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Brief Report

Prevalence of Pathogenic *Leptospira* Spp. in Non-Volant Small Mammals of Hutan Lipur Sekayu, Terengganu, Malaysia

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Abstract: Leptospirosis is an important zoonotic disease that is transmitted worldwide through infected small mammals such as rodents. In Malaysia, there is paucity of information on the animal reservoirs that are responsible for leptospirosis transmission, with only few studies focusing on leptospirosis risk in recreational areas. Therefore, in this study we characterized the species composition and the prevalence of pathogenic *Leptospira* spp. in non-volant small mammals of Hutan Lipur Sekayu, Terengganu. We performed ten trapping sessions totaling 3,000 trapping efforts between September 2019 and October 2020. Kidney samples from captured individuals were extracted for the PCR detection of pathogenic *Leptospira* spp. Overall, we captured 45 individuals from 8 species (1.56% successful trapping effort), with 9 individuals testing positive for pathogenic *Leptospira,* that is 20% (n = 9/45) prevalence rate. *Rattus tiomanicus* (n = 22) was the most dominant captured species and was found to harbour the highest positive individual with pathogenic *Leptospira* (44.4%, n = 4/9). Despite the low successful trapping effort in this study, the result shows that the non-volant small mammals of Hutan Lipur Sekayu are capable of maintaining and transmitting pathogenic *Leptospira,* thus making this recreational area a potential infestation ground for leptospirosis.

Keywords: Animal reservoirs; Leptospirosis; recreational area; rodents; Malaysia

Introduction

The Southeast Asian rainforests are the earth's oldest, with countless biodiversity [1], and the majority of rainforests in this region are considered biodiversity hotspots [2]. Malaysia as one of the world's twelve most biologically diverse countries [3] supports extensive diversity of fauna. Its mammalian diversity is noteworthy with approximately 440 species, out of which about 15% are endemic [4]. Also, there are approximately 185 species of non-volant small mammals in Southeast Asia from different families, with about 62 of those endemic in the region [5].

However, some species of small mammals are of concern to public health as they are carriers of zoonotic diseases for example; rats are reservoirs for the bacteria (*Leptospira*) that cause disease (leptospirosis) [6, 7]. Rodents are considered the main host of leptospires bacteria and the major source of human infections [8, 9]. Pathogenic *Leptospira* spp. is the causative agent of leptospirosis [10]. This pathogenic *Leptospira* is comprised

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of eighteen identified species, out of which only the Icterohaemorrhagiae complex causes the most severe disease [11, 12, 13].

Leptospirosis outbreaks are common in the tropical region since this area provides favorable conditions such as high levels of rainfall and flooding that are necessary for leptospires survival [14]. Usually, exposure to pathogenic *Leptospira* depends on the contact between humans and infected animals or contaminated environments or surfaces [15]. On the other hand, leptospirosis is commonly associated with individuals that have frequent contact with contaminated surfaces, a process that could be easily activated during leisure activities in an infected recreational area [6].

In the past, studies on the prevalence of pathogenic *Leptospira* in small mammals of Malaysia have been limited to the urban areas [16, 17] suburban areas [18, 19] and oil palm plantations [18], with few or none directly reporting the prevalence of pathogenic *Leptospira* in recreational areas, thereby limiting our understanding of the disease transmission potential in these areas. Only one study has reported the presence of pathogenic *Leptospira* spp. in water samples obtained from selected recreational areas of Terengganu [20], however, there is lack of information on the species composition of non-volant small mammals and the prevalence of pathogenic *Leptospira* spp. in these species in Terengganu. Therefore, this study examines the species composition of non-volant small mammal species in a recreational forest located in Kuala Berang, Hulu Terengganu, Malaysia, and the prevalence of pathogenic *Leptospira* in these small mammals in an attempt to guide potential future interventions.

Methods

Study area

The study was carried out in Hutan Lipur Sekayu (N04° 57.85' E102° 56.71'), a recreational forest located in Kuala Berang, Hulu Terengganu, Malaysia (Fig 1), with an estimated population of 89,000 inhabitants of low to moderate standard of living [21]. The recreational area has a total landmass of about 30 hectares, and boasts facilities like waterfall, rest areas, public toilets, camping sites, playgrounds and food stalls for the visitors.

