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Research Article

MOLECULAR DETECTION OF LEPTOSPIRAL DNA IN KIDNEY AND LIVER SAMPLES OF WILD LONG-TAILED MACAQUES IN SELANGOR, WEST MALAYSIA

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ABSTRACT: Leptospirosis is a zoonotic bacterial disease that is re-emerging and is caused by pathogenic species of the genus *Leptospira*. Although rodents are acknowledged as the most significant reservoirs of the disease, domestic and wild animals play a substantial role in the epidemiology of leptospirosis by acting as either natural or incidental hosts. Malaysia is the epicentre of leptospirosis, with a significant population of wild long-tailed macaques. The investigation collected 120 samples from 14 wildlife-human conflict zones in Selangor, with 60 samples each from kidney and liver tissues. Each tissue was subjected to PCR after DNA extraction, with a focus on the 16S rRNA gene for intermediate serovars and lipL32 for pathogenic serovars. When exclusively examining kidney tissues, Leptospiral DNA was detected in 16.67% (10/60) of kidney samples that targeted the 16S rRNA gene, while no Leptospiral DNA was detected in the liver tissues. The Leptospiral DNA detection rate was 8.33% (10/120) when all samples from the liver and kidney were combined. Although 24% (6/25) of male and 11.4% (4/35) of female macaque kidney samples tested positive, no statistically significant association between Leptospiral detection and sex was observed (p > 0.05). Despite the fact that the DNA detected was of intermediate *Leptospira*, it is important to not underestimate its potential to significantly contribute to the epidemiology of leptospirosis. The invasion of households and public buildings in certain regions of the study area by long-tailed macaques is a cause for concern not only for public health, but also for the potential transmission of *Leptospira* to domestic animals, wild animals, and humans through their urine contamination.

Keywords: *Leptospira*, Kidney, Liver, Long-tailed macaques, PCR, Zoonosis.

INTRODUCTION

Leptospirosis is a zoonotic bacterial disease that affects humans, domestic animals, and wildlife and is caused by pathogenic species of the genus *Leptospira* [1,2,3]. The disease is regarded as a worldwide threat to public health and is more prevalent in tropical and subtropical countries where the environment is conducive to the survival of the organisms and the spread of the disease [4,5,6]. Leptospirosis is responsible for 1.03 million cases worldwide annually, with over 500,000 severe cases and 58,900 fatalities [7,8]. Nevertheless, it is posited that the cases are underrated as a result of the absence of specific symptoms, the brief or insignificant manifestations in certain cases, the low levels of knowledge among medical professionals, and the readily accessible diagnostics [9,10]. The primary sources of the disease are contaminated water, soil, sediment, feed, aborted fetuses, and the urine of infected or carrier animals [11]. The organism enters the body through the mucous membranes of the nose, eyes, vagina, or abraded epidermis [12]. The transmission of the infection can be either direct or indirect, contingent upon the source. The transmission of leptospirosis is believed to be influenced by a variety of factors, including the degree of interaction between incidental and maintenance hosts, environmental climate, and population density [11].

The genus *Leptospira* has been recently redefined, and it is now known to contain 66 species and over 300 pathogenic serovars. The serovars are categorized based

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on the antigens of their outer lipopolysaccharide [13,14]. Serovars that are antigenically similar are classified into similar serogroups [15]. The pathogenicity of the species in the genus *Leptospira* is categorised into three groups: saprophytic, intermediate, and pathogenic [16]. The pathogenic and intermediate species of *Leptospira* are the causative agents of the disease, while saprophytic species exist in the environment and have a weak relationship with mammalian hosts [17,18,19], or exist as free-living organisms in water and soil [20]. In contrast to the conventional belief that pathogenic leptospires could only proliferate in infected individuals, a recent study has demonstrated that both saprophytic and pathogenic leptospires increased their numbers in waterlogged soil, not just in the soil or water [21]. Leptospires, despite their fragility, have been demonstrated to endure and even preserve their virulence for as long as 20 months in unfavourable environments, such as frigid, nutrient-poor acidic waters [22,23]. Khairani-Bejo *et al*. [24] Reported that leptospires can persist in water under the conditions of the Malaysian field. They also live longer in shady areas than in areas with direct sunlight.

