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SHORT COMMUNICATION

Prevalence of *Leptospira* Species in Environmental Soil and Water from National Parks in Sarawak, Malaysia

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ABSTRACT

In Malaysia, there is an increasing number of reported leptospirosis cases which led to mortality. The infection is usually spread through the urine of infected animals and may contaminate the environmental soil and water. This study was conducted to determine the prevalence of *Leptospira* spp. in two national parks in Sarawak. A total of 50 soil and 60 water samples were collected from Tanjung Datu National Park (TDNP) and Bako National Park (BNP). The samples were filtered through sterile membrane filter prior to inoculation into modified semisolid Ellinghausen-McCullough-Johnson-Harris (EMJH) media added with 5-fluorouracil. The cultures were incubated at room temperature (28-30 °C) for 30 days before specific polymerase chain reaction (PCR) was conducted. PCR-positive samples were subjected to DNA sequencing. Out of the 110 environmental samples from two national parks in Sarawak, the prevalence of pathogenic, intermediate and saprophytic *Leptospira* was 0.9%, 5.5% and 0%, respectively. Results indicated that *Leptospira borgpetersenii* serovar Mini and *Leptospira wolffii* serovar Khorat were the pathogenic and intermediate *Leptospira* circulating in these study areas, respectively. Due to the ability of *Leptospira* to survive for months in environment, there is a risk of exposure to the public and tourists who visit these national parks. Increased awareness, continuous monitoring and effective preventive measures should be taken by local authorities to control leptospirosis outbreak.

Keywords: *Leptospira*, national park, soil, Sarawak, water

Leptospirosis, more commonly known as "rat urine disease", is a zoonotic disease caused by *Leptospira* sp. This disease is usually transmitted through direct contact with infected animal urine or indirect contact with soil and water contaminated with urine from infected animals (Lim *et al.*, 2011; Villanueva *et al.*, 2010). It is a helically coiled, highly motile, flexible and filamentous spirochete with hook-shaped end.

Based on DNA-DNA hybridization, there are 17 species of *Leptospira* (Lim *et al.*, 2011). These species can be further divided into pathogenic, intermediate or opportunistic and non-pathogenic *Leptospira*. Pathogenic *Leptospira* are normally found in the renal tubules of host animals but they can be spread through the urine of carrier animals to the environment while saprophytic *Leptospira* are commonly isolated from humid environments. On the other hand, there is a lack of information on the pathogenicity of the intermediate *Leptospira* as their phenotypic characteristics are different from pathogenic *Leptospira* (Lim *et al*., 2011; Slack *et al.*, 2006).

The most severe form of leptospirosis is often characterized by jaundice, continuous fever, azotaemia, renal failure, consciousness disturbances, haemorrhages and thrombocytopenia. The incubation period varies from 2 to 20 days (Guidugli *et al.*, 2000). In Malaysia, leptospirosis has been gazetted as a notifiable disease in 2010 under the Prevention and Control of Infectious Diseases Act 1988. According to Sarawak Health Department (2015), a total of 616 cases and 24 deaths resulted from leptospirosis had been notified in 2014 whereas 163 cases with six deaths was reported as of 7 March 2015. This is of public health concern and hence this issue should be a cause of concern to health authorities and the community at large.

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Several occurrence studies on *Leptospira* from environmental samples had been conducted in Malaysia. Benacer and co-workers (2013) studied the presence of *Leptospira* in environment from recreational parks and drain effluents of residential homes in Kuala Lumpur, Selangor and Johor. Ridzlan *et al.* (2010) reported the presence of *Leptospira* in soil and water from National Service Training Centres in Kelantan and Terengganu. Meanwhile, in Sarawak, Thayaparan *et al.* (2013) reported the occurrence of *Leptospira* in wildlife around tourism areas whereas Su'ut *et al.* (2011) highlighted the distribution of *Leptospira* in Rejang Basin. To the best of our knowledge, there is no published data on the distribution of *Leptospira* species in environmental soil and water from national parks in Sarawak.

