

ASSOCIATION BETWEEN MELIOIDOSIS AND OPISTHORCHIASIS IN THE NORTHEAST THAILAND

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Abstract

A carcinogenic liver fluke, *Opisthorchis viverrini* (Ov), infection is endemic in the Greater Mekong Subregion, particularly in the Northeast Thailand where melioidosis is also endemic as an emerging infectious disease. Here, we report the association between melioidosis and opisthorchiasis. For this purpose, we collected serum samples of 145 Ov infected and 145 Ov non-infected individuals from the Northeast Thailand, and anti- *Burkholderia pseudomallei* antibody level was measured using an indirect hemagglutination (IHA) test. Of 290 sera, overall serologically diagnosed (IHA $\geq$ 1:160) melioidosis cases was 10.3% (30/290 cases). The number of participants with any detectable antibody titer (IHA $\geq$ 1:20) against *B. pseudomallei* was significantly higher in Ov-infected group than in Ov-uninfected group ($p = 0.001$). There were also borderline evidence that seroprevalence of melioidosis was two-fold higher in Ov-infected than in Ov-uninfected group ($p = 0.054$). When anti-*B. pseudomallei* IHA titer of Ov-infected individuals were followed up one year after anti-helminthic praziquantel (PZQ) treatment to Ov infections, antibody titers of most participants have risen after treatment ($p=0.0001$). To investigate whether Ov can be a reservoir for *B. pseudomallei*, we tried to detect *B. pseudomallei* in adult Ov worms and bile of Ov-infected hamsters and Ov metacercariae from fish using PCR. So far, we were unable to detect *B. pseudomallei* in the materials examined. The underlying mechanisms of this phenomenon are discussed.

Keywords: *Burkholderia pseudomallei*, *Opisthorchis viverrini*, association, indirect hemagglutination, seroprevalence, Northeast Thailand

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Introduction

Melioidosis is an emerging disease caused by the bacteria *Burkholderia pseudomallei*, which is endemic mainly in Southeast Asia and Northern Australia (Limmathurotsakul *et al.*, 2016). The pathogen is spreading in soil and water at large in endemic regions. Skin inoculation is the major route of infection but airborne transmission can occur during extreme weather conditions in endemic areas (Chen *et al.*, 2015). It can cause fatal pneumonia, multiple abscesses, and, in some cases, multi-organ failure. The US Centers for Disease Control and Prevention (CDC) assigned melioidosis as a disease with bioterrorism potential category B (Wiersinga *et al.*, 2012). Globally, it was estimated that 165,000 (68,000 - 412,000) new human melioidosis cases per year with the mortality of 89,000 (36,000 - 227,000) cases per year (Limmathurotsakul *et al.*, 2016). In 2017, morbidity in Northeast Thailand was 10.73/100,000 population which is much higher than that of other places in Thailand (Ministry of Public Health, Thailand, 2017).

The geographical distribution pattern of melioidosis is similar to those of opisthorchiasis caused by infection with *Opisthorchis viverrini* (Ov), which is transmitted by ingesting undercooked fish particularly among farmers (Thaewongiew *et al.*, 2014). Most opisthorchiasis patients are asymptomatic but <1% of patients may proceed to cholangiocarcinoma (Sripa *et al.*, 2011). The prevalence of opisthorchiasis in Thailand in 2009 was 16.6%, 10%, 1.3%, and 0.0% in the Northeast, North, Central, and South regions, respectively (Sithithaworn *et al.*, 2012). Based on the survey in 2013, the prevalence of opisthorchiasis in the upper Northeast Thailand was 22.7% (Thaewongiew *et al.*, 2014). These results indicate that Ov infection is still endemic in Northeast Thailand.

