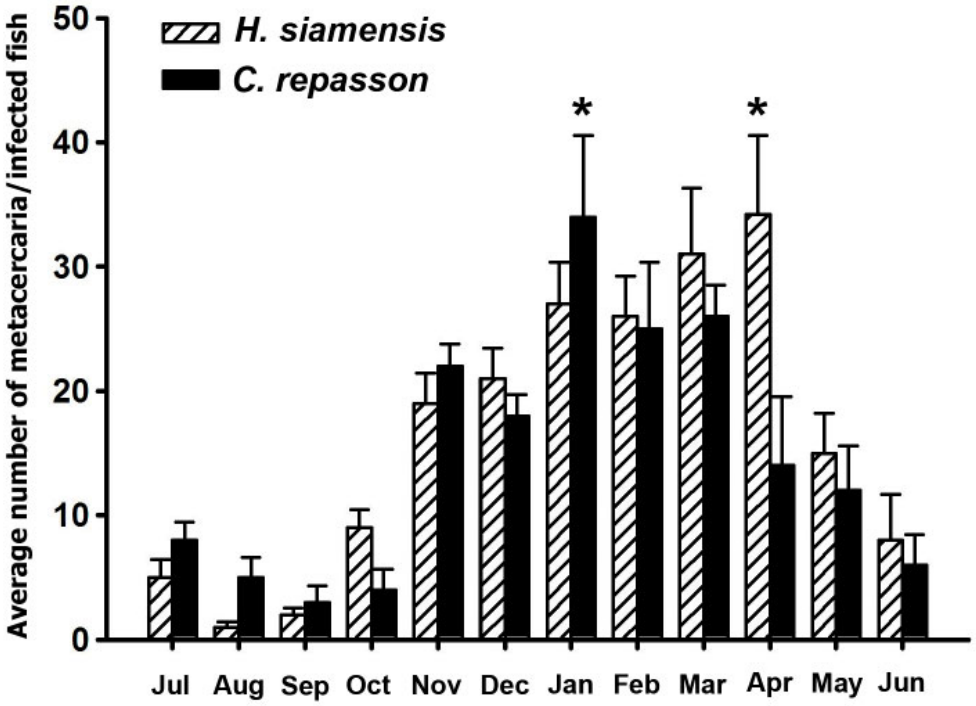

Fig. 2. Seasonal distribution of metacercariae in two fish species.
(* – significant $p < 0.05$)

area for the development of cholangiocarcinoma.

We proved that the risk factors for *O. viverrini* infection were significantly related with habits of eating raw or undercooked infected-fish (Sripa *et al*., 2007) an activity that is common in all areas in which opisthorchiasis is spread (Grundy-Warr *et al*., 2012). This habits suggest that the selection of food in this area is based on tradition and not on nutritional or other health values.

The higher parasite numbers in males than females (305.1 EPG in males versus 43.2 EPG in females) can be accounted for the significant differences in food habits between genders, where male consumption of a raw fish (*koi pla*) with alcohol drinking is associated with masculine behaviour (Grundy-Warr *et al*., 2012). This probably also explains the association with age found in the present study ($p \leq 0.035$; OR = 2.7), as compounded by the cumulative effects of the adult trematode living for over 10 years (Harinasuta & Harinasuta, 1984). The higher parasite quantities have been exacerbated by the observation that a considerable fraction of the surveyed population has never been treated with antihelminthics. As previously demonstrated all these risk factors can also be linked with the education level (Chaiputcha *et al*., 2015). Our survey indicates that a lesser education, up to the level of primary school or lower, was associated with higher risk of infection. In contrast, the education beyond secondary school level or above was a factor with significant protective outcome.

Table 4. Prevalence and intensity of *O. viverrini* metacercariae in cyprinid fishes.

Host	Number of fish	Number testing positive	Prevalence (%)	Average metacercaria/gram (range)	Average metacercaria/fish (range)
Henicorhynchus siamensis	946	219	23.1	3.1 (2 – 24)*	25.9 (1 – 125)*
Cyclocheilichthys repasson	843	134	15.8	1.9 (1 – 17)*	15.4 (2 – 33)*
Hampala dispar	276	15	5.4	0.3 (1 – 12)	2.4 (1 – 15)
Barbonymus gonionotus	124	3	2.4	ND	ND
Total/Average	2,189	371	16.9	1.7 (1 – 24)	14.3 (1 – 125)

* – significant $p < 0.05$., ND = Not determined

Fig. 3. Dogs eating fish in Huay Luay dam. A ; dogs waiting for discarded fish from the fishermen's, B ; dog eating decay fish, C ; collected fish for metacercaria detection.

