Tropical Biomedicine 36(2): 443–452 (2019)

Serological survey of Leptospirosis in high-risk rangers and wild animals from ex-situ captive centers

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Received 17 October 2018; received in revised form 24 January 2019; accepted 28 January 2019

Abstract. Leptospirosis is a bacterial disease caused by pathogenic *Leptospira* that infect both human and animals. This study was conducted to determine the seroprevalence of leptospirosis among rangers and wild animals in two ex-situ captive centers, Bukit Merah Orangutan Island (BMOUI) and Taiping Zoo, Perak and to identify the risk factors responsible for the leptospiral seropositivity. Blood samples from rangers and animals of BMOUI and Taiping Zoo were taken to determine the presence of antibodies against *Leptospira* through microscopic agglutination test (MAT) using 21 serovars of *Leptospira* commonly found in Malaysia as antigens. Structured surveys in identifying risk factors were given to each ranger from both study sites. It was observed that *Rattus exulans* (1/10) (10.0%), *Rattus rattus* (1/5) (20.0%), *Niniventer fulvescens* (1/1) (100.0%), *Callosciurus notatus* (0/6) (0.0%), *Tupaia tana* (1/1) (100.0%), *Pongo pygmaeus* (5/10) (50.0%) and BMOUI rangers (8/18) (44.4%) were positive for leptospiral antibodies. Samples obtained from Taiping Zoo also revealed the presence of leptospiral antibodies in *R. rattus* (0/19) (0.0%), *R. exulans* (1/2) (50.0%) and rangers (2/5) (40.0%). Among the positive cases, most human and animal samples from both study sites reacted with serovar Lepto 175. Our surveys indicated no significant associations between seroprevalence of leptospiral antibodies with rangers' age ($p = 0.82$), sex ($p = 0.85$), ethnicity ($p = 0.65$), educational level ($p = 0.88$) and working experience ($p = 0.82$). In terms of risk factors, no significant associations between seroprevalence of leptospiral antibodies with knowledge on leptospirosis ($p = 0.82$), working hours ($p = 0.53$), smoking ($p = 0.85$), crossing rivers/pools/stagnant water while working ($p = 0.90$) and wearing full personal protective equipment (PPE) ($p = 0.73$). This study provides epidemiological data on leptospirosis in rangers and animals from BMOUI and Taiping Zoo which is of paramount importance for improving strategies in prevention of the disease.

INTRODUCTION

Leptospirosis is a zoonotic disease caused by pathogenic spirochetes of the genus *Leptospira* that infect all mammals including human (Dietrich *et al.*, 2015). This disease has a worldwide distribution but is endemic in tropical and subtropical regions (Levett, 2001; Bharti *et al.*, 2003; LaRoque *et al.*, 2005; Slack *et al.*, 2006). Recently, leptospirosis has been recognised as a re-emerging public health problem in Malaysia (Thayaparan *et al.*, 2014). The country's wet and humid climate promotes long survival of *Leptospira* in the environment (Azali *et al.*, 2016). Currently, there are nearly 300 known serovars (Valverde *et al.*, 2008) and most of them have their primary reservoirs in wild and domestic animals, of which rodents are the most common source worldwide (Green-Mckenzie & Shoff, 2012). *Leptospira* can be transmitted between hosts through direct contact with urine or damaged skin of

infected organisms or indirectly via environment through the ingestion of contaminated water (Dechner, 2014).

Microscopic agglutination test (MAT) is commonly used as serological diagnosis of leptospirosis as it allows for the detection of group specific antibodies (Brandão *et al.*, 1998) although they can react with more than one serovar (Haake & Levett, 2015). It is performed by mixing a patient's serum with a panel of *Leptospira* serovars that are considered endemic in a given region (Levett 2001). The test is considered positive when there is leptospiral agglutination of at least 50%. Information obtained through MAT has been used in epidemiological studies to infer the possible prevalent serovars in a region (Smythe *et al.*, 2009; Chirathaworn *et al.*, 2014; Yaakob *et al.*, 2015).

