Serological evidence of high *Leptospira* exposure among indigenous people (Orang Asli) in Peninsular Malaysia using a recombinant antigen-based ELISA

Loong, S.K.[1], Khor, C.S.[1], Chen, H.W.[2,3], Chao, C.C.[2,3], Ling, I.S.C.[1,4], Abdul Rahim, N.F.[1,4], Hassan, H.[1,4], Nellis, S.[1,4], Ching, W.M.[2,3] and AbuBakar, S.[1,4*]

[1]Tropical Infectious Diseases Research & Education Centre (TIDREC), University of Malaya, 50603 Kuala Lumpur, Malaysia
[2]Naval Medical Research Center, 503 Robert Grant Avenue, Silver Spring, Maryland 20910, United States of America
[3]Department of Preventive Medicine and Biostatistics, Uniformed Services University of the Health Sciences, Bethesda, Maryland 20814, United States of America
[4]Department of Medical Microbiology, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia
*Corresponding author e-mail: sazaly@um.edu.my
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Abstract. The lifestyles of the indigenous people (Orang Asli) of Peninsular Malaysia who traditionally live close to the forest, put them at higher risk of exposure to zoonotic diseases. Leptospirosis has recently emerged as one of the most important diseases of public health concern. Here, we aimed to obtain a baseline data on the level of *Leptospira* exposure among the 107 Orang Asli volunteers using a recombinant antigen-based ELISA, previously shown to have sensitivity of ~90.0% in comparison to the microscopic agglutination test (MAT). Among the Orang Asli volunteers in this study, 60.7% had IgM against *Leptospira* and 57.9% were anti-*Leptospira* IgG positive. Of these seropositive individuals, 29.9% had both anti-*Leptospira* IgM and IgG antibodies. Age was found to be a significant predictor for exposure to *Leptospira* ($P < 0.05$) with the younger Orang Asli population more likely to be tested positive for anti-*Leptospira* IgM. The finding of high *Leptospira* exposure among the Orang Asli volunteers could be due to their socio-economic practices and dependency on the forest for their livelihood. The rapid and sensitive recombinant antigen-based ELISA used in the study, could possibly complement MAT for the epidemiological surveillance of leptospirosis, especially among the underserved populations.

INTRODUCTION

Leptospirosis is a significant global zoonotic disease (El Jalii & Bahaman, 2004), particularly affecting people living in tropical and subtropical regions (World Health Organization, 2003). Countries in the Southeast Asia as well as the Central and South American regions are recognized as hot spots for leptospirosis (Benacer *et al.*, 2016a). This disease is endemic in Malaysia, with the first documented human leptospirosis case reported in 1925 (El Jalii & Bahaman, 2004). In recent years, the number of leptospirosis cases, including the number of deaths, have exponentially increased and these data have prompted the Malaysian government to gazette leptospirosis as a notifiable disease (Yaakob *et al.*, 2015; Benacer *et al.*, 2016b). Human infections are usually transmitted through contact with *Leptospira* contaminated environment or the blood, urine or infected tissues from animal hosts (Benacer *et al.*, 2016a). Rodents are recognized as an important maintenance hosts, central in the transmission of *Leptospira* to humans and other mammals (El Jalii & Bahaman,

2004). These maintenance hosts may excrete *Leptospira* in their urine during their life span (Lim *et al.*, 2011). *Leptospira* flourishes in the wet and humid tropical climate (El Jalii & Bahaman, 2004; Benacer *et al.*, 2016a; Benacer *et al.*, 2016b), and the leaching of *Leptospira* from the environment after heavy rains and floods have contributed to the spread of leptospirosis (Badrul Hisham *et al.*, 2009).

