Published in final edited form as:

Parasitol Int. 2017 August ; 66(4): 503–509. doi:10.1016/j.parint.2016.10.006.

Association between *Opisthorchis viverrini* and *Leptospira* spp. infection in endemic Northeast Thailand

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Abstract

Opisthorchiasis caused by *Opisthorchis viverrini* is an important foodborne trematodiasis in Thailand, Laos and Cambodia. Interestingly, the opisthorchiasis endemic region overlaps with an area of leptospirosis emergence. Here we report an association between opisthorchiasis and leptospirosis in Thailand. Of 280 sera collected from villagers living around the Lawa wetland complex in Khon Kaen province, 199 (71%) were seropositive for leptospirosis by immunochromatography. Individuals with *O. viverrini* infection had a significantly higher rate of leptospirosis than those without ($P = 0.001$). Significant higher leptospirosis prevalence was found in males than females ($P = 0.002$). However, females but not males with *O. viverrini* infection showed a significantly higher seroprevalence of leptospirosis. Twenty-one of 35 environmental samples from the lake (water, mud and fish skin mucus) were positive for *Leptospira* spp. DNA sequencing, sequence alignment, and phylogenetic analysis of some positive nested PCR products revealed both pathogenic and intermediate pathogenic strains of *Leptospira* in the samples. Strikingly, *O. viverrini* metacercariae from the fish were positive for *L. interrogans*. These results suggest a close association between opisthorchiasis and leptospirosis. Contact with water, mud or eating raw fish harboring liver fluke metacercariae may be risk factors for *Leptospira* infection.

Graphical Abstract

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Keywords

Opisthorchis viverrini; *Leptospira* spp.; leptospirosis; metacercariae; Lawa wetland

1. Introduction

Human liver fluke infection caused by *Opisthorchis viverrini* is a major waterborne/foodborne trematodiasis in Thailand, the Lao People's Democratic Republic (Lao PDR) and Cambodia with over 10 million people infected [1–3]. The parasite life cycle is complex. *O. viverrini* eggs are ingested by fresh water *Bithynia* snails and, after several developmental stages, the mature free swimming cercariae are released into the water. The cercariae infect cyprinid fish by penetration of the flesh through mucous membrane [4] where the parasite develops into an infective stage metacercariae [1]. Humans acquire the infection by eating raw or undercooked fish that harbor *O. viverrini* metacercariae, which completes the cycle. The infection is associated with several hepatobiliary complications, such as cholangitis, hepatomegaly, gallstones, periductal fibrosis, and cholangiocarcinoma–bile duct cancer [2, 5].

In Thailand, *O. viverrini* is endemic in the northern and northeastern regions of the country with an estimated 6–8 million infected [3]. These regions are also endemic for leptospirosis [6, 7], the waterborne zoonotic disease caused by spirochetal bacteria of the *Leptospira* family [8]. The common species that causes leptospirosis in Thailand is *Leptospira interrogans* [9]. Leptospirosis is commonly found in tropical and subtropical regions and has its greatest impact on public health in developing countries [8]. The infection occurs either from direct exposure to infected animals and their products or indirectly through contact with water of soil contaminated with urine of a *Leptospira* spp. shedder [9]. Leptospiral infection often has minimal or no clinical manifestations [10, 11]. The clinical features of symptomatic infection in humans vary from mild to severe including headaches, muscle pain and fever [11]. More severe clinical manifestations may involve vital organs such as the liver, lung, and kidney [8, 11].

To our knowledge there are no studies on any direct relationship between *O. viverrini* and *Leptospira* spp. Several reports suggest that *Leptospira* spp. are abundant in aquatic hosts such as freshwater fish, which might explain how the pathogens disseminate to humans [12,

13]. Human leptospirosis cases associated with the freshwater environment, especially stagnant water and fishponds, have been reported [7, 14]. Since opisthorchiasis and leptospirosis are water-borne zoonotic diseases, co-infection of the two pathogens in endemic areas is possible. Given that *O. viverrini* requires cyprinid fish as a second intermediate host, we hypothesized that the parasite cercariae may carry *Leptospira* from water or fish skin during penetration, and encyst in the flesh. *Leptospira* can enter the gastrointestinal tract and bile ducts when people eat raw fish containing the metacercariae. We recently reported the presence of leptospiral bacteria in *O. viverrini*-infected hamster feces and in worms [15]. This study aimed to investigate the relationship between *O. viverrini* and *Leptospira* spp. infection in humans and in the environment—water, mud, and fish in the Lawa wetland region, Khon Kaen province, Thailand.