Recently, a high number of confirmed cases and associated mortalities around the nature reserves and recreational parks have been documented in Malaysia (Arif, 2013; Thayaparan *et al.*, 2014). For example, in 2016, a student who suffered from leptospirosis after bathing in Jeram Mak Nenek, Selising, Kelantan died after experiencing a prolonged fever, poor appetite and vomiting (Alias 2016). In the same year, a teacher who had been diagnosed with leptospirosis after participating in kayaking activities in Sungai Ketil, Baling, Kedah also died due to brain hemorrhage and major organs failure (Ghazali, 2016). In 2018, nine families believed to have symptoms of leptospirosis after a holiday at Gunung Belumut Recreational Forest, Kluang, Johor (Noh, 2018).

However, despite all the positive cases recorded in Malaysia, there is insufficient information on leptospirosis among high-risk workers in the country's wildlife reserves and ex-situ captive centers. Rangers are of high risk due to daily exposure/ contact with potential infected wild animals and rodents. This study was carried out to determine the seroprevalence of leptospirosis among rangers and wild animals in Bukit Merah Orangutan Island (BMOUI) and Taiping Zoo, Perak and to identify the risk factors responsible for the leptospiral seropositivity. This study provides epidemiological data which is important in the planning and implementation control measures.

METHOD

Sample collection

Rodents from both study sites were trapped alive and anaesthetized using 30% v/v isoflurane in propylene glycol (Nagate *et al.*, 2007). Their length and weight were recorded. Species identifications were carried out according to Francis (2008) based on the phenotypic characteristics, such as fur color (side, ventral and dorsal), ears, tail, hind foot, body weight and head-body length. Blood extracted by cardiac puncture using 3 ml syringes and 23G needles.

Ten orangutans (aged between 4-22 years old) from BMOUI were mechanically and chemically restrained before 5-10 ml of blood was drawn from the femoral vein of each individual by certified veterinarian. No blood samples were obtained from the orangutans in Taiping Zoo due to health risks.

Information sheet regarding study scope was provided and consent form was given to 23 rangers of BMOUI and Taiping Zoo. Blood samples of 5 ml each were taken by medical assistant. All blood samples collected were stored in plain tube and centrifuged at 4000 rpm for 15 minutes to obtain the sera. The sera were then stored under -20°C for preservation.

Questionnaires

Structured questionnaires to identify the sociodemographic and risk factors were given to 21 rangers from both BMOUI and Taiping Zoo (two rangers were excused from the evaluation due to personal matters).

Microscopic agglutination test (MAT)

The MAT was performed according to Faine (1982) to check for *Leptospira*-specific antibodies from rangers and animals. Each serum was tested against

21 endemic serovars obtained from Institute for Medical Research (IMR): serovars Patoc, Whitcombi, Hardjobovis, Malaya, Pomona, Hardjo, Bataviae, Pyrogenes, Fugis, Bangkinang, Jonsis, Birkini, Biggis, Copenhageni, Lai, Grippotyphosa, Coxi, Lepto 1, Lepto 115, Lepto 175 and HOSHAS. Fifty microliters of two-fold diluted serum samples ranging from 1:50 to 1:800 were prepared on 96-well microtiter plates, and fifty microliters of live antigens were added to each well respectively. The serum-antigen mixtures were examined under dark-field microscope after two hours of incubation for leptospiral agglutination of at least 50% when compared with control (WHO 2003). A titer ≥1:100 on the MAT was considered as evidence of recent or past infection with *Leptospira* (Thayaparan *et al.*, 2015a).

Data analysis

Statistical data was analyzed using Minitab 18 software. Pearson Chi-Square test was used to test the association between factors studied and seropositivity of leptospiral antibodies among BMOUI and Taiping Zoo rangers. Due to small sample sizes, Yate's correction was included in the calculation. The p-value of less than 0.05 was considered as statistically significant.

