



RESEARCH ARTICLE

# Distribution of pathogenic *Leptospira* in environmental water and soils of selected recreational forests in Perak, Malaysia

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## ABSTRACT

Leptospirosis is an emerging zoonotic disease endemic in tropical regions. Aiming at assessing the potential infection risks via recreational exposure, the molecular prevalence of pathogenic *Leptospira* in 14 amenity forests in five selected districts of the state of Perak was determined. Water and soil samples along streams and waterfalls were subjected to culture of leptospires and the pathogenic *Leptospira* spp. was detected by *lipL32*-based polymerase chain reaction (PCR). Twenty out of 154 samples (13%) that tested positive for leptospires were mostly soils and still water recorded with tolerable temperatures (22.2-26.5°C) and pHs (5.73-6.70). The localised prevalence was highly varied among eight positive forests (6.7-41.7%), particularly higher in Kampar and Kinta districts which are the more populated urban areas. The importance of public health surveillance should not be underrated given the high prevalence of *Leptospira* spp. in forests in close proximity to indigenous settlements, even where the places are clean. Overall, this study discovered a wide distribution of pathogenic *Leptospira* spp. in recreational areas.

**Keywords:** Pathogenic *Leptospira*; Perak; environment; recreational forest; prevalence.

## INTRODUCTION

Leptospirosis is an emerging zoonosis of global distribution with high endemicity in tropical and subtropical regions (Bharti *et al.*, 2003). This potentially fatal bacterial disease is caused by highly motile spirochaete of the genus *Leptospira* (Levett, 2001). Rats and rodents are the primary reservoir animals despite the fact that virtually all mammals are susceptible hosts (Haake & Levett, 2015). They maintain the bacteria in the renal tubules and chronically shed them in their urine into the natural environment. Infection occurs when the pathogens enter the host's body through abrasion or cut in the skin or mucosal membranes and disseminate to multiple organs via the bloodstream (Bharti *et al.*, 2003; Haake & Levett, 2015). Acute kidney and liver failures and pulmonary hemorrhage are frequently reported in severe cases of leptospirosis or Weil's disease.

Pathogenic *Leptospira* is an occupational hazard for people who are frequently exposed to infected animals and their bodily fluids, or wet soil or water contaminated with urine of infected animals (Levett, 2001; Bharti *et al.*, 2003). Other important determinants of transmission are recreational and avocational, such as freshwater aquatic activities and adventure sports, extreme weather events

(heavy rainfalls, flooding), rat infestations, and poor sanitation, which can place the entire community at risk of infection (Bharti *et al.*, 2003; Mwachui *et al.*, 2015). The exposure frequency to infection sources is related to renal carriage in animals, survival and persistence of infectious *Leptospira* in ambient environment, and human behaviours and socioeconomic factors (Haake & Levett, 2015). Walking barefoot, having skin wounds, washing in streams, performing activities in forests, presence of wet soil, rat sighting, inadequate waste disposal facility, and the accumulation of refuse were found associated with occurrence of human leptospirosis (Bovet *et al.*, 1999; Reis *et al.*, 2008).

A number of eco-parks have been established in amenity forests in Malaysia and become popular destinations for ecotourism and leisure activities. However, being in proximity to wild animals' habitats has made the forest environment a potential source of infection. The first description of Malaysian primary forest as an endemic focus for leptospirosis was made by McCrumb and coworkers (1957), where the infectious leptospires were successfully isolated from a hamster inoculated with water and soil washing (Baker & Baker, 1970). Leptospirosis outbreaks associated with recreational water have been increasingly reported

during the past two decades. The high morbidity among athletes who participated in the Eco-Challenge-Sabah 2000 multisport race (Sejvar *et al.*, 2003) and eight deaths after a rescue operation in Lubuk Yu recreational forest in 2010 (Sapian *et al.*, 2012) highlighted the significance of investigating the environmental source of infection in active surveillance. In order to protect the public from potential health threats, it is important to identify high-risk sites, as well as environmental and human factors that are conducive to leptospirosis transmission.