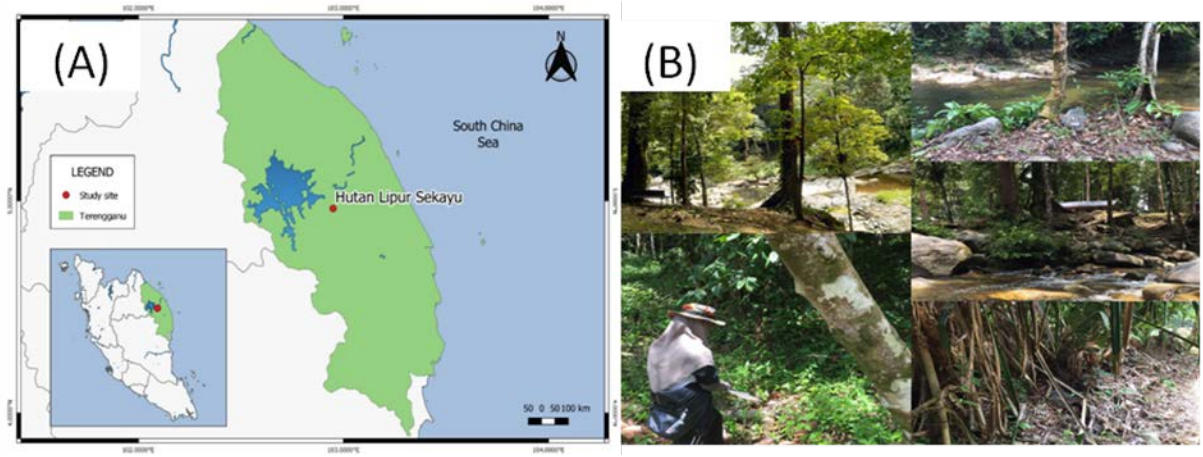

Figure 1. Map of Hutan Lipur Sekayu, Terengganu, Malaysia. (A) Location of the study site in Peninsular Malaysia, Terengganu state; (B) Location of the trapping sites.

Animal trapping and sampling collection

Animal trapping was performed using wire-mesh live traps (25 cm x 15 cm x 12 cm) which were set up randomly along the forest trails/streams with an approximately 10m distance between traps. There were 20 trapping points with 5 live traps set at each point for three consecutive nights, making a total of 300 trapping efforts per trapping session. Overall, a total of 10 trapping sessions were conducted between September 2019 and October 2020. Ripe banana baited traps were set at dusk and checked at dawn each day.

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Baits were replaced each day to maintain freshness. The captured animals were kept in an individual cloth bag and transported to the field station, with their weight and sex recorded. A Field Guide to the Mammals of South-East Asia [22] was used for the identification process.

All captured animals were humanely euthanized using diethyl ether and processed for kidney removal. The kidney from individual animal was placed in a sterile specimen container and stored in a Panasonic Ultra-Low Freezer at -86℃ until further investigation. All animal procedures were carried out according to the protocol previously described by Mills et al. [23].

Ethical statement

This study was conducted in accordance with the Malaysian laws regarding ethics in research. The Ethics Review Committee Board of the Universiti Malaysia Terengganu gave the approval and permission to conduct research on small mammals with Project Number: UMT/JKEPHT/2019/30.

DNA extraction

DNA extraction procedure was performed on kidney samples according to the technical manual for genomic DNA purification/ Extraction Kit (QIAamp DNA DNeasy Blood and Tissue Kit, Qiagen, California, USA). Briefly, kidney samples were rinsed with a sterile phosphate-buffered saline solution to reduce possible bacterial contaminants. A small piece of kidney tissue was cut (25 mg) and placed into a 1.5ml micro-centrifuge tube containing 180µl Buffer ATL and 20µl proteinase K to break the cell membrane and release DNA into the solution. Then, the tube was incubated at 56℃ until samples were completely lysed.

A 200µl Buffer AL was added and the samples incubated at 56℃ for 10 minutes. A volume of 200µl ethanol (96-100%) was added to enhance the binding of DNA to silica. The precipitated DNA was filtered through DNeasy mini spin column by centrifugation for 1 minute at 8,000rpm. The flow-through was discarded and the filtered DNA placed in a new spin column with 500µl AW1 and AW2 buffer. The flow through and collection tube were removed after 3 minutes of centrifuge at 14,000rpm. DNA was eluted in a new 2ml micro-centrifuge tube with 100µl Buffer AE. The quality and the quantity of the DNA were recorded using Nanodrop™.

PCR detection of pathogenic Leptospira spp.