Study ethics

The study and structured questionnaires used were approved by Universiti Kebangsaan Malaysia Human Ethics Committee: UKM/PPI/111/8/JEP-2017-243, Universiti Kebangsaan Malaysia Animal Ethics Committee (UKMAEC): FST/2016/HANI/25-JAN./822-JAN.-2017-JUNE-2018 and Department of Wildlife and National Parks (PERHILITAN) with special permit: B-00245-16-17.

RESULTS

Microscopic agglutination test (MAT)

A total of ten polynesian rats (*Rattus exulans*), five black rats (*Rattus rattus*), a chestnut white-bellied rat (*Niniventer fulvescens*), six plantain squirrels (*Callosciurus notatus*) and a large treeshrew (*Tupaia tana*) were caught in BMOUI. Ten semi-captive orangutans (*Pongo pygmaeus*) were restrained and 18 rangers volunteered to participate in the study. It was observed that *R. exulans* (1/10) (10.0%), *R. rattus* (1/5) (20.0%), *N. fulvescens* (1/1) (100.0%), *C. notatus* (0/6) (0.0%), *T. tana* (1/1) (100.0%), *P. pygmaeus* (5/10) (50.0%) and rangers (8/18) (44.4%) were shown to be positive for leptospiral antibodies. From the positive cases, 15/26 (57.7%) of the samples showed positive titer with Lepto 175, 4/26 (15.4%) with Hardjoprajitno, 3/26 (11.5%) with Lepto 115, 2/26 (7.7%) with Fugis, 1/26 (3.8%) with Lai Langkawi and 1/26 (3.8%) with Patoc. Titers of antibodies to different serovars found in animals and rangers from BMOUI is shown in Table 1.

Nineteen *R. rattus* and two *R. exulans* were caught in the primate sections of Taiping Zoo and five rangers volunteered to participate in the study. Observation revealed that *R. rattus* (0/19) (0.0%), *R. exulans* (1/2) (50.0%) and rangers (2/5) (40.0%) from Taiping Zoo were positive for leptospiral antibodies. From the positive cases, 2/3 (66.7%) of the samples showed positive titer with Lepto 175, 1/3 (33.3%) with Lepto 115. Titers of antibodies to different serovars found in animals and rangers from Taiping Zoo is shown in Table 2.

Table 1. Antibody titers of the seropositive samples from BMOUI against several leptospiral serovars

Serovars	Titers			
	1:100	1:200	1:400	1:800
Lepto 115	3	0	0	0
Lepto 175	10	5	0	0
Hardjoprajitno	3	0	1	0
Fugis	2	0	0	0
Lai Langkawi	1	0	0	0
Patoc	1	0	0	0

Table 2. Antibody titers of the seropositive samples from Zoo Taiping against several leptospiral serovars

Serovars	Titers			
	1:100	1:200	1:400	1:800
Lepto 115	1	0	0	0
Lepto 175	2	0	0	0

Risk factors for exposure to *Leptospira* evaluation survey

a) Sociodemographic factors

A total of 21 healthy respondents from BMOUI and Taiping Zoo participated in the survey. A slightly higher number of the respondents aged below 30 (52.4%). Majority of the respondents were male (57.1%). In terms of ethnicity, most of the respondents were Malay (81.0%). More than half of the respondents have lower educational level (66.7%). Most respondents have working experience less than 30 years (52.4%).

b) Seroprevalence

Among 21 healthy respondents, 47.6% serum samples showed positive reaction against *Leptospira* serovars. From 21 serovars tested, five reacted with the samples. Out of ten respondents who showed positive results, seven of them showed positive reaction with one serovar, two with two serovars and one with three serovars. From all serovars tested, seven sera reacted with serovar Lepto 175, three sera reacted with Lepto 115, two sera with Fugis, one serum with serovar Hardjoprajitno and one serum with serovar Patoc.

