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Research Article

Occurrence of *Leptospira* Species from Rodents, Soil and Water from an Oil Palm Plantation in Northern Sarawak

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Abstract

Background and Objectives: Leptospirosis is a death-causing disease caused by corkscrew-shaped bacteria, *Leptospira* especially in tropical countries. Current study was aimed to detect pathogenic, intermediate and saprophytic *Leptospira* species using polymerase chain reaction (PCR) assay from an oil palm plantation in Borneo, specifically in Miri, Malaysia. **Materials and Methods:** A total of 63 samples from rodents (n = 3), water (n = 30) and soil (n = 30) were isolated from an oil palm estate in Northern Sarawak, Borneo. All samples were inoculated into modified semisolid Ellinghausen-McCullough-Johnson-Harris (EMJH) broth with 5-fluorouracil and incubated for a month. Polymerase chain reaction (PCR) was performed using primer targeting *lipL32* (423 bp) for pathogenic, *16S rRNA* (331 bp) for intermediate and *rrs* (240 bp) for saprophytic species. **Results:** pathogenic *Leptospira* was found in 33.3% rodents (1/3) *Rattus tiomanicus*, 23.3% soil samples (7/30) and 16.7% water samples (5/30). Intermediate species were demonstrated in the other 66.7% rodents (2/3), *Sundamys muelleri* and *Rattus exulans* and 10% soil samples (3/30). Saprophytic species was found in only 3.3% soil sample (1/30). Results from DNA sequencing analysis indicated that the most dominant pathogenic *Leptospira* species discovered in the study was *Leptospira interrogans,* followed by *Leptospira noguchii* and *Leptospira weilii*. **Conclusion:** These preliminary findings provide baseline data on the occurrence of *Leptospira* species in captured rodents and the environment. These findings could assist in control and prevention of leptospirosis among oil palm estate workers in Sarawak. Awareness and knowledge on leptospirosis should be promoted among oil palm workers for prevention and mitigation.

Key words: *Leptospira*, oil palm plantation, polymerase chain reaction, DNA sequencing, leptospirosis

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Lesptospirosis is one of the emerging zoonotic diseases worldwide. Initially, *Leptospira* species was differentiated using DNA-DNA hybridisation, which found 17 species overall. Nonetheless, the latest data based on analysis of *16S rRNA* gene sequencing revealed 22 species which included 10 pathogenic, 5 intermediate and 7 saprophytic *Leptospira* species[1]. *LipL32* gene encodes for 32-kDa lipoprotein, the leptospiral outer membrane protein which is highly conserved among the pathogenic[2] and intermediate species[3]. The *16S rRNA* gene encodes for component of small ribosomal subunit in prokaryotes is used for identification of intermediate species[4]. While, a primer designed by Murgia *et al.*[5] based on *rrs* gene encoding 16S rRNA was used to specifically detect saprophytic bacteria.

The incidence of leptospirosis is frequent in Southeast Asia and known to be endemic in Thailand, Cambodia, Laos, Vietnam and Malaysia[6]. Infection with pathogenic and intermediate leptospires may induce non-specific symptoms such as fever, muscle pain, headache, while severe infection may lead to multiple organs failure and fatality[7] while saprophytic species does not cause infections. Nevertheless, intermediate species is not ensured of its pathogenicity. This matter arises when it shows different infectivity in different host species[8]. Leptospires may be transmitted to humans directly by contact with infected urine or indirectly via contaminated soil or water. Rodent is one of the most important *Leptospira* reservoirs and can transmit the bacteria to farmed animals, pets and humans. This disease is often related to occupational exposures specifically the persons involved in agricultural pursuits including oil palm workers[9].

Oil Palm industry has profited Malaysia as the second largest palm oil supplier after Indonesia. According to the recent statistics from Malaysian Palm Oil Board, Malaysia had exported 7,291,597 tonnes of palm oil valued at USD 4173.25 million from January-June, 2016[10]. Sarawak is considered as the last frontier in oil palm expansion in Malaysia where it contributes to 24% of the total oil palm areas in Malaysia. The total oil palm area in Sarawak exceeded 1.24 million ha in 2015. Leptospirosis is also a challenge to agricultural sectors and oil palm plantation workers. Oil palm plantation workers showed the highest seroprevalences (32.6%) among 18 occupational groups from northeastern Malaysia[9] while a seroprevalence rate of 28.6% was found in another study in southern Malaysia[11]. The relationship between occupation and leptospirosis has been reported in many studies[12-14]. Due to the high prevalence of antibodies in oil palm workers, the study of *Leptospira* distribution in oil palm estates in Sarawak is timely. This preliminary study was undertaken to detect pathogenic, intermediate and saprophytic *Leptospira* species present in a selected oil palm estate.

