

# LEPTOSPIRA SPP. SEROPREVALENCE OF CO-INFECTION WITH OPISTHORCHIS VIVERRINI INFECTION IN NORTHEAST, THAILAND

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## Abstract

Leptospirosis caused by *Leptospira* species bacteria is an emerging infectious zoonotic disease with high morbidity and mortality in both tropical and subtropical regions. In Thailand, leptospirosis is endemic in the Northeastern region, where carcinogenic liver fluke, *Opisthorchis viverrini* (Ov), infection is also highly endemic. Here, we report the effects of Ov infection on seroprevalence of *Leptospira* infection at the community level. Sera were collected from asymptomatic Ov-positive and negative people from Khon Kaen and Kalasin Provinces in Northeast Thailand. Anti-*Leptospira* IgM antibody detection using immunochromatography test (ICT) kits showed overall seropositivity against *L. interrogans* of 265 (59.2%) out of 448 samples. *Leptospira* seroprevalence rate in the Ov-positive group was comparable with that in the Ov-negative group without a statistical difference, although the *Leptospira* seropositive rate of males was significantly higher than that of females. When 265 ICT-kit seropositive samples were examined for serovars using the Microscopic Agglutination Test (MAT), 24 sera were MAT-positive and 31 serovars were identified due to multiple serovar infection in some individuals. Among 24 MAT-positive sera, 18 were in the Ov-positive group and 6 were Ov-negative group. The most predominant serovar in this study was Australis (32%) followed by Shermani (26%), Cynopteri (23%) while the detection rates of other serovars, Icterohaemorrhagiae, Autumnalis, Grippotyposa, and Pomona, were <10%. Among 31 serovars detected, 23 were in the Ov-positive group and 8 were in the Ov-negative group. These results indicate that Ov infection might modulate the susceptibility/immunoreactivity of humans to different serovars of *Leptospira*.

**Keywords:** *Opisthorchis viverrini*, *Leptospira*, Leptospirosis, seroprevalence, serovar

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## Introduction

Leptospirosis is a leading zoonotic disease with high morbidity and mortality in humans (Torgerson *et al.*, 2015). About one million cases are estimated to occur worldwide every year (Torgerson *et al.* 2015), especially in the tropical regions and the world's poorest countries. Clinical features in humans ranging from mild or asymptomatic infection to fulminant hepato-renal failure (Weil's disease) (Seguro and Andrade 2013). The disease spreads to humans via contamination of the environment by infected animal urine. The majority of cases appeared after flooding and heavy rainfall in Northeast Thailand as well as other tropical countries (Chadsuthi *et al.*, 2012, Hinjoy 2014). Rodents are the most important animal reservoirs and brown rats (*Rattus norvegicus*) play an important role as reservoir hosts that transmit this disease to humans (Haake and Levett 2015). Excreted bacteria can remain viable in water or soil for many weeks; flooding is commonly followed by an increase of cases.

Previous study demonstrated that *Leptospira* infection is associated with *Opisthorchis viverrini* (Ov) infection (Van *et al.*, 2017). Serum antibody titer to *Leptospira* was higher in the Ov-positive group than in Ov-negative group. Indeed, *Leptospira interrogans* was detected in fish mucus, Ov metacercariae, and adult worms in the co-endemic area of Northeastern Thailand. While Ov infection is a chronic disease leading progressive hepatobiliary abnormalities to bile duct cancer (Sripa *et al.*, 2018), leptospirosis is rather an acute disease with high morbidity and mortality in severe cases (Haake and Levett 2015). Therefore, we hypothesized that chronic Ov infection might affect *Leptospira* infection in the co-endemic areas.

For this purpose, we determined *Leptospira* seroprevalence in Ov-positive and negative groups using the ICT kit (rapid IgM lateral flow chromatography). Then, all the ICT-positive sera were subjected to detect and identify *Leptospira* serovars using the Microscopic Agglutination Test (MAT). The results show that, although Ov infection did not affect the seroprevalence of *Leptospira* using ICT kit screening, Ov infection seems to modulate the susceptibility of humans to various *Leptospira* serovars. This study will allow us to consider further the effect of chronic Ov infection on the seroprevalence and the distribution patterns of *Leptospira* serovars in the co-endemic areas.