Leptospirosis has a wide range of clinical presentations ranging from asymptomatic infections to the more severe manifestations involving kidney and liver failure (World Health Organization, 2003). However, the majority of the infections are mild, with the presence of fever, chills, headache and muscle pain (Mohammed *et al.*, 2011). Despite the increase of reported cases in Malaysia, the true incidence rate is likely to be higher because leptospirosis tend to be misdiagnosed, as it shares similar clinical symptoms with many other diseases common in Malaysia including dengue fever and malaria (El Jalii & Bahaman, 2004; Benacer *et al.*, 2016b). Correct diagnosis of leptospirosis is onerous as the organism is difficult to isolate and culture in the laboratory (Mohammed *et al.*, 2011). This presents a challenge to clinicians as *Leptospira* infection requires immediate treatment before the disease develops into a more disseminated infection (Lim *et al.*, 2011; Yaakob *et al.*, 2015).

The microscopic agglutination test (MAT) is currently the standard method for the diagnosis of leptospirosis. However, it is laborious and cumbersome to perform, considering that the method requires live *Leptospira* cultures of multiple serovas (Chen *et al.*, 2013). Laboratory personnel have to be trained and take special precautions to prevent laboratory-acquired *Leptospira* infection when performing the MAT (Chen *et al.*, 2013; Benacer *et al.*, 2016b). MAT requires visualization of results under dark field microscopy and it is limited by the subjective interpretation of laboratory personnel (Yaakob *et al.*, 2015). Commercially available ELISA kits for the detection of *Leptospira*-specific antibodies that are more rapid, safer and easier to perform have been reported (Yaakob *et al.*, 2015), however the accuracy can be variable in different geographical regions (Desakorn *et al.*, 2012). Recently, Chen *et al.* (2013) developed an ELISA assay using *Leptospira*-specific recombinant antigens with an overall sensitivity of almost 90.0%. The assay used recombinant antigens rLipL32, rLipL41 and rLigA-Rep, chosen on the basis of their high amino acid sequence homology across a broad range of pathogenic *Leptospira* species (Chen *et al.*, 2013). Recombinant antigens offer an advantage over the MAT as they can be easily prepared and standardized, contributing to batch-to-batch assay consistency (Chen *et al.*, 2013). For these reasons, the recombinant antigen-based ELISA is preferred for population-based epidemiological studies of leptospirosis and to investigate past exposure to *Leptospira*.

In the present study, we undertook a seroepidemiological investigation of leptospirosis among the indigenous people (Orang Asli) of Peninsular Malaysia. The Orang Asli populations mostly live near forest fringe areas where they depend on swidden agriculture, hunting, fishing and the trading of forest products for their livelihood (Kari *et al.*, 2016). Although their living conditions have improved over the years, these economically marginalized populations are still burdened with zoonotic diseases including leptospirosis (Hotez *et al.*, 2015) due to their lifestyle of forest foraging and hunting for wildlife (Khor & Zalilah, 2008). This study was designed to obtain a baseline level of *Leptospira* exposure in a high risk population of Orang Asli. The findings would be useful for public health management of leptospirosis and, for comparison with other Orang Asli ethnic groups living in other parts of Malaysia.

MATERIALS AND METHODS

Ethical consideration

This study received approval and permission from the Department of Orang Asli Development (JAKOA) (Ref. No. JHEOA.PP.30.052 Jld. 6 (19)) and the Medical Ethics Com-

mittee, University Malaya Medical Centre (MEC Ref. No. 824.11). The villagers were informed that their personal information will be kept confidential, their participation in the study was entirely voluntary and they have the right to withdraw from the study at any given time. Written informed consent was obtained and an additional assent form was completed by the parents or guardians of volunteers below the age of 18 years old.

Collection of Orang Asli sera

Sera were collected from healthy Orang Asli volunteers of the Temuan sub-ethnic group, living in Kampung Dusun Kubor, Jelebu, Negeri Sembilan (3.0929°N, 102.0787°E). A total of 107 individual serum samples collected from September 2012 to February 2013 were included in the present study. Other demographic data, including the age and sex of every volunteer were also recorded.

