ORIGINAL ARTICLE

Detection of Pathogenic *Leptospira* in Rats and Phylogenetic Analysis Using Outer Membrane Lipoprotein *Lipl32* Gene at Two Major Public Markets

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ABSTRACT

Introduction: Limited studies have been documented on the presence of pathogenic *Leptospira* in public markets serving the community in sub-districts of Selangor. The aim of this study was to detect the presence of pathogenic *Leptospira* in rats using a gene encoding an outer membrane lipoprotein *LipL32*. **Methods:** Polymerase chain reaction (PCR) was performed using *LipL32* primers on sixty kidney samples of rats trapped at two locations of study; Pasar Borong Selangor in Seri Kembangan and Pasar Basah Bandar Baru Bangi in Bangi. **Results:** Out of 60 samples analysed, 36.7% were positive for the presence of *LipL32*. All positive samples highly matched (>94%) nucleotide sequence for *LipL32* of pathogenic *Leptospira* and related to the pathogens through phylogenetic analysis. **Conclusion:** The detection of *LipL32* indicates the potential presence of pathogenic *Leptospira* species at public markets. Although only 60 rats were successfully trapped, the rats are mobile and might further transmit the pathogenic organisms to other areas.

Keywords: *Leptospira*, Rats, *LipL32*, Polymerase chain reaction, Phylogenetic analysis

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INTRODUCTION

Leptospira species are spirochetes that are free-living saprophytes found in the environment, while some are pathogenic living in an animal host and cause zoonotic infection (1). Leptospires may be shed into the environment through the urine of infected host (2). Leptospirosis is an infectious disease caused by leptospires where it can be suspected in human based on certain clinical symptoms varying from flu-like illness such as fever and headache to severe complications including organ failure (3).

Leptospira interrogans and *Leptospira borgpetersenii* are the main infecting species causing leptospirosis (4). The ability of the bacteria to cause infection may be contributed by the virulence factors that represent adhesins of multifunctional biological activities. These virulence factors include lipopolysaccharides (LPS), hemolysins and outer membrane proteins (OMPs). More than 900 putative genes which are potentially implicative for virulence properties are unique to either *L. interrogans* or *L. borgpetersenii* (5) . From the identified 900 putative genes, a large number of the genes encode for proteins of unknown functions, consistent with the hypothesis that *Leptospira* species have no virulence factor homologues among protein with known functions found in other bacterial species. These suggest that *Leptospira* may possess virulence mechanism not present in other bacteria. Among these genes, *LipL32* encodes for the major outer membrane protein in these bacteria (5). Functional studies have shown that *LipL32* lipoprotein binds to several extracellular matrix proteins, including laminin, multiple collagens, and the plasma proteins suggesting a role in adhesion to host tissues (6, 7).

Leptospira species are diverse, that their prevalence in potential high risk areas need to be assessed for prophylactic measures. Various studies on *Leptospira* in Malaysia focused mainly on environment and humans following outbreak and cases in high-risk areas and population (8, 9). It is well known that human infection

can result from a complex interaction between pathogen, animal reservoirs, human and environment (10). From more than 37 serovars identified in Malaysia, half of them are carried by rats and are pathogenic (11, 12).

Studies on *Leptospira* in rat populations were commonly centred in the highly populated metropolitan city of Kuala Lumpur (12, 13) and other districts associated with the outbreak (14) . Only a few data are available on detection of *Leptospira* in the state of Selangor, which is the most industrialized and highly populated state in Malaysia, but limited studies are found for detection of leptospires from rats in residential areas and environmental samples from recreational areas (15, 16) . There are 1,832 of reported leptospirosis cases in Selangor for the year 2014 and this state ranked fourth in a number of leptospirosis outbreaks (17).

Thus, the aim of this study is to detect the presence of pathogenic *Leptospira* in rats caught in two major public markets located in sub-districts of Selangor using *LipL32* as the target gene of convenience. Detection of pathogenic *Leptospira* and phylogenetic analysis will provide data on its occurrence and the potential dominant circulating *Leptospira* species in the selected locations.