## Materials and Methods

### Ethical Statement

The study protocol was approved by the Human Ethics Committee of Khon Kaen University (HE611236). Each patient was recruited to the study after written informed consent was obtained.

### Study Populations

The serum samples for this project are the leftover specimens from the previous study (Sripa *et al.*, 2009) that has been approved by KKKU Human Ethics Committee (HE 480528). The samples were retrieved from the bio-bank of the Tropical Disease Research Center (TDRC). Ov infection was assessed by stool examination using the formalin-ethyl acetate concentration technique at the time of serum collection in Kalasin and Khon Kaen Provinces, Thailand. The required sample size was calculated based on the preliminary MAT test results with a cross-sectional study design using the n4Studies (Ngamjaras, 2016), with the following formula.

$$n_{case} = \left[ \frac{z_{1-\frac{\alpha}{2}} \sqrt{\bar{p}\bar{q} \left(1 + \frac{1}{r}\right)} + z_{1-\beta} \sqrt{p_1 q_1 + \frac{p_2 q_2}{r}}}{\Delta} \right]^2$$

$$p_1 = P(\text{exposure}|\text{case}), q_1 = 1 - p_1$$

$$p_2 = P(\text{exposure}|\text{control}), q_2 = 1 - p_2$$

$$\bar{p} = \frac{p_1 + p_2 r}{1 + r}, \bar{q} = 1 - \bar{p}, r = \frac{n_{control}}{n_{case}}$$

$$p_1 = \frac{p_2 OR}{1 + p_2 (OR - 1)}$$

n = Sample size

$z_{1-\alpha/2}$  = depend on a confidence level (1- $\alpha$ )

$\alpha$  = confidence level (5%)

z = 1.96

r = error (0.05)

p = A proportion of event in an outcome from previous study

According to the preliminary study by MAT.

The proportion in group OV+ rate = 14.91%

The proportion in group OV- rate = 6.65%

Alpha ( $\alpha$ ): 0.1-0.5,

Confidence level 1- $\alpha$  = 95%

Total is n  $\approx$  442, Case 221, and control:221

The sampling was performed using a computer-based random sampling method. The inclusion criteria are based on the project entitled "Pathogenesis of liver fluke induced cancer in

Thailand” that has been approved by the KKU ethics committee (HE480528) with the following inclusion criteria: 1) Participant age 20-60-year-old, with and without Ov infection; 2) Agree to participate in the project with the signed informed consent. Exclusion criteria include: 1) Studying or working outside of the research area; 2) Women with pregnancy; 3) Individuals with mental disorder.

### Detection of Anti- *Leptospira* Igm Antibody in The Sera

All sera were tested to determine anti-*Leptospira* specific IgM antibody using an IgM-based immunochromatography assay (*Leptospira* IgM) produced by the Department of Medical Science, Ministry of Public Health, Thailand, following the manufacturer’s instruction. Briefly, 10 µl of test sera was placed on the sample pad followed by 100 µl sample running buffer and the results were examined visually within 15 min. Positivity was determined by the development of two bands in the internal control and test area. Negative samples developed a single band in the internal control area alone. The sample was re-examined if the control band was not 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 band.