Detection of *Leptospira* specific antibodies using ELISA

ELISA was performed to detect the binding of *Leptospira* specific IgM and IgG from the collected sera to each of the three recombinant antigens; rLipL32, rLipL41 and rLigA-Rep. This assay has previously been shown to have ~90% sensitivity based on comparison to the MAT and high specificity (>90%) when the recombinant antigens were run individually against serum samples. Each serum was tested in duplicate and was scored as positive as long as it was positive against either one of the recombinant antigen (Chen *et al.*, 2013). 11 patient sera from urban towns confirmed to be infected with dengue or Rubella virus were used as negative controls to calculate the cut-off values for the ELISA assay. Cut-off values at 99.0% confidence level, for the individual recombinant antigens were derived by the addition of the mean negative control readings to the multiplied values of the standard deviation and the standard deviation multiplier (Frey *et al.*, 1998). The ELISA protocols used in this study have been previously published by Chen *et al.* (2013).

Statistical analysis to determine predictors for *Leptospira* specific IgM and IgG

SPSS version 24.0 (IBM Corp., New York, USA) was used to perform Binary Logistic Regression with *Leptospira* specific IgM and IgG (dependant variables) against age and gender (independent variables). Independent variables with *P*-value of <0.05 are considered as significant predictors.

RESULTS

A total of 107 Orang Asli from the Temuan sub-ethnic group volunteered for this study. They consisted of 47 males (43.9%) and 60 females (56.1%) with a mean age of 24.6 (Standard deviation; ± 17.7) and median age of 18.0 years. Amongst the volunteers, 65 individuals (60.7%) had IgM immuno-globulins against *Leptospira* and 62 individuals (57.9%) were anti-*Leptospira* IgG positive. Of these seropositive individuals, 32 (29.9%) had both anti-*Leptospira* IgM and IgG antibodies and their mean age was 19.8 (SD; ± 14.7) (Table 1).

Among the IgM positive samples, 42 (64.6%), 31 (47.7%) and 22 (33.8%) samples had specific antibodies against rLipL41, rLigA-Rep and rLipL32, respectively. For the IgG positive samples, 50 (80.6%), 31 (50.0%) and 5 (8.1%) had specific antibodies against rLigA-Rep, rLipL41 and rLipL32, respectively. There were 12 (11.2%) individuals who tested negative for all the antigens, suggesting no previous *Leptospira* exposure (Table 1).

Overall, age was found to be a significant predictor for anti-*Leptospira* IgM sero-positivity (*P* < 0.05), where the younger Orang Asli were more likely to be exposed to the pathogen. Binary logistic regression analysis found that for every one year increase in age, the Orang Asli will be less likely to be tested positive for anti-*Leptospira* IgM by 3.5%. Gender however, was not a significant predictor (*P* = 0.31) for *Leptospira* exposure among our studied population. Both, the age (*P* = 0.48) and gender (*P* = 0.17) were not found to be significant predictors for

Table 1. Seroprevalence of *Leptospira* IgG and IgM antibodies among the Orang Asli at Kampung Dusun Kubor

Serology results	Age, Mean ± SD	Male, N (%)	IgM Seropositive, N (%)			IgG Seropositive, N (%)			Seroprevalence, N (%)
			rLigA-Rep	rLipL41	rLipL32	rLigA-Rep	rLipL41	rLipL32	
IgG positive	25.9±18.2	31 (50.0)	–	–	–	50 (80.6)	31 (50.0)	5 (8.1)	62 (57.9)
IgM positive	20.4±15.3	26 (40.0)	31 (47.7)	42 (64.6)	22 (33.8)	–	–	–	65 (60.7)
IgG and IgM positive	19.8±14.7	14 (43.8)	14 (43.8)	21 (65.6)	15 (46.9)	25 (78.1)	16 (50.0)	2 (6.3)	32 (29.9)
IgG and IgM negative	27.8±18.8	4 (33.3)	–	–	–	–	–	–	12 (11.2)
Overall	24.6±17.7	47 (43.9)	31 (29.0)	42 (39.3)	22 (20.6)	50 (46.7)	31 (29.0)	5 (4.7)	107 (100)

Leptospira exposure when tested with binary logistic regression using IgG as dependent variable.

