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Detection and distribution of putative pathogenicity-associated genes among serologically important *Leptospira* strains and post-flood environmental isolates in Malaysia

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ABSTRACT

Aims: Leptospirosis is an infectious disease that is endemic to many tropical regions. Large epidemics usually happen after heavy rainfall and flooding. This potentially fatal zoonosis is caused by pathogenic bacteria belonging to the genus *Leptospira*. Leptospirosis can be diagnosed using specific biomarkers such as target genes and virulence indicators that are well preserved across various *Leptospira* spp., including those that are prevalent in clinical samples and in the environment. To date, several pathogenicity-determinant genes, including *lipL32* and *lipL41*, have been described and used for diagnosing leptospirosis. However, prevalence of these genes in leptospiral strains is unclear.
Methodology and results: In the present study, we assessed the distribution of eight pathogenicity-determinant genes in reference *Leptospira* strains and environmental isolates in Malaysia, by polymerase chain reaction (PCR). We found that only *lipL32* and *ligB* were consistently expressed in all pathogenic *Leptospira* strains compared with the other tested genes. Moreover, our results suggested that the use of *lipL41*, *lipL21*, *ompL1*, *lfb1*, *ligA*, and *ligC* as biomarkers could incorrectly misdetect pathogenic *Leptospira* strains present in the environment.
Conclusion: Thus, our results suggest that the pathogenicity-determinant genes *lipL32* and *ligB* can be used as biomarkers for detection pathogenic *Leptospira*.

Keywords: Leptospira, leptospirosis, polymerase chain reaction (PCR)

INTRODUCTION

Leptospirosis is caused by *Leptospira* spirochaetes, is an important zoonotic disease that annually infects 1.03 million people worldwide (Costa *et al*., 2015). Leptospirosis is considered to be an endemic disease in Malaysia, with the number of reported cases and outbreaks increasing significantly over the years (Benacer *et al*., 2016). The mortality rate of leptospirosis ranges broadly between 5% and 83.3% and depends on several factors, including sociodemographic status, disease manifestation, and age (Edilane *et al*., 2008; Victoriano *et al*., 2009; Costa *et al*., 2015; Widiastuti *et al*., 2016). Symptoms of leptospirosis usually include fever, headache, myalgia, vomiting, and jaundice (Haake and Levett, 2015).

Animals may serve as an intermediate and/or a natural reservoir of *Leptospira* strains depending on their species. In livestock animals such as cattle, leptospirosis causes a significant economic loss because of abortion, infertility, weight loss, reduced milk production, and death (Villanueva *et al*., 2016). Other animals, especially rodents, may carry *Leptospira* strains asymptomatically (Perra *et al*., 2002) and may continuously excrete leptospires into the environment (Picardeau, 2013). Human infections usually originate from indirect exposure to a contaminated environment, such as contact with contaminated water and soil (Thibeaux *et al*., 2017). Freshwater recreation, farming, jungle trekking, and other outdoor activities increase the risk of leptospirosis because of the ability of pathogenic *Leptospira* strains to persist in the environment (El Jalii and Bahaman, 2004; Andre-Fontaine *et al*., 2015).

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Effective surveillance, management, and control of leptospirosis significantly depend on the detection of *Leptospira* strains in both the clinical setting and environment (Yuszniahyati *et al*., 2015). Underestimation of leptospirosis cases because of misdiagnosis and limited availability of diagnostic assays, especially in resource-limited areas, is a major concern (Musso and La Scola, 2013; Picardeau, 2013; Yuszniahyati *et al*., 2015). Furthermore, many aspects of leptospiral epidemiology, especially the role of the environment in the persistence of leptospires, are unclear (Jorge *et al*., 2015).

Conventionally, *Leptospira* strains can be isolated from the environment through culturing. However, isolation or detection of pathogenic *Leptospira* strains through culturing is difficult because both pathogenic and non-pathogenic *Leptospira* strains have similar morphology, thus making it difficult to differentiate between these strains. Moreover, non-pathogenic *Leptospira* strains grow rapidly compared with pathogenic *Leptospira* strains, thus resulting in the predominance of non-pathogenic *Leptospira* strains in a culture (Cerqueira *et al*., 2009). Moreover, besides being time-consuming, culturing is associated with a high risk of contamination by other microorganisms, particularly fast-growing bacteria (Rawlins *et al*., 2014). These limitations can be overcome by using molecular methods that detect pathogens by targeting their specific genes (Adler and de la Pena Moctezuma, 2010).

