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Detection of *Leptospira wolffii* in Water and Soil on Livestock Farms in Kelantan After a Massive Flood

(Pengesanan *Leptospira wolffii* dalam Air dan Tanah di Ladang Ternakan di Kelantan Selepas Banjir Besar)

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ABSTRACT

Pathogenic leptospires cause leptospirosis in both animals and humans around the world. Humans can be infected by leptospires through direct contact with infected animals or indirect contact with leptospires contaminated water or soil. Outbreaks of leptospirosis usually occur after heavy rainfall and flooding, particularly in endemic areas. The aim of this study was to detect the presence of leptospires in water and soil in livestock farms after a massive flood in Kelantan. Samples were collected from 28 livestock farms in Kelantan, comprising 62 water samples and 62 soil samples, respectively. The samples were filtered and inoculated into a semisolid EMJH medium containing 5-FU for the isolation of *Leptospira* spp. The *Leptospira* spp. isolates were then identified using classical methods (1M NaCl, 8-azaguanine, and 13 °C), serology (MAT), multiplex polymerase chain reaction (mPCR), and DNA sequencing. The classical identification methods showed varying results and failed to differentiate between pathogenic and non-pathogenic leptospires. MAT showed the isolates reactions against serovars Autumnalis, Hebdomadis, Pyrogenes, Bataviae, Patoc, and Wolffii. However, mPCR showed that all isolates were non-pathogenic *Leptospira* spp. Further identification using DNA sequencing found that all isolates were identified as *Leptospira wolffii*, an intermediate species of *Leptospira*. The presence of *L. wolffii* in water and soil provides evidence that this species is circulating in the environment and could potentially transmit to humans and animals.

Keywords: Flood; Kelantan; Leptospirosis; soil; water

ABSTRAK

Patogen *Leptospira* menyebabkan Leptospirosis pada haiwan dan manusia di seluruh dunia. Manusia boleh dijangkiti bakteria ini melalui sentuhan langsung dengan haiwan yang dijangkiti atau melalui sentuhan tidak langsung dengan air atau tanah yang tercemar. Wabak leptospirosis biasanya berlaku selepas hujan lebat dan banjir, terutamanya di kawasan endemik. Matlamat kajian ini adalah untuk mengesan kehadiran *Leptospira* dalam air dan tanah di ladang ternakan selepas banjir besar di Kelantan. Sampel diperoleh daripada 28 ladang ternakan di Kelantan yang masing-masing terdiri daripada 62 sampel air dan 62 sampel tanah. Sampel ditapis dan diinokulasi ke dalam medium EMJH separa pepejal yang mengandungi 5-FU untuk pemencilan *Leptospira* spp. Pencilan *Leptospira* spp. kemudiannya dikenal pasti menggunakan kaedah klasik (1M NaCl, 8-azaguanine, dan 13°C), serologi (MAT), tindak balas berantai polimerase multipleks (mPCR) dan penjujukan DNA. Kaedah pengesanan klasik memberikan hasil yang berlainan dan gagal membezakan antara *Leptospira* patogen dan bukan patogen. MAT menunjukkan tindak balas terpencil terhadap serovars Autumnalis, Hebdomadis, Pyrogenes, Bataviae, Patoc, dan Wolffii. Walau bagaimanapun, mPCR menunjukkan bahawa semua pencilan adalah *Leptospira* spp. yang tidak patogen. Pengecaman lanjut menggunakan penjujukan DNA mendapati bahawa semua pencilan dikenal pasti sebagai *L. wolffii*, spesies perantaraan *Leptospira*. Kehadiran *L. wolffii* dalam air dan tanah menunjukkan bahawa spesies ini beredar dalam alam sekitar dan berpotensi untuk menular kepada manusia dan haiwan.

Kata kunci: Air; banjir; Kelantan; Leptospirosis; tanah

Introduction

Leptospirosis is one of the most common zoonotic diseases in the world. It has been recognised as a re-emerging infectious disease among animals and humans (Levett 2004). It has also been reported that the whole region of Southeast Asia is an endemic area for leptospirosis (Laras et al. 2002), including Malaysia, as several outbreaks have been recorded. Outbreaks usually occur after heavy rainfall and floods. In December 2014, Kelantan experienced a massive flood following continuous heavy rainfall where many reservoirs, carriers, or maintenance hosts for leptospires died and contaminated the water. Humans and animals can then contract leptospirosis from the contaminated water or soil during or even after the flood. During this period, the incidence of leptospirosis among humans in Kelantan was reportedly high (MOH, 2015). Altogether, 1229 leptospirosis cases in humans were reported before, during, and after the flood (Mohd-Radi et al. 2018). In addition, an outbreak occured, with 62 confirmed leptospirosis cases reported among humans from Kelantan after the flood (MOH 2015). However, the presence of leptospires in the environment in Kelantan after the flood is still under-reported. Therefore, this study was conducted to isolate and identify *Leptospira* spp. in water and soil on livestock farms in Kelantan after the 2014 flood using multiple laboratory tests.

Polymerase chain reaction (PCR) is a molecular biological technique to amplify DNA for adequate testing and is commonly used in the diagnosis of infectious diseases, including leptospirosis. PCR has previously been used to detect leptospiral DNA in samples obtained from animals (Bomfim et al. 2008; Lilenbaum et al. 2008) and humans (Fonseca et al. 2006; Ooteman, Vago & Koury 2006). In this study, multiplex polymerase chain reaction (mPCR) has been used, which will aid in the specific and sensitive detection of *Leptospira* spp. The mPCR targets the well-known *16S rRNA* gene (Merien et al. 1992) and the *LipL32* gene (Ahmed et al. 2012).

In a previous study, blood and urine samples were obtained from livestock in Kelantan and tested directly using mPCR (Sabri et al. 2019). Water and soil samples were not directly tested using mPCR as contaminants and commensal organisms present in the samples could possibly affect the PCR results. For this study, the isolation of *Leptospira* spp. was carried out to detect *Leptospira* spp. in the water and soil samples obtained from livestock farms in Kelantan. Further identification was carried out using classical, serological, and molecular methods.

