

## TECHNICAL REPORT

### Isolation by culture and PCR identification of *LipL32* gene of pathogenic *Leptospira* spp. in wild rats of Kuala Lumpur

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#### Abstract

**Background:** A study was conducted to confirm the status of rats as the carrier of pathogenic leptospira in Kuala Lumpur, Malaysia. **Method:** A total of 140 urine samples were collected from trapped rats. These samples were cultured in EMJH enriched media and 18 of these samples (12.9%) were found to be positive when observed under x40 by dark field microscope. Genomic DNA was extracted from all the 18 native isolates for PCR. **Result:** All the 18 isolates generated the expected 786 base pair band when the set of primers known to amplify *LipL32* gene were utilized. These results showed that the primers were suitable to be used for the identification of pathogenic leptospira from the 18 rat samples. **Conclusion:** The sequencing of the PCR products and BLAST analysis performed on each representative isolates confirmed the pathogenic status of all these native isolates as the *LipL32* gene was detected in all the *Leptospira* isolates. This indicates that the rats are carriers of the pathogenic leptospira in the study area, and therefore are of public health importance.

**Keywords:** wild rats, urine isolates, pathogenic, leptospira, polymerase chain reaction, *LipL32* gene

#### INTRODUCTION

Leptospirosis, an infectious disease caused by the pathogenic strains of the spirochetes bacteria, *Leptospira* spp. is a zoonotic disease. The transmission can be direct or indirect from animals to humans, such as through contact with infective urine of animals through contamination of the environment by the pathogens.<sup>1</sup> Diagnosis for leptospirosis infection has to be confirmed by laboratory testing because the infection can present with different clinical symptoms. These symptoms of infection may resemble other diseases, such as dengue fever, bleeding disorders and jaundice caused by different viruses. However, as the testing service is not always available in many parts of the world, the prevalence of the disease is often under-reported.<sup>2</sup> In Malaysia, cases of leptospirosis infections are increasing. 12,325 cases of leptospirosis were reported from 2004 to 2012, with the highest number in 2012. 338 deaths were reported during this period.<sup>3</sup> The Ministry of Health Malaysia in

May 2015 reported 15 cases of leptospirosis and 1 death from secondary school students in Negeri Sembilan.<sup>4</sup> Rodents especially rats were found to be the common host for the *Leptospira* spp., as rats and mice tend to live in close proximity to human habitats for water and food supply. Mice infected with *Leptospira* spp. spread the disease at specific time intervals.<sup>5</sup> Rats were also capable of secreting high concentrations of *Leptospira* spp. (107 organisms/ml) for 9 months after being infected.<sup>6</sup>

The capital city of Malaysia, Kuala Lumpur has a large human population and abundance of food supply which attracts rats and makes it a suitable breeding habitat for them.<sup>7</sup> Kuala Lumpur City Hall estimated a population of about 6.8 million rats in Kuala Lumpur and this is 4 times the total human population of the city.<sup>8</sup>

*Leptospira* spp. is divided into 21 species and has been categorized into three major subgroups based on 16S rRNA sequences. The subgroups are pathogenic (group I), half pathogenic (group II) and saprophytic (group III). Pathogenicity of

*Leptospira* spp. is influenced by a protein from the outer surface of the membrane that plays a role in the interaction between the bacteria and the host tissue.<sup>9</sup> This outer surface protein has been shown to bind with various components of the extracellular matrix *in vitro*.<sup>10</sup>

Pathogenic *Leptospira* spp. has different profiles of protein and lipopolysaccharide on the outer surface of its membrane.<sup>11</sup> About 145 genes are known to produce this lipoprotein, including the extracellular and membrane proteins on the outer surface.<sup>12,13</sup> LipL32 is found abundantly on the outer surface of the membrane of *Leptospira* spp. Thus, it was considered suitable as a marker to determine pathogenic *Leptospira* spp.<sup>14</sup>