Over recent years, investigations on environmental prevalence of pathogenic *Leptospira* spp. have been reported in Kelantan (Ridzlan *et al.*, 2010; Azali *et al.*, 2016), Terengganu (Ismail *et al.*, 2014), Sarawak (Pui *et al.*, 2015, 2017), Kuala Lumpur and Selangor (Benacer *et al.*, 2013). However, comprehensive epidemiologic information about leptospirosis and its aetiology is still greatly lacking in the Perak state, despite recording the third highest average incidence rate and second highest case-fatality rate between 2004 and 2012 (Benacer *et al.*, 2016). Furthermore, only a few studies have described the environmental conditions of the samples (Benacer *et al.*, 2013; Pui *et al.*, 2015), therefore restricting our understanding of the microenvironment that may impact the survival of pathogen.

Aiming to measure the likelihood of environmental exposure and infection risk, this study determines the presence and distribution of pathogenic *Leptospira* spp. in amenity forests and eco-parks in Perak. In addition, this study further details the environmental conditions of pathogen-positive samples and forests to better understand the factors conducive to the establishment of the infection source.

## MATERIALS AND METHODS

### Study sites and sample collection

Perak is located in the north-western region of Peninsular Malaysia and endowed with large tracks of forests distributing from the sea to the mountains with mangroves along the coastal zone. Fourteen recreational or amenity forests and eco-parks in Perak, Malaysia were selected (Table 1 and Figure 1) for sampling between November 2016 and March 2017.

Depending on the coverage area, 8 to 13 samples were collected from each study site. Damp soils, stagnant water, and puddles located in a shaded place, in proximity to rubbish, an animal sighting, or the shoreline were favourable. All sampling spots were accessible or frequented by the public. Approximately 50 ml of surface water sample was taken by dipping a sterile tube into the water at a 5-cm depth. Approximately 40 cm<sup>3</sup> of damp soil was taken within an area of 15 cm × 15 cm and 3 cm underneath the ground. Temperature and pH were measured in the field for water samples using a multiprobe (Professional Plus Multiparameter Instrument, YSI, USA). As for the soil samples, only temperature was recorded and measured using a portable thermometer. All sampling was carried out in the morning at a temperature of between 24°C and 28°C. The samples were then transported at ambient temperature in a lightproof container to the laboratory and processed within 12 hours.