### Microscopic Agglutination Test (MAT)

Specific IgM antibody positive sera were subjected for *Leptospira* serovar determination using MAT. The MAT was performed as described previously (Suwancharoen *et al.*, 2013). Briefly, serum samples were serially diluted in phosphate buffer saline (PBS), pH 7.2 in a 96-well micro-plate. To the serially diluted serum samples, a panel of

23 *Leptospira* reference serovars were added and mixed. The plate was incubated for 2 h at room temperature and agglutination was observed under a dark-field microscope. The serovar positive is determined when agglutination is observed at least 1:100 dilution for any serovars (Desvars *et al.*, 2012). The panel of *Leptospira* serovars included Australis (AUS), Autumnalis (AUT), Ballum (BAL), Bataviae (BAT), Canicola (CAN), Celledoni (CEL), Cynopteri (CYN), Djasiman (DJA), Grippotyphosa (GRI), Hebdomadis (HEB), Icterohaemorrhagiae (ICT), Javanica (JAV), Louisiana (LOU), Manhao (MAN), Mini (MIN), Panama (PAN), Pomona (POM), Pyrogenes (PYR), Ranarum (RAN), Sarmin (SAR), Sejroe (SEJ), Shermani (SHE), and Tarassovi (TAR).

### Data Analysis

Data were entered into Microsoft Excel 2013 spreadsheet. Seroprevalence of leptospirosis was calculated based on the ICT test. Chi square test was used to evaluate the significant level between categorical variables and the final diagnosis. P value <0.05 was considered as statistically significant difference.

## Results and Discussion

### Demographic Data and Distribution

Demographic data of a total of 448 participants consisting of Ov-uninfected (n = 225) and Ov-infected (n = 223) groups with age range from 20-60 years were shown in Table 1. There were 205 males and 243 females with the mean age of 47.5 (47.5±7.9) years.

Table 1. Demographic characteristics of study population

| Characteristic | Groups           | Ov-Positive      | Ov-Negative      |
|----------------|------------------|------------------|------------------|
| Mean age (±SD) | 47.5±7.9         | 47.98±7.6        | 47.02±8.1        |
| Gender (%)     |                  |                  |                  |
| Male           | 205/448 (46%)    | 111/205 (54.10%) | 94/205 (45.90%)  |
| Female         | 243/448 (54%)    | 112/243 (46.10%) | 131/243 (53.90%) |
| Occupation     |                  |                  |                  |
| Not Farming    | 83/448 (18.52%)  | 24/83 (28.90%)   | 59/83 (71.10%)   |
| Farming        | 365/448 (81.47%) | 199/365 (54.50%) | 166/365 (45.50%) |
| Province       |                  |                  |                  |
| Kalasin        | 332/448 (74.10%) | 107/332 (32.20%) | 225/332 (67.80%) |
| Khon Kaen      | 116/448 (25.89%) | 116/116 (100%)   | -                |

Table 2. Demographic characteristics of study population

| Group | Anti- <i>Leptospira</i> Igm antibody |             | Total | P-value |
|-------|--------------------------------------|-------------|-------|---------|
|       | Negative                             | Positive    |       |         |
| Ov-   | 87 (38.7%)                           | 138 (61.3%) | 225   | 0.346   |
| Ov+   | 96 (43.0%)                           | 127 (57.0%) | 223   |         |
| Total | 183 (40.8%)                          | 265 (59.2%) | 448   |         |

### ***Leptospira* Seroprevalence in Ov-positive and -Negative Groups Using Anti-*Leptospira* Igm ICT Kit**

A total of 448 sera were screened for anti-*Leptospira* IgM antibody using the ICT kits (anti-*Leptospira* IgM detection). We found that 265/448 samples (59.2%) were seropositive in this method. There was no significant difference of *Leptospira* seropositivity between Ov-positive (127/223, 57%) and Ov-negative groups (138/225, 61.3%) Table 2.

### **Effects of Age and Gender on *Leptospira* Seroprevalence using ICT Kit**

The seropositivity of *Leptospira* infection in males (134/205, 65.4%) was significantly higher than that in females (131/243, 53.9%) in Table 3. The age did not affect the seropositivity of *Leptospira* infections (data not shown).