PCR is used for detecting pathogenic *Leptospira* strains from soil and water (Saito *et al*., 2013; Muñoz-Zanzi *et al*., 2014). Although several pathogenic leptospiral biomarker genes such as *lipL32*, *lipL41*, *ligA*, *ligB*, and *ligC* have been identified, distribution of these genes in environmental *Leptospira* strains is unclear. This increases the risk of false-negative results and misdetection in cases where infecting *Leptospira* strains lack the targeted genes (Mayer-Scholl *et al*., 2011). Therefore, the present study assessed the distribution of selected pathogenic leptospiral genes among serologically important *Leptospira* strains and post-flood environmental isolates in Malaysia.

MATERIALS AND METHODS

Reference leptospiral strains

Twenty-two *Leptospira* reference strains were obtained from the Institute for Medical Research, Kuala Lumpur (isolates were subcultured since 2012), and Universiti Putra Malaysia, Serdang, Malaysia (Table 1). All the *Leptospira* strains were maintained in liquid EMJH medium (Difco, USA) supplemented with 200 µg/mL 5-fluorouracil (Merck, Germany) and were incubated at 30 °C for 1 week.

Study site

Environmental sampling was conducted from June to September 2015 from flood-affected areas, namely, Pasir Mas and Rantau Panjang. These two areas are located in the northern part of Kelantan, Malaysia, and were selected as sampling sites based on the reported leptospirosis cases

Collection and processing of environmental samples

Ninety-nine environmental samples, including water samples (*n*=16) and soil samples (*n*=83), were collected and processed using a method described by Azali *et al*. (2016). Water samples were isolated by collecting approximately 100 mL water from 1 ft below the water surface. Soil samples were collected in sterile containers containing sterile water and were mixed by shaking vigorously. Both the water and soil samples were filtered using 0.2 µm Nalgene® filter unit (Thermo Fisher Scientific, USA), and the filtrates were centrifuged at 4,000 × *g* and 27 °C for 20 min. Next, 2 mL samples were inoculated in the liquid EMJH medium supplemented with 5-fluorouracil and were incubated at 30 °C for 1 week. The suspension cultures were examined daily for 28 days by performing dark-field microscopy. Presence of *Leptospira* strains in the culture was confirmed based on their active motility and hook-like end morphology, which distinguishes them from other spirochaetes. Details of environmental *Leptospira* isolates were summarized in Table 2.

Genomic DNA isolation

Ten mL suspension culture was centrifuged at 8,000 × *g* for 5 min. The pellet obtained was used for isolating genomic DNA by using NucleoSpin® tissue kit (Macherey-Nagel, Germany), according to the manufacturer's recommendation. DNA was eluted using 50 µL TE buffer, and the quantity of genomic DNA was measured using Biophotometer (Eppendorf, Germany). For subsequent experiments, the concentration of genomic DNA was adjusted to 20 ng/µL.

Polymerase chain reaction (PCR)

PCR was performed to determine the distribution of the eight pathogenic genes (*lipL41*, *lipL32*, *lipL21*, *ompL1*, *lfb1*, *ligA*, *ligB*, and *ligC*) and the two housekeeping genes (*flaB* and *secY*) in all the reference *Leptospira* strains and environmental isolates. Moreover, PCR was performed to amplify the 16S rRNA gene in all the environmental isolates for molecular characterization. All oligonucleotides used in the present study are listed in Table 3.

PCR was performed in a 20 µL reaction mixture containing 1× PCR buffer, 2.5 mM dNTPs, 2.5 mM $MgCl_2$, 0.75 U Taq DNA polymerase, oligonucleotides (Table 3), 2 µL extracted genomic DNA, and PCR-grade water. All the above-mentioned PCR reagents, except the oligonucleotides (Integrated DNA Technologies, Singapore), were purchased from Thermo Fisher Scientific. PCR was performed in Eppendorf Mastercycler (Germany) by using the following conditions : initial

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activation (95 °C for 5 min); 30 cycles of denaturation (95 °C for 30 sec), annealing (temperature for each oligonucleotide pair is shown in Table 3), and extension (72 °C for 30 sec); and final extension (72 °C for 5 min). PCR products obtained were analyzed by performing electrophoresis on 2% agarose gel (First Base Laboratories, Malaysia) with RedSafe stain (Intron Biotechnology, Korea) for 45 min and were visualized under UV illumination by using ChemiImager 5500 UV illuminator (Alpha Innotech, USA) with an image-capturing unit.