### Isolation of leptospires by culture

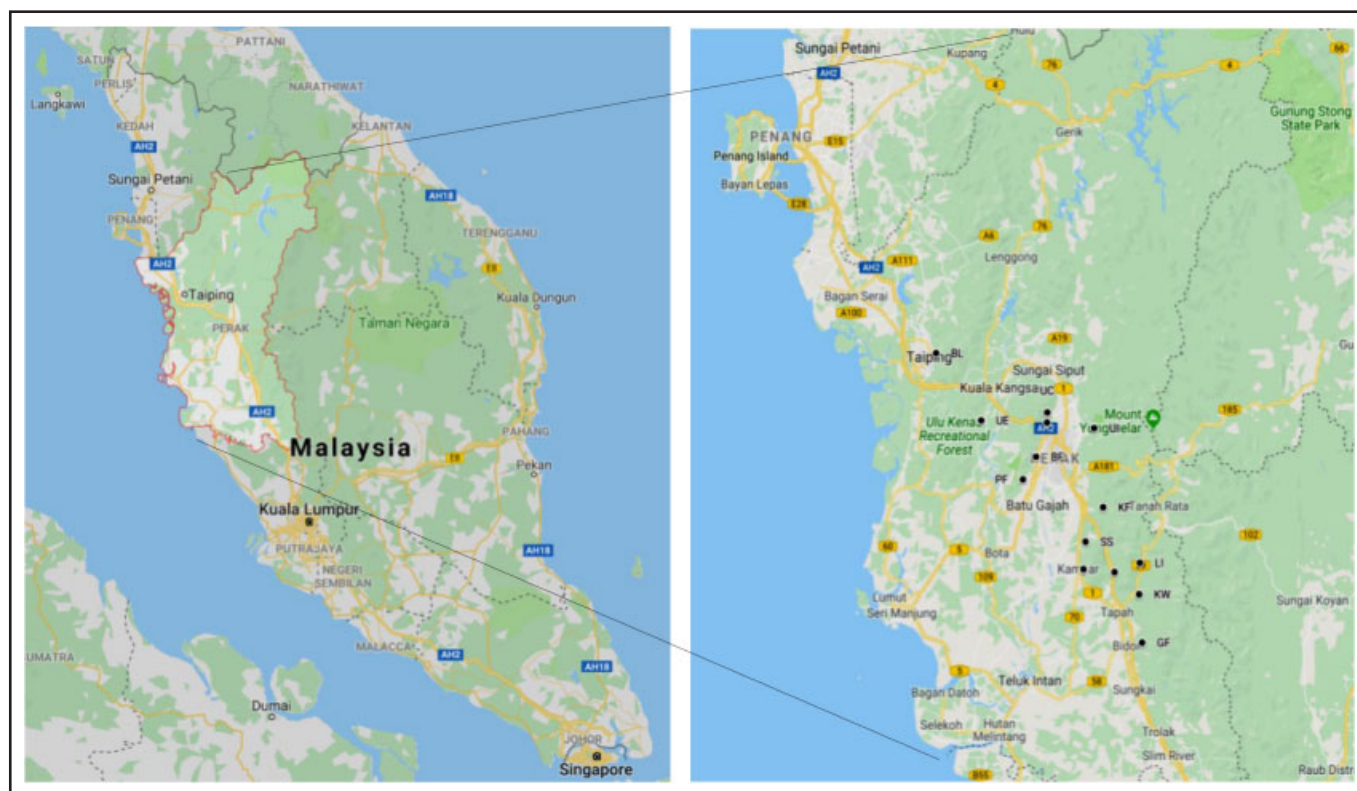
Soil samples were processed by suspending the soil in 10 ml of sterile phosphate-buffered saline and allowed to settle for one hour. Next, 5 ml of each water sample or soil supernatant were filtered through a nitrocellulose syringe filter with 0.45-µm pore size. A volume of 2 ml of filtrate was inoculated into 5 ml of Ellinghausen-McCullough-Johnson-Harris (EMJH) liquid medium (Difco™, USA) supplemented with 10% (v/v) *Leptospira* enrichment medium (Difco™, USA) and 5-fluorouracil at 200 µg/ml. All bacterial cultures were then incubated at room temperature in the dark for at least four weeks.

### Genomic DNA extraction

Bacterial genomic DNA was extracted from 1.5 ml of each culture using the EZ-10 Spin Column Bacterial Genomic DNA Mini-Preps kit (BioBasic, Canada). Cell pellet was obtained via centrifugation at 15000 × g for 10 min at 4°C followed by resuspension in 200 µl of cold Tris-EDTA buffer. The extraction adhered to protocol prescribed by the manufacturer for Gram-negative bacteria. The DNA extracts were suspended in 50 µl of elution buffer, quantified, and stored at -20°C.

**Table 1.** Amenity forests and respective environmental prevalence of leptospiral *lipL32* gene

Forest	Water source	District	Number and types of samples screened			Number of positive samples (prevalence, %)	Garbage heap
			Total	water	soil		
KF	River	Kampar	12	10	2	5 (41.7)	+
UC	Stream cascade	Kinta	12	8	4	4 (33.3)	-
BB	Waterfall	Kampar	13	8	5	4 (30.8)	+
UKI	Stream cascade	Kinta	8	8	0	2 (25.0)	-
LK	Waterfall	Batang Padang	11	10	1	2 (18.2)	-
BL	Stream cascade	Larut, Matang, Selama	8	6	2	1 (12.5)	+
UKE	Stream cascade	Kuala Kangsar	11	10	1	1 (9.1)	-
KS	Stream cascade	Kinta	12	10	2	1 (8.3)	-
BF	Waterfall	Kinta	10	7	3	0 (0)	-
GF	Waterfall	Batang Padang	12	5	7	0 (0)	+
KW	River	Batang Padang	12	12	0	0 (0)	-
LI	Waterfall	Batang Padang	13	9	4	0 (0)	-
PF	Stream cascade	Kinta	10	9	1	0 (0)	-
SS	Waterfall	Kampar	10	6	4	0 (0)	-
			154	118	36	20 (13)	



**Figure 1.** Location of amenity forests of study in Perak, Malaysia. The map was constructed at [www.google.com/maps](http://www.google.com/maps).