Related to the environment, the population living within two kilometers of water had a closer relationship with fishing activities and consequently a higher probability of consuming parasitized fish. Additionally, the transmission from human/dog/cat feces to *Bithynia* snails inhabiting the dam should be more likely. Therefore the elimination of fecal contamination from water is imperative to interrupt the transmission of disease.

Most of the participants did not know about the praziquantel effectivity and significant success in the history of disease treatment. Despite the question not being included in the questionnaire most of the surveyed population was aware of the antihelmintic drug albendazole. The praziquantel is the only drug that has been shown to be effective against *O. viverrini* infection in the eastern Thailand where a single dose (40 mg/kg) shows 98 % efficiency rate (Pungpark, Bunnag, & Harinasuta, 1984). Albendazole is not approved for the treatment of *O. viverrini* infection by the U.S. Food and Drug Administration (CDC, 2012). These may be related to the cost of treatment. Praziquantel treatment is relatively expensive (80 bahts) in comparison to albendazole (30 bahts). A better communication between the Health Care Units and the affected communities would enhance the antihelminthic treatment and will help avoid of an inappropriate self-treatment.

As previously reported by Sithithaworn *et al.* (1997) our results indicate on a seasonal variation in the prevalence of metacercariae

(Fig. 2). Despite these differences during the year, the metacercariae concentrations were always high enough to sustain life cycle, and the probability of disease transmission to humans must be considered all year around. It should mentioned that dams have much higher probability of finding infected fish than in ponds, lakes and rivers (Pinlaor *et al*., 2013). Our results fit with results from Huay Luang dam. The infection rate varies according to fish species as well as habitats and this explains the differences in the infection prevalences and intensities observed (Pinlaor *et al*., 2013). Our results demonstrate the association between the intensity of metacercariae in fish and prevalence of flukes in humans, which can vary from one province to another. Similar features were observed also by Pinloar *et al*. (2013). Study from another Thailand area found that a reduction in the prevalence of *O. viverrini* in humans to undetectable levels reduced also the prevalence in fish to 1 % (Sripa *et al*., 2015; personal communication).

The high, statistically significant correlation between dog, cat and human *O. viverrini* infection is of particular interest. Previous survey conducted in Thailand (Aunpromma *et al*., 2012) showed that infected cats (35.51 %) represent the main reservoir in an endemic areas and dogs were well behind (prevalence = 0.37 %). Earlier studies showed similar results regarding infected cats and dogs: 22.6 % and 1.9 % respectively (Impand *et al*., 1983) and 36.4 % and 3.9 % respectively (Enes *et al*., 2010). Despite dogs usually ignoring fish scraps (Aunpromma *et al*., 2012), dogs are apparently well accustomed to fish eating in the areas we have studied (Fig. 3). Our results show that the infected dogs (17.3 %) should also be taken into consideration as a significant reservoir. The zoonotic role of dogs should not be underestimated and should be considered in disease control strategies under similar circumstances. As stated by Sripa *et al*. (2015), all aspects of the life cycle/transmission process should be properly determined in regarding the contribution of other fish eating vertebrates, but may not have been adequately determined in human-wetland complexes. Moreover, despite parasite load being lower and according to our results dogs can be good indicators for the presence of the Asiatic liver fluke in endemic areas with similar characteristics to that which we recently studied.

Our results allow us to demonstrate that the ecology of transmission between vertebrates (reservoirs as well as the intermediate hosts) is based on direct associations between the elements involved in the parasite life cycle. Consequently, measures to control opisthorchiasis should consider into account various environmental components. As regards to the administration of antihelminthics to humans the treatment is only effective in the short term. It is due to parasites survival in the environment as well as in the other hosts. The other prevention strategies and control programs should be considered to break the epidemiological cycle. Analysis of the risk factors in the villages including the people education is critical element to control and eradicate opisthorchiasis (Petney *et al*., 2013; Sripa *et al*., 2015). In conclusion, an integrative approach to *O. viverrini* infection is essential to achieve the best results on the elimination or reduction of this parasite among the population living in endemic areas.

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