Molecular characterization of the environmental isolates

All the cultures yielding positive results were verified by performing DNA sequencing based on the amplified 16S rRNA gene. Amplicons were purified using QIAquick PCR purification kit (Qiagen, USA) and were submitted to First BASE Laboratories for sequencing. Resulting DNA sequence data were compared with the GenBank database by using the BLAST algorithm, which available on the National Center for Biotechnology Information website (https://www.ncbi.nlm.nih.gov/genbank/). Sequences obtained in this study were submitted to GenBank.

RESULTS

Identification of *Leptospira* strains in environmental samples by EMJH culture and 16S rRNA sequencing

After culturing, 57.6% (57/99) environmental samples yielded positive results for *Leptospira* strains, which showed a typical morphology and active motility when examined under dark-field microscopy. Of the positive samples, 15.8% (9/57) were isolated from water and 84.2% (48/57) were isolated from soil. Furthermore, 16S rRNA sequencing showed that 94.7% (54/57) positive samples contained *Leptospira* strains and 5.3% (3/57) positive samples contained a non-*Leptospira* spirochaete (*Leptonema illini*). Of the positive samples, 22.2% (12/54) contained pathogenic *Leptospira* strains, 55.6% (30/54) contained intermediate-pathogenic *Leptospira* strains, and 22.2% (12/54) contained non-pathogenic *Leptospira* strains. Moreover, 16S rRNA sequencing showed that all the pathogenic *Leptospira* strains were *L. kmetyi*; 80% (24/30), 6.7% (2/30), 6.7% (2/30), and 6.7% (2/30) intermediate-pathogenic *Leptospira* strains were *L. wolffii*, *L. fainei*, *L. inadai*, and *L. licerasiae*, respectively; and 91.7% (11/12) and 8.3% (1/12) non-pathogenic *Leptospira* strains were *L. meyeri* and *L. idonii*, respectively. GenBank accession numbers of all the *Leptospira* strains are listed in Table 2.

Distribution of pathogenic genes among reference *Leptospira* strains

Two pathogenicity-determinant genes, namely, *lipL32* and *ligB*, were well conserved across all the pathogenic *Leptospira* strains (Table 1). The other tested genes were also detected in all the pathogenic *Leptospira* strains, except serovars Australis, Javanica, Celledoni, and Tarassovi. In contrast, *lipL32*, *lipL41*, *lipL21*, *ompL1*, *lfb1*, *flaB*, and *secY* were not detected in any of the intermediate and non-pathogenic *Leptospira* strains, whereas all the leptospiral immunoglobulin-like (*lig*) genes were detected in *Leptospira* serovars Hurstbridge, Varillal, and Terengganu. Amplification products of *lipL32*, *lipL41*, *lipL21*, *ompL1*, *flaB*, *lfb1*, *ligA*, *ligB*, *ligC*, and *secY* on electrophoresis gels, with *L. interrogans* serovar Canicola genes as a reference, are shown in Figure 1. All observed bands corresponded to the expected sizes obtained using each respective primer set.

Distribution of pathogenic genes among environmental *Leptospira* strains

Of the six pathogenic genes, *lipL32*, *lipL21*, *flaB*, and *ligB* were detected in all the samples containing the pathogenic environmental strain *L. kmetyi* and *lfb1* and *lipL41* were detected in 8.33% (1/12) and 50% (6/12) samples, respectively, containing the pathogenic environmental strain *L. kmetyi*. None of the tested pathogenic genes was detected in the intermediate and non-pathogenic *Leptospira* strains as well as in *L. illini*. Moreover, *secY* was not detected in any of the tested environmental isolates. These results are summarized in Table 2.

DISCUSSION

Increasing incidence of leptospirosis is usually associated with recreational activities, heavy rainfall, flooding, occupational exposure, and poor sanitation (Victoriano *et al*., 2009). The causative agent of leptospirosis, i.e., pathogenic *Leptospira* strains, are shed by animal reservoirs through urination and disperse in the environment, including water and soil. These bacteria persist in the environment until they enter a new reservoir host or humans. Because the contaminated environment is the major source of leptospirosis, appropriate control and preventive measures are necessary to minimize the risk of this infection.