DISCUSSION

The ELISA used in the study consisted of three different recombinant *Leptospira* antigens; rLipL32, rLipL41 and rLigA-Rep (Chen *et al.*, 2013). These antigens were earlier shown to exhibit high specificity and sensitivity in a laboratory study (Chen *et al.*, 2013). These antigens however, have not been evaluated for use in the field even though *Leptospira* from different geographical regions were expected to elicit comparable immune responses. We found in our study that sera of the Orang Asli volunteers showed presence of IgM (60.7%) and IgG (57.9%) antibodies against these *Leptospira* antigens, suggesting high levels of exposure to leptospires in the community. This finding was comparable to the study among the forest dwelling Irula tribe in South India where 61.1% of the population were tested positive for leptospiral antibodies (Gnanasekaran *et al.* 2013). In Sarawak, the rural communities sharing similar socio-economic traits as the Orang Asli, showed 37.4% seroprevalence of leptospirosis by MAT (Suut *et al.*, 2016), much higher than those found in hospital-based studies conducted elsewhere in Malaysia (Thayaparan *et al.*, 2015). The seroprevalence rate however, could actually be higher as MAT in general, overlooks serovars which are not cultured and maintained in the testing laboratory. This suggests that populations living at forest fringe areas are at high risk to contract leptospirosis and despite the public health threat of this disease, no study has been conducted to assess the base level of *Leptospira* exposure among the Orang Asli.

Seroepidemiological studies of leptospirosis in Malaysia have been documented and associations with occupation (El Jalii & Bahaman, 2004), gender, rainfall and age group (Benacer *et al.*, 2016a) were identified as risk factors. Army personnel (Supramaniam, 1979), town service workers (Shafei *et al.*, 2012) and paddy planters (Tan, 1970) are male-dominated occupations with high risk of exposure to *Leptospira* infections. Leptospirosis cases were reported to peak at rainy seasons with the highest number of cases involving people between the age of 20 and 49 years old (Benacer *et al.*, 2016a). Similarly, age was found to be associated to *Leptospira* exposure in our study, with the younger Orang Asli population more likely to be exposed to leptospires. To the contrary, Benacer *et al.* (2016a) found that children between the age of <1 and 9 years had the lowest leptospirosis incidence. The divergent finding was probably reflecting the different sampled populations, whereby Benacer *et al.* (2016a) studied confirmed clinical leptospirosis cases instead of the otherwise healthy Orang Asli population in our study. Initial *Leptospira* exposure in the Orang Asli children were likely to occur earlier in life, hence explaining the correlation with IgM and subsequent exposures later in life may not illicit strong serological responses as a result of high *Leptospira* background in an endemic area (Millar *et al.*, 1987). A census performed in 1991 found that on average, over 15% of male and female Orang Asli aged between 10 and 14 years were actively employed (Khor & Zalilah, 2008), and they were most likely working in oil palm plantations (Tay *et al.*, 2013). Ridzuan *et al.* (2016) reported that oil palm plantation workers were at high risk of contracting leptospirosis with a seroprevalence rate of 28.6%. Not surprisingly, oil palm plantation workers can be exposed to *Leptospira* from the urine shed by infected cows, as plantation companies allow cattle grazing on their lands to reduce weeding costs (Devendra, 1997). Apart from rodents and cows, *Leptospira* could also be transmitted to the Orang Asli by other farm animals (Bahaman *et al.*, 1987) or even wild animals (Thayaparan *et al.*, 2013). Chicken rearing may be a risk factor since rodents are attracted to chicken feed and waste (Reis *et al.*, 2008), facilitating the transmission of *Leptospira* through infected urine. Poverty has also been demonstrated to be a determinant for the increase likelihood of *Leptospira* exposure (Ashford *et al.*, 2000;

Reis *et al.*, 2008). The high seroprevalence rate of *Leptospira* exposure in the studied Orang Asli population could be contributed by these factors.