c) Risk factors

In this study, 11 out of 21 respondents (52.4%) have little knowledge on leptospirosis. Fifteen respondents (71.4 %) worked more than 5 hours per day. Nine respondents (42.9%) were smokers. Majority of respondents (76.2%) had to cross rivers/ any stagnant water while working. However, only six out of 21 respondents (28.6%) wore full PPE (long sleeves, long pants, gloves, safety boots, face mask and goggles) while working at the primate sections and mostly agreed that PPE provided were in good conditions.

d) Sociodemographic factors and risk factors with seroprevalence of leptospiral antibodies among respondents from BMOUI and Taiping Zoo

Based on Table 3, there were no significant associations between seroprevalence of leptospiral antibodies with age ($p = 0.82$), sex ($p = 0.85$), ethnicity ($p = 0.65$), educational level ($p = 0.88$) and working experience ($p = 0.82$). In terms of risk factors, there were no significant associations between seroprevalence of leptospiral antibodies with knowledge on leptospirosis ($p = 0.82$), working hours ($p = 0.53$), smoking ($p = 0.85$), crossing rivers/pools/stagnant water while working ($p = 0.90$) and wearing full personal protective equipment (PPE) ($p = 0.73$).

DISCUSSION

Our findings showed that among 21 serovars tested, Lepto 175 was the most prevalent serovar reacted with antibodies of the orangutans and rodents, thus, there might be transmission of *Leptospira* from one host to another. Primates that are kept in captivity, may get infected when they encountered infected rodents. According to rangers in BMOUI and Taiping Zoo, rodents reportedly seen roaming at proximity of the exhibition/ caging areas and food storage area. Jung *et al.* (2007) has speculated that free-living rodents carrying *Leptospira* might infect zoo animals by nearing their cages as they were the only free-living mammals that were able to come into contact with captive animals in zoos. Previous study by Thayaparan *et al.* (2014, 2015b) also showed many primates, rodents and bats in Sarawak displayed high levels of antibodies to Lepto 175.

Besides wild animals, Lepto 175 was also the most predominant serovar identified in serologic surveys of rangers from both study sites. This is due to their daily encounter/ contact with infected primates and rodents making them more susceptible to *Leptospira* infection. Thus, zoological parks can be a potential source of human leptospirosis since the transmission of *Leptospira* may occur from animals to humans and Romero *et al.* (2011) has reported it positively between Neotropical monkeys and zookeepers in Columbian Zoo. Another prevalence studies conducted among oil palm plantation workers in

Table 3. Association between sociodemographic and risk factors with prevalence of antibody reactions against *Leptospira* among respondents from BMOUI and Taiping Zoo