Materials and Methods

Environmental samples

Water and soil samples were collected from 28 livestock farms in 10 districts of Kelantan after a massive flood in 2014. The sampling period began in March and lasted until December 2015, following a surveillance programme conducted by the Department of Veterinary Services (DVS), the governmental organisation responsible for the health, productivity, and welfare of animals in Malaysia. One hundred and fourteen samples (57 water samples and 57 soil samples) were collected from flood-affected livestock farms in eight districts of Kelantan, whereas 10 samples (5 water samples and 5 soil samples) were collected from livestock farms in Pasir Putih and Bachok that were not affected by the flood. Altogether, 124 samples were collected, consisting of 62 water samples and 62 soil samples (Table 1).

A total of 62 water samples were collected from puddles on farms. Approximately 30 mL of water samples were collected into sterile universal bottles that were then filtered through a pore membrane (0.45 µm, Millex®, Ireland). Meanwhile, 62 soil samples were collected from moist soil on the farms where the animals were directly contacting the areas. Approximately 30 g of soil was collected into sterile universal containers that were then suspended and mixed in sterile distilled water at approximately three times the volume of the sample. The suspension was then filtered through filter paper (Whatman™, UK) followed by a pore membrane (0.45 µm, Millex®, Ireland). The samples were kept in the chiller at 4 °C in the nearest DVS laboratories before being dispatched to the Bacteriology Laboratory, Faculty of Veterinary Medicine for further analyses.

Isolation of *Leptospira* spp. from water and soil

Isolation of *Leptospira* spp. in this study was performed according to the protocol described by the OIE (2014). One mL of filtered water and soil samples were immediately inoculated into semisolid Ellinghausen–McCullough–Johnson–Harris (EMJH) medium containing 200 µg/mL 5-fluorouracil (5-FU) as primary cultures. These primary cultures were kept in a 30 °C incubator for 12 weeks with periodic monitoring for

TABLE 1. Environmental samples composed of 62 water samples and 62 soil samples collected in 28 farms in Kelantan

Location (District)	Farm	Water sample	Soil sample
Gua Musang	GM1	2	2
	GM2	2	2
	GM3	2	2
	GM4	2	2
	GM5	2	2
Kuala Krai	KK1	2	2
	KK2	2	2
	KK3	2	2
	KK4	2	2
	KK5	2	2
	KK6	2	2
Jeli	J1	3	3
	J2	3	3
Tanah Merah	TM1	3	3
	TM2	2	2
	TM3	3	3
Pasir Mas	PM1	2	2
	PM2	2	2
Tumpat	T1	2	2
	T2	2	2
Machang	M1	2	2
	M2	2	2
Kota Bharu	KB1	3	3
	KB2	2	2
	KB3	2	2
	KB4	2	2
Pasir Putih	PP1	2	2
Bachok	B1	3	3
Total	28 farms	62	62

leptospires every two weeks under darkfield microscopy. If leptospires were present within 12 weeks, the primary cultures were subcultured multiple times into a new semisolid EMJH medium to minimise contamination. If there was no sign of leptospire growth within 12 weeks, the cultures were discarded after careful examination. Subcultured leptospires were transferred into liquid EMJH medium to enhance growth and then filtered by pore membrane (0.45 µm, Millex®, Ireland) until pure isolates were obtained. All isolates were examined under darkfield microscopy to confirm leptospire morphology and purity. Pure isolates were then maintained in liquid EMJH medium for further identification.

DETECTION OF PATHOGENIC AND SAPROPHYTIC LEPTOSPIRAL ISOLATES USING CLASSICAL METHODS

Conversion of cells to spherical forms in 1M NaCl solution

Freshly prepared cultures of isolates were inoculated into sterile test tubes containing freshly prepared solutions of 1M NaCl. Pathogenic reference serovar Hardjobovis strain 117123 and saprophytic reference serovar Patoc strain Patoc I were selected as control groups. All inoculated tubes were incubated at 30 °C for 2 h and then examined under darkfield microscopy. After 2 h of incubation in 1M NaCl, pathogenic leptospires converted into spherical forms, while saprophytic leptospires remained in their original morphology (thin, helical, and spiral) (Johnson & Faine 1984).

Growth inhibition of leptospires in the presence of 8-azaguanine

All isolates and control groups of both pathogenic reference serovar Hardjobovis and saprophytic reference serovar Patoc were subcultured into liquid EMJH medium (225 µg/mL 8-azaguanine) and incubated at 30 °C. Growth rate was observed at day 4, day 7, and day 10 by comparing isolate growth against the growth of reference control groups. In the presence of 8-azaguanine, pathogenic leptospires were not able to propagate more than 10^8 cells/mL (compared with McFarland standard 0.5), while saprophytic leptospires grew abundantly at the same time point (Johnson & Gary 1962).

Leptospire growth at 13 °C

All isolates and control groups were subcultured into liquid EMJH medium. Inoculums were incubated at 13 °C and examined twice a week for up to three weeks. Pathogenic leptospires were unable to propagate, whereas saprophytic leptospires were able to propagate in this temperature environment (Noubade et al. 2002).

IDENTIFICATION OF LEPTOSPIRAL ISOLATES USING MICROSCOPIC AGGLUTINATION TEST

Isolates grown in liquid EMJH medium were tested against 17 panels of hyperimmune sera, as shown in Table 2. Darkfield microscopy was used to examine isolates for evidence of agglutination against hyperimmune sera: a particular serological reaction to hyperimmune serum (titre equivalent to or more than 1:5120) identifies isolates to their particular leptospiral serovar (Hartskeerl et al. 2001). Reference serovars, Hardjobovis strain 117123 and Patoc strain Patoc I, were used as reference controls.