2. Materials and methods

2.1 Study site

This study was carried out in the Lawa wetland complex, which is located about 30 km south of the city of Khon Kaen along the Chi River in northeastern Thailand (Fig. 1). This wetland consists of three lakes interconnected with the Chi River and covers an area of approximately 100 km² encompassing four districts of the province. Lawa Lake is the biggest among the three lakes. There are 11 villages and over 15,000 inhabitants residing around this lake, most of whom engage in crop farming and fishing as a livelihood. Lawa Lake is well known as an endemic area for *O. viverrini* and cholangiocarcinoma [16, 17].

2.2 Human sera

Human sera (n = 280) from subjects residing around the Lawa wetland were randomly retrieved from the biobank of the Tropical Disease Research Center (TDRC). *O. viverrini* infection was assessed by stool examination using the formalin-ethyl acetate concentration technique at the time of serum collection [18]. These sera were examined for anti-leptospiral antibodies using immunochromatography.

2.3 Immunochromatography

Detection of IgM antibody specific to pathogenic *Leptospira interrogans* was determined by a IgM-based immunochromatography assay (LEPkit-ICT) as described [18]. Briefly, 10 microliters of test sera was placed on the sample pad followed by 100 µl sample running buffer [19], and examined within 15 minutes. Positivity was determined by the development of two bands in the internal control and test area. Negative samples developed a single band in internal area alone. The sample was re-examined if no control band developed. Positivity was read out as weak (grade 1), moderate (grade 2), or strong (grade 3) color band of the test band compared to the control reference band (Fig. 2).

2.4 Immunoblotting

To elucidate cross-reactivity between *O. viverrini* and *L. interrogans*, we performed immunoblotting of the *L. interrogans* lysate by using specific antibodies [20]. Briefly, lysate of multiple *L. interrogans* serovars (as used in the LEPkit preparation) was electrophoresed and the proteins were transferred to nitrocellulose membrane using transblot (Bio-Rad). The

membrane was probed with rabbit antibodies against - *L. interrogans* [19] or anti - *O. viverrini* somatic antigens [21] and color was developed.

2.5 Environmental and fish sample collection

Water and mud were collected from eight different spots around the lake (Fig. 1). Cyprinid fish were bought from fishermen at the lake. All samples were brought to TDRC on the same day. Fish mucus was scraped from its skin. Water and mud as well as skin mucus were immediately processed for DNA extraction. The fish were minced and digested with pepsin-HCl for metacercarial collection as previously described [22]. The digested slurry was serially sieved and sedimented. Metacercariae of *O. viverrini* and other trematodes were morphologically examined under a stereomicroscope. Specie confirmation was done by PCR amplification as previously described [22].

2.6 DNA extraction

DNA from mud samples was extracted using a Soilmaster DNA extraction kit (Epicentre® Illumina Company) according to the manufacturer's recommendation. For water samples, 500 ml water was centrifuged at $10,000 \times g$ and pooled pellets were subjected to DNA extraction using a QIAamp DNA tissue kit (QIAGEN®, Germany). DNA from the fish mucous, parasite metacercariae and adult *O. viverrini* were extracted using standard lysis buffer (10 mM Tris-HCl pH 8.5, 10 mM EDTA, 100 mM NaCl, 0.5% SDS) - proteinase K and ethanol precipitation.