A recent study demonstrated that Ov infection modifies the microbial communities in the gastrointestinal tract (GIT) of the host (Jordan *et al.*, 2013). Indeed, several bacteria such as *Lactobacillus*, *Pseudomonas*, *Flavimonas*, *Acinetobacter*, *Enterobacteriaceae* family members, and *Burkholderia* were found in the gut of Ov adult worms and the bile of infected hamsters. In addition, *Leptospira* bacteria were detected in the metacercariae, an infective stage, of Ov (Van *et al.*, 2017). Anti-*Leptospira* antibody titer was higher in Ov-infected patients than in Ov uninfected individuals. Moreover, the *Leptospira*-seropositive rate in females is significantly higher in Ov infected individuals than in Ov uninfected individuals. They hypothesized that the culture of eating Ov-contaminated raw fish may induce the

seropositivity of leptospirosis owing to the presence of *Leptospira* in Ov metacercariae. Such association would also occur between opisthorchiasis and melioidosis because both pathogens resided in soils and rice paddy water sharing the same environment in Northeast Thailand (Thaewongiew *et al.*, 2014; Ong *et al.*, 2017). We, therefore, hypothesized that *O. viverrini* infection might affect *B. pseudomallei* infection as well.

The present study aimed to investigate whether there is an association between *O. viverrini* and *B. pseudomallei* infections. The anti-*B. pseudomallei* antibody titer was determined in Ov-infected and uninfected individuals using an indirect hemagglutination (IHA) test. Furthermore, the antibody titer was also measured before and one year after praziquantel (PZQ) treatment for Ov infection. In addition, using polymerase chain reaction (PCR), we have tried to detect *B. pseudomallei* DNA in Ov metacercariae collected from freshwater fishes in the endemic area, Ov adult worms and the bile from Ov infected hamsters, and also fecal samples of Ov-infected and uninfected individuals.

Materials and Methods

Ethics Statement

This study was approved by the Human and Animal Ethics Committee of Khon Kaen University (HE611198). Recruitment of participants was done after receiving written informed consents.

Study Design and Study Site

This study was a case-control study in order to compare anti-*B. pseudomallei* antibody titer in Ov-infected and uninfected group. Then, the project was continued as a prospective study to compare the antibody titer in Ov-infected individuals before and one year after PZQ treatment. This study was conducted in Khon Kaen, Mahasarakham, Roi-Et, and Kalasin Provinces in Northeast Thailand where both opisthorchiasis and melioidosis are endemic.

Samples and Specimens Used

We recruited a total of 290 left-over serum samples from Ov-infected (n = 145) and uninfected (n = 145) individuals of the previous project entitled "Pathogenesis of liver fluke induced cancer in Thailand" that has been approved by the KKU Human Ethics Committee (HE480528) in the study areas. Ov-infected and uninfected individuals were identified by stool examination for Ov eggs using

Table 1. Summary of possible risk factors

Potential risk factors	Seropositive (titer $\geq$ 160)	Seronegative (titer <160)	p-value (IHA titer $\geq$ 1:160)	p-value (IHA category)	p-value (IHA titer $\geq$ 1:20)
Total (n = 290)	30	260			
Age					
Mean	48.67 $\pm$ 7.8	47.28 $\pm$ 8.1	0.719		0.211
Median	50.5	48			
Age, years					
$\leq$ 48	13	131	0.469	0.546	0.772
>48	17	129			
Gender					
Male	17	123	0.331	0.119	0.124
Female	13	137			
Ov infection					
Positive	20	125	0.054	0.003*	0.001*
Negative	10	135			OR=2.35 (1.42-3.88)
EPG					
0	10	235	0.152	0.001*	0.002*
1-99	15	91			
$\geq$ 100	5	34			
PZQ treatment					
Before	20	125	0.019*	0.0001*	
After	31	114			

a formalin-ether concentration technique (FECT). We selected 140 males and 150 females who were chosen by age and sex matching. The mean age of participants in this study was 48 years in both Ov-infected and uninfected groups. The median age of the participants was 50 years Table 1. To Ov-infected individuals, PZQ was prescribed and they were followed up one year later. A paired serum of Ov-infected group before and one-year after PZQ treatment (clearance of Ov infections confirmed by stool examination) were used to determine IHA titer against *B. pseudomallei*. In addition, fecal samples of 80 Ov-infected and 50 uninfected individuals were collected to detect *B. pseudomallei* using the copro-PCR method.