IDENTIFICATION OF LEPTOSPIRAL ISOLATES USING MULTIPLEX POLYMERASE CHAIN REACTION

DNA extraction from leptospiral isolates

DNA extraction from leptospiral isolates was performed using the Dneasy® Blood & Tissue Kit (QIAGEN, Germany) according to the manufacturer's instructions. The extracted DNA was stored at -30 °C until further analysis. To ensure that DNA was successfully extracted from the sample, 5 µL of the eluted DNA template was subjected to gel electrophoresis on 1.5% agarose in half-strength 0.5x tris-borate-EDTA (TBE) buffer at 80 volts for 30 min. The gel was pre-stained with SYBR® Safe DNA gel stain (Invitrogen™, North America) and examined using GelDoc (AlphaImager™).

Polymerase chain reaction (PCR) primers

Two sets of PCR primers targeting the *16S rRNA* gene and *LipL32* gene were employed in this study. The forward and reverse primers for *16S rRNA* are 5′–CAT GCA AGT CAA GCG GAG TA–3′ and 5′–AGT TGA GCC CGC AGT TTT C–3′, respectively, with an amplicon size of 541 bp. The forward and reverse primers for *LipL32* are 5′–GTC GAC ATG AAA AAA CTT TCG ATT TTG–3′ and 5′-CTG CAG TTA CTT AGT CGC GTC AGA AGC–3′, respectively, with amplicon size of 756 bp. Both genes are present in pathogenic *Leptospira* spp., but only the *16S rRNA* gene is present in non-pathogenic *Leptospira* spp. (Tansuphasiri et al. 2006). Furthermore, the 16S rRNA primer used in this study has been able to amplify intermediate *Leptospira* spp. and saprophytic *Leptospira* spp. (Ahmed et al. 2011; Sabri et al. 2019).

TABLE 2. Panel of hyperimmune sera used in MAT for identification of *Leptospira* sp. isolates

Species	Serovar	Strain
L. borgpetersenii	Hardjobovis	117123
L. Interrogans	Hebdomadis	Hebdomadis
L. weilii	Celledoni	Celledoni
L. kmetyi	Malaysia	Bejo-ISO9
L. interrogans	Pomona	Pomona
L. borgpetersenii	Tarassovi	Perepelitsin
L. interrogans	Pyrogenes	Salinem
L. kirschneri	Cynopteri	3522C
L. interrogans	Lai	Lai
L. interrogans	Icterohaemorrhagiae	RGA
L. interrogans	Bataviae	Swart
L. borgpetersenii	Javanica	Veldrat Bataviae 46
L. interrogans	Autumnalis	Akiyami A
L. borgpetersenii	Ballum	Mus 127
L. interrogans	Djasiman	Djasiman
L. biflexa	Patoc	Patoc I
L. interrogans	Wolffii	3705

Multiplex polymerase chain reaction (mPCR) amplification

In this study, mPCR was performed using a mixture of 16S rRNA and LipL32 primers to detect pathogenic and non-pathogenic *Leptospira* spp. simultaneously. Amplification by mPCR was performed in freshly prepared 25 µL reaction volumes consisting of 20 µL PCR working mixture (12.5 µL of TopTaq Master Mix; 1.25 µL of forward and reverse primers for *16S rRNA* and *LipL32* genes; 2.5 µL RNAse-free water) and 5 µL DNA template. The solution was mixed well by short spin using a mini spin centrifuge (Eppendorf, Germany) and then immediately transferred into Mastercycler Pro S® (Eppendorf, Germany). Amplification was optimised with an initial denaturation of 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 1 min, primer annealing at 58 °C for 45 s, and DNA extension at 72 °C for 30 s, before the final extension step at 72 °C for 6 min to complete the synthesis of all strands. The PCR product amplicons were subjected to gel electrophoresis on 1.5% agarose in half-strength tris-borate-EDTA (TBE) buffer (80 volts, 1.5 h). The gel was pre-stained with SBYR® Safe DNA gel stain (Invitrogen™, North America) and examined using GelDoc (AlphaImager™). Amplicons were identified by band size: the *16S rRNA* gene at 541 base pairs (bp) and *LipL32* gene at 756 bp.

PARTIAL 16S rRNA SEQUENCING

Amplicons were sequenced by First BASE Laboratories Sdn Bhd, Malaysia, and compared against the GenBank database using nucleotide BLAST from the National Centre for Biotechnology Information (NCBI). Sequences generated alongside representative sequences from the genus *Leptospira* (pathogenic, intermediate, and

saprophytic) and *Leptonema illini* strain Habaki (as an outgroup) deposited to GenBank were aligned with CLUSTAL OMEGA and subjected to phylogenetic analysis. The phylogenetic tree was inferred by using the maximum likelihood method based on the General Time Reversible model conducted in MEGA7 (Kumar, Stecher & Tamura 2016).

STATISTICAL ANALYSIS

Detection of *Leptospira* spp. in water and soil was represented by means of descriptive statistics using MedCalc Statistical Software version 2014.

RESULTS

From the 124 samples collected, only 21 *Leptospira* spp. isolates (16.9%; 21/124) were obtained. Among these, 14 were soil samples, and seven were water samples. Fourteen soil isolates were obtained from Gua Musang, Kuala Krai, Jeli, Tanah Merah, Pasir Mas, and Kota Bharu. Seven water isolates were obtained from Kuala Krai, Jeli, Tanah Merah, Pasir Mas, and Tumpat (Table 3). All *Leptospira* spp. isolates obtained were from flood-affected environments. Leptospire distribution was detected in water and soil in seven districts of Kelantan, except Machang, Bachok, and Pasir Puteh (Figure 1). Districts of Gua Musang, Jeli, and Kota Bharu showed higher *Leptospira* spp. distribution at 3.23% (4/124), while detection at Kuala Krai and Tanah Merah was at 2.42% (3/124), and districts of Pasir Mas and Tumpat were at 1.61% (2/124) and 0.81% (1/124), respectively.