MATERIALS AND METHODS

Sampling site: Sampling was conducted from 11-16 April, 2015. A total of three captured rats, thirty soil and thirty water samples were collected and examined from a selected oil palm estate in Miri, Sarawak. Sampling was conducted at Bukit Durang in PBB Oil Palm Plantation Saremas in Miri, Sarawak (Fig. 1). Saremas Estate has two main habitats, oil palm plantation and fragmented forests. There are also riparian areas within the estate. Additionally, there are longhouses and other oil palm estates located near the sampling site. Bukit Durang Conservation area is a fragmented forest with an area of 474.88 ha dominated by secondary forest. Soil samples were collected randomly from the estate and water samples were collected from stream, drainage and nearby ponds.

Rat/rodent trappings: Wire mesh cage traps were used to catch the non-volant small mammals. They were randomly set on the ground or branches of trees along three transects throughout the sampling sessions. Twenty-eight cage traps were set up at each transect and set at every 100 m up to 300 m away from the edge and into the oil palm plantation and the forest. Each trap was tagged with flagging tape and the nearby tree was also tagged. Four types of baits were used, namely banana, pineapple, peanut and oil palm fruit. The traps were checked twice daily, around 08:00 and 16:00 and re-baited when needed. The traps were relocated if there was no capture for 2 days.

Captured rodents were removed from the cages and taken out for morphological measurements. The identification of species was based on morphological features according to guidelines of Francis[15] and Payne *et al.*[16]. Centers for Disease Control and Prevention[15] which includes head-and body length (HB), ear length (E), total length (TL), hind foot length (HF) and tail (TV) were measured using Mituyo™ digital calipers and steel rulers. Weight of the individuals was recorded by using Pesola scale. Sexes of caught rodents were also recorded.

Dissection procedure was based on the recommended procedure by Centers for Disease Control and Prevention[17] with minor modifications. The caught rats were treated with

Fig. 1: Sampling site in Miri, Sarawak

Table 1: Primers used for detection of pathogenic, intermediate and saprophytic *Leptospira* using specific PCR

Target species	Gene target	Primer sequence (5′-3′)	Size of amplicon (bp)	References
Pathogenic *Leptospira*	*lipL*32	CGCTGAAATGGGAGTTCGTATGATT CCAACAGATGCAACGAAAGATCCTTT	423	Vein[21]
Intermediate *Leptospira*	*16s rRNA*	GGCGGCGCGTCTTAAACATG TTCCCCCCATTGAGCAAGATT	331	Cetinkay[22]
Saprophytic *Leptospira*	*rrs*	AGAAATTTGTGCTAATACCGAATGT GGCGTCGCTGCTTCAGGCTTTCG	240	Murgia[23]

chloroform as inhalant anesthetics and dissected aseptically. The kidneys and liver were removed, meshed into small pieces of about 2 mm using sterile blade. The kidneys and liver samples were inoculated into Ellinghausen-Mc-Cullough-Johnson-Harris (EMJH) media added with 100 µg mL^{-1} 5-Fluorouracil. These enriched samples were incubated aerobically at room temperature in the dark for 1 month[18].

Collection of soil and water samples: Approximately, 20 g soil and 50 mL water samples were collected and processed according to the method by Ridzlan *et al.*[19] and Benacer *et al.*[20] with little modifications. The soil samples were mixed vigorously with sterile distilled water before allowed to settle for 15 min and filtered through sterile 0.2 µm membrane filters (Sartorious AG, Germany). The water samples were filtered using the similar type of membrane filter. One millilitre of the samples was inoculated into modified EMJH broth with 100 µg mL^{-1} 5-Fluorouracil, followed by aerobic incubation for one month at room temperature[1]. Later, DNA extraction and PCR assay were performed only on the positive grown cultures.

DNA extraction and PCR assay: Leptospiral DNA was extracted using Wizard™ Genomic DNA purification Kit (Promega Corporation, USA) in accordance to the manufacturer's instructions. Specific PCR assay was performed using three sets of primer as displayed in Table 1. The PCR reaction mixture (25 µL) included 0.4 µM of each reverse and forward primers (0.1 µL each), 5 µL DNA template, 5 µL of 5X green PCR buffer, 25 mm $MgCl_2$, 0.2 mM of dNTP mix and 1.25 µL *Go Taq* DNA Polymerase (Promega Corporation, USA) distilled water was added until 25 µL.