In addition, to see whether Ov might act as a vector for *B. pseudomallei*, Ov metacercariae (200 metacercariae) from cyprinoid fish in the endemic area, Ov adult worms (10 worms) and 500 μ l of pooled bile sample of infected hamsters were collected from the left-over specimens of the previous studies, which have been approved by the KKU Animal Ethics Committee (AEKKU 21/2552), and were stored at -80°C in the Tropical Disease Research Centre (TDRC). The bacteria, *B. pseudomallei* isolated in Northeast Thailand were obtained from the Melioidosis Research Centre, Khon Kaen University.

Indirect Hemagglutination (IHA) Test

IHA test was conducted using a Melioidosis-IHA Test kit (a Rapid Test for Melioidosis) produced by the Department of Medical Science (DMSc), Ministry of Public Health, Thailand,

following the manufacturer's instruction. Briefly, patients' sera (25 μ l) were heat-inactivated in a water bath at 56°C for 30 min and prepared for 2-fold serial dilution starting from 1:10 in 96-microwell plates. Melioidosis test cells (25 μ l) were added into serially diluted patients' sera. After incubation at room temperature for 2 h, antigen-antibody complex in positive sera can be seen as agglutination. IHA titers of $\geq$ 1:20 were considered as evidence of exposure to *Burkholderia pseudomallei* (Peacock *et al.*, 2011), and IHA titer of $\geq$ 1:160 were considered as seropositive for melioidosis (Cheng *et al.*, 2006).

Sample Preparation for Polymerase Chain Reaction (PCR)

DNA samples were extracted from Ov adult worms, bile from infected hamsters, Ov metacercariae, and *B. pseudomallei* using a Gentra Puregene Tissue Kit (QIAGEN) following the manufacturer's instruction. To detect *B. pseudomallei* in human feces, DNA samples were purified from the feces using a stool purification kit (TIANamp Stool DNA Kit, TIANGEN, catalog no. #DP328-02). The purified DNA samples were stored at -20°C until use.

Polymerase Chain Reaction (PCR)

PCR was performed to detect *B. pseudomallei* DNA in the purified total DNA from samples mentioned above. For PCR positive control, *B. pseudomallei* DNA was included as a template. Distilled water was used as negative control. The PCR reaction was conducted in a final volume of

25 µl containing 2 µl of DNA sample (40 ng/µl), 2.5 µl 10X PCR buffer, 0.5 µl of deoxynucleoside triphosphates (10 mM, 0.5 µl of primers (100 µM), 0.5 µl of 1U Taq polymerase (1U), and 18.5 µl of distilled water (DW). The *B. pseudomallei* specific primer set, forward Bp1 (5'-CGATGATCGTTGGC GCTT-3') and reverse Bp4 (5'-CGTTGTGCCGTA TTCCAAT-3'), was used with the following PCR thermal cycles; initial denature for 4 min at 94°C, followed by 45 cycles of denaturing for 30 sec at 94°C, annealing for 30 sec at 50° and extension at 72°C for 45 sec, with a final extension for 7 min at 72°C (Couto *et al.*, 2009). PCR products were separated on a 1.5% agarose gel electrophoresis with ethidium bromide and the results were visualized under UV transilluminator. The amplification product has an approximate size of 302 bp.

Data Analysis

All data are entered into Microsoft excel 2013 and analyzed using SPSS version 22 (Vandana *et al.*, 2016). Continuous data were analyzed using descriptive statistics, categorized data by Chi-square test and dependent data by Wilcoxon Signed Ranks test and McNemar test. Seropositivity was considered as a dichotomous variable (positive, titer level $\geq 1:160$, negative, titer level < 160). Association with other risk factors such as age and sex were also analyzed. A p-value of < 0.05 was considered as statistically significant.

Results and Discussion

Seroepidemiology of Melioidosis

Using the cut-off point $\text{IHA} \geq 1:160$ titer, we found 20 seropositives in 145 Ov-infected individuals (13.8%) and 10 seropositives in 145 Ov-negative individuals (6.9%) Figure 1. Hence, the overall seroprevalence of melioidosis in these study areas was 10.3% (30/290 cases). Although statistically not significant ($p = 0.054$), seroprevalence of melioidosis was two-fold higher in the Ov-infected than in the Ov-uninfected group.