The DNA of *Leptospira* used in PCR analysis was standardized to 20ng/µl using the manufacturer's guidelines as contained in the Qiagen kit. LipL32 primer set was used to detect the presence of pathogenic *Leptospira* spp. DNA with a forward sequence of LipL32 -45F 5′ -AAG CAT TAC CGC TTG TGG TG -3′ and reverse sequence of LipL32 -286R 5′ -GAA CTC CCA TTT CAG CGA TT -3′ [24]. The positive and negative controls were included in each PCR run. DNA of pathogenic *Leptospira, L. interrogans* serovar Copenhageni was used as the positive control whereas distilled water was used as the negative control. Amplification of the DNA was conducted in a total volume of 25µl consisting of 12.5µl ready to use Mastermix, 1µl forward and 1µl reverse primer and 0.5µl probes at a concentration of 10µM, and diluted to a final volume of distilled water and 5µl DNA templates. These protocols of amplification were enhanced according to Eppendorf real-time PCR system and consisted of 2- minute denaturation at 94℃ followed by 35 cycles of amplification at 94℃ for 3s, 58℃ for 15s, 72℃ for 1 minute and final extension at 72℃ for 10 minutes. Afterward, the reaction was stopped at 4℃. The results of the data were analyzed using the software provided by real-time PCR system (Eppendorf realplex 2.2). Gel electrophoresis in a 1.0% TAE agarose gel stained was run to analyze the amplification of products, with results deemed only valid if the positive and negative controls produced the expected results. Samples were interpreted as positive for pathogenic *Leptospira* spp. if a band corresponding to 242 base-pair (bp) was obtained or otherwise interpreted as negative. 16S rRNA sequencing was performed for

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positive samples to identify *Leptospira* species [25]. Primers Lep1 and Lep2 which related to 16S rRNA gene has forward sequence of 5′-GGA ACT GAG ACA CGG TCC AT -3′ and reverse sequence of 5′- GCC TCA GCG TCA GTT TTA GG -3′. PCR was performed using primers to amplify 412 base-pair (bp) fragment correspond to 16S rRNA genes. The PCR protocol designed for both LipL32 and 16S rRNA genes were same.

Data analysis

We used descriptive analysis to report species diversity and abundance, and analyzed the association between *Leptospira* positive individuals and species, age and sex using Fisher exact test for small samples. All analyses were performed in R 4.0.0 version [26].

Results

We had total trapping effort of 3,000 over ten trapping sessions. Out of this, 121 traps were either lost or damaged, thus leaving us with a total of 2,879 effective trapping efforts. Overall, we captured 45 individuals (1.56% trapping success) from 8 species, comprising of 30 (66.7%) rats, 10 (22.2%) tree shrews, and 5 (11.1%) squirrels (Table 1). The captured non-volant small mammals belonged to two orders (Scandentia and Rodentia) and three families (Tupaiidae, Muridae and Sciuridae). The majority (67%) of the captured individuals were from the family Muridae followed by Tupaiidae, (22%), and then Sciuridae (11%).

Table 1. Number of captured non-volant small mammal species and positive individuals in Hutan Lipur Sekayu recreational area.

Order & Family	Scientific name	Common name	Total individuals captured	Total positive individuals (%)
Scandentia Tupaiidae	*Tupaia glis*	Common Treeshrew	10	2 (20)
Rodentia Muridae	*Rattus tiomanicus*	Malaysian Wood Rat	22	4 (18.2)
	Rattus rattus	Black Rat	1	0 (0)
	Maxomys rajah	Rajah Spiny Rat	4	1 (25)
	Maxomys whiteheadi	Whitehead's Spiny Rat	1	1 (100)
	Sundamys muelleri	Muller's Giant Sunda Rat	2	1 (50)
Rodentia Sciuridae	*Collasciurus notatus*	Plaintain Squirrel	4	0 (0)
	Sundasciurus tenuis	Slender Squirrel	1	0 (0)
	Total		**45**	**9 (20)**

Out of the 45 captured individuals, 9 (20%) from four species of rodents and one species of tree shrew tested positive for pathogenic *Leptospira* (Table 1). *R. tiomanicus* (n = 22) was the most dominant captured species, followed by *T. glis* (n = 10). Also, we observed a non-significant association in the gender of the nine positive individuals (Supplementary material 1). In the same vein, the Fisher exact test showed no significant difference between the eight adults and one juvenile that tested positive for pathogenic *Leptospira*.

As shown in (Fig 2), all nine isolates amplified by LipL32 primers were confirmed as *Leptospira interrogans* with results from gene sequencing also identifying all isolates as *Leptospira interrogans*.