2.7 Molecular detection of *Leptospira* by PCR

DNA (50 ng) from each sample was used as a template in all PCR assays. The reaction mixture contained $1 \times$ I-Taq DNA polymerase containing 0.2 mM dNTP, 1.5 mM MgCl₂, and 1.25 U of *tag* DNA polymerase and 0.2 mM primers. The specific primer sequences and PCR conditions for detection of *O. viverrini* (ITS2) [23], *Leptospira* spp. (16S rRNA) [24], pathogenic *Leptospira* (single tube nested PCR) [25] were described elsewhere [23–25]. Another amplification targeting lipoprotein L32 (*lipL32*) gene for diagnosis of pathogenic *Leptospira* including *L. interrogans* was also done [26]. PCR was performed in duplicate for each sample using a thermocycler GeneAmp® PCR system 9700 (Applied Biosystems CO., LTD). Positive and negative controls were included in each run. The positive control was genomic DNA extracted from cultured *L. interrogans* serovar Lai strain. The negative control was reaction mixture with no DNA template. The amplified products were visualized using 1.5% gel electrophoresis followed by staining with ethidium bromide under a UV illuminator. A positive PCR result was defined as the visualization of a band of predicted size. Pathogenic *Leptospira* was confirmed by sequencing of the purified PCR products (1st BASE Inc., Malaysia) using the *rrs*-inner-F and *rrs*-inner-R primers [25]. Obtained sequences were compared with pathogenic, intermediate and non-pathogenic *Leptospira* spp. retrieved from GenBank database. Multiple sequence alignment was assembled with clustalW using BioEdit software [27]. The aligned sequences were trimmed to 426 base pairs covering the amplified region. Phylogenetic analysis was performed in MEGA6 program (version 6.06) [28], using Neighbor-Joining method and displayed in a bootstrap consensus tree inferred from 1000 replicates.

2.8 Statistical analysis

The chi-square (X^2) or the Fisher exact tests (SPSS 16.0 for Windows SPSS, Inc., Chicago, IL) were used to compare association in leptospiral status between *O. viverrini* infected and uninfected subjects, as well as between females and males. A *P*-value of less than 0.05 was considered statistically significant.

2.9 Ethical statement

The study protocol was approved by the Human Ethics Committee of Khon Kaen University (HE851170). Each patient was recruited to the study after written informed consent was obtained.

3. Results

3.1 Demographic data

We recruited 280 sera from subjects with *O. viverrini* - uninfected ($n = 124$) and - infected ($n = 156$) from Lawa wetland. There were 126 males and 154 females with overall mean age of 46.5 ± 9.1 years.

3.2 Seroepidemiology of leptospirosis

Immunochromatographic results showed overall positivity for *L. interrogans* in 199 out of 280 (71%) serum samples. The majority of the positivity was grade 1 (117/280, 41.8%) while a strong antibody response to *L. interrogans* (grade 3) was found in 25/280 (8.9%) (Table 1). Males had a significantly higher prevalence of leptospirosis than females ($P = 0.002$) (Table 1). Sero-positivity for *L. interrogans* infection did not correlate with age in this study ($P > 0.05$).

3.3 Relationship of *O. viverrini* infection and leptospirosis

The relationship of *O. viverrini* infection with antibody to *L. interrogans* in humans around the Lawa wetland region is shown in Table 2. The seroprevalence of *L. interrogans* was significantly higher in the *O. viverrini* positive group than in the negative one (Chi-square test, $P = 0.002$). Interestingly, seropositivity for *L. interrogans* infection was significantly increased with the intensity of *O. viverrini* infection as stratified by egg counts per gram (EPG) (Chi-square for trend, $P = 0.013$) (Table 2).

3.4 Relationship of genders, *O. viverrini* infection and leptospirosis

The analysis of the association between the prevalence of *Leptospira* infection with *O. viverrini* showed that the *Leptospira* infection rate was significantly higher ($P < 0.05$) in *O. viverrini*-infected females than uninfected identical sex counterparts (Table 3). However, this rate was not significantly different between *O. viverrini* infected and uninfected males ($P > 0.05$).

3.5 No cross reactivity between *O. viverrini* and *L. interrogans*

Immunoblotting of *L. interrogans* lysate revealed no cross reaction with *O. viverrini*. The membrane strip showed intense bands when probed with antibody to *L. interrogans* but not for antibody to *O. viverrini* somatic antigens (Fig. 3).