The present study involved collection of environmental samples from flood-affected areas in Kelantan, Malaysia, to detect pathogenic *Leptospira* strains in these areas because leptospirosis outbreaks are usually associated with increased rainfall and flooding (Garba *et al*., 2017). The 16S rRNA sequencing performed in the present study detected pathogenic *Leptospira* strains in 21% (12/57) environmental samples and showed that all these strains belonged to *L. kmetyi*. Several previous studies have detected pathogenic and intermediate *Leptospira* strains belonging to *L. interrogans*, *L. borgpetersenii*, *L. kmetyi*, *L. alstonii*, and *L. wolffii* in environmental samples (Alexander *et al*., 1975; Slack *et al*., 2009; Ridzlan *et al*., 2010; Benacer *et al*., 2013; Mohd Ali *et al*., 2017). Thus, our results suggest that pathogenic *Leptospira* strains are widely distributed in the environment. The environment is the natural habitat for non-pathogenic

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Table 1 : Distribution of 8 pathogenic genes and 2 housekeeping genes among *Leptospira* reference strains from Malaysia.

Reference strains	Screened genes										Source
	lipL32†	lipL41†	lipL21†	ompL1†	lfb1†	ligA†	ligB†	ligC†	secY††	flaB††	
Clade: Pathogenic											
L. interrogans serovar:											
Canicola	+	+	+	+	+	+	+	+	+	+	UPM
Pyrogenes	+	+	+	+	+	+	+	+	+	+	UPM
Icterohemorrhagiae	+	+	+	+	+	+	+	+	+	+	UPM
Hebdomadis	+	+	+	+	+	+	+	+	+	+	UPM
Australis	+	+	+	+	+	+	+	+	+	-	UPM
Bataviae	+	+	+	+	+	+	+	+	+	+	IMR
Pomona	+	+	+	+	+	+	+	+	+	+	IMR
Autumnalis	+	+	+	+	+	+	+	+	+	+	IMR
Pyrogenes	+	+	+	+	+	+	+	+	+	+	IMR
Copenhageni	+	+	+	+	+	+	+	+	+	+	IMR
Djasiman	+	+	+	+	+	+	+	+	+	+	IMR
Hardjoprajitno	+	+	+	+	+	+	+	+	+	+	IMR
Javanica	+	+	+	-	-	-	+	+	-	-	IMR
Tarassovi	+	+	+	-	-	-	+	-	-	-	IMR
Celledoni	+	-	-	-	-	-	+	-	-	-	IMR
L. borgpetersenii serovar Ballum	+	-	+	+	+	+	+	-	+	+	UPM
Clade: intermediate											
L. fainei serovar Hurstbridge	-	-	-	-	-	-	+	-	-	-	UPM
L. licerasiae serovar Varillal	-	-	-	-	-	+	+	+	-	-	IMR
L. wolffii serovar Sarawak (Lepto 175)	-	-	-	-	-	-	-	-	-	-	IMR
Clade: Non-pathogenic											
L. meyeri serovar:											
Melaka	-	-	-	-	-	-	-	-	-	-	IMR
Terengganu	-	-	-	-	-	+	+	-	-	-	IMR
L. biflexa serovar Patoc	-	-	-	-	-	-	-	-	-	-	UPM

†Pathogenic gene;
††Housekeeping gene
+: Presence of the gene
- : Absence of the gene

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Table 2: Distribution of 8 pathogenic genes and 2 housekeeping genes among environmental isolates from Malaysia.