In general, national parks are established for recreational and educational purposes due to the extensive diversity of flora and fauna. This helps to promote the eco-tourism in Malaysia (Backhaus, 2003). However, an outbreak due to leptospirosis was reported among athletes participating in Eco-Challenge-Sabah 2000 held in Malaysian Borneo. It was reported to be associated with sport activities such as jungle trekking, prolonged swimming and kayaking (Sejvar *et al.*, 2003). Consequently, surveillance of leptospirosis in these tourism areas are of significant importance as an effective public health tool in preventing large outbreaks of the disease (Victoriano *et al.*, 2009).

A variety of methods have been employed in the detection of *Leptospira* sp. including conventional culturing method as well as molecular techniques. Polymerase chain reaction (PCR) is the preferred molecular method to detect fastidious microorganisms including *Leptospira*. Different primer pairs targeting specific genes, especially the *16S* or *23S rRNA* genes have been designed for the rapid detection of *Leptospira* (Uavechanichkul *et al.*, 2011). PCR assay using sets of primers that target the *LipL32*, *16S rRNA* and *rrs* genes were useful and could differentiate the pathogenic, intermediate and saprophytic isolates (Ridzlan *et al.*, 2010). It should be noted that PCR offers some advantages over gold standard of culture and microscopic agglutination test (MAT), due to its speed and does not require any reference hyperimmune antisera (Ahmed *et al.*, 2012).

The objective of this study was to determine the prevalence of pathogenic, intermediate and saprophytic *Leptospira* in environmental soil and water from two national parks in Sarawak, Malaysia. Through this study, we aimed to gain an insight on the survival of *Leptospira* sp. in the diverse environmental conditions, and contribute to the current knowledge regarding the distribution of the organism, especially in national park.

The study areas were chosen because national parks had been successfully attracting both local and foreign tourists to engage in eco-tourism activities, for example hiking, jungle trekking and bird watching. The two selected national parks, Tanjung Datu National Park (TDNP) and Bako National Park (BNP) are situated at N02°03'18.97" E109°38'31.93" and N01°42'59.83" E110°28'0.23", respectively (Figure 1). As one of the most beautiful national parks in Sarawak, TDNP is an important reservoir of biodiversity with undisturbed beach and patches of coral reef. It is also one of Sarawak's smallest national parks with an area of about 14 square kilometers. On the other hand, as the oldest national park in Sarawak, BNP offers visitors an excellent place for jungle trekking with its rainforest offering and abundant wildlife. It covers an area of 27 square kilometers at the tip of Muara Tebas peninsula.

Sample collection was conducted at TDNP and BNP from April 2014 to June 2014. The methodologies employed by Ridzlan *et al.* (2010) and Benacer *et al.* (2013) were adapted with some modifications. A total of 50 soil (20 from TDNP, 30 from BNP) and 60 water samples (30 from TDNP, 30 from BNP) were collected from the two study areas. About 20 g soil and 50 ml water samples were placed in different 50 ml sterile falcon tubes. The pH and temperature of all the samples were recorded using portable pH/temperature meter (Extech PH100 ExStik, USA).