3.6 *Leptospira* spp. detected in *O. viverrini*, water, mud and fish

The present study showed that 21 out of 35 (60%) water and mud and fish mucus samples were positive for *Leptospira* spp. by 16S rRNA amplification; 4/12 (33.3%), 9/12 (75%), 8/11 (73%), respectively. *Leptospira* species were detected at a higher rate in mud and fish mucous samples. DNA samples from 16S rRNA positive cases were further investigated using nested PCR (Fig. 4). Positive and negative samples for pathogenic *Leptospira* including *L. interrogans* specific PCR targeting *lipL32* gene are shown in Fig. 5. The pathogenic *Leptospira* was detected in *O. viverrini* metacercariae, adult worms, fish mucus (data not shown), and water samples from certain villages. Adult *O. viverrini* showed faint PCR amplified band due to long storage of the DNA.

Samples with a positive nested PCR were cloned and sequenced. Phylogenetic analysis of the sequences revealed that 3 of 5 samples (water from Chi Kok Kor, *O. viverrini* metacercariae from Chi Kok Kor, and water from Donpordang) contain pathogenic *Leptospira* sequences, while the other two samples (mud from Ban Tad and water from Kok Sam Ran village) contain the non-pathogenic strain as shown in Fig. 6.

4. Discussion

Opisthorchiasis is a major health problem for countries in the watershed of the Mekong River, particularly in Northeast Thailand [1]. Control of liver fluke infection has become an important national campaign as infection strongly associates with an increasing risk of cholangiocarcinoma [17]. However, to our knowledge, there are no published reports describing an association between *O. viverrini* infection and other diseases. Given that the two diseases are co-endemic as of up to 90% of reported cases of leptospirosis in Thailand were from Northeast Thailand where *O. viverrini* is endemic [1, 9], we tried to find a link between the two diseases in the Lawa wetland. We found that liver fluke infection is significantly associated with a higher frequency of antibody positivity to the pathogenic *L. interrogans*. Moreover, *Leptospira* spp. were found in the environment, i.e. water, mud, fish skin mucus, and, strikingly, in *O. viverrini* metacercariae.

We report here a high seroprevalence of *Leptospira* infection using IgM immunochromatography in *O. viverrini* endemic areas of Northeast Thailand. All participants were clinically healthy and males had an overall higher frequency of seropositivity than females. Although clinical manifestations of leptospirosis, if present, may vary from a mild to a severe disease including jaundice, renal failure, or pulmonary hemorrhage [8, 11, 29], the majority of people infected by *Leptospira* have subclinical disease [8, 11]. Asymptomatic infection is commonly found in endemic areas and has been reported in several studies [29–31]. Bovet et al. reported 9–48% of healthy subjects were diagnosed as having asymptomatic leptospiral infection by serology (ELISA-IgM) and PCR

[30]. Northeast Thailand is the endemic area for leptospirosis with over 90% of the cases in Thailand are reported from this region [9]. The main risk factors for infection in this region is occupation in agriculture, which involves contact with water or soil contaminated with the urine of domestic and wild animals that have persistent infection by *Leptospira* [6, 7, 32, 33]. Our study clearly demonstrated that several pathogenic, intermediate, and pathogenic strains of *Leptospira* including *L. interrogans* were present in water and mud/soil from Lawa Lake. Males working outdoors in the fields or lake risk exposure to the bacteria more than females [9, 32, 34].

However, this study reports for the first time the significant association between seropositivity for *L. interrogans* and *O. viverrini* infection in the co-endemic area of Lawa wetland. The common risk factor is water contact since *Leptospira* and *O. viverrini* are both water-borne zoonotic diseases [5, 7, 29]. But this is not the sole explanation since the seroprevalence of leptospirosis was exceedingly high (71%) compared with other studies [35–37]. Is there cross reactivity between antigenic epitopes of *O. viverrini* and *L. interrogans* given that the maximum prevalence of *O. viverrini* in the Lawa Lake region is as high as 74% [17] and that over 70% of participants showed positive antibody to *O. viverrini* excretory-secretory antigens by ELISA (Sripa, unpublished)? In this report, we show that there is no cross reaction between the two pathogens by Western blotting, indicating co-infection. We previously reported that a higher prevalence of spirochetal bacteria in *O. viverrini*-infected hamster feces compared with uninfected controls in a microbiome study [15]. Infection with the liver fluke may favor conditions for *Leptospira* infection and therefore increase the antibody response in infected people. This correlates with our observation that seropositivity to *L. interrogans* significantly declined after praziquantel treatment for one year in *O. viverrini*-infected people (Sripa, data not shown).