No	Samples name	Accession no	Sequencing result	Clade	lipL32†	lipL41†	lipL21†	ompL1†	lfb1†	ligA†	ligB†	ligC†	secY††	flaB††
1	LS 0002/15	KX452324	*L. kmetyi*	Pathogenic	+	-	+	-	-	-	+	-	-	+
2	LS 0008/15	KX452323	*L. kmetyi*	Pathogenic	+	+	+	-	-	-	+	-	-	+
3	LS 0009/15	KX452322	*L. kmetyi*	Pathogenic	+	-	+	-	-	-	+	-	-	+
4	LS 0023/15	KX452321	*L. kmetyi*	Pathogenic	+	-	+	-	-	-	+	-	-	+
5	LS 0024/15	KX452325	*L. kmetyi*	Pathogenic	+	+	+	-	-	-	+	-	-	+
6	LS 0031/15	KX452320	*L. kmetyi*	Pathogenic	+	+	+	-	-	-	+	-	-	+
7	LS 0037/15	KX452319	*L. kmetyi*	Pathogenic	+	-	+	-	-	-	+	-	-	+
8	LS 0050/15	KX452318	*L. kmetyi*	Pathogenic	+	+	+	-	-	-	+	-	-	+
9	LS 0054/15	KX452317	*L. kmetyi*	Pathogenic	+	-	+	-	-	-	+	-	-	+
10	LS 0072/15	KX452315	*L. kmetyi*	Pathogenic	+	+	+	-	-	-	+	-	-	+
11	LS 0075/15	KX452314	*L. kmetyi*	Pathogenic	+	+	+	-	-	-	+	-	-	+
12	LW 0005/15	KX452313	*L. kmetyi*	Pathogenic	+	-	+	-	+	-	+	-	-	+
13	LW 0006/15	KX452310	*L. fainei*	Intermediate	-	-	-	-	-	-	-	-	-	-
14	LW 0013/15	KX452309	*L. fainei*	Intermediate	-	-	-	-	-	-	-	-	-	-
15	LW 0003/15	KX452311	*L. inadai*	Intermediate	-	-	-	-	-	-	-	-	-	-
16	LW 0010/15	KX452312	*L. inadai*	Intermediate	-	-	-	-	-	-	-	-	-	-
17	LS 0011/15	KX452307	*L. licerasiae*	Intermediate	-	-	-	-	-	-	-	-	-	-
18	LS 0063/15	KX452308	*L. licerasiae*	Intermediate	-	-	-	-	-	-	-	-	-	-
19	LS 0010/15	KX452302	*L. wolfii*	Intermediate	-	-	-	-	-	-	-	-	-	-
20	LS 0018/15	KX452301	*L. wolfii*	Intermediate	-	-	-	-	-	-	-	-	-	-

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Table 2: Continued

21	LS 0019/15	KX452304	*L. wolfii*	Intermediate	-	-	-	-	-	-	-	-	-	-
22	LS 0027/15	KX452305	*L. wolfii*	Intermediate	-	-	-	-	-	-	-	-	-	-
23	LS 0033/15	KX452300	*L. wolfii*	Intermediate	-	-	-	-	-	-	-	-	-	-
24	LS 0035/15	KX452299	*L. wolfii*	Intermediate	-	-	-	-	-	-	-	-	-	-
25	LS 0039/15	KX452298	*L. wolfii*	Intermediate	-	-	-	-	-	-	-	-	-	-
26	LS 0048/15	KX452297	*L. wolfii*	Intermediate	-	-	-	-	-	-	-	-	-	-
27	LS 0049/15	KX452306	*L. wolfii*	Intermediate	-	-	-	-	-	-	-	-	-	-
28	LS 0057/15	KX452296	*L. wolfii*	Intermediate	-	-	-	-	-	-	-	-	-	-
29	LS 0059/15	KX452295	*L. wolfii*	Intermediate	-	-	-	-	-	-	-	-	-	-
30	LS 0066/15	KX452294	*L. wolfii*	Intermediate	-	-	-	-	-	-	-	-	-	-
31	LS 0067/15	KX452303	*L. wolfii*	Intermediate	-	-	-	-	-	-	-	-	-	-
32	LS 0069/15	KX452293	*L. wolfii*	Intermediate	-	-	-	-	-	-	-	-	-	-
33	LS 0070/15	KX452292	*L. wolfii*	Intermediate	-	-	-	-	-	-	-	-	-	-
34	LS 0071/15	KX452291	*L. wolfii*	Intermediate	-	-	-	-	-	-	-	-	-	-
35	LS 0074/15	KX452290	*L. wolfii*	Intermediate	-	-	-	-	-	-	-	-	-	-
36	LS 0079/15	KX452289	*L. wolfii*	Intermediate	-	-	-	-	-	-	-	-	-	-
37	LS 0080/15	KX452288	*L. wolfii*	Intermediate	-	-	-	-	-	-	-	-	-	-
38	LS 00081/15	KX452287	*L. wolfii*	Intermediate	-	-	-	-	-	-	-	-	-	-
39	LW 0004/15	KX452286	*L. wolfii*	Intermediate	-	-	-	-	-	-	-	-	-	-
40	LW 0008/15	KX452285	*L. wolfii*	Intermediate	-	-	-	-	-	-	-	-	-	-
41	LW 0009/15	KX452284	*L. wolfii*	Intermediate	-	-	-	-	-	-	-	-	-	-