The amplification was performed with initial denaturation at 95°C for 2 min, 35 cycles each of denaturation at 95°C for 1 min, primer annealing at 55°C for 30 sec and extension at 72°C for 1 min, further extension at 72°C for 5 min and indefinite holding period at 4°C[2]. The DNAs of *Leptospira noguchii* strain LT796, *Leptospira wolffii* serovar Khorat strain Khorat-H2 and *Leptospira meyeri* strain Sant-1 were used as positive controls for pathogenic, intermediate and saprophytic strains of *Leptospira*, respectively.

Electrophoresis was performed using 1% agarose gel electrophoresis with 1X TBE buffer for 1 h 15 min at 90 V. A

Table 2: Occurrence of pathogenic, intermediate and saprophytic *Leptospira* in rats captured, soil and water samples collected in an oil palm estate in Northern Sarawak

	Pathogenic		Intermediate		Saprophytic	
Samples	Number[a]	%[b]	Number[a]	%[b]	Number[a]	%[b]
Rat	1/3	33.3	2/3	66.7	0/3	0.0
Soil	7/30	23.3	3/30	10.0	1/30	3.3
Water	5/30	16.7	0/30	0.0	0/30	0.0
Total	13/63	20.6	5/63	7.9	1/63	1.6

[a]Number of positive samples/number of samples collected, [b]Prevalence (in %) of positive samples among the samples collected

Table 3: Total score of DNA sequencing for pathogenic *Leptospira*

Sample ID	Sample types	Accession numbers	Description	Maximum scores	Total scores	Query coverage (%)
W09	Water	CP011934.1	*Leptospira interrogans* serovar Manilae strain UPMMC-NIID HP	28.2	1513.0	87
W09	Water	CP011934.1	*Leptospira interrogans* serovar Manilae strain UPMMC- NIID HP	33.7	65.7	33
W14	Water	KF2997610.1	*Leptospira weilii*	462.0	462.0	86
W14	Water	CP013137.1	*Leptospira interrogans* serovar Autumnalis strainRTCC 2802	431.0	431.0	90
S28	Soil	AY461919.1	*Leptospira noguchii* strainLSU2580	451.0	451.0	86
S28	Soil	AY461920.1	*Leptospira noguchii* strainLT796	473.0	473.0	91
Control	Soil	L14263.1	*Leptospira borgpetersenii*	48.2	48.2	1

W09 and W14 represents water isolates, S28 represents soil isolates

100 bp ladder was used as molecular marker and DNA bands were visualized by using ultraviolet transilluminator (Maestrogen). The PCR products were sent for sequencing and analyzed using NCBI BLAST.

RESULTS AND DISCUSSION

This preliminary study revealed the occurrence and distribution of *Leptospira* spp. from the collected samples (rat, water and soil samples) using PCR assay. Overall, 20.6% (13/63) samples produced *lipL*32 gene , 7.9% (5/63) samples produced *16S rRNA* gene and 1.6% (1/63) samples produced *rrs* gene. Pathogenic *Leptospira* was present in only one rat (*Rattus tiomanicus*) (33.3%), seven soil (23.3%) and five water samples (16.7%). A total of two rat species (*Sundamys muelleri* and *Rattus exulans*) and three soil samples were positive towards intermediate *Leptospira*. Saprophytic *Leptospira* was detected in only one soil sample. On the other hand, none of the water samples was positive for the presence of intermediate and saprophytic *Leptospira*. None of the rat species was positive for the presence of saprophytic *Leptospira*. The occurrence of pathogenic, intermediate and saprophytic *Leptospira* in captured rats, soil and water samples collected in this study is shown in Table 2. The gel images are shown in Fig. 2, 3 and 4. Representatives for DNA sequencing total score for pathogenic *Leptospira* are shown in Table 3.