The absence of fever is possible in human leptospirosis (Ashford *et al.*, 2000) and asymptomatic individuals from endemic areas have been tested to have anti-*Leptospira* IgM (Gonsalez *et al.*, 1998). This explains the observation that all the Orang Asli volunteers in our study did not have fever and was otherwise healthy, especially among children. A higher likelihood of *Leptospira* exposure among the younger Orang Asli population probably reflects frequent exposure of children involved in water and agricultural activities (Khor & Zalilah, 2008). We hypothesized that the Orang Asli adults are relatively protected by immunity resulting from previous exposures to *Leptospira* common in the area (World Health Organization, 2003). It is also possible that the 32 Orang Asli individuals tested positive for both anti-*Leptospira* IgM and IgG is a reflection of constant exposure to *Leptospira* contaminated environments (Cumberland *et al.*, 2001). It is reasonable to believe because Kampung Dusun Kubor is located near Kenaboi River (Figure 1), where it is popular for recreational and water activities. Parts of the river however, is polluted with rubbish and leftover waste from visitors (Economic Planning Unit, 2002), attracting rodents and subsequently contaminating the area with *Leptospira*. Further research to determine the serovars circulating in the surrounding areas should be performed and novel serovars included into the existing MAT panels for future surveillance. The rapid and sensitive recombinant antigen-based ELISA used in the study could complement MAT as reference for public health laboratories for epidemiological surveillance of lepto-spirosis, especially among the underserved populations.
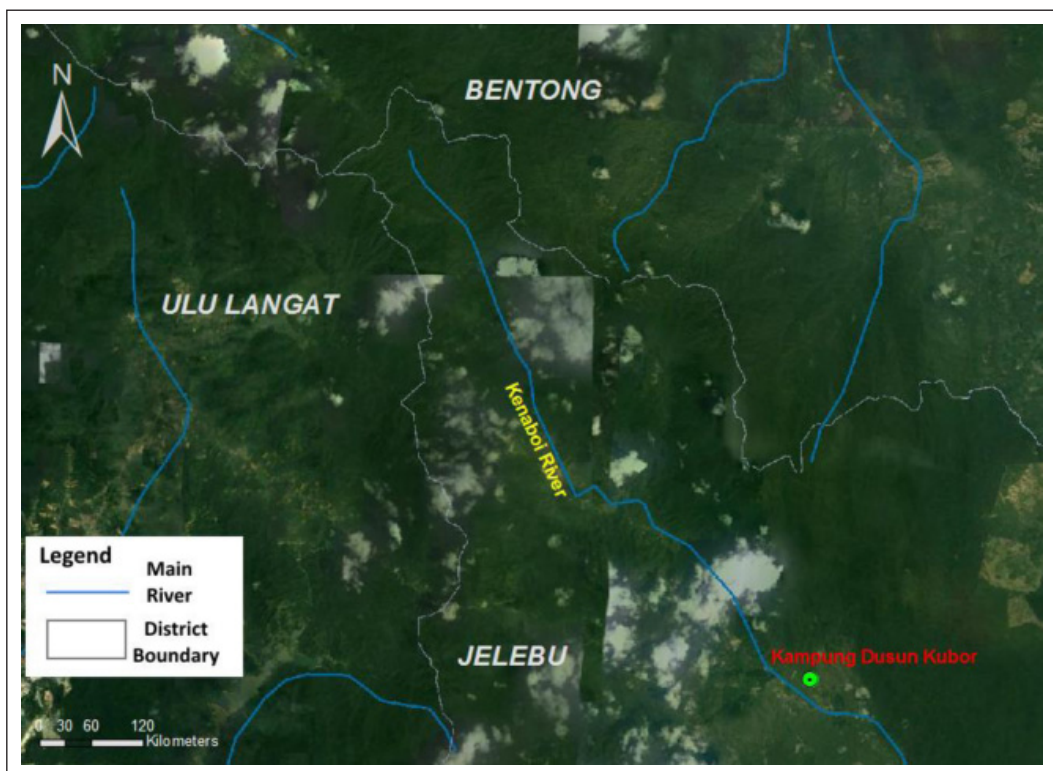

Figure 1. Map of the study site, Kampung Dusun Kubor located at Jelebu district, Negeri Sembilan.

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