IDENTIFICATION OF *Leptospira* spp. ISOLATED FROM WATER AND SOIL

Table 4 shows the differences between pathogenic and saprophytic leptospires from the 21 *Leptospira* spp. isolates using 1M NaCl, 8-azaguanine, and 13°C methods. Five isolates were classified as saprophytic, while 16 isolates were classified as undefined. Table 5 shows the isolates reactions towards 17 hyperimmune sera by using microscopic agglutination test (MAT). Ten isolates reacted against six serovars, namely Autumnalis (*n*=1), Bataviae (*n*=2), Hebdomadis (*n*=1), Pyrogenes (*n*=2), Patoc (*n*=3) and Wolffii (*n*=1) with titre ranges from 1:100 to 1:400. However, 11 isolates showed no reaction to any hyperimmune sera.

Multiplex polymerase chain reaction (mPCR) showed that only the *16S rRNA* gene was amplified in the isolates. Two bands were observed at 541 bp and 756 bp in the positive control *L. borgpetersenii* serovar Hardjobovis strain 117123, while only one band was observed at 541 bp in all isolates. Figure 2 shows six out of the 21 isolates presenting one band, with similar findings for the remaining 15 isolates. This result indicates that all *Leptospira* spp. isolates obtained from water samples and soil samples were non-pathogenic *Leptospira* spp.

TABLE 3. Positive *Leptospira* sp. isolation from water (n=62) and soil (n=62) samples in Kelantan

Location	Water	Soil	Total, N=124 (%)
Gua Musang	-	4	4 (3.23)
Kuala Krai	1	2	3 (2.42)
Jeli	2	2	4 (3.23)
Tanah Merah	2	1	3 (2.42)
Pasir Mas	1	1	2 (1.61)
Tumpat	1	-	1 (0.81)
Kota Bharu	-	4	4 (3.23)
Total	7	14	21 (16.9)

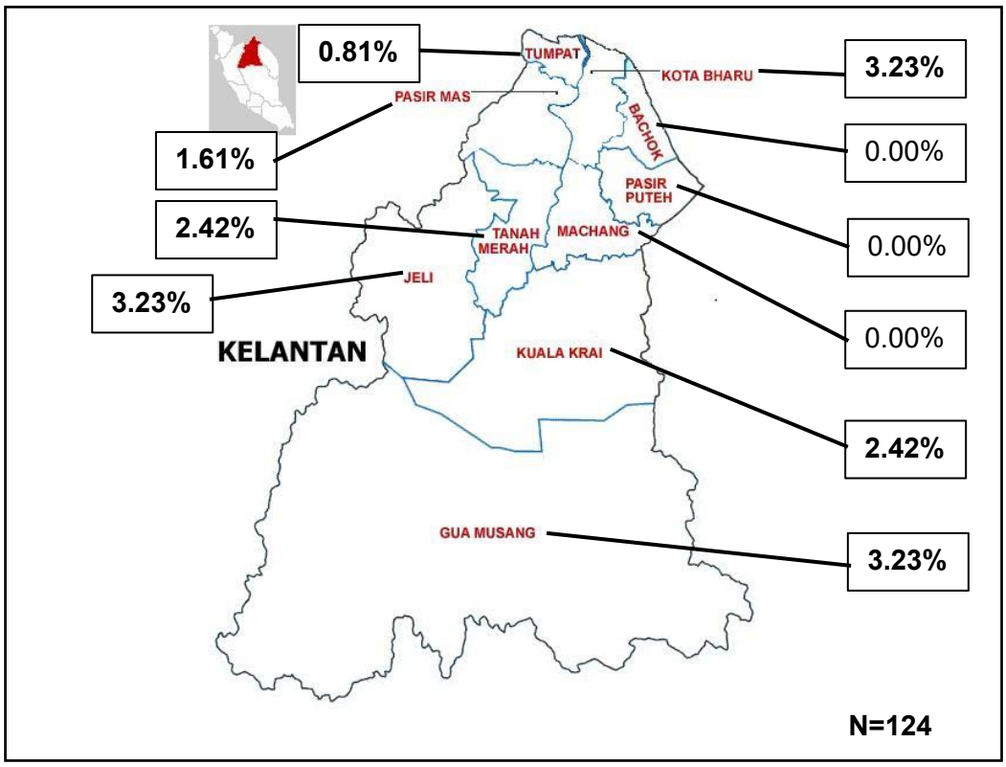

FIGURE 1. Distribution of *Leptospira* sp. isolated from water and soil from livestock farms in Kelantan. The data were represented by % where number of positive in an area divided with total number of sample

BLAST (www.ncbi.nlm.nih.gov/blast) analysis of 16S rRNA gene sequences from all isolates were confirmed as *L. wolffii*. All isolates had both query coverage and maximum identity of more than 95% against *L. wolffii* strain Khorat-H2^T (GenBank accession no: EF025496.1) with query lengths of 426-493 bp. The sequences have been submitted to GenBank (GenBank accession no: MF040635.1 - MF040642.1 & MF091696.1 - MF091708.1). Phylogenetic analysis showed three distinct clades related to pathogenic, intermediate, and saprophytic *Leptospira* species, as shown in Figure 3. These *L. wolffii* strains were then designated as UPM strains, and alongside the newly reported *L. wolffii* strain Khorat-H2^T (GenBank accession no: EF025496.1), were placed within the intermediate clade, showing close relations between all strains.

DISCUSSIONS

Twenty-one environmental samples out of the 124 total samples exhibited leptospire growth. Leptospires detection by water and soil isolation was higher in this study than previously reported by Pui et al. (2017a), at 5.95% (25/420). This could be due to a smaller sample size and different sampling areas in the previous study. All *Leptospira* spp. isolates for this study were isolated from environments that were previously affected by the 2014 flood in seven districts of Kelantan. The districts of Gua Musang, Kota Bharu, and Jeli showed higher distribution of leptospires in livestock farms, which suggests high leptospires contamination in the soil and water as a result of a massive flood. However, Machang, Bachok, and Pasir Puteh districts showed no distribution of leptospires in livestock farms. This situation was expected, as Bachock and Pasir Puteh were previously not affected by the flood (Wan-Yussof 2015). However, Machang was formerly affected by the flood. This situation might be due to the absence of leptospires in the sampling area. Although floods could play a major role in spreading *Leptospira* spp., leptospires still need favourable conditions (clean and stagnant water) to survive, which may explain their absence in these flood-affected areas.