*LipL21* and *LipL32* genes were reported to be present in all pathogenic serovars. Efficacy of primers capable of amplifying the genes have been tested against serum and tissue samples collected from cattle, buffaloes and experimentally infected guinea pigs.<sup>14-16</sup> Primer sets of Lep1132F and Lep1132R are derived from major surface outer membrane surface proteins of *LipL32* gene. It can detect major outer membrane LipL32 specific to *Leptospira* spp. and has been able to distinguish the pathogenic from non-pathogenic forms.<sup>15</sup>

In this study we attempted to isolate *Leptospira* spp. from the urban wild rats by culture technique, and test them with LipL32 primer sets to detect for pathogenic *Leptospira* harvested from the culture. More specifically, the objectives of this study were to (1) isolate and identify the isolated strains of *Leptospira* from urban wild rats, (2) determine whether they were pathogenic or non-pathogenic by the PCR method utilizing LipL32 primer sets of Lep1132F and Lep1132R which targeted the *LipL32* gene, and (3) confirm the strain's identity by sequencing the PCR products.

## MATERIALS AND METHODS

### Sampling

#### *Selection of study area*

Two areas in Kuala Lumpur were chosen for the study and the selections were based on suitability of habitat for food, rat breeding and the possibility of spreading the disease. Datuk Keramat (03 ° 09'57.00 " N, 101 ° 43'52.00 " E) had an abundance of roadside stalls selling cooked food. Chow Kit (03 ° 09'53.75 " N, 101 ° 41'56.84 " E) had the largest fresh food wet market in Kuala Lumpur. The market provides a

variety of raw foods, including fruits, vegetables, seafood and meat to the public.

#### *Trapping of rats*

The trapping was carried out for 5 days using 100 metal cages measuring 18 cm x 12 cm x 28 cm which were readily available from most hardware shops, and baited with coconut, sweet potatoes or bread. Traps were placed in the evening and were collected early in the morning of the next day, with the assistance of Kuala Lumpur City Hall (DBKL) staff before these areas were crowded with people.

#### *Preparation of EMJH media*

About 2.3 g of EMJH *Leptospira* spp. Difco™ basic media was dissolved with 900 ml distilled water in a 1 L Scotch bottle at pH 7.5, autoclaved for 15 min, left at room temperature overnight and filtered through a 0.2 µm filter unit. About 100 ml EMJH *Leptospira* spp. Difco™ enriched media was added to the 900 ml basic media prepared earlier. Next 5 ml of the media was aliquoted into each of the 10 ml culture tubes and 5-fluorouracil (200 µg/ml) was also added to it. These culture tubes were then kept in 4°C until used.

#### *Sampling techniques*

In the laboratory, each of the trapped rats was transferred into a cloth bag and anaesthetised with chloroform in a glass jar for about 30 seconds. Urine samples were collected by direct puncture of the bladder of the rats and 2 drops of each sample placed in culture tubes containing 5 ml EMJH media and 5-fluorouracil (200 µg/ml) prepared earlier. Samples were then incubated for 8 weeks in the dark at 28°C-30°C.

### Identification of pathogenic *Leptospira*

#### *Microscopic examination*

During incubation, samples were observed using dark field microscopy on day 1 until day 3, followed by the first week until the eighth week for the presence of *Leptospira* spp.

#### *Isolation of Leptospira spp. by culturing*

On the 8<sup>th</sup> week, once the presence of *Leptospira* spp. was detected, sub-culturing was done by transferring 1.5 ml of the culture into a new 10 ml culture tube containing a 5 ml fresh media of EMJH to obtain a pure culture. Contaminated cultures were filtered with 0.45 µm syringe filter prior to sub culture.

*Genomic DNA extraction and PCR*

Cultures were centrifuged 3 times by centrifugation at 3000 rpm for 10 minutes each time and processed immediately for DNA extraction. The DNA was extracted by following Qiagen DNA Extraction Kit (USA) protocol. Extracted genomic DNA was used as template for PCR with modification on the primers to detect *LipL32* gene generating the 786 base pair product.<sup>15</sup> The PCR was performed with 2.5 units of the *Taq* DNA polymerase (Fermentas AB, Lithuania) in a reaction mixture (100 µl) containing dNTPs (200 µM) and 2.5 mM MgCl<sub>2</sub>, subjected to 40 cycles of 50 sec at 94°C (denaturation), 1 min at 62°C (annealing) and 1 min at 72°C (extension). The amplified PCR product was purified using High Pure PCR Product Purification Kit (Boehringer Mannheim, Germany).