Another hypothesis to explain the link between *O. viverrini* and *Leptospira* infection is that the fluke is infected by *Leptospira* and the two organisms co-migrate to the liver. This is not impossible as we recently demonstrated *Helicobacter* spp. in the gut of *O. viverrini* [38]. Other parasites such as filarial nematodes including *Brugia malayi*, *Dirofilaria immitis* and *Onchocerca volvulus* can be reservoirs of *Wolbachia* endobacteria [39]. We demonstrated in this study that the liver fluke metacercariae from Lawa Lake contained pathogenic *Leptospira interrogans*. *Leptospira* may present in the gut as several spiral bacteria were found in the gut of *O. viverrini* [38]. Our data indicate that consumption of metacercariae by ingestion of raw fish enhances leptospiral infection. This would explain why *O. viverrini*-infected females have higher rates of leptospiral seropositivity compared with uninfected females, while seropositivity in males is similar regardless of *O. viverrini* infection status. This finding is consistent with males having an additional exposure to leptospiral infection from the environment; females generally work at home, have less exposure to environmental sources of infection and are exposed to infection by *O. viverrini* and *Leptospira* mostly through the consumption of raw fish.

Acquisition of the bacteria by *O. viverrini* may be from water or fish skin mucus during penetration of the parasite cercariae as we can demonstrate pathogenic *Leptospira* including *L. interrogans* in the water and in fish skin mucus from Lawa Lake. In line with our findings, [13] showed a high prevalence of *Leptospira* spp. infection in catfish (58.3%) and tilapia fish

(48.7%). Moreover, Maestroni and Benjaminson [12] reported that goldfish, a cyprinid fish, are susceptible to *Leptospira* spp. when living in contaminated water. We report here that over 70% of the cyprinid fish caught from Lawa Lake contained *Leptospira* spp. Given that Isaan (northeastern) people have a deep culture or tradition of eating raw or undercooked fish such as *koi pla* (fish salad) or fish products such as *pla-som* (fermented sour fish) [5], *Leptospira* can infect these people and give rise to a high seropositivity in Northeast Thailand.

However, we understand that this study has limitation in definite diagnosis of leptospirosis that may require microscopy agglutination test (MAT), bacterial culture, other molecular diagnosis or ELISA. Acute leptospirosis may also need clinical evidence. In our cases, all were asymptomatic leptospirosis.

5. Conclusion

The water-borne *O. viverrini* and *Leptospira* infection co-endemic in the same area can be due to sharing of the same environment. However, our findings indicate that the liver fluke can enhance *Leptospira* infection through eating raw fish that harbor *O. viverrini* with *Leptospira*. The culture of eating raw cyprinid fish may also enhance the risk of leptospirosis because the majority of the fish contain *Leptospira* spp.

Acknowledgments

This work was supported by the Thailand Research Fund (TRF) grant number RTA 5680006 and partially from the National Institute of Allergy and Infectious Diseases (NIAID), NIH, grant number P50AI098639. We thank Manop Sripa for technical assistance. John T. Cathey is gratefully acknowledged for editing English. CDV received scholarship from the Faculty of Medicine, Khon Kaen University. BS is a TRF Senior Research Scholar. The content is solely the responsibility of the authors and does not necessarily represent the official views of the TRF, NIAID or the NIH or the funders.

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Highlights

- *Opisthorchis viverrini* infected people had high seropositivity for leptospirosis
- The liver fluke metacercariae contained pathogenic *Leptospira*
- Eating raw fish can be risk of *Leptospira* infection
- Opisthorchiasis may enhance persistent *Leptospira* transmission in co-endemic areas

Fig. 1.