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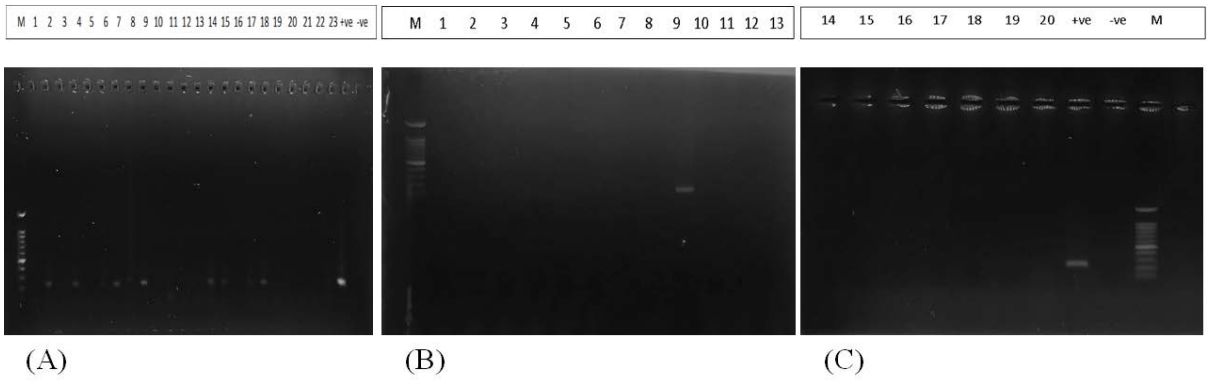

Figure 2. Results from PCR. (A) Agarose gel electrophoresis showing PCR products amplified by LipL32 primer; Lane M: DNA size marker, 100 bp DNA ladder; Lane 1-23: Sample of cultured kidneys; Lane -ve: Negative control; Lane +ve: Positive control (*L. interrogans* serovar Copenhageni), (B & C) Agarose gel electrophoresis showing PCR products amplified by LipL32 primer; Lane M: DNA size marker, 100 bp DNA ladder; Lane 1-20: Sample of cultured kidneys (Second batch); Lane -ve: Negative control; Lane +ve: Positive control (*L. interrogans* serovar Copenhageni).

Discussion

Our findings of family Muridae being the most abundant in the study area is not surprising, considering they are the most abundant family of rodents, with more than 560 species representing 126 genera [27]. Their abundant status in the study area might also be because the majority of the species are forest and habitat generalists that will typically exploit any habitat type and thrive in a wide range of conditions [28]. Moreover, most of the habitat generalist species from this family feed on large groups of leaves, seeds, fruits and roots that are usually available in the study area, thus enhancing their survival rate in the study area [29]. Apart from the family Muridae, family Tupaiidae (treeshrew) and Sciuridae (squirrel) were also recorded in this study. Both of these families shared the same characteristics; diurnal species with their main diet including insects and a wide variety of fruits and seeds [22], while they can also be found in a wide range of forest types such as plantations, cultivated areas and gardens.

The most dominant species recorded in this study is in line with the previous study by Rahim et al. [30], probably due to these species being habitat generalists, nocturnal species capable of inhabiting forested/agricultural areas that are capable of spending time on trees and similarly exploring the ground [22]. They hide under fallen trees/branches in low woody vegetation, these attributes altogether probably contribute to their high capture rate.

The prevalence of pathogenic *Leptospira* in the five species is similar to the result of a previous study conducted in a recreational area by Yusof et al. [31] who found *Leptospira* in all species reported in the study except for *M. rajah*. This shows that the non-volant small mammals of Hutan Lipur Sekayu have the potential to maintain the pathogenic *Leptospira* transmission cycle in the study area. Also, the 20% (n = 9/45) prevalence rate of pathogenic *Leptospira* reported here is comparatively similar to that reported from a previous study (19.4%, n =18/93) also from non-volant small mammals in recreational areas of Selangor, Malaysia [31]. Even though we could not statistically make inference from our result owing to the low sample size, but the result shows that the study area serves as a potential infestation ground for leptospirosis. According to the Ministry of Health Malaysia [32], recreational areas are categorised as one of the most important hotspots for leptospirosis outbreaks after settlement areas. The somewhat poor garbage disposal by visitors at the centre could indirectly attract rats, which in turn shed the pathogenic bacteria into the water/soil that could later infect visitors visiting the park [33].

In conclusion, although our sample size limits the generalizability of our study, the result nevertheless illustrates that the non-volant small mammals could maintain and transmit patho-

genic *Leptospira* to tourists in the study area. Therefore, appropriate waste management should be implemented in the study area in order to avoid possible rat propagation and the subsequent rat-human interaction. Likewise, public awareness programmes should be conducted to increase tourists' knowledge about leptospirosis to avoid future outbreaks in recreational areas.

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Data availability statement: All data and code used in this study is available in Zenodo under the Creative Common 4.0 license, accessible through https://doi.org/10.5281/zenodo.5156867

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