#### Molecular detection of pathogenic *Leptospira* by *lipL32*-PCR

The PCR amplification was performed in 25  $\mu$ l: 12.5  $\mu$ l of MyTaq™ mix (Bioline, UK), 1  $\mu$ l of each forward and reverse primers at final concentration of 0.4 nM, and 2  $\mu$ l of DNA extract. The primers used (Stoddard *et al.*, 2009) amplified 242-bp *lipL32*, a conserved leptospiral gene that is exclusive to pathogenic species of the genus *Leptospira* and encodes a major outer membrane lipoprotein. The specificity of primers was validated on reference *Leptospira* strains comprising 19 serovars, of which four were pathogenic, two were intermediate, and one was saprophytic species obtained from WHO/FAO/OIE Collaborating Centre for Reference and Research on Leptospirosis (The Netherlands). The reaction was performed in a thermal cycler (Bio-Rad, UK) using the following conditions: one cycle of pre-denaturation at 95°C for 3 min, followed by 35 cycles of amplification at 95°C for 15 s, 55°C for 15 s, and 72°C for 10 s, and a final extension at 72°C for 10 min. *Leptospira interrogans* serovar Icterohaemorrhagiae and *Escherichia coli* were used as a positive control (PC) and negative control (NC), respectively. A control without a DNA template (NTC) was also included in each reaction. PCR amplification was performed in duplicates for reproducibility. The amplicons were separated on 1.8% (w/v) agarose gel electrophoresis and stained with HealthView™ Nucleic Acid Stain (Genomics, Taiwan).