TABLE 4. Differences between pathogenic and saprophytic leptospires from the 21 *Leptospira* sp. isolates using 1M NaCl, 8-azaguanine, and 13 °C methods

Isolate	Sample and location	1M NaCl	8-azaguanine	13 °C	Saprophytic/Pathogenic/ Undefined
1	Soil-Gua Musang	Non-spherical	Propagating	Propagating	Saprophytic
2	Soil-Tanah Merah	Non-spherical	Not propagating	Propagating	Undefined
3	Soil-Kota Bharu	Spherical	Propagating	Not propagating	Undefined
4	Soil-Kota Bharu	Spherical	Propagating	Not propagating	Undefined
5	Soil-Pasir Mas	Non-spherical	Propagating	Propagating	Saprophytic
6	Water-Tanah Merah	Non-spherical	Propagating	Propagating	Saprophytic
7	Soil-Kota Bharu	Spherical	Propagating	Propagating	Undefined
8	Water-Tanah Merah	Spherical	Propagating	Propagating	Undefined
9	Water-Jeli	Non-spherical	Not propagating	Propagating	Undefined
10	Water-Kuala Krai	Spherical	Propagating	Propagating	Undefined
11	Water-Pasir Mas	Non-spherical	Propagating	Not propagating	Undefined
12	Soil-Jeli	Non-spherical	Not propagating	Propagating	Undefined
13	Water-Jeli	Non-spherical	Propagating	Propagating	Saprophytic
14	Soil-Gua Musang	Non-spherical	Not propagating	Not propagating	Undefined
15	Soil-Jeli	Non-spherical	Propagating	Propagating	Saprophytic
16	Soil-Gua Musang	Spherical	Propagating	Not propagating	Undefined
17	Soil-Gua Musang	Non-spherical	Propagating	Not propagating	Undefined
18	Soil-Kuala Krai	Non-spherical	Propagating	Not propagating	Undefined
19	Soil-Kuala Krai	Non-spherical	Propagating	Not propagating	Undefined
20	Soil-Kota Bharu	Non-spherical	Not propagating	Not propagating	Undefined
21	Water-Tumpat	Non-spherical	Not propagating	Not propagating	Undefined
22	Patoc (reference)	Non-spherical	Propagating	Multiply	Control
23	Hardjobovis (reference)	Spherical	Not propagating	Not propagating	Control

TABLE 5. *Leptospira* sp. isolate reaction towards 17 hyperimmune sera by using MAT

Isolate	Sample-location	Serovar (MAT titre)
1	Soil-Gua Musang	Autumnalis (1:100)
2	Soil-Tanah Merah	No reaction
3	Soil-Kota Bharu	No reaction
4	Soil-Kota Bharu	No reaction
5	Soil-Pasir Mas	No reaction
6	Water-Tanah Merah	No reaction
7	Soil-Kota Bharu	Bataviae (1:100)
8	Water-Tanah Merah	Hebdomadis (1:100)
9	Water-Jeli	Pyrogenes (1:100)
10	Water-Kuala Krai	No reaction
11	Water-Pasir Mas	No reaction
12	Soil-Jeli	No reaction
13	Water-Jeli	No reaction
14	Soil-Gua Musang	Patoc (1:400)
15	Soil-Jeli	Pyrogenes (1:100)
16	Soil-Gua Musang	Bataviae (1:200)
17	Soil-Gua Musang	Wolffii (1:100)
18	Soil-Kuala Krai	Patoc (1:200)
19	Soil-Kuala Krai	Patoc (1:400)
20	Soil-Kota Bharu	No reaction
21	Water-Tumpat	No reaction
22	Patoc (saprophytic control)	Patoc (1:6400)
23	Hardjobovis (pathogenic control)	Hardjobovis (1:6400)