*DNA sequencing*

DNA reaction cycle was done according to the protocol of ABI PRISM DYE terminator sequencing Ready Reaction kit with Ampli *Taq* DNA polymerase. Then, automated DNA sequencing was carried out using ABI PRISM 377 machine (Applied Biosystem Inc).

*BLAST analysis of DNA sequences*

DNA sequences were compared against the European Molecular Biology Laboratory (EMBL) and Gen Bank nucleotide databases

using the BLAST programme, from the 'National Centre for Biotechnology Information' (<http://www.ncbi.nlm.nih.gov>).

**RESULTS AND DISCUSSION**

Of 140 urine samples taken from trapped urban wild rats, 18 urine samples showed growth of *Leptospira* spp. (12.9%) when cultured in EMJH enriched media, and observations carried out under x40 dark field microscopy. Figure 1 shows the image of one of the native isolates of *Leptospira* spp. that was observed in the urine of an urban wild rat.

Figure 2a shows the successful optimization of the PCR technique prior to amplification of the 18 isolates from infected animals. In this figure, PCR utilizing primer sets of Lep1132F and Lep1132R which targeted the *LipL32* gene showed amplification of the pathogenic leptospires of *Leptospira interrogans* serovar Bataviae, *Leptospira interrogans* serovar Australis and *Leptospira interrogans* serovar Javanica (Lane 2, 5 and 6) by generating the 786 base pair product. This set of primers did not amplify the saprophytic leptospires of *Leptospira interrogans* serovar Patoc (Lane 3) and the parasite species of *Giardia duodenalis* (Lane 7) and *Toxoplasma gondii* (Lane 8).

Figures 2b and 2c show the PCR amplification results of the 18 isolates of the infected urban

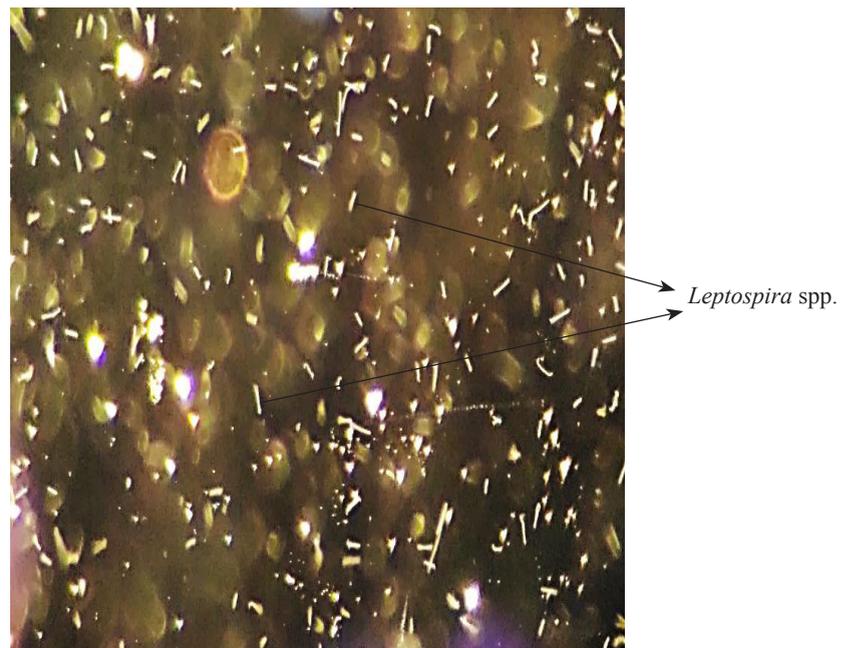


FIG. 1: Image of *Leptospira* spp. observed under x40 magnification using dark field microscope