Because of the high prevalence of infections (nearly 90%) and the remarkably high level of spirochetes in rodent urine compared to other animal species, rats particularly *Rattus norvegicus* are considered the major and most significant reservoir hosts of leptospirosis globally [25,26,27]. All mammals are susceptible to infection from any serovar, aside from the multitude of domestic and wild animals that might act as asymptomatic reservoir hosts of leptospirosis [28,6]. Cattle, pigs, dogs, small ruminants, horses, camelids, and occasionally certain wildlife species are infected by pathogenic leptospires [29,30]. Despite having the ability to act as unintentional hosts for leptospirosis, wild long-tailed macaques may not contract the disease to a significant degree like other wildlife species. Therefore, the study sought to explore the possible contribution of wild long-tailed macaques to the existence and spread of leptospires, considering the macaques' expanding population and their near proximity to human settlements in both rural and urban regions, which may pose a risk to public health.

MATERIALS AND METHODS

Study site and sample collection

Based on opportunistic sampling caused by macaques-human conflicts (WHC) from 14 areas under six out of nine districts of Selangor state, the Department of Wildlife and Natural Park Peninsular Malaysia (PERHILITAN) collected and provided a total of 60 carcasses of wild long-tailed macaques (*Macaca fascicularis*) of various ages. Pulau Indah, Teluk Panglima Garang, Cheras, Pulau Meranti, Cyberjaya, Puncak Alam, Klang, Ulu Jenderam, Ulu Langat, SG Merab, Shah Alam, and Ulu Jenderam are among the localities. During the necropsy of the 60 long-tailed macaques, a total of 120 samples (60 liver samples and 60 kidney samples) were taken. The liver and kidney samples were processed and put into a collecting tube, each measuring approximately 1 cm by 1 cm. The tissues were all stored at -20°C prior to the extraction of DNA.

Molecular PCR protocol for *Leptospira* DNA detection

The DNeasy@ Blood and Tissue reagent (Qiagen, Germany) was employed to extract genomic DNA from the kidney and liver in accordance with the manufacturer's instructions. In summary, 25 mg of each tissue was cut into small fragments and transferred to a 1.5 mL microcentrifuge tube. 180 µL and 20 µL of tissue lysis buffer (ALT) and proteinase K were added, respectively, and the mixture was thoroughly mixed by vortexing. The tissue was completely lysed after the mixtures were incubated at 56 0C. The 16S rRNA gene of the genus *Leptospira* was detected by subjecting the entire extracted DNA template to a conventional PCR. The PCR was conducted using a 25 µL reaction volume, 12.5 µL taq polymerase master mix, 1.25 µL of forward and reverse primers (details in Table 1), 5 µL of RNase-free water, and 5 µL of DNA template. The PCR was conducted in an Eppendorf master cycler (Germany) according to the following protocol: The initial denaturation was conducted at 94ºC for 5 minutes, followed by 30 cycles of denaturation at 94ºC for 1 minute, annealing at 58ºC for 45 seconds, and DNA extension at 72ºC for 30 seconds. The final extension step was conducted at 70ºC for 6 minutes [31]. The solvent for electrophoresis and the preparation of a 1.5% agarose gel was 0.5X Tris borate EDTA (TBE) buffer. The initial three wells were supplied with a 100 bp ladder, a negative control (RNase-free water), and a positive control (Bataviae), respectively, and were subsequently followed by the samples. The 430 bp DNA products of PCR or amplicons for non-pathogenic leptospires were obtained from the amplifiable DNA template. The pathogenic leptospires were anticipated to have a 756 bp genome, as evidenced by the positive control. A constant voltage of 80 volts was used to perform an electrophoresis on a 1.5% agarose gel for

one hour. The Alpha Innotech gel imaging systemTM machine was employed to visualize the amplicons that were produced under UV light. Depending on the primer employed, bands that corresponded with the positive control (Bataviae) were classified as positive. In order to prevent the occurrence of false positive results due to contamination, a negative control (RNase-free water) was implemented.

RESULTS AND DISCUSSION

Molecular result revealed that *Leptospira* DNA, which targets the 16S rRNA gene, was found in 16.67% (10/60) of kidney samples on its own. On the other hand, the percentage of positive molecular detection of *Leptospira* DNA when the liver and kidney are combined is 8.33% (10/120). The fact that *Leptospira* DNA could only be successfully detected molecularly in kidney samples means that the positive samples came from macaques that were infected for an extended period of time. These macaques could act as carrier hosts, continuously excreting leptospires in their urine and potentially contaminating the surrounding area.