Overall prevalence of *Leptospira* spp. in the oil palm estate: Current study provides the first account on the occurrence of *Leptospira* spp. in oil palm plantation in Sarawak with pathogenic *Leptospira* was found to be the most frequent (20.6%). Results from DNA sequencing analysis indicated that the most dominant pathogenic *Leptospira* species discovered in the study was *Leptospira interrogans*, followed by *Leptospira noguchii* and *Leptospira weilii*. Substantial concern should be focused on both pathogenic and intermediate *Leptospira* because these two groups have been documented to infect humans and animals. Another study also focused in Sarawak where *Leptospira* spp. had been detected in environmental soil and water from local national parks[1]. While, Thayaparan *et al.*[23] reported that

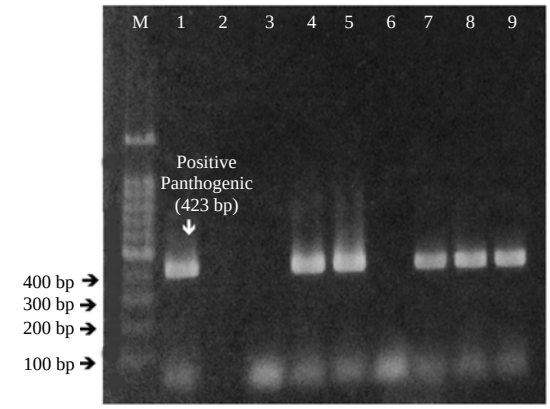

Fig. 2: Representative of PCR detection for pathogenic *Leptospira* on 1.0% agarose gel. Lane M denotes 100 bp DNA ladder, lane 1 denotes positive control, lane 2 denotes negative control, lanes 3 and 6 denote negative samples, lanes 4-5 and 7-9 denote positive samples

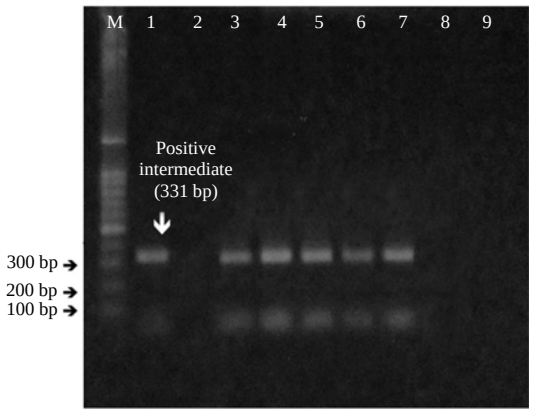

Fig. 3: Representative of PCR detection for intermediate *Leptospira* on 1.0% agarose gel. Lane M denotes 100 bp DNA ladder, lane 1 denotes positive control strain, lane 2 denotes negative control, lanes 3-7 denote positive samples, lanes 8-9 denote negative samples

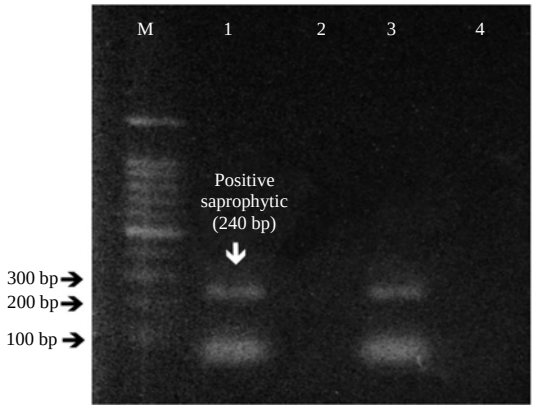

Fig. 4: Representative of PCR detection for saprophytic *Leptospira* on 1.0% agarose gel. Lane M denotes 100 bp DNA ladder, lane 1 denotes positive control, lane 2 denotes negative control, lane 3 denotes positive sample, lane 4 denotes negative sample

wildlife found nearby caves, in national park and wildlife centres in Sarawak were positive for *Leptospira*. The risk of infections to human due to pathogenic leptospires is usually the main cause of leptospirosis. Leptospirosis outbreak is plausible in the existence of animal reservoirs and infecting organism within the environment and humans. Previously, leptospirosis outbreak was documented in Beaufort, Sabah in 1999[24]. An epidemiological investigation revealed that the outbreak was associated with swimming in the creek near an oil palm plantation in Kampung Kebatu, Beaufort. The findings indicated that the water in the creek could have been contaminated by infected animal urine.

Rodents as vector for leptospiral transmission: In present study, only three rodent species were captured. The reason of the scarcity might be complex, but it might be linked to food source availability within the area. Rodent is the dominant pest in palm oil estate. They eat ripe fruit from bunches and bottom leaves of young plants. Some ripe fruits are also readily available on the ground after detachment from bunches. Oil palm estate serves food foundation for rodent was even highlighted by Puan *et al.*[18] where obliteration number of fruit bunches has association with the number of rodent in palm oil estate. Therefore, probability of successfully capturing the rodent was low during the sampling trip as they would not get into the cage traps even given with fruit baits. The presence of pathogenic *Leptospira* from one rodent sample suggested that rats were probably a reservoir of the bacteria in Borneo and that they could contaminate the environment. However, larger number of rodent sample should be tested in future to comprehensively study occurrence of the bacteria from rodent.