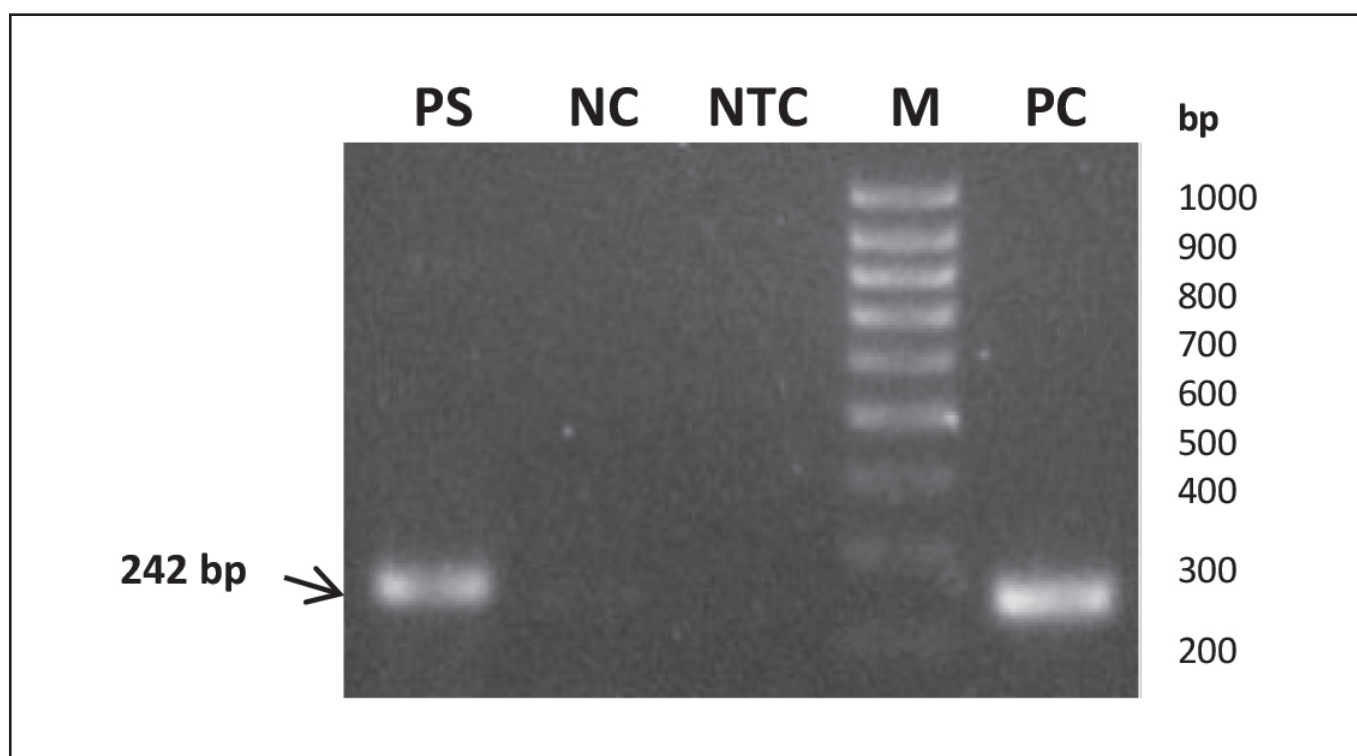
#### RESULTS

All sampling sites are located in proximity to different forest reserves, alongside a stream, river, or waterfall. During sampling, the public were seen picnicking, swimming, bathing, wading or submerging themselves in water, and

jungle trekking. Indigenous people were seen living upstream of the waterfall or river in sites KF, BB, and UKI. No animals were sighted, except macaques gathered in abundance in site BL.

A successful amplification of 242-bp *lipL32* gene fragment by PCR from the cultures indicated the presence of DNA of pathogenic *Leptospira* (Figure 2). All pathogenic species but none of the intermediate and saprophytic species of reference strains were amplified, indicating a satisfactory analytical specificity and sensitivity of the primers used. The overall prevalence of pathogenic *Leptospira* was 13% (20 of 154 samples) (Table 1). Over half of the sites studied ( $n=8$ , 57%) were contaminated, among which the localised prevalence varied widely ranging between 8.3% and 41.7% (standard deviation 14.6). Pathogenic *Leptospira* was found in at least one site from each of the five districts studied. Both districts of Kampar and Kinta showed relatively higher prevalence at 25.7% (9/35) and 13.5% (7/52) respectively, in relative to Larut, Matang and Selama (12.5%, 1/8), Kuala Kangsar (9.1%, 1/11), and Batang Padang (4.2%, 2/48). Consistently, all high-risk sites with prevalence above 25% are located in either Kampar or Kinta district.

Pathogenic *Leptospira* was detected in the majority of forests sighted with garbage heaps (75%, 3/4) (Table 1). Given the odd ratio exceeding 1.0 [(3/4)÷(5/10)], the garbage heaps is positively linked to the presence of pathogenic *Leptospira*. However, it is worth noting that a considerable proportion of the pathogen-positive sites (62.5%, 5/8) has maintained satisfactory cleanliness where regular waste management service is provided by responsible institutions. For instance, forests UC and UKI were clean and clear from rubbish although they are among the sites with highest prevalence.



**Figure 2.** Agarose gel electrophoresis of 242-bp *lipL32* gene fragment amplified from culture of environmental samples. Lanes PS: positive sample collected from forest BB; NC, negative control; NTC, non-template control; M, MassRuler™ DNA ladder mix (Thermo Scientific); PC, positive control.

Pathogenic *Leptospira* was mostly detected in soil (19%, 7/36), instead of in water (11%, 13/118) (Table 2). Slightly low temperatures (22.2-26.5°C) and acidic pHs (5.73-6.70) were recorded among the positive samples, out of the entire range of temperature and pH at 20.0-29.9°C and 5.04-7.43, respectively. Most of the positive samples were collected from shaded places with brief exposure to direct sunlight, and also from stagnant and still water (Table 2).