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Table 2: Continued

42	LW 0015/15	KX452283	*L. wolfii*	Intermediate	-	-	-	-	-	-	-	-	-	-
43	LS 0001/15	KX452326	*L. idonii*	Non-pathogenic	-	-	-	-	-	-	-	-	-	-
44	LS 0006/15	KX452338	*L. meyeri*	Non-pathogenic	-	-	-	-	-	-	-	-	-	-
45	LS 0007/15	KX452337	*L. meyeri*	Non-pathogenic	-	-	-	-	-	-	-	-	-	-
46	LS 0015/15	KX452336	*L. meyeri*	Non-pathogenic	-	-	-	-	-	-	-	-	-	-
47	LS 0030/15	KX452335	*L. meyeri*	Non-pathogenic	-	-	-	-	-	-	-	-	-	-
48	LS 0036/15	KX452334	*L. meyeri*	Non-pathogenic	-	-	-	-	-	-	-	-	-	-
49	LS 0047/15	KX452333	*L. meyeri*	Non-pathogenic	-	-	-	-	-	-	-	-	-	-
50	LS 0052/15	KX452331	*L. meyeri*	Non-pathogenic	-	-	-	-	-	-	-	-	-	-
51	LS 0068/15	KX452330	*L. meyeri*	Non-pathogenic	-	-	-	-	-	-	-	-	-	-
52	LS 0077/15	KX452327	*L. meyeri*	Non-pathogenic	-	-	-	-	-	-	-	-	-	-
53	LS 0082/15	KX452329	*L. meyeri*	Non-pathogenic	-	-	-	-	-	-	-	-	-	-
54	LS 0083/15	KX452328	*L. meyeri*	Non-pathogenic	-	-	-	-	-	-	-	-	-	-
55	LS 0046/15	KX452340	*Leptonema illini*	Non-leptospira	-	-	-	-	-	-	-	-	-	-
56	LS 0053/15	KX452341	*Leptonema illini*	Non-leptospira	-	-	-	-	-	-	-	-	-	-
57	LS 0065/15	KX452343	*Leptonema illini*	Non-leptospira	-	-	-	-	-	-	-	-	-	-

†Pathogenic gene
††Housekeeping gene
+: Presence of the gene
- : Absence of the gene

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Table 3: List of oligonucleotide primers used in the present study.

Target genes	Oligonucleotide sequence (5' → 3')	Annealing temp.	Amplicon size	Source
lipL32	F: AAGCATTACCGCTTGTGGTG R: GAACTCCCATTTCAGCGATT	60 °C	243 bp	This study
lipL41	F: ATTGGAGCGGAAGCAATT R: GGAATTAACATCATACGTACTCC	56 °C/*	1050 bp	(Lin *et al*., 2009)
lipL21	F: CGCGGTCGACATGATCAATAGACTTATAGCTC R: CGCGCTGCAGTTATTGTTTGGAAACCTCTTG	55 °C	561 bp	(Cheemaa *et al*., 2007)
ompL1	F: TTGATTGAATTCTTAGAGTTCGTGTTTATA R: AAGGAGAAGCTTATGATCCGTAACATAAGT	56 °C	960 bp	(Patricia *et al*., 2014)
lfb1	F: CATTCATGTTTCGAATCATTTCAAA R: GGCCCAAGTTCCTTCTAAAAG	61 °C	350 bp	(Bourhy *et al*., 2011)
ligA	F: CKGAWCTTGTRACYTGGARKTCYTC R: TTGTTAATGTTTTCATRTTAYGGC	54 °C	211 bp	(Cerqueira *et al*., 2009)
ligB	F: ACWRVHVHRGYWDCCTGGTCYTCTTC R: TARRHDGCYBTAATATYCGRWYYTCCTAA	54 °C	380 bp	(Cerqueira *et al*., 2009)
ligC	F: GAGAAATAYAATCTCCTTCTTCCGG R: CCTRTTCGTGTTGGARGAATTCC	54 °C	304 bp	(Cerqueira *et al*., 2009)
secY	F: GCGATTCAGTTTAATCCTGC R: GAGTTAGAGCTCAAATCTAAG	60 °C	203 bp	(Ahmed *et al*., 2009)
flaB	F: TCTCACCGTTCTCTAAAGTTCAAC R: CTGAATTCGGTTTCATATTTGCC	59 °C	793 bp	(Natarajaseenivasan *et al*., 2010)
16S rRNA	F: AGTTTGATCMTGGCTCAG R: GGACTACHAGGGTATCTAAT	52 °C	796 bp	(Hookey *et al*., 1993)

*Slight modification from the original article.