Moreover, cases with any detectable antibody titer by IHA ($\geq 1:20$) were 62/145 (42.8%) in the Ov-infected group and 35/145 (24.13%) in the Ov-uninfected group Figure 1. The overall antibody detection rate was 33.45% (97/290 cases). The antibody detection rate was significantly higher in the Ov infected group than in the Ov uninfected group ($p=0.001$) with the crude odds ratio of 2.35 (1.42-3.88).

The antibody titer was significantly higher in high-intensity Ov infection than in the low-intensity infection ($p = 0.001$). A positive correlation was

seen between the intensity of Ov infection (EPG) and IHA antibody levels ($p<0.0003$) Figure 2. However, the antibody detection rate did not correlate with age ($p = 0.546$) or sex ($p = 0.119$) in this study Table 1.

Effects of Gender on Ov Infection and Seroprevalence of Melioidosis

There was no difference in antibody titer between males and females ($p = 0.124$). However, when Ov infection status is considered, the statistical analysis between Ov infection and anti-*B. pseudomallei* antibody titer ($\geq 1:160$) showed that seroprevalence of melioidosis was significantly higher in Ov-infected females than in Ov-uninfected females ($p = 0.002$) (Figure 3). In contrast, the seroprevalence was not significantly different between Ov-infected and Ov-uninfected males ($p = 0.944$). When Ov infection and any detectable antibody titer against *B. pseudomallei* by IHA ($\geq 1:20$) were analyzed, the antibody detection was also significantly higher in Ov-infected females than in Ov-uninfected females ($p = 0.0003$) but not

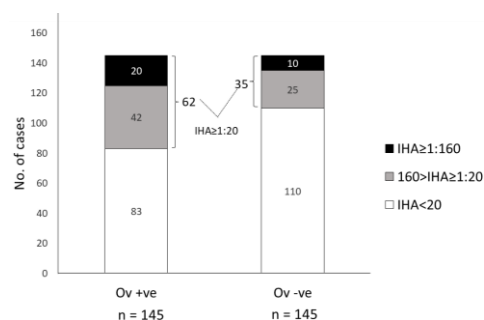


Figure 1. Number of seroprevalence of melioidosis in 145 Ov infected sera and 145 Ov uninfected sera samples, IHA = Indirect Hemagglutination Test

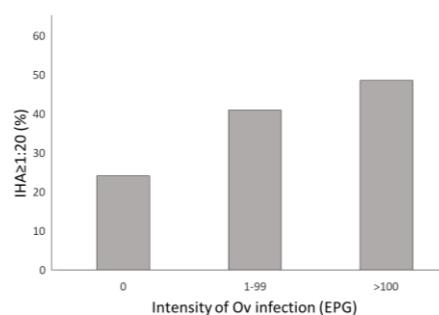


Figure 2. Relationship between intensity of Ov infection (EPG) and anti-*B. pseudomallei* antibody detection by IHA ($\geq 1:20$) Linear by linear association, IHA vs EPG, $p = 0.001$ Positive Spearman correlation, $p < 0.0003$

different between Ov-infected and Ov-uninfected males ($p = 0.226$) (Figure 3).

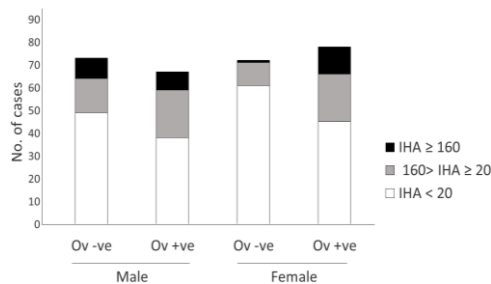


Figure 3. Effect of gender on Ov infections and anti-*B. pseudomallei* antibody detection

O. viverrini and *B. pseudomallei* are co-endemic and their endemicity is highest in Northeast Thailand compared with other regions (Sithithaworn *et al.*, 2012; Ministry of Public Health, Thailand, 2017). While *O. viverrini* is a carcinogenic liver fluke that causes hepatobiliary diseases including bile duct cancer, *B. pseudomallei* causes melioidosis with fatal pneumonia, multiple abscesses and, in some cases, multi-organ failure. To our knowledge, there was no research studying on the association between these two deadly pathogens. Thus, we aimed to explore the relationship between opisthorchiasis and melioidosis.