Sociodemographic and risk factors	Prevalence of antibody reactions against *Leptospira* among respondents (%)		n	% (95% CI)	χ^2-value	df	p-value
	Positive	Negative					
Age							
a) < 30 years	5 (45.5)	6 (54.5)	11	16.7-76.6	0.053	1	0.82
b) ≥ 30 years	5 (50.0)	5 (50.0)	10	18.7-81.3			
Sex							
a) Male	6 (50.0)	6 (50.0)	12	21.0-78.9	0.036	1	0.85
b) Female	4 (44.4)	5 (55.6)	9	13.7-78.8			
Ethnicity							
a) Malay	8 (47.1)	9 (52.9)	17	23.0-72.2	0.203	1	0.65
b) Non-Malay	2 (50.0)	2 (50.0)	4	6.8-93.2			
Educational level							
a) Lower	6 (42.9)	8 (57.1)	14	17.7-71.1	0.024	1	0.88
b) Higher	4 (57.1)	3 (42.9)	7	18.4-90.1			
Working experience							
a) < 30 years	5 (45.5)	6 (54.5)	11	16.7-76.6	0.053	1	0.82
b) ≥ 30 years	5 (50.0)	5 (50.0)	10	18.7-81.3			
Knowledge on leptospirosis							
a) Good	5 (50.0)	5 (50.0)	10	18.7-81.3	0.053	1	0.82
b) Poor	5 (45.5)	6 (54.5)	11	16.7-76.6			
Working hours							
a) < 5 hours	4 (66.7)	2 (33.3)	6	22.3-95.7	0.387	1	0.53
b) ≥ 5 hours	6 (40.0)	9 (60.0)	15	16.3-67.7			
Smoker							
a) Yes	5 (55.6)	4 (44.4)	9	21.2-86.3	0.036	1	0.85
b) No	5 (41.7)	7 (58.3)	12	15.2-72.3			
Crossing rivers/pools/ stagnant water while working							
a) Yes	7 (43.8)	9 (56.3)	16	19.8-70.1	0.015	1	0.90
b) No	3 (60.0)	2 (40.0)	5	14.7-94.7			
Wearing full personal protective equipment (PPE)							
a) Yes	3 (50.0)	3 (50.0)	6	11.8-88.2	0.119	1	0.73
b) No	7 (46.7)	8 (53.3)	15	21.3-73.4			

Melaka and Johor (Mohd Ridzuan *et al.*, 2016) and farmers in northeastern region of Malaysia (Daud *et al.*, 2018) also showed Lepto 175 as the main infecting serovar (the information on Lepto 175 is still scarce but best considered an intermediate strain, as it is closely related *L. wolffii*). However, different study in northeastern region of Malaysia by Mohd Nazri *et al.* (2012) found Patoc 1 as the most common identified serovar among town service workers while Rafizah *et al.* (2013) have shown Sejroe was the most prevalent serovar detected among febrile cases. The variations in prevalent serovars may be related to different types of maintenance hosts and their geographical distributions. Therefore, animal surveys should also be carried out as they are helpful

in determining the primary reservoirs in a community (Espi *et al.*, 1999; WHO, 2003).

In our study, despite the presence of the workers' antibodies against *Leptospira*, there were no significant associations between seroprevalence of leptospiral antibodies with sociodemographic and risk factors. The insignificance probably be due to a small number of respondents in our study. However, the seroprevalence in males (50.0%) was higher than females (44.4%) as most rangers from BMOUI and Taiping Zoo who dealt with primates were men. Previous studies by Alavi & Khoshko (2014) and Sakinah *et al.* (2015) have presented similar findings where although there were no significant differ-ence between genders, higher seroprevalence was noted in male groups due to outdoor occupation ($p > 0.05$). However, numerous studies have pointed out the significant difference in incidence between males and females attributable to the same reason ($p < 0.05$) (Kawaguchi *et al.*, 2008; Vanasco *et al.*, 2008; Dezhbord *et al.*, 2014; Dupouey *et al.*, 2014; Puca *et al.*, 2016). According to Caruso *et al.* (2013), besides occupational factor, the higher number of incidence in males was also due to the difference in immune response to *Leptospira* between genders. A sexual dimorphism in the immune response means that females were more resistant to infections (Caruso *et al.*, 2013). In terms of age, our study reported the seroprevalence in workers that aged ≥ 30 years (50.0%) was higher than < 30 years (45.5%). Majority of the positive cases worldwide also belonged to age group 41-50 and 21-30 years, which falls into productive age group (WHO, 2003; Kamath *et al.*, 2014; Oliveira *et al.*, 2017).

Regarding the seroprevalence in good and poor knowledge respondents on leptospirosis, Sakinah *et al.* (2015) has reported a higher prevalence in respondents who had poor knowledge than respondents who had good knowledge although there was no significant difference between both groups ($p > 0.05$). According to Mohd Rahim *et al.* (2011) people will hardly recognize the danger of leptospirosis with poor knowledge on the disease, thus making it impossible for them to adopt good preventive practice without knowing the risk factor. However, our findings really contradicted with Sakinah *et al.* (2015) as the seroprevalence was higher in those who had good knowledge on leptospirosis (50.0%) compared to those who had little knowledge (45.5%).