At the 5% significance level, there was no statistically significant association between Leptospiral detection and the sex of macaques, although 24% (6/25) of kidney samples from males tested positive

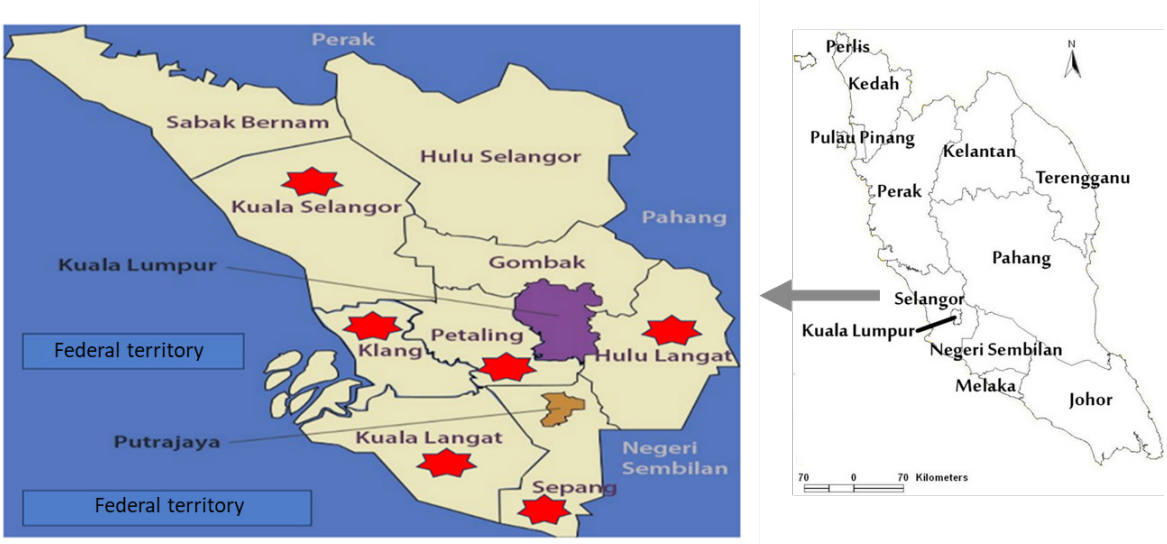

Fig. 1. The map of Selangor showing 6 districts (with red stars) out of 9 districts of the state from where sample of macaques were collected. The two federal territories, Kuala Lumpur and Putrajaya are situated within the state of Selangor.