First, we compared the seropositivity to *B. pseudomallei* in Ov infected versus Ov uninfected groups and found that participants with *O. viverrini* infection have significantly higher seropositivity against *B. pseudomallei*. This indicates that people with *O. viverrini* infection are prone to exposure to *B. pseudomallei* or vice versa. This result is similar to our previous report, in that Ov infected people have higher seropositivity to *Leptospira* when compared with Ov negative people (Van *et al.*, 2017). These results indicate that there are associations among these three diseases that share the same endemic area. Indeed, *O. viverrini* infection might favor the condition of bacterial infection by certain mechanisms that need further investigations.

B. pseudomallei seropositivity rate was higher in Ov-infected than in Ov-uninfected females, although such a difference was not observed in males. This result is also similar to our previous report of *Leptospira* infection in *O. viverrini* infected people (Van *et al.*, 2017). This phenomenon might be explained by the fact that males are working outdoor and are continuously exposed to bacterial contaminated environments. On the other hand, females are working indoors having less chance to be exposed to pathogens.

Females might only contact to both bacterial species by eating raw fishes. Indeed, most of the fishes in endemic areas are contaminated with *Leptospira*. In a similar fashion, fishes are likely contaminated with *B. pseudomallei* because the bacteria were detected in freshwater reservoir (Chuah *et al.*, 2017). In addition, Ov-infected females might be exposed to *B. pseudomallei* through raw fish preparation.

Comparison of IHA antibody titers in Ov infected individuals before and after PZQ treatment

In order to investigate the interaction between Ov and *B. pseudomallei* infections, anti-Bp antibody levels were analyzed for 145 Ov infected individuals before and one year after PZQ treatment. It showed that one-third of participants had significantly higher anti-*B. pseudomallei* antibody titer after treatment ($p = 0.0001$) Figure 4. Especially, 23 cases (15.9%) appeared as new infections with *B. pseudomallei* after one year. Including 23 new

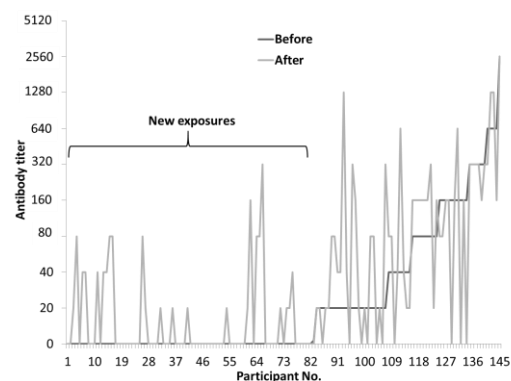


Figure 4. Comparison of antibody titer against *B. pseudomallei* in 145 Ov infected individuals before and one year after PZQ treatment. The X-axis is the participant numbers sorted from low to high titer according to the antibody titer before treatment. The Y-axis is the antibody titer against *B. pseudomallei* before (black line) and after (grey line) treatment. New exposures are those participants whose titer was negative before treatment and became positive after treatment

Table 2. Relationship between PZQ treatment and seroprevalence (IHA≥1:160)

PZQ treatment	IHA status		Total	p-value
	<1:160	≥1:160		
Before	125	20	145	0.019*
After	114	31	145	

* McNemar test

infection cases, 48 cases (33.1%) showed increased antibody titer level, whereas 17 cases (11.7%) had decreased Ab titer. In addition, seroprevalence (IHA titer $\geq 1:160$) of melioidosis became significantly higher after PZQ treatment ($p = 0.019$) Table 2.