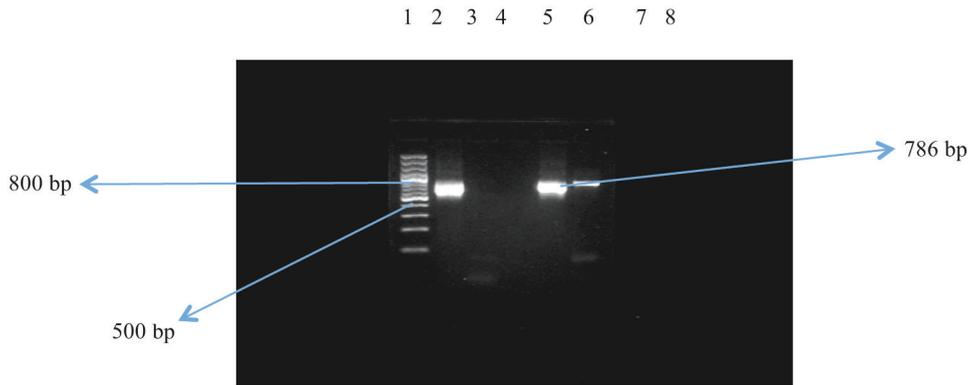


FIG. 2a: Ethidium bromide stained agarose gel electrophoresis of PCR amplified DNA using Primer Lep1132F and Lep1132R. Lane 1: 1kb ladder. Lane 2: *Leptospira interrogans* serovar Bataviae. Lane 3: *Leptospira biflexa* Patoc. Lane 4: Negative control. Lane 5: *Leptospira interrogans* serovar Australis. Lane 6: *Leptospira interrogans* serovar Javanica. Lane 7: *Giardia duodenalis*. Lane 8: *T. gondii*



FIG. 2b: Ethidium bromide stained agarose gel electrophoresis of PCR amplified DNA using primer Lep1132F and Lep1132R. Lane 1: 1 kb ladder. Lane 2-10: local isolates originated from urban wild rats S1, S2, S3, S4, S5, S6, S7, S8, and S9. Lane 11: *Leptospira biflexa* Patoc. Lane 12: *Leptospira interrogans* serovar Bataviae

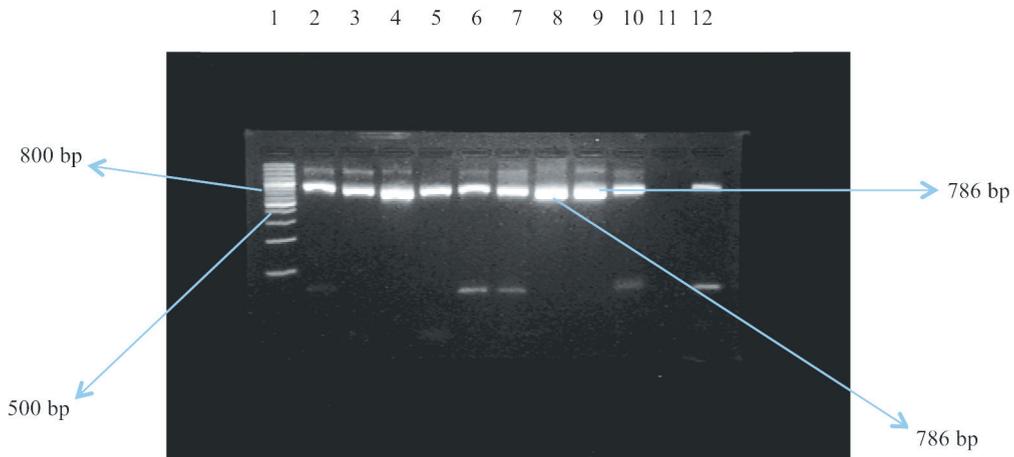


FIG. 2c: Ethidium bromide stained agarose gel electrophoresis of PCR amplified DNA using primer Lep1132F and Lep1132R. Lane 1: 1 kb ladder. Lane 2-10: local isolates originated from urban wild rats S10, S11, S12, S13, S14, S15, S16, S17, and S18. Lane 11: *Leptospira biflexa* Patoc. Lane 12: *Leptospira interrogans* serovar Bataviae

wild rats (S1 to S18). All of them (100%) showed the targeted band of 786 base pair PCR products in ethidium bromide stained agarose gel. In this study, saprophytic leptospire of *Leptospira biflexa* Patoc (Lane 11) was used as non-pathogenic negative control whereas *Leptospira interrogans* serovar Batavia was used as pathogenic positive control (Lane 12).