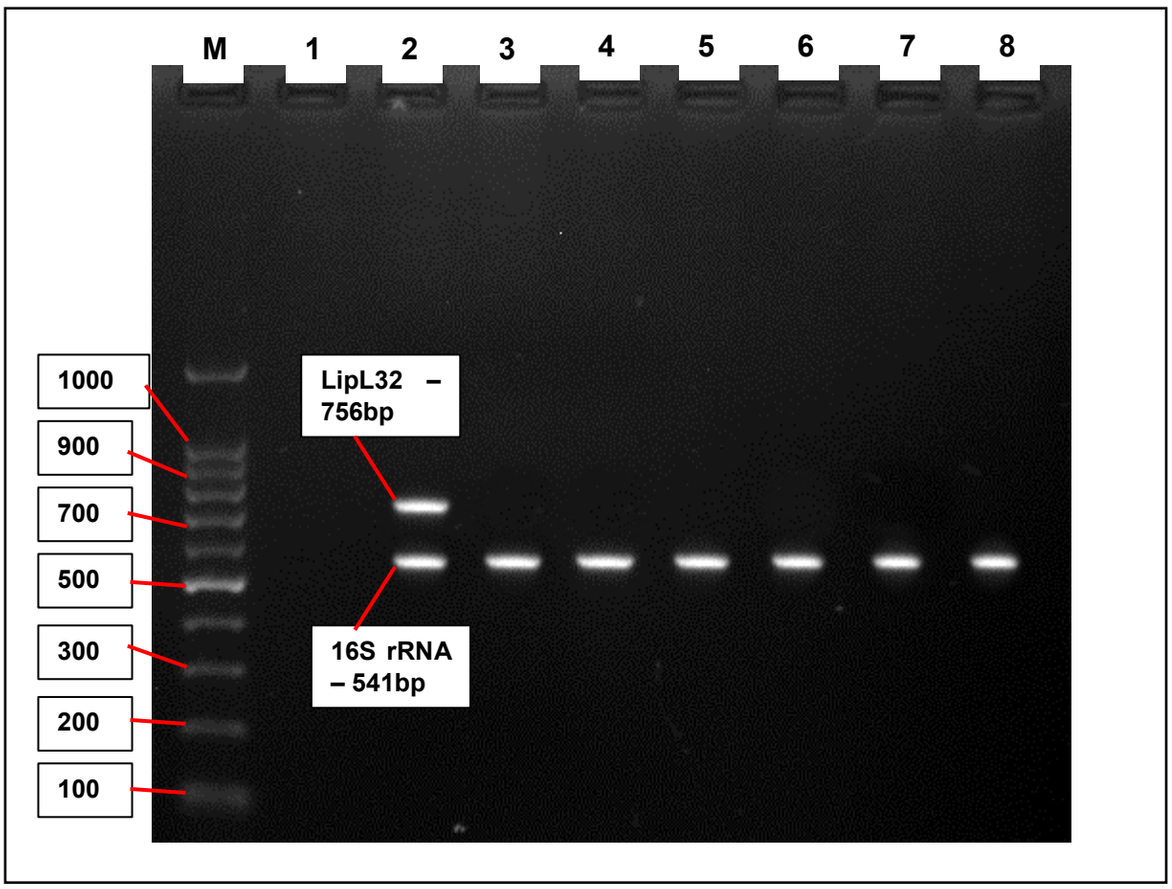

FIGURE 2. Six *Leptospira* sp. isolates from environmental samples identified by using mPCR. **(M)** 100bp DNA ladder; **(1)** negative control (RNase free water); **(2)** positive control *L. borgpetersenii* serovar Hardjobovis strain 117123; **(3-6)** *Leptospira* sp. isolates from environmental samples

Classical methods to differentiate pathogenic or saprophytic leptospires identified five isolates as saprophytic, while the other 16 isolates were unidentified. This may indicate that classical methods can only differentiate between saprophytic and pathogenic leptospires but not intermediate species. *Leptospira* spp. isolates were then identified by MAT, showing ten isolates with serological reactions towards six hyperimmune sera, but MAT titres were less than or equal to 1:400. A cross-reaction between the isolates with their respective serovars may have occurred. All isolates were further identified as being non-pathogenic by mPCR and confirmed by partial 16S rRNA sequencing as *L. wolffii*, an intermediate or opportunistic *Leptospira* species. In a previous study, *L. wolffii* serovar Khorat strain Khorat-H2^T was isolated from the urine of a human patient with suspected leptospirosis in Thailand (Slack et al. 2008). The authors reported that the *L. wolffii* strain Khorat-H2^T showed typical *Leptospira* motility and morphology under darkfield microscopy and did not grow at 13 °C nor in the presence of 8-azaguanine.

Serological identification using MAT also showed that *L. wolffii* strain Khorat-H2^T had no cross-reaction with any recognised *Leptospira* serogroups (Slack et al. 2008). For this study, BLAST analysis showed that all the isolates had 95% similarity to *L. wolffii* serovar Khorat strain Khorat-H2^T and were closely related in the phylogenetic tree. However, all the *Leptospira* spp. isolates were unique and may be genetically different based on phenotype identification (growth or