In this study, higher prevalence can also be seen in smokers (55.6%) compared to non-smokers (41.7%). An evaluation made by Cook *et al.* (2016) among high-risk workers in slaughterhouses showed that smoking was significant for leptospirosis seropositivity ($p < 0.05$). As stated by Rusin *et al.* (2002), smoking can increase the possibility of transmitting *Leptospira* from contaminated hands to the mucous membranes of the mouth. Besides smoking, there was also a significant association between skin wounds and leptospirosis infection in human as wounds were portals of entry for *Leptospira* into the body ($p < 0.05$) (Sasaki *et al.*, 1993; Phraisuwan *et al.*, 2002; Leal-Castellanos *et al.*, 2003; Sharma *et al.*, 2006; Monahan *et al.*, 2009; Sugunan *et al.*, 2009; Victoriano *et al.*, 2009; Kamath *et al.*, 2014). However, in our study, none of the participants suffered from any injuries while working.

Occupational factor such as outdoor jobs involve direct contact with soil, mud or water during work putting individuals at risk and previous studies from Thailand (Phraisuwan *et al.*, 2002) and Andaman Island (Sambasiva *et al.*, 2003) have documented it positively. Studies in other Asia-Pacific countries also showed that contact with stagnant water, flood or river water were significantly associated with leptospiral infection in humans ($p < 0.05$) (Tangkanakul *et al.*, 2000; Thai *et al.*, 2006). However, our findings revealed that the seroprevalence in respondents who crossed rivers/pools/stagnant water while working was lower (43.8%) than those who did not (60.0%). This can also be seen in previous study in Indonesia by Anies *et al.* (2009) that documented a weak association between positive cases and contact with stagnant water and flood ($p > 0.05$). Thus, we believed

that most of the past infections in BMOUI and Taiping Zoo rangers could be caused by frequent contacts with the primates as exposure to animals was known to be a potential risk factor for the disease (Haake & Levett, 2015).

Furthermore, our data also showed a higher prevalence of leptospiral antibodies (50.0%) in respondents that did not use full PPE while working than those who did (46.7%). Sakinah *et al.* (2015) has demonstrated a similar result among respondents who frequently exposed to rodents, where the prevalence of leptospiral antibodies is higher among those who did not use any protective equipment although the significant association was not found ($p > 0.05$). Nevertheless, Cook *et al.* (2016) proved that workers in slaughterhouses with protective aprons were at lower risk of testing seropositive for leptospirosis ($p < 0.05$). Therefore, wearing specific protective measures should be recommended among high occupational risk community since they significantly reduce the risk of infection particularly those who suffered lesions on their body (Leal-Castellanos *et al.*, 2003; WHO, 2003; Johnson *et al.*, 2004; Mohd Rahim *et al.*, 2011).

CONCLUSION

Although it is not possible to eliminate the occupational risk of exposure to *Leptospira*, the risk of infection can be alleviated through early recognition and appropriate management of infected or potentially infected animals, as well as proper animal handling. Personal protection must be encouraged among high-risk workers especially when dealing with wildlife. Furthermore, maintaining cleanliness of the primates' habitats and their water sources should be carried out at the earliest possible stage to reduce the possibility of getting infected. Although this study had its limitation with small sample size due to the small number of orangutan conservation sites in Peninsular Malaysia, we are expanding our research to the Borneo islands of Sabah and Sarawak.

Acknowledgements. The authors would like to thank researchers at the Institute for Medical Research for providing support and advice during the study period. Our gratitude and appreciation to the veterinarians and staffs of BMOUI and Taiping Zoo for their participation in the study. This study was supported by Universiti Kebangsaan Malaysia grant, GUP-2016-021.

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