Leptospira strains. Results of the 16S rRNA sequencing showed that 21% (12/57) and 53% (30/57) environmental samples contained non-pathogenic and intermediate *Leptospira* strains, respectively. The total number of non-pathogenic and intermediate-pathogenic *Leptospira* strains was higher than the number of pathogenic *Leptospira* strains isolated from the environmental samples. This finding is consistent with that of previous studies that detected a higher number of saprophytic *Leptospira* strains than pathogenic *Leptospira* strains in water and soil samples (Azali *et al*., 2016). The present study included samples isolated from selected sites, including recreational lakes, waterfalls, and markets, in Peninsular Malaysia. Therefore, our results strongly suggest that pathogenic *Leptospira* strains are less adapted to environmental conditions than saprophytic *Leptospira* strains.

Detection of pathogenic *Leptospira* strains in human-exposed environments may indicate a public health risk. However, the absence of reliable pathogenic gene markers limits the detection of pathogenic *Leptospira* strains in environmental studies even though these strains survive in water and soil for prolonged periods under favourable environmental conditions (Rawlins *et al*., 2014). In all, 10 genes were selected to determine the best gene for detecting pathogenic *Leptospira* strains. Of the selected genes, eight genes (*lipL32*, *lipL21*, *lipL41*, *ompL1*, *ligA*, *ligB*, *ligC*, and *lfb1*) were pathogenicity-determinant genes that are conserved in all pathogenic *Leptospira* strains and two were housekeeping genes (*secY* and *flaB*) that are present in all pathogenic *Leptospira* strains.

Of the tested pathogenicity-determinant genes, *lipL32* and *ligB* were detected in all pathogenic *Leptospira* strains, including the reference strains and environmental isolates. Several studies have reported the usefulness of

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lipL32 as a pathogenicity-determinant gene (Muñoz-Zanzi *et al*., 2014; Rawlins *et al*., 2014; Riediger *et al*., 2016). This gene encodes an outer membrane lipoprotein (OMP), which is the most abundant surface protein in pathogenic *Leptospira* strains. OMP is an essential component that can be used for the molecular characterization of various bacteria. However, mechanisms underlying leptospiral pathogenesis are not well understood, OMP is suggested to play several important roles in leptospiral pathogenesis, including evasion of the immune response, acquisition of host proteins, and survival in the host (Haake and Matsunaga, 2010; Patricia *et al*., 2014).

The present study also included other genes involved in lipoprotein biosynthesis and expression, including *lipL21* and *lipL41*. Surprisingly, these genes could not be detected in one of the reference pathogenic serovar, namely Celledoni. However, *lipL21* was detected in all the pathogenic environmental isolates, whereas *lipL41* was detected in only 50% (6/12) pathogenic environmental isolates. The gene *lipL41* encodes the third most abundant surface lipoprotein that provides synergistic immunoprotection along with OmpL1 (Haake and Matsunaga, 2010). However, *lipL41* alone is not essential for inducing acute leptospirosis (King *et al*., 2013). OmpL1 porin is a transmembrane OMP expressed in pathogenic *Leptospira* strains and promotes the diffusion of hydrophilic solutes from the external membrane to the periplasm (Haake and Matsunaga, 2010). Another conserved pathogenic OMP, i.e., fibronectin-binding protein Lfb1, is predicted to mediate the attachment of *Leptospira* strains to host cells (Lehmann *et al*., 2014).