### ***Leptospira* and *O. viverrini* Co-infection Rates in Males and Females**

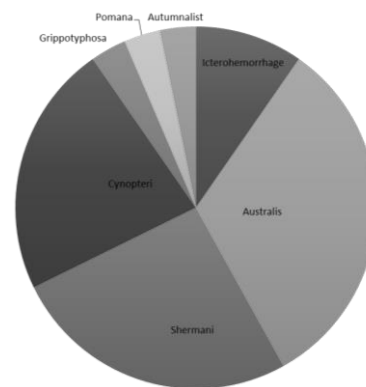
Association between the rates of *Leptospira* infection with Ov infection in males and females were analyzed separately and the data were presented in Table 4. There was no significant difference between the *Leptospira* infection rate in Ov-positive and -negative groups in males ( $P = 0.1$ ) and the same in the females ( $P = 0.7$ ) Table 4.

Contradict to the previous study (Van *et al.*, 2017), our results showed no statistical difference in *Leptospira* infection by ICT in Ov-positive and negative groups, although it was slightly higher in the Ov-positive group than in the negative group. Still, similar to the previous study (Van *et al.*, 2017), seroprevalence by ICT was significantly higher in males than in females. This phenomenon might be explained by the fact that, regardless of *O. viverrini* infection, males are prone to be exposed to *Leptospira* infection by frequent works outdoor compared to females who generally work at home. Another reason for the difference in the seropositivity in this study and the previous study

may be due, at least in part, to the difference of the screening test used in these studies. Although both of them detected IgM antibody to *Leptospira*, the Lepkit immunochromatography used in the previous study (Doungchawee *et al.*, 2017) was constructed with the LPS antigens prepared from six combined *Leptospira* serovars of locally prevalent ones (including Autumnalis, Bratislava, Canicola, Pomona, Sejroe, and Shermani), in this study we used immunochromatography prepared from *Leptospira* serovar Australis and *L. biflexa* serovar Pathoc (Personal communication, Buddhirakkul *et al.*, Department of Medical Science, National Institute of Health of Thailand). The number of antigens included in the LPS preparation might affect the sensitivity of detection. Indeed, Lepkit immunochromatography yielded high sensitivity (97.4%) than the kit used in this study (92%) (Doungchawee *et al.*, 2017).

### **Serovars Detected by MAT Test**

When all 265 ICT positive samples were subjected to serovar detection using MAT, 24 samples were MAT positive (24/265, 9.1%). Since some samples were MAT positive against more than



**Figure 1. Frequency of *Leptospira* serovars observed by MAT**

**Table 3. Rate of *Leptospira* infection in males and females**

| Gender  | <i>Leptospira</i> infection rate |                 | P-value |
|---------|----------------------------------|-----------------|---------|
|         | Negative (%)                     | Positive (%)    |         |
| Females | 112 (46.1%)                      | 131 (53.9%)     | 0.01*   |
| Males   | 71 (34.6%)                       | 134 (65.4%)     |         |
| Total   | 183/448 (40.8%)                  | 265/448 (59.2%) |         |

\*Statistical significance ( $P < 0.05$ )

**Table 4. Association of Gender and *O. viverrini* infection and *Leptospira* infection by ICT**

| Gender | OV status | <i>Leptospira</i> infection rate |              | P-value |
|--------|-----------|----------------------------------|--------------|---------|
|        |           | Negative (%)                     | Positive (%) |         |
| Male   | positive  | 43 (38.7%)                       | 68 (61.3%)   | 0.1     |
|        | negative  | 28 (29.8%)                       | 66 (70.2%)   |         |
| Female | positive  | 53 (47.3%)                       | 59 (52.7%)   | 0.7     |
|        | negative  | 59 (45%)                         | 72 (55%)     |         |

one serovars, the total number of serovars found in this study was 31. The most predominant serovar was Australis (10/31, 32%) followed by Shermani (8/31, 26%), Cynopteri (7/31, 23%) and Icterohaemorrhagiae (3/31, 10%). Autumnalis, Grippytyposa and Pomona were found in only one sample each (1/31, 3%) Figure 1.