Percentage abundance of rat species in palm oil estate was significantly influenced by obtainability of food source and competency to adapt to the ambient for building nets from the crop-based residues and breeding. Besides vegetation areas, rats were most encountered inhabit near to junkyards where they rummage waste for food and shelter. A longitudinal study done in Peninsular Malaysia (Kelantan, Terengganu, Malacca, Selangor, Negeri Sembilan and Perak) revealed *R. tiomanicus* was the highest number of rodent species caught (420/480 rats)[25] and it was in accordance with what was reported by Hafidzi and Saayon[26]. In tandem to that, current study successfully detected pathogenic *Leptospira* from *R. tiomanicus.* Involvement of rodent was vital for leptospiral dissemination as renal tubule of the animal is the best reservoir for the bacteria[1]. A cross sectional study by Mohamed-Hassan *et al.*[25] which was conducted in the selected states in Malaysia obtained relatively high occurrence of pathogenic *Leptospira*, 42 isolates was detected from 60 isolates (70%) from rodent samples. This suggests rodents as a main reservoir of the bacteria.

Incidences of leptospirosis with occupation: Leptospirosis has long been considered as an occupational disease. The presence of *Leptospira* spp. in this oil palm plantation poses a risk of infection to the oil palm plantation workers. These findings were consistent to a study in West Malaysia which

was conducted from 1961-1971 year on 18 occupational groups to survey for leptospiral antibodies. Out of the 4646 serum samples tested, the highest leptospiral antibody rate of 32.6% (30/92) was exhibited among oil palm plantation workers[27]. Among 11 occupational groups, the highest seroprevalence rate was detected among agricultural workers (29/84 respondents) based on microscopic agglutination test (MAT) on febrile cases admitted in hospital in Northeastern Malaysia[9].

In a recent study, a 28.6% seroprevalence of leptospiral antibodies was reported among 350 asymptomatic oil palm plantation workers in Melaka and Johor in Malaysia[11]. It was assumed that the high positivity might be related to rats, which are the main carriers for leptospires. Rats are generally attracted to the fresh oil palm fruit which can be found in abundance in oil palm plantations, thus contaminating the environmental areas including soil and water. Rodents move among habitats either as part disperse process or in response to the seasonal variation habitat quality, which likelihood of human contracting the illness is increased along with the risk leptospiral transmission via urine as the rodents migrate. A part of that, *Leptospira* may also be introduced by other animals such as bats and birds due to their flight mode and spatially distributed. Even though other animals were not tested in this study, however the authors would suggest study on other animals besides bats for potential of harbouring *Leptospira* in palm oil estate. In another study, Ridzuan *et al*.[28] observed that most oil palm plantation workers use the river, trench or swamp water available in the plantations for swimming, washing hands or feet and cleaning work equipment. All of these practices increase the risk of leptospiral infection to plantation workers.

This study revealed potential risks to the oil palm workers who might come in close contact with the infected rats or contaminated environment. Therefore, the susceptible group should be warned of potential leptospirosis and proper hygienic practices. Limitations of this study were lack of sampling frequency and small sample size which would not accurately represent the whole population. Subsequently, this prompts a comprehensive study involving larger number of sample, climatic effect and longitudinal study in agricultural settings including oil palm estates in Sarawak and Borneo.

CONCLUSION

The findings of this preliminary study showed that the presence of *Leptospira* from soil and water in oil palm estates was relatively high. Even though three rodent samples were captured, it is significant to highlight the presence of pathogenic *Leptospira* from the mammal. Knowledge and awareness regarding leptospirosis among oil palm plantation workers are crucial as parts of leptospirosis preventative measures.

SIGNIFICANCE STATEMENTS

This study discovered the different prevalence of pathogenic, intermediate and saprophytic *Leptospira* species from environmental samples which were water, soil and rodent at oil palm estate in Miri, Sarawak and that can be beneficial for leptospirosis prevention measures especially among the related agricultural workers as the high-risk groups. This study will help researchers to uncover the critical area of *Leptospira* distribution in the environment that many researchers were not able to explore. Thus, a new theory on variance of diversity on these species in the environment, may be arrived at.

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