A satellite image of Lawa wetland and 6 sites around the big lake from where samples were collected (Yellow spots indicate mud and water collection sites). CKK = Chi Kok Kor, KSR = Kok Sam Ran, DPD = Donpordang, BT = Ban Tad, PAO = Ban Pao, NNK = Nongnakwan, Red circles = fish reservation areas. Scale bar = 1 km

Fig. 2.
Grading of immunochromatography. Lane 1 = negative, Lane 2 = +, Lane 3 = ++, Lane 4 = +++
C = internal control, T = test sample

Fig. 3. Immunoblotting of *L. interrogans* strips with rabbit anti – *L. interrogans* (2), anti – *O. viverrini* somatic antigens (3). Lane 4 is *O. viverrini* somatic antigens probed with anti-*O. viverrini* somatic antigens. Lane 1 is negative control (PBS).

Fig. 4. Representative nested PCR amplified products of different samples. 1 = 100 bp DNA ladder, lane 2 = mud from control site, lane 3 = water from control site, lane 1 = *L. interrogans* positive control, Lai strain 56601, lane 2 = water sample CKK, lane 3 = water sample KSR-1, lane 4 = *O. viverrini* metacercariae, lane 5 = water sample KSR-2, lane 6 = mud from Ban-tad

1 = Positive control, 2 = OV adult, 3 = MC1, 4 = MC2, 5 = Mucus, 6 = BP(w), 7 = BT (w), 8 = CKK(w), 9 = DPD(w), 10 = KSR(w), 11 = Distilled water, M = 100 bp marker

Fig. 5.

Representative pathogenic *Leptospira*-specific for lipL32 PCR amplified products of different samples. M = 100 bp DNA ladder, lane 1 = *L. interrogans* positive control, Lai strain 56601, lanes 2–11 = samples from different sources including negative control (DW). OV = *O. viverrini*, MC = metacercariae, Mucus = fish mucus, w = water sample

Fig. 6. Phylogenetic tree of known *Leptospira* sequences retrieved from GenBank (accession number indicated after each sequence) and *Leptospira* sequences identified from the current study. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the *Leptospira*. Branches corresponding to partitions that were reproduced in less than 50% of bootstrap replicates are collapsed. The bootstrap values are indicated at the branch points. Pathogenic, intermediate and non-pathogenic *Leptospira* spp. are indicated as vertical lines. *Leptospira* spp. identified from this study are boxed.

Table 1

Seroprevalence of leptospirosis using immunochromatography and sex.

	ICG grades *			
	0	1	2	3
<hr/>				
				Total
Male	26	51	31	18
				126
Female	55	66	26	7
				154
<hr/>				
Total	81	117	57	25
				280

* ICG = Immunochromatography,

** Chi-square = 14.9, df = 3, $P=0.002$

Table 2

Relationship between intensity of *O. viverrini* infection stratified by egg counts per gram of feces (EPG) groups and grading of antibody response to *L. interrogans* using immunochromatography.

ICG [*] grades	<i>O. viverrini</i> infection intensity (EPG) ^{**}					Total
	0	1–99	100–999	1000		
0	48	16	11	6		81
1	42	38	27	10		117
2	26	15	10	6		57
3	8	6	6	5		25
Total	124	75	54	27		280

* ICG = Immunochromatography,

** Chi-square for trend, EPG vs. ICG grades, $P = 0.013$

Table 3Relationship between sex and *O. viverrini* infection and leptospirosis.

Gender	OV status	<i>Leptospira</i> infection		P value
		Positive (%)	Negative (%)	
Male	<i>O. viverrini</i> -infected	64 (83.1%)	13 (16.9%)	0.259
	<i>O. viverrini</i> -uninfected	36 (73.5%)	13 (26.5%)	
Female	<i>O. viverrini</i> -infected	59 (74.7%)	20 (25.3%)	0.007
	<i>O. viverrini</i> -uninfected	40 (53.3%)	35 (46.7%)	