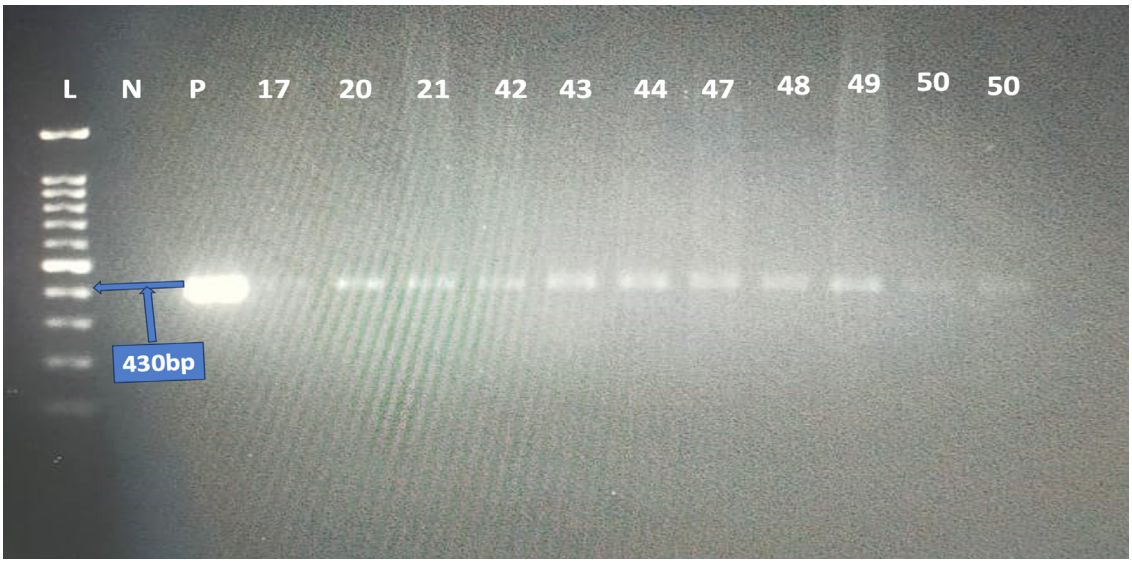

Fig. 2. The image displays a 1.5% agarose gel stained with ethidium bromide, showing the specific PCR products amplified with primers 16SrRNA. The gel electrophoresis setup includes a 100-bp DNA ladder marker in lane L), negative control in lane N), a positive control in Lane P), and DNA samples from kidney tissues that tested positive for Leptospira (Lane 17 to 50).

Table 1. Oligonucleotide information for the detection of Leptospiral DNA in macaques.

Primer name	Sequence (5' →3')	Amplicon size (bp)	Length	Reference
16SrRNA-F	GAACTGAGACACGGTCCAT	430	19	**32**
16SrRNA-R	GCCTCAGCGTCAGTTTTAGG	430	20	
LipL32-F	ATCTCCGTTGCACTCTTTGC	756	20	**33**
LipL32-R	ACCATCATCATCATCGTCCA	756	20	

compared to 11.4% (4/35) from females. The current study is consistent with previous investigations of leptospirosis in humans, which indicated that men were at a ninefold increased risk of contracting the disease compared to females [35]. This risk was attributed to their occupations, including banana plantation farmers, sugarcane workers, paddy farmers, veterinarians, animal shelter employees, hunters, damp market workers, and abattoir workers [36,37], which frequently resulted in skin injuries and abrasions [38]. Research has demonstrated that the male is frequently associated with the risk of canine leptospirosis in dogs [39]. This association may be attributed to the fact that male dogs are more likely to roam, which increases their susceptibility to diseases [40]. In male primates, it may be because of their aggressive behaviours, including resource protection between groups, predation, and intragroup social situations such as competition for mates, status, food, and other resources, as well as reproduction [41].

One-third of the macaques (33.33%) were younger than a year old, 9.09% (1/11) were between one and three years old, 22.72% (5/22) were between three and six years old, 16.67% were between six and nine years old, and 25% (1/4) were aged twelve and above, based on the age-based PCR positive result. However, none of the macaque samples from nine to twelve years old tested positive. This study has demonstrated that age plays a significant role in leptospirosis detection. The age group of less than a year-old macaques had the highest detection rate, with 33.33% (1/3) positive cases. This age group was followed by those aged 12 years and beyond, with a detection rate of 25% (1/4). This result is consistent with earlier research on dogs, which demonstrated that leptospirosis is 16.5 times more common in dogs under a year old and 11 times more common in dogs one year of age and older [42]. It implies that young macaques may experience significant levels of pressure in their early years, presumably as a result of social interactions that hinder colonies from associating with adult possible carriers and a contaminated environment when they are feeding and foraging.

40% (4/10) of the positive cases were identified in macaques obtained from Puncak Alam, 20% (2/10) from Teluk Panglima Garang, and 10% each from Ulu Jenderam, Hulu Ulu Jenderam, ABI UPM, and Teluk Panglima Garang. All of the animals were captured in the vicinity of populated areas and permitted to roam freely in areas where they could have encountered other animals, including livestock, and potentially their urine. This has the potential to either expose macaques to the risk of contracting leptospirosis from livestock that is infected with the disease, or it can serve as a source for macaques to introduce leptospires into human and animal environments.

CONCLUSION

The macaques were chronically infected, carriers, and potentially discharging leptospires in their urine, as evidenced by the molecular detection of Leptospiral DNA in their kidneys rather than their livers in this study. The urine's potential to contaminate the environment (soil and water) could consequently pose a significant health risk to other animals, both domestic and wild. Given that tong-tailed macaques are frequently observed in the study areas and occasionally infiltrate public structures and residences. Consequently, the results of this investigation can be employed to inform and educate the public regarding the zoonotic implications of environmental contamination caused by their urine.

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