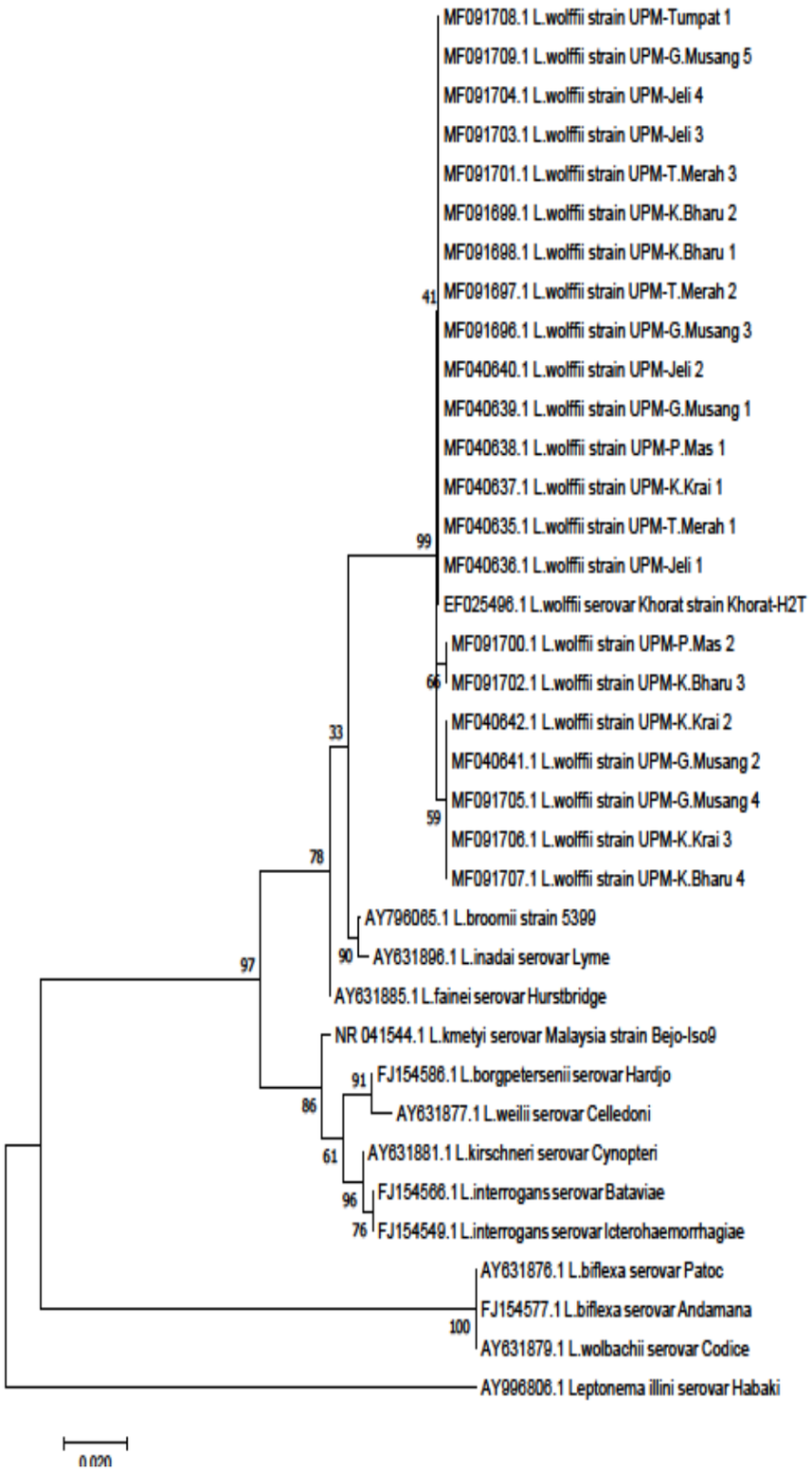

FIGURE 3. Evolutionary relationships of taxa based on 16S rRNA gene for *L. wolffii* isolates obtained from soil and water in Kelantan and designated with UPM strains

inability to grow in 1M NaCl, 8-azaguanine, or 13 °C, respectively) and serological identification. All isolated *L. wolffii* designated UPM strains, including *L. wolffii* serovar Khorat strain Khorat-H2^T, were located within the intermediate clade in the phylogenetic analysis and showed significant divergence from other intermediate species, namely *L. inadai*, *L. fainei*, and *L. broomii*. Furthermore, based on the phylogenetic tree, these intermediate species could be potentially pathogenic to humans and animals because they diverged from the pathogenic clade.

Abd-Rahman et al. (2021) reported that *L. wolffii* was the most predominantly found in humans compared to the other intermediate species, which were *L. inadai* and *L. broomii*. Another study reported that *L. wolffii* was detected in dogs for the first time, with 100% identity in clinical human samples (Zakeri et al. 2010). It suggests that intermediate species of *Leptospira* still cause diseases in animals and humans, though the disease burden of the intermediate leptospires is still unclear.

Findings from this study suggest that *L. wolffii* has started circulating in Malaysia. The *L. wolffi* that was identified from the Gua Musang district may be the non-pathogenic *Leptospira* spp. that was previously reported in two goats from Gua Musang (Sabri et al. 2019). Isolation of *L. wolffii* serovar Khorat strain Khorat-H2^T has also been reported in Sarawak (Pui et al. 2017b, 2015). Moreover, Kelantan is geographically closer to Thailand, where the *L. wolffii* serovar Khorat strain Khorat-H2^T was first isolated. As such, the 2014 flood could have caused the circulation of the *L. wolffii* strain in Malaysia.

Differentiation between pathogenic and saprophytic *Leptospira* spp. was carried out by classical methods like pathogenicity to animals, growth response to 8-azaguanine (225 µg/mL), growth at low temperatures, and/or conversion to spherical forms by 1M NaCl. The low temperature test makes use of the fact that the minimum growth temperature for different *Leptospira* spp. ranges from 13 °C to 15 °C for pathogenic *Leptospira* spp. and 5 °C to 10 °C for saprophytes. However, this criterion could be misleading as some pathogenic *Leptospira* spp., like serovar icterohaemorrhagiae, can also grow at 10 °C (Kmety et al. 1966). PCR was found to be a simpler and more rapid method of differentiating pathogenic and non-pathogenic *Leptospira* spp. when compared to classical methods. Nowadays, classical methods are less commonly used because they are laborious, time-consuming, require multiple tests, and require viable organisms to be tested, while PCR has the advantage of detecting DNA from both viable and non-viable organisms.

Conclusion

Twenty-one out of 124 environmental samples (16.94%) were positive for *Leptospira* spp. The districts of Gua Musang, Kota Bharu, and Jeli showed higher *Leptospira* spp. distribution in livestock farms as compared to other districts. All *Leptospira* spp. isolates were identified as non-pathogenic *Leptospira* spp. and confirmed as *L. wolffii* by DNA sequencing. Detection of *Leptospira* spp. in livestock farms may indicate the survivability of *Leptospira* spp. in the environment, possibly becoming a source of infection for humans and animals.

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References

Abd-Rahman, A.N., Hasnul-Hadi, N.H., Sun, Z., Thilakavathy, K. & Joseph, N. 2021. Regional prevalence of intermediate *Leptospira* spp. in humans: A meta-analysis. *Pathogens* 10: 943.

Ahmed, A., Grobush, M.P., Klatser, P. & Hartskeerl, R.A. 2012. Molecular approaches in the detection and characterization of *Leptospira*. *Journal of Bacteriology & Parasitology* 3: 133.

Ahmed, S.A., Doblin, A.S., Musa, S., Hoe, C.H., Riadzi, M., Lau, K.L. & Tang, T.H. 2011. Rapid diagnosis of leptospirosis by multiplex PCR. *Malaysian Journal of Medical Sciences* 19(3): 9-16.

Bomfim, M., Barbosa-Stancioli, E.F. & Koury, M.C. 2008. Detection of pathogenic leptospires in urine from naturally infected cattle by nested PCR. *Veterinary Journal* 178(2): 251-256.

Fonseca, C., Teixeira, M., Romero, E.C., Tengan, F.M., da Silva, M.V. & Shikanai-Yasuda, M.A. 2006. Leptospiral DNA detection for the diagnosis of human leptospirosis. *Journal of Infections* 52(1): 15-22.

Hartskeerl, R.A., Smits, H.L., Korver, M., Goris, M.G. & Terpstra, W.J. 2001. *International Course on Laboratory Methods for the Diagnosis of Leptospirosis.* Netherlands: Royal Tropical Institute Department of Biomedical Research.