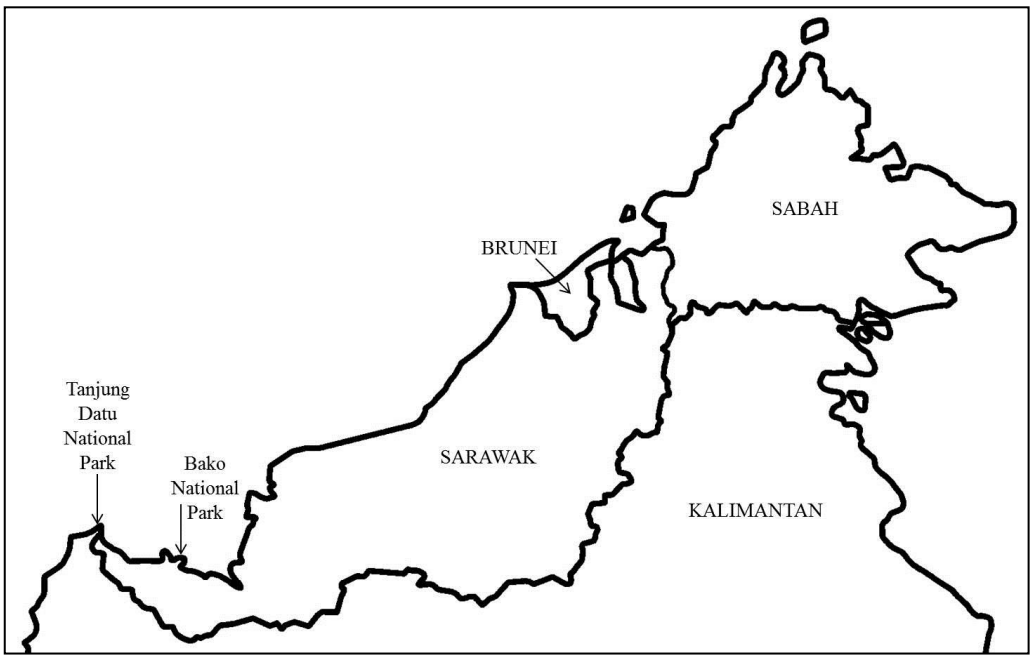

Figure 1. Location of study areas.

Water samples were passed through sterile 0.2 µm pore size membrane filter whereas soil samples were soaked in sterile distilled water, mixed vigorously and allowed to settle for 15 minutes before being filtered using same type of membrane filter. Following that, about 1 ml of the samples were inoculated into modified semisolid Ellinghausen-McCullough-Johnson-Harris (EMJH) media with 100 µg/mL 5-fluorouracil to minimize bacterial contamination. All the enriched cultures were incubated aerobically at room temperature for 30 days.

After 30 days of incubation, genomic DNA was extracted using Wizard™ Genomic DNA purification Kit (Promega Corporation, USA) following the manufacturer's instructions.

Three sets of primers specific to pathogenic, intermediate and saprophytic *Leptospira* were used to target *LipL32* gene, *16S rRNA* gene and *rrs* gene, respectively (Table 1). All the primer sets were synthesized commercially. Specific PCR amplification was carried out using Veriti™ 96-Well Thermal Cycler (Applied Biosystems, USA).

The reaction was performed in 25 µL reaction mixtures containing 5 µL of 5x PCR buffer, 0.2 mM of deoxynucleoside triphosphate mix, 0.4 µM of each primer pair, 2.0 mM $MgCl_2$, 1.25 U of *Taq* DNA polymerase (Promega Corporation, USA) and 5 µL of DNA template. The reference *Leptospira noguchii* strain LT796, *Leptospira wolffii* serovar Khorat strain Khorat-H2 and *Leptospira meyeri* strain Sant-1 were included as positive controls in specific PCR for pathogenic, intermediate and saprophytic *Leptospira*, respectively. The following conditions were used for amplification: Initial denaturation at 95°C for 2 mins, followed by 35 cycles of denaturation at 95°C for 1 min, primer annealing at 55°C for 30 sec and extension at 72°C for 1 min. Lastly, a final extension at 72°C for 5 min and indefinite holding period at 4°C was employed.

A 5 µL aliquot of each amplification product was electrophoresed on 2% agarose gel in 1 x TBE buffer. The gel was then stained with ethidium bromide and viewed under ultraviolet (UV) transilluminator. A 100-bp ladder (Promega Corporation, USA) was included in each gel as molecular weight marker.

Table 1. Primer sequences for the detection of pathogenic, intermediate and saprophytic *Leptospira* using specific PCR.