PCR products of the isolates of *Leptospira* originating from all infected urban wild rat were sequenced. The sequence of the 786 base pairs of the PCR product was compared against the European Molecular Biology Laboratory (EMBL) and Gen Bank nucleotide databases using the BLAST programme, from the 'National Centre for Biotechnology Information' (<http://www.ncbi.nlm.nih.gov>). The results of BLAST analysis of the sequences are shown in Table 1. The results showed that Open Reading Frame (ORF) of all the 18 isolates of *Leptospira* studied matched with the major outer membrane lipoprotein of *LipL32* gene.

### Conclusion

In this study, *Leptospira* spp. from 18 infected urine samples was successfully isolated from culture. *LipL32* gene was detected in all (100%) of these native isolates, suggesting that the sets of primers used were appropriate for the identification of the pathogenic strains in urban wild rat's populations. This study supports the use of PCR as a method for detection of infection. All 18 native isolates were confirmed to be pathogenic strain based on ORF of BLAST analysis of DNA sequence results.

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**TABLE 1: Nucleotide similarity using BLAST of various *Leptospira* isolates from urban rats**

Leptospira isolates	Identification	E value	Accession	Max-ident
S1	LipL32 ( <i>Leptospira borgpetersenii</i> )	$5 \times 10^{-157}$	AAS21763	97
S2	LipL32 ( <i>Leptospira weilli</i> )	$4 \times 10^{-160}$	AAS21800	99
S3	LipL32 ( <i>Leptospira interrogans</i> serovar Hardjo)	$2 \times 10^{-164}$	AFC76104	100
S4	LipL32 ( <i>Leptospira interrogans</i> serovar Hardjo)	$2 \times 10^{-157}$	AFC76104	100
S5	LipL32 ( <i>Leptospira interrogans</i> serovar Hardjo)	$3 \times 10^{-159}$	AFC76104	100
S6	LipL32 ( <i>Leptospira interrogans</i> serovar Carnicola)	$1 \times 10^{-161}$	AAY630.1	99
S7	LipL32 ( <i>Leptospira interrogans</i> serovar Carnicola)	$1 \times 10^{-163}$	AAY630.1	100
S8	LipL32 ( <i>Leptospira interrogans</i> serovar Carnicola)	$5 \times 10^{-161}$	AAY630.1	100
S9	LipL32 ( <i>Leptospira interrogans</i> serovar Carnicola)	$6 \times 10^{-146}$	AAY630.1	100
S10	LipL32 ( <i>Leptospira interrogans</i> serovar Carnicola)	$3 \times 10^{-144}$	AAY630.1	100
S11	LipL32 ( <i>Leptospira interrogans</i> serovar Carnicola)	$4 \times 10^{-144}$	AAY630.1	100
S12	LipL32 ( <i>Leptospira interrogans</i> serovar Carnicola)	$2 \times 10^{-142}$	AAY630.1	100
S13	LipL32 ( <i>Leptospira noguchii</i> )	$8 \times 10^{-125}$	AAS21788	99
S14	LipL32 ( <i>Leptospira interrogans</i> serovar Carnicola)	$5 \times 10^{-142}$	AAY630.1	100
S15	LipL32 ( <i>Leptospira interrogans</i> serovar Hardjo)	$7 \times 10^{-158}$	AFC76104	100
S16	LipL32 ( <i>Leptospira interrogans</i> serovar Hardjo)	$7 \times 10^{-141}$	AFC76104	100
S17	LipL32 ( <i>Leptospira interrogans</i> serovar Hardjo)	$1 \times 10^{-41}$	AFC76104	100
S18	LipL32 ( <i>Leptospira interrogans</i> serovar Carnicola)	$1 \times 10^{-142}$	AAY630.1	100

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