The *lig* genes *ligA*, *ligB*, and *ligC* encode an important family of OMPs that are characterized by the presence of immunoglobulin-like domains, which are virulence determinants of leptospirosis (McBride *et al*., 2009). Both

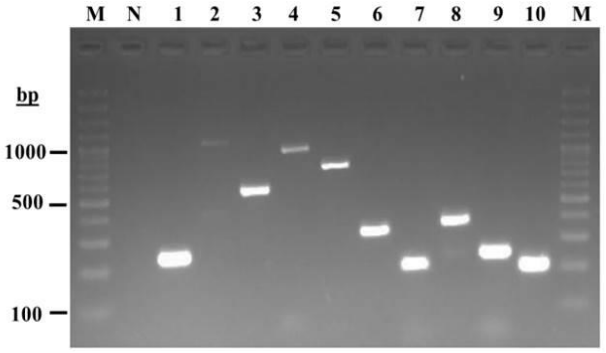

Figure 1: Representative agarose gel electrophoresis of *L. interrogans* serovar Canicola screened by using 10 sets of PCR primers. Lane M, 100 bp PLUS DNA ladder; Lane N, Non-template control; Lane 1, *lipL32* gene (243 bp); Lane 2, *lipL41* gene (1050 bp); Lane 3, *lipL21* gene (561 bp); Lane 4, *ompL1* gene (960 bp); Lane 5, *flaB* gene (793 bp); Lane 6, *lfb1* gene (350 bp); Lane 7, *ligA* gene (211 bp); Lane 8, *ligB* gene (380 bp); Lane 9, *ligC* gene (304 bp); Lane 10, *secY* gene (203 bp).

LigA and LigB bind to the same extracellular matrix and plasma proteins, indicating their role in the colonization and dissemination stages of leptospirosis. On the other hand, *ligC* functions as a pseudogene in several *Leptospira* strains (Cerqueira *et al*., 2009). The *lig* genes were detected in the majority of pathogenic *Leptospira* strains and some intermediate-pathogenic *Leptospira* strains but not in environmental isolates. Of the three *lig* genes, only *ligB* was consistently detected in all the pathogenic samples. Several studies have assessed the involvement of intermediate *Leptospira* strains in human infection (Petersen *et al*., 2001; Arzouni *et al*., 2002). The mechanism underlying leptospiral pathogenesis is not completely understood. However, our results suggest that some of the reported pathogenic genes are present in intermediate strains, which may cause human infection.

Some studies have used housekeeping genes for diagnosing leptospirosis (Ahmed *et al*., 2009; Bourhy *et al*., 2011). In the present study, we selected two housekeeping genes of *Leptospira*, namely, *secY* and *flaB*, and examined their presence in the reference *Leptospira* strains. Primers used for detecting the housekeeping genes were designed using conserved regions among the pathogenic genes. The housekeeping gene *secY* is located within S10-*spc*-α locus that encodes ribosomal proteins (Zuerner *et al*., 2000). This gene encodes a preprotein translocase in *Leptospira*. Alternating conserved and variable regions in this gene make it a suitable marker for differentiating between pathogenic and saprophytic *Leptospira* strains (Ahmed *et al*., 2009). Benacer *et al*. (2013) has showed the usefulness of *secY* in determining the pathogenicity of *Leptospira* strains present in environmental samples. However, the present study did not detect *secY* in the three pathogenic serovars Javanica, Tarassovi, and Celledoni of the reference *Leptospira* strains. Moreover, this gene was not detected in any of the environmental isolates. These results suggest that *secY* is not suitable for diagnosing leptospirosis because it was not detected in environmental isolates.

The other housekeeping gene, i.e., *flaB*, encodes one of the two major flagellar proteins, which are involved in flagella assembly and mortality (Picardeau *et al*., 2001). The genes of the periplasmic flagellum are conserved in both saprophytic and pathogenic leptospires and are considered to be core genes because the flagellum is essential for the motility of leptospires (Picardeau *et al*., 2008). Although *flaB* was detected in all the pathogenic environmental isolates, it was absent in the four pathogenic serovars Australis, Javanica, Tarassovi, and Celledoni of the reference *Leptospira* strains. These results suggest that *flaB* cannot be used for detecting pathogenic *Leptospira* strains.

CONCLUSION

In summary, we found that two pathogenic genes, namely, *lipL32* and *ligB*, are present in all the pathogenic *Leptospira* strains. Our results indicate that *lipL32* and *ligB* can be used as molecular markers for diagnosing

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leptospirosis. Our future study will determine serovars among the environmental *Leptospira* isolates and will assess the distribution of pathogenicity-determinant genes among these isolates.

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