Target species	Primer sequence 5' to 3'	Amplicon size (bp)	Reference
Pathogenic *Leptospira*	CGCTGAAATGGGAGTTCGTATGATT CCAACAGATGCAACGAAAGATCCTTT	423	Vein *et al.*, 2012
Intermediate *Leptospira*	GGCGGCGCGTCTTAAACATG TTCCCCCCATTGAGCAAGATT	331	Cetinkaya *et al.*, 2000
Saprophytic *Leptospira*	AGAAATTTGTGCTAATACCGAATGT GGCGTCGCTGCTTCAGGCTTTCG	240	Murgia *et al.*, 1997

The amplified PCR products from positive samples were subjected to commercial facility for sequencing (First BASE Laboratories Sdn Bhd, Malaysia). The analysis of nucleotide sequences for homology search was performed using nucleotide BLAST, which is available from National Center for Biotechnology Information (NCBI).

The gel electrophoresis image from specific PCR conducted in this study was shown in Figure 2. The target genes specific to saprophytic, intermediate and pathogenic *Leptospira* produced amplicons at 240 bp, 331 bp and 423 bp, respectively. The primer sets used are highly conserved and specific to their own target genes. *LipL32* gene is highly conserved in pathogenic *Leptospira* and expressed during human infection (Evangelista & Coburn, 2010). Primer derived from *16S rRNA* gene is universal to *Leptospira* sp. (Tansuphasiri *et al.*, 2006). Murgia *et al.* (1997) designed the primer set specific to saprophytic *Leptospira* from ribosomal 16S genes (*rrs*) available in databases.

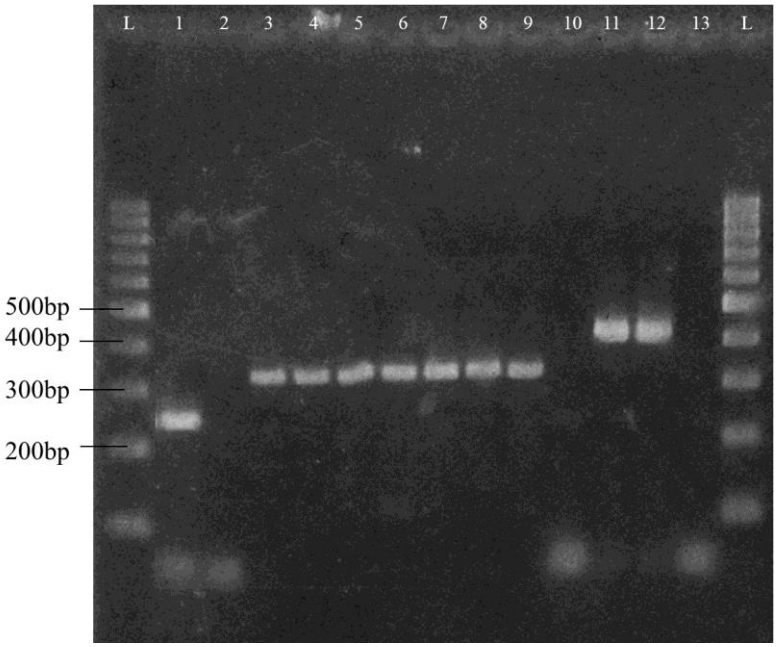

Figure 2. Representative amplicons for the identification of saprophytic, intermediate and pathogenic *Leptospira*. Lanes L show the 100-bp DNA ladder. *Leptospira meyeri* strain Sant-1 (lane 1), *Leptospira wolffii* serovar Khorat strain Khorat-H2 (lane 9) and *Leptospira noguchii* strain LT796 (lane 12) are positive controls. Lanes 2, 10 and 13 are negative controls. Lanes 3 to 8 show the positive amplicons specific to intermediate *Leptospira* at 331 bp whereas lane 11 shows the positive amplicon specific to pathogenic *Leptospira* at 423 bp.

A total of three soil samples were found to be positive towards the *16S rRNA* gene specific to intermediate *Leptospira*. Out of these three samples, two of them were samples from TDNP whereas one sample was from BNP. Besides, only one soil sample from BNP was shown to be positive towards *LipL32* gene specific to pathogenic *Leptospira*. Nonetheless, all the soil samples were negative towards *rrs* gene specific to saprophytic *Leptospira*. Table 2 indicated the prevalence of pathogenic, intermediate and saprophytic *Leptospira* in 50 soil samples collected.