The most predominant *Leptospira* serovars in humans in Northeast Thailand in the hospital-based study was Shermani, followed by Bratislava, Panama, and Sejroe using Microscopic Agglutination Test (MAT) (Chadsuthi *et al.*, 2017). In this study, we conducted a community-based study to investigate whether the serovars of *Leptospira* circulating in the community is the same as what has been found at the hospital level. We found that serovars Australis was the highest followed by Shermani, Cynopteri, Icterohaemorrhagiae, Autumnalis, Grippytyposa and Pomona. It should be noted that the serovars Bratislava and Australis are included in the serogroup Australis (Bharti *et al.*, 2003).

#### Detection of *Leptospira* Serovars Antibody Using MAT Test and Its Association to *O. viverrini* Infection

Although *Leptospira* seroprevalence by ICT was comparable between Ov-positive and negative groups, serovar detection rate by MAT was much higher in Ov-positive (18/24) than in Ov-negative group (6/24) Table 5. The MAT positive rate in Ov-positive group (14.2%) was significantly ( $P = 0.005$ ) higher than that in Ov-negative group (4.3%) Table 5. Among 31 serovars from 24 sera, the most predominant serovars was Australis, followed by Shermani and Cynopteri. Other serovars, Icterohaemorrhagiae, Autumnalis, Grippytyposa, and Pomona are rather rare. While the most predominant serovars, Australis, was commonly found in both Ov-positive and negative groups, Shermani and Cynopteri were predominated in Ov-positive group Figure 2.

In this study, MAT results show that the Ov-infected group showed higher positive rate of *Leptospira* infection than the uninfected group. While Australis is the only one leading serovar in the Ov-negative group, Shermani, Cynopteri and Australis are almost equally distributed in the Ov-infected group. These results suggest that

*O. viverrini* infection increase the diversity of *Leptospira* strains, which might be explained by the following mechanisms. First, *O. viverrini* metacercaria have been proven to carry *Leptospira* bacteria so that Ov-infection might increase the chance of exposure to *Leptospira* (Van *et al.*, 2017). Moreover, Ov metacercariae might select and carry specific serovar as has been demonstrated in case of *Cag A+* *Helicobacter pylori* (Deenonpoe *et al.*, 2017). If this is the case, Shermani and Cynopteri are two seravars that are likely harbored by *O. viverrini* because they are prevalent in the Ov-infected group. Second, *Leptospira* diversity might be driven by altered immune response of the host humans due to *O. viverrini* infection induces regulator T cells that could inhibit or suppress T helper 1 response responsible for elimination of bacterial infection (Kaewraemruaen *et al.*, 2016). This is not uncommon because helminth infection has been shown to have profound effect on host microbiota (Lee *et al.*, 2014).

In Thailand, a previous study demonstrated that while the incidence of leptospirosis was highest in the Northeast Thailand, the mortality rate in Northeast Thailand is lower than in other regions (Hinjoy, 2014). The underlying mechanism of this phenomenon is currently unknown. Indeed, the correlation between *Leptospira* serovar and disease severity is still an issue of debate (Dupont *et al.*, 1997; Chidambaram *et al.*, 2007). In addition, there are different serovars that are endemic in the

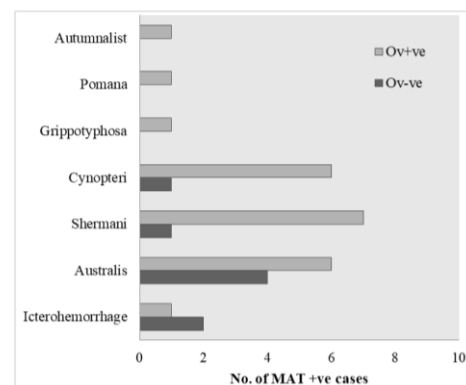


Figure 2. Comparison of *Leptospira* serovars in Ov-positive and negative groups

Table 5. MAT positive cases in Ov-positive and negative groups

| Groups | MAT         |            | Total | P-value |
|--------|-------------|------------|-------|---------|
|        | Negative    | Positive   |       |         |
| Ov-    | 132 (95.7%) | 6 (4.3%)   | 138   | 0.005*  |
| Ov+    | 109 (85.8%) | 18 (14.2%) | 127   |         |
| Total  | 241 (90.9%) | 24 (9.1%)  | 265   |         |