## DISCUSSION

The existence of the infectious *Leptospira* serves as an important evidence of infection source in clinical case investigations and epidemiological surveillance. Environmental prevalence of pathogenic *Leptospira* spp. and the likelihood of human exposure to it are among the factors that contribute to leptospirosis. Overall, this study revealed a higher prevalence in relation to other local studies performed in recreational settings, including the lakes in Selangor and Kuala Lumpur (0%, 0/60) (Benacer et al., 2013), waterfalls in Terengganu (7.5%, 3/40) (Ismail et al., 2014) and Kelantan (0%, 0/72) (Azali et al., 2016), and national parks in Sarawak (0.9%, 1/110) (Pui et al., 2015). This finding is likely attributed to spatial and temporal factors, localities, and sources of samples (Ganoza et al., 2006; Benacer et al., 2013; Lall et al., 2016; Pui et al., 2017). Moreover, there are differences in the protocol design and materials used between our study and others, especially in the use of PCR primers which present a variation in detection sensitivity in the context of environmental samples (Yap et al., 2019). Considering the low sensitivity of dark-field microscopy, subjecting the cultures that only showed spirochete growth to PCR detection of pathogenic *Leptospira* might lead to false-negative samples and a low positivity rate (Levett, 2001; Bharti et al., 2003; Schreier et al., 2013). It is important to note that although a

particular site has been tested negative for *Leptospira* genome, this does not guarantee that it is risk-free at another time.

One of the significances of this study is the considerable number of forests sampled, allowing a comprehensive analysis of the distribution of pathogenic *Leptospira* across the state. Batang Padang, Kinta, Larut, Matang and Selama, and Kuala Kangsar were targeted as samples because these districts were worst hit by leptospirosis based on epidemiological data provided by the Perak Department of Health. The higher prevalence in Kinta and Kampar districts very likely resulted from urbanisation, defined by massive and rapid land conversion, and housing development, which seems more intense in the central region than other regions of Perak. This is supported by the finding that the association of leptospirosis incidence and concentrations of *Leptospira interrogans* in surface water was higher in urban settings than in rural ones (Ganoza et al., 2006), and that land use and water infrastructure were significantly associated to leptospirosis incidence rates (Rood et al., 2017).

It is also worth noting that three high-prevalence forests (KF, BB, and UKI) are in the vicinity of indigenous community households, suggesting a potentially high infection risk to neighbouring settlements, apart from the public who occasionally visit the sites. The risk should not be underestimated given the high seroprevalence of anti-*Leptospira* antibodies among the indigenous people in Negeri Sembilan (Loong et al., 2018) and Sarawak (Thayaparan et al., 2015b).

Similar to this study, several local studies also reported a higher prevalence of pathogen in soils than water (Benacer et al., 2013; Pui et al., 2015, 2017). *L. kmetyi*, the first described pathogenic species that was not classically sourced from animals or humans, was isolated from soil (Slack et al., 2009). A higher concentration of shed leptospires is expected in small water bodies than in large ones after taking into consideration the bacteria would be contained within a

**Table 2.** Sample type and environmental conditions of pathogenic *Leptospira*-positive samples

Forest	Sample no.	Sample type	Temperature (°C)	pH*	Water flow**a	Site shadiness <sup>b</sup>	Description
KF	1	water	22.5	6.34	2	2	Backyard of household where domestic chicken and pet cat sighted
	4	water	22.3	6.25	1	4	Still water by waterfall
	6	soil	26.0	–	–	4	Dark brown sandy soil, adjacent to running water and garbage heap
	10	water	22.2	6.31	2	2	Rapid-flowing stream
	11	water	22.5	6.70	1	2	Rapid-flowing stream
UC	1	soil	24.0	–	–	4	Narrow gap flanked by large rocks, covered up by rubbish
	3	water	23.7	5.73	2	3	Still water by stream cascade
	4	soil mix with water	24.0	–	–	5	Burnt, dark, sandy soil, under bamboo bush, adjacent to toilet and bathroom
	8	water	23.8	6.55	2	3	Surrounded by rocks, source from jungle
BB	1	soil	24.0	–	–	4	Brown soil, adjacent to garbage heap
	3	soil	24.5	–	–	4	Wet and light brown in color, adjacent to garbage heap
	9	soil	25.0	–	–	3	Sandy soil, adjacent to garbage heap
	12	soil	25.0	–	–	4	Adjacent to a cottage and burnt garbage heap
UKI	2	water	25.3	6.41	2	4	Still water by river
	7	water	25.3	6.52	1	5	Still water by river
LK	3	water	24.0	–	1	4	Puddle on ground with muddy water, beside staircase
	7	water	23.5	6.45	1	3	Pool of standing water on rock
BL	7	soil	26.5	–	–	2	Adjacent to garbage heap
UKE	3	water	24.2	6.68	1	2	Still water by river
KS	1	water	24.7	6.44	1	4	Still water by stream cascade

## Legend:

\*hydrology parameters, applicable on water samples only.