Johnson, R.C. & Faine, S. 1984. Genus I. leptospira noguchi 1917. In *Bergey's Manual of Systematic Bacteriology*, edited by Krieg, N.R. & Holt, J.G. Baltimore: Williams & Wilkins. pp. 62-67.

Johnson, R.C. & Gary, N.D. 1962. Nutrition of *Leptospira pomona*. 1. A chemically defined substitute for rabbit serum ultrafiltrate. *Journal of Bacteriology* 83(3): 668-672.

Kmety, E., Plesko, I., Bakoss, P. & Chorvath, B. 1966. Evaluation of methods for differentiating pathogenic and saprophytic leptospiral strains. *Annales des Sociétés Belges de Médecine Tropicale, de Parasitologie, et de Mycologie* 46: 111-122.

Kumar, S., Stecher, G. & Tamura, K. 2016. MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. *Molecular Biology and Evolution* 33: 1870-1874.

Laras, K., Cao, B.V., Bounlu, K., Nguyen, T.K.T., Olson, J.G., Thongchanh, S., Nguyen, V.A.T., Hoang, K.L., Punjabi, N., Ha, B.K., Ung, S.A., Insisiengmay, S., Watts, D.M., Beecham, H.J. & Corwin, A.L. 2002. The importance of Leptospirosis in Southeast Asia. *The American Journal of Tropical Medicine and Hygiene* 67(3): 278-286.

Levett, P.N. 2004. Leptospirosis: A forgotten zoonosis? *Clinical & Applied Immunology Reviews* 4: 435-448.

Lilenbaum, W., Varges, R., Brandão, F.Z., Cortez, A., de Souza, S.O., Brandão, P.E., Richtzenhain, L.J. & Vasconcellos, S.A. 2008. Detection of *Leptospira* spp. in semen and vaginal fluids of goats and sheep by polymerase chain reaction. *Theriogenology* 69(7): 837-842.

Merien, F., Amouriaux, P., Perolat, P., Baranton, G. & Saint Girons, I. 1992. Polymerase chain reaction for detection of *Leptospira* spp. in clinical samples. *Journal of Clinical Microbiology* 30: 2219-2224.

MOH. 2015. Epidemiology and current situation of Leptospirosis in Malaysia. Dr. Zainudin AW. *Persidangan Kesihatan Persekitaran Pihak Berkuasa Tempatan* 2015, 8-9 September 2015, WP Labuan.

Mohd-Radi, M.F., Hashim, J.H., Jaafar, M.H., Hod, R., Ahmad, N., Mohammed Nawi, A., Baloch, G.M., Ismail, R. & Farakhin Ayub, N.I. 2018. Leptospirosis outbreak after the 2014 major flooding event in Kelantan, Malaysia: A spatial-temporal analysis. *The American Journal of Tropical Medicine and Hygiene* 98(5): 1281-1295.

Noubade, R., Krishnamurthy, G., Murag, S., Venkatesha, M. & Krishnappa, G. 2002. Differentiation of pathogenic and saprophytic Leptospires by polymerase chain reaction. *Indian Journal of Medical Microbiology* 20(1): 33-36.

OIE. 2014. *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals*. http://www.oie.int/manual-of-diagnostic-tests-and-vaccines-for-terrestrial-animals/. Accessed on 29 January 2017.

Ooteman, M.C., Vago, A.R. & Koury, M.C. 2006. Evaluation of MAT, IgM ELISA and PCR methods for the diagnosis of human leptospirosis. *Journal of Microbiology Methods* 65(2): 247-257.

Pui, C.F., Bilung, L.M., Apun, K. & Su'ut, L. 2017a. Diversity of *Leptospira* spp. in rats and environment from urban areas of Sarawak, Malaysia. *Journal of Tropical Medicine* 2017: 1-8.

Pui, C.F., Bilung, L.M., Su'ut, L., Chong, Y.L. & Apun, K. 2017b. Detection of *Leptospira* spp. in selected national service training centres and paddy fields of Sarawak, Malaysia using polymerase chain reaction technique. *Tropical Agriculture Science* 40(1): 99-110.

Pui, C.F., Bilung, L.M., Su'ut, L. & Apun, K. 2015. Prevalence of *Leptospira* species in environment soil and water from national parks in Sarawak, Malaysia. *Borneo Journal Resource Science and Technology* 5(1): 49-57.

Sabri, A.R., Khairani-Bejo, S., Zunita, Z. & Hassan, L. 2019. Molecular detection of *Leptospira* sp. in cattle and goats in Kelantan, Malaysia after a massive flood using multiplex polymerase chain reaction. *Tropical Biomedicine* 36(1): 165-171.

Slack, A.T., Kalambaheti, T., Symonds, M.L., Dohnt, M.F., Galloway, R.L., Steigerwalt, A.G., Chaicumpa, W., Bunyaraksyotin, G., Craig, S., Harrower, B.J. & Smythe, L.D. 2008. *Leptospira wolffii* sp. nov., isolated from a human with suspected leptospirosis in Thailand. *International Journal of Systematic and Evolutionary Microbiology* 58(10): 2305-2308.

Tansuphasiri, U., Chanchai, T., Duangporn, P. & Charnchudhi, C. 2006. Duplex PCR-hybridization based detection of pathogenic *Leptospira* in environmental water samples obtained from endemic areas in northeast region of Thailand. *Southeast Asian Journal of Tropical Medicine Public Health* 37(4): 729-741.

Wan-Yussof, W.N. 2015. *Flood in Kelantan in 2014: An Introspection*. Report from Centre for Strategic Studies, the State of Kelantan, Malaysia.

Zakeri, S., Khorami, N., Ganji, Z.F., Sepahian, N., Malmasi, A.A., Gouya, M.M., Djadid, N.D. 2010. *Leptospira wolffii*, a potential new pathogenic *Leptospira* species detected in human, sheep and dog. Infection, genetics and evolution. *Journal of Molecular Epidemiology and Evolutionary Genetics in Infectious Diseases* 10(2): 273-277.

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