PCR Detection of *Burkholderia Pseudomallei* in Worms, Bile and *Ov Metacercariae*

In the previous microbiome study (Jordan *et al.*, 2013), *Burkholderia* species bacteria was detected in *Ov* adult worms, and the bile of infected hamsters but they did not determine the species. Therefore, using PCR assay with *B. pseudomallei* species-specific primers (Bp1 & Bp4), we examined whether *Ov* worms carry *B. pseudomallei* species and any *B. pseudomallei* in bile of *Ov* infected hamsters. As a negative control, the worms and bile of uninfected hamsters were used. *Ov* metacercariae from fish were also examined for the presence of *B. pseudomallei* using PCR. However, we were unable to detect any *B. pseudomallei* DNA in worms or bile samples of neither *Ov* infected and control hamsters, nor did in *Ov* metacercariae from fish (data not shown).

Detection of *Burkholderia Pseudomallei* in *Ov*-positive and Negative Human Fecal Samples

Since serological study revealed an association of *B. pseudomallei* infection and *Ov* infection, we examined the presence of *B. pseudomallei* in the stool samples of *Ov*-positive and negative individuals. The results showed that *B. pseudomallei* was detected slightly, though statistically not significant, higher ($p = 0.569$) in fecal samples of *Ov* infected (7/80, 8.3%) than in *Ov* non-infected (3/50, 6%) individuals.

In the *Ov*-infected group, there was an increase in antibody titer against *B. pseudomallei* one year after PZQ treatment. This result is contradicted that of *Leptospira* infection, in that the antibody titer was decreased after worm removal (Van *et al.*, 2017). This difference might be because *O. viverrini* has been proved as one of the reservoir hosts for *Leptospira*, worm removal also removes *Leptospira*, which in turn will reduce the antibody response to *Leptospira*. On the other hand, in this study, *B. pseudomallei* was not detected in metacercariae, adult worms, or *O. viverrini* infected bile duct, which indicates that *O. viverrini* is not a reservoir of *B. pseudomallei*. Therefore, worm removal did not affect on *B. pseudomallei*.

Elevation of anti-*B. pseudomallei* antibody titer one year after PZQ treatment for *O. viverrini* might be simply explained by the fact that those people in the endemic area are continuously

exposed to *B. pseudomallei*. Another possibility is *O. viverrini* suppresses the immune response to *B. pseudomallei*, which is exaggerated after the removal of *O. viverrini* worms. This is not impossible because the Treg-like cells producing TGF- β and IL-10 are detected in hamster spleens and mesenteric lymph nodes (MLNs) during *O. viverrini* chronic infection (Kaewraemruaen *et al.*, 2016). In addition, the reduction of TGF- β and IL-10 is correlated with a low number of worms recovered in *O. viverrini* infected hamster, indicating that immune suppression is essential for worm survival (Jittimanee *et al.*, 2012). Furthermore, the immunosuppressive effect was reversible after PZQ treatment (Wongratanaheewin *et al.*, 1987).

We understand that this study has a limitation in the diagnosis of melioidosis, of which definite diagnosis requires patients' symptoms and positive bacterial culture. However, all study participants are asymptomatic residents from the community of endemic areas. This study used a leftover specimen from the previous project, and the sera at a year follow-up could not be obtained from *O. viverrini* uninfected individuals. Consequently, we could not determine that background exposure to *B. pseudomallei* in the endemic area. Furthermore, the serum samples and fecal samples were collected from different groups and thus the correlation between PCR detection of Bp from human feces and antibody level from human serums could not be examined.

Conclusions

This study reports the immunological association between melioidosis and opisthorchiasis in Northeast Thailand where both diseases are co-endemic. Our findings indicate that, unlike *Leptospira*, *O. viverrini* is not a reservoir for *B. pseudomallei*. Instead, *O. viverrini* infection may enhance the *B. pseudomallei* infections through immune suppression mechanism. We are aware that our study has some limitations as mentioned above; however, we anticipate that our data have set the ground for further investigation of the relationship between these two pathogens, especially how *O. viverrini* infection enhance *B. pseudomallei* infection and vice versa. Given they share the endemic area in the Northeast Thailand, better understanding as to how these two diseases interact and their effects on human health will provide a clue to develop an effective control and prevention strategies for these two deadly diseases.

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