As for the 60 water samples collected from TDNP and BNP, none of them generated *LipL32* gene at 423 bp and *rrs* gene at 240 bp. However, three water samples collected showed positive band at 331 bp, indicating the presence of *16S rRNA* gene specific to intermediate *Leptospira*. Out of these, one of them was collected from TDNP whereas the remaining two were collected from BNP. The prevalence of pathogenic, intermediate and saprophytic *Leptospira* in 60 water samples collected was shown in Table 3.

Table 2. Prevalence of pathogenic, intermediate and saprophytic *Leptospira* in soil samples collected.

Study areas	Soil					
	Pathogenic		Intermediate		Saprophytic	
	No.[a]	%[b]	No.[a]	%[b]	No.[a]	%[b]
TDNP	0/20	0.0	2/20	10.0	0/20	0.0
BNP	1/30	3.3	1/30	3.3	0/30	0.0
Total	1/50	2.0	3/50	6.0	0/50	0.0

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

Table 3. Prevalence of pathogenic, intermediate and saprophytic *Leptospira* in water samples collected.

Study areas	Water					
	Pathogenic		Intermediate		Saprophytic	
	No.[a]	%[b]	No.[a]	%[b]	No.[a]	%[b]
TDNP	0/30	0.0	1/30	3.3	0/30	0.0
BNP	0/30	0.0	2/30	6.7	0/30	0.0
Total	0/60	0.0	3/60	5.0	0/50	0.0

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

For the 50 soil samples, 2% and 6% of them were PCR positive for pathogenic and intermediate *Leptospira* genes, respectively. On the other hand, 5% of the 60 water samples were PCR positive for intermediate *Leptospira* gene. The results highlighted the presence of pathogenic *Leptospira* (0.9%) and intermediate *Leptospira* (5.5%) in the environmental soil and water from two national parks in Sarawak. This finding documented a low prevalence of pathogenic and intermediate *Leptospira* in the study areas. This result was consistent with the findings by Benacer *et al.* (2013) who detected 1.33% pathogenic *Leptospira*, 0.66% intermediate *Leptospira* and 3.31% saprophytic *Leptospira* from 151 environmental samples in West Malaysia using PCR. Furthermore, PCR detection of *Leptospira* in 145 environmental samples from Kelantan and Terengganu also indicated the low prevalence of pathogenic *Leptospira* (2.07%) in the samples examined (Ridzlan *et al.*, 2010).

The average pH and temperature of environmental samples at the study areas were summarized in Table 4. Soil pH at TDNP and BNP had the average value of pH 5.69 and pH 4.46 while the average water pH were pH 6.90 and pH 6.48, respectively. On the other hand, the average soil temperature were found to be 28.4°C and 29.0°C whereas the average water temperature were 27.3 °C and 29.2°C, respectively at TDNP and BNP. This indicated the ability of *Leptospira* sp. to survive in a wide range of environmental conditions. This observation was supported by Fentahun & Alemayehu (2012) who stated that the survivability of *Leptospira* sp. in the environment is influenced by the variation in soil and water conditions at contaminated areas. It has also been found that different *Leptospira* spp. have different tolerance levels for some of the environmental factors (Diesch *et al.*, 1969). For example, *Leptospira interrogans* can survive in low-nutrient environments, such as moist soil and fresh water for long time, with salt concentration, pH and viscosity as critical factors (Evangelista & Coburn, 2010). Mohan *et al.* (2009) also stated that the soil during raining season is usually moist and can lead to collection of water pools in the environment, which sustains the growth of *Leptospira* in the environment.

Table 4. The average pH and temperature of environmental samples collected at study areas.

Study area	Soil pH	Soil temperature (°C)	Water pH	Water temperature (°C)
TDNP	5.69±0.59	28.4±0.8	6.90±1.02	27.3±1.3
BNP	4.46±0.91	29.0±1.1	6.48±1.51	29.2±1.7

Value indicated the average measurement of all the samples, mean ± standard deviation.