\*Statistically significance ( $P < 0.05$ )

**Table 6. Association between Microscopic Agglutination Test (MAT) positivity and the degree of *O. viverrini* infection**

| MAT      | EPG*      |           |           | Total | P-value |
|----------|-----------|-----------|-----------|-------|---------|
|          | 1-50      | 51-100    | >100      |       |         |
| Negative | 142       | 14        | 49        | 205   | 0.245   |
| Positive | 9 (5.96%) | 2 (12.5%) | 7 (12.5%) | 18    |         |
| Total    | 151       | 16        | 56        | 223   |         |

\*EPG = egg per gram

**Table 7. Association between Sex and *Leptospira* infection by MAT**

| Gender | MAT         |            | Total | P-value |
|--------|-------------|------------|-------|---------|
|        | Negative    | Positive   |       |         |
| Female | 126 (96.2%) | 5 (3.8%)   | 131   | 0.003*  |
| Male   | 115 (85.8%) | 19 (14.2%) | 134   |         |
| Total  | 241         | 24         | 265   |         |

\* Statistically significance (P&lt;0.05)

**Table 8. Association between anti-*Leptospira* IgM screening test with MAT**

| ICT   | MAT      |           | Total | P-value |
|-------|----------|-----------|-------|---------|
|       | Negative | Positive  |       |         |
| 1     | 162      | 13 (7.4%) | 175   | 0.001*  |
| 2     | 53       | 5 (8.6%)  | 58    |         |
| 3     | 26       | 6 (18.8%) | 32    |         |
| Total | 241      | 24        | 265   |         |

\*Statistically significance (P&lt;0.05)

different time periods and the environment factors (Della Rossa *et al.*, 2016). The effect of *O. viverrini* infection on *Leptospira* serovar distribution might affect the outcome of *Leptospira* infection in Northeast Thailand. This issue warrant requirement of further investigation.

#### Association Between Seropositivity by MAT and the Degree of *O. viverrini* Infection

Since the seropositivity of *Leptospira* infection by MAT was significantly higher in Ov-positive group than that in Ov-negative group (P = 0.008), correlation between seroprevalence for *Leptospira* infection by MAT and the intensity of *O. viverrini* infection was examined. As shown in Table 6, there was no significant difference in seropositivity of *Leptospira* infection by MAT among Ov-positive groups with different infection intensities. The result might be due to most of the *O. viverrini* infection are light infection. Another reason is due to the low number of MAT positive samples. Nevertheless, there is a trend of increasing MAT positive samples in higher *O. viverrini* infection intensities. This phenomenon warrants further investigation with a larger population.

#### Effect of Gender on the Incidence of MAT Positive Cases

The seropositivity of *Leptospira* infection by MAT in males (19/134, 14.2%) was significantly higher than in females (5/131, 3.8%) Table 7 with statistically significant association between gender and *Leptospira* infection by MAT (p = 0.003). As mentioned above, more seropositive in males than females might be explained by the fact that male are more active in outdoor activities, which are prone to contact with the *Leptospira* contaminated environments.

#### Correlation Between Anti-*Leptospira* IgM Screening Test with MAT

Although only 10% of ICT positive were MAT positive, there was a statistically significant (p = 0.001) correlation between the anti-*Leptospira* IgM test and MAT (Table 8).

In the present study, the detection rate of *Leptospira* antibody by ICT was about 10 times higher than that by MAT. This difference might be reasoned by the fact while ICT detects both antibodies against intracellular antigen and LPS on the cell surface, MAT detects only antibody against

surface antigen including LPS (Doungchawee *et al.*, 2008; 2017). Another possibility is that a higher antibody titer is required for agglutination in MAT than that required for immunoreactivity in ICT as described elsewhere (Doungchawee *et al.*, 2008). Nonetheless, the results of ICT and MAT were well correlated.

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