– no information available.

<sup>a</sup> Score 1 to 5: stagnant or still water, to fast moving water.<sup>b</sup> Score 1 to 5: full of shade, to fully exposed to sunlight.

relatively small area on the ground and not diluted like those flushed into a running stream (Barragan *et al.*, 2017). This finding supports the speculation that infection sources are derived from the soils when excess rainwater flushes down the pathogen from the bank into river or streams and facilitates a wide dispersal into the environment.

The environmental conditions of all samples in this study were not detrimental for survival of leptospire as they can live at a pH between 5.5 and 7.6, and temperatures between 4°C and 40°C under controlled laboratory conditions (Khairani-Bejo *et al.*, 2004; Saito *et al.*, 2013; Andre-Fontaine *et al.*, 2015). The lowest sample temperature (22.2°C) recorded in this field study is acceptable although the bacteria can survive longer in warm temperatures than in the cold (Andre-Fontaine *et al.*, 2015). Interestingly, all pathogen-positive water samples in this study were acidic in contrast to a study on ornamental water fountain in Columbia where all samples were found to be alkaline (pH 7.69-8.9) (Escandün-Vargas *et al.*, 2018). Overall, this finding suggests that pathogenic *Leptospira* may tolerate a broader range of pH although leptospire survived longer in alkaline water than in acidic water (Smith & Turner, 1961). Motility of the spirochetes

facilitates their survival by avoiding detrimental environments (Stamm *et al.*, 1988; Khairani-Bejo *et al.*, 2004) and enabling tropism towards more favourable conditions.

Given that most local leptospirosis outbreaks occurred in public recreational areas, people are urged to maintain environmental cleanliness as the scraps left behind may promote harbourage for rats or wild animals, whose urine end up contaminating the water and soils. It is a common perception that a clean place provided with proper waste disposal facility would keep away pathogenic *Leptospira*. However, our findings do not support this hypothesised statement. By taking the high-prevalence forests UC and BB as examples, cleanliness of the former has been well maintained in contrast to the horrendous conditions in the latter. Apparently, the distribution of pathogenic leptospire has no strong relation with environmental cleanliness, but may rather be with density and seroprevalence of inhabiting wild animals (Barragan *et al.*, 2017). Several surveillance studies reported high seroprevalence (up to 47%) among wild mammals in Sarawak living close to human settlements (Thayaparan *et al.*, 2015a; Su'ut *et al.*, 2018). Predicting the presence of pathogenic *Leptospira* and making a conclusion

on infection risk merely based on environmental cleanliness is subjective and might lead to misinterpretation. The findings raised a concern on public health at tourist destinations involving aquatic activities, taking note of the increased publicity of ecotourism in Perak. It is crucial for relevant authorities to devise and implement interventions accordingly. Closing down a park for clearing infection sources may be impractical given the huge coverage area in a jungle and abundance of potential reservoir animals wandering in it. The public should be well informed of the high-risk recreational sites and given sufficient knowledge about leptospirosis transmission and effective preventive measures.

In conclusion, pathogenic *Leptospira* spp. was widely distributed in the environmental water and soils of amenity forests. This study provides valuable epidemiological information about the general characteristics that influence the distribution of the pathogen. Further researches should be conducted to verify if environmental prevalence of pathogenic *Leptospira* reflects the local index of human leptospirosis. It is also suggested to seek evidence that ties a clinical case to a specific environmental site suspected to be the source of infection by establishing a matching genotype and contact history. The phenotype, genotype, and virulence of the environmental isolates should be further characterised to provide more insight on epidemiology and evolutionary relationships.

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#### Conflict of interest

The authors declare that they have no conflict of interest.

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