In Malaysia particularly in Sarawak, there is still a paucity of information on the circulating *Leptospira* serovars in the environment. The understanding of the circulating serovars is important in providing us an insight on the prevalence of at least the most dominant *Leptospira* serovar in the environment (Collares-Pereira *et al.*, 2000). Further to DNA sequencing, the nucleotide sequences for the positive samples were subjected to nucleotide BLAST search (Table 5). The results revealed a high degree of homology with other pathogenic and intermediate *Leptospira*, varying from 84% to 98% similarity. This demonstrated the presence of pathogenic and intermediate *Leptospira* in these two national parks.

The presence of *Leptospira borgpetersenii* serovar Mini in Bako National Park highlights the possibility of infection to occur among the national park visitors and may cause mild to severe illness. Since *Leptospira borgpetersenii* serovar Mini is the main serogroup circulating in animals (Desvars *et al.*, 2012), there is a possibility that the soil in BNP is contaminated with the urine from infected animals. The direct contact with urine or tissue from infected animals, or indirect contact with soil or water contaminated with *Leptospira* sp. will expose human to the risk of infection by this spirochete (Sergio *et al.*, 2012). On the other hand, though less frequent, intermediate *Leptospira* also has the ability to cause disease in animals and human (Ricaldi *et al.*, 2012).

From the present study, *Leptospira wolffii* serovar Khorat is the dominant intermediate *Leptospira* found circulating in both national parks. *Leptospira wolffii* serovar Khorat strain Khorat-H2 was isolated from the urine of an adult male patient with suspected leptospirosis from Thailand (Slack *et al.*, 2008). Since little is known about the pathogenicity of intermediate *Leptospira* group (Slack *et al.*, 2006), it is postulated that *Leptospira wolffii* serovar Khorat strain Khorat-H2 has the ability to evolve into life-threatening complications. Therefore, the detection of this intermediate *Leptospira* strain from the environment indicated that precautionary action should be taken in handling the soil and water samples at the study areas.

Table 5. Results of nucleotide BLAST showing similarity with *LipL32* gene of pathogenic *Leptospira* and *16S rRNA* gene of intermediate *Leptospira* from GenBank.

Sample ID	Accession number	Description	Maximum score	Total score	Query coverage	Maximum identity
G1	NR_044042.1	*Leptospira wolffii* serovar Khorat strain Khorat-H216S ribosomal RNA gene, partial sequence	569	569	97%	98%
G2	NR_044042.1	*Leptospira wolffii* serovar Khorat strain Khorat-H216S ribosomal RNA gene, partial sequence	577	577	100%	97%
G3	NR_044042.1	*Leptospira wolffii* serovar Khorat strain Khorat-H216S ribosomal RNA gene, partial sequence	538	538	93%	96%
G4	AY631891.1	*Leptospira inadai* serovar Aguaruna strain MW4 16S ribosomal RNA gene, partial sequence	501	501	99%	94%
G5	KC662454.1	*Leptospira wolffii* strain LS0914U 16S ribosomal RNA gene, partial sequence	276	276	95%	84%
G6	NR_044042.1	*Leptospira wolffii* serovar Khorat strain Khorat-H216S ribosomal RNA gene, partial sequence	460	460	95%	92%
P1	AY609333.1	*Leptospira borgpetersenii* serovar Mini major outer membrane protein (*LipL32*) gene, complete cds	545	545	98%	90%

G1 and G2 denoted soil samples from TDNP, G3 denoted soil sample from BNP, G4 denoted water sample from TDNP, G5 and G6 denoted water samples from BNP, P1 denoted soil sample from BNP.

In conclusion, *Leptospira* sp. can survive under a wide range of pH and temperature. This study demonstrated very low prevalence of pathogenic and intermediate *Leptospira* at two national parks in Sarawak as shown by PCR detection. Although it was at low level, it is of public interest that increased awareness regarding the organism is propagated to the public or community. Proper health education regarding the appropriate preventive measures to be taken, such as chemoprophylaxis and wearing protective clothing especially footwear should be emphasized as to avoid any leptospirosis outbreak which may affect the tourism industry in Sarawak.

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