MATERIALS AND METHODS

Sampling collection

Pasar Borong Selangor (2°59′6″N 101°40′15″E) and Pasar Basah Bandar Baru Bangi (2.9445° N, 101.7692° E) which serve the community in the densely populated district of Seri Kembangan and Bangi respectively were chosen as the study sites. Seri Kembangan and Bangi are the two sub-districts in the district of Hulu Langat, Selangor. These public markets are located close to the vicinity of residential areas. Fruits, vegetables, meat and seafood are the major commodities in the market centre. In addition, surrounding the centre areas, there are hawker stalls selling cooked food. Clogged drains in near distance from hawker stalls and moist ground, due to excess garbage at the rubbish dumping site, may provide favourable environment for rats to breed and *Leptospira* to proliferate. Steel traps were placed at the market, supplied with baits such as sweet potatoes and dried fish. Trapping was conducted over a period of two days per session with a total of 22 sessions from June 2016 to November 2017. The captured rats were placed in non-transparent container to reduce stress and transported to the laboratory. The rats were then euthanized with carbon dioxide in a closed glass chamber (13). Euthanized rats were immediately killed and the kidney organ was collected for further analysis (Institutional Animal Care and Use Committee approval number; Universiti of Malaya-ISB/10/06/2016/NHMI(R).

DNA extraction

Prior to DNA extraction, the kidney was prepared following sample preparation steps of an extraction kit. A 25 mg of kidney specimens were cut up to into small pieces using a blade. The samples were then placed in a 1.5 ml microcentrifuge tube, 180 µl of tissue lysis buffer (ATL) and 20 µl proteinase K provided with the extraction kit (QIAGEN, Malaysia) were added respectively. The mixture was mixed by vortexing and incubated at 56°C until the tissue was completely lysed. Total DNA was extracted from the homogenized sample using DNeasy Blood and Tissue kit (QIAGEN, Malaysia) per manufacturer's recommended protocol. Extracted DNA was used as template in PCR.

DNA amplification for the detection of *LipL32* gene

DNA extracts were screened by PCR to detect *LipL32* using PCR primers as described previously (4); F: ATCTCCGTTGCACTCTTTGC, R: ACCATCATCATCATCGTCCA with the expected size of 474bp. Pathogenic *Leptospira* serovar Canicola was used as the positive control, and DNA was substituted with nuclease-free water of the same volume as the negative control. The 25 µL reaction mixtures consisted of 12.5 µL GoTaq Green Master Mix (2.5mM $MgCl_2$; 200 µM dNTPs; 0.2U/µL DNA polymerase) (Promega, USA), 5 µL sterile water, 1.25 µL of each reverse and forward primers and 5 µL of DNA was prepared. The amplification was conducted using the following PCR conditions; initial denaturation at 94°C for 5 minutes, 30 cycles of denaturation at 94°C for 30 seconds, annealing at 58°C for 45 seconds, extension at 72°C for 30 seconds and final extension at 72°C for 6 minutes. The products were checked for amplification by electrophoresis using 1.5% (Tris base, acetic acid and EDTA [TAE]) - agarose gel. The gel was visualized by gel documentation system with Quantity One software (Biorad, USA) and photographed. Products of positive results for PCR were subjected to DNA sequencing using commercial service (1st BASE Laboratories Sdn. Bhd., Selangor, Malaysia). The sequences were then analysed by sequence alignment tool in MEGA 7.1v software (http://www.megasoftware.net). BLAST analysis was conducted for homology search of the sequenced data (www.ncbi.nlm.nih.gov/BLASTN). Sequences were submitted to BankIt for accession number assignation.

Phylogenetic analysis

Sequenced PCR amplicon and gene sequences of *LipL32* from GenBank were used to assemble a phylogenetic tree. Phylogenetic trees with 1000 bootstrap replicates were constructed by the neighbour-joining method using the Maximum Composite Likelihood model on MEGA 7.1v software (http://www.megasoftware.net).

RESULTS

From 60 rat's kidney, 22 samples were found positive for *LipL32*. Based on locations, ten positive samples originated from rats trapped in Pasar Borong Selangor, while 12 samples were found in rats trapped in Pasar

Basah Bandar Baru Bangi. Representative PCR products of expected size 474 bp corresponded to *LipL32* amplification were displayed in 1.5% agarose gel as shown in Figure 1. The BLAST analysis (www.ncbi.nlm.nih.gov/BLASTN) showed that all sequenced amplicons demonstrated more than 90% identity with *LipL32* sequences of pathogenic species of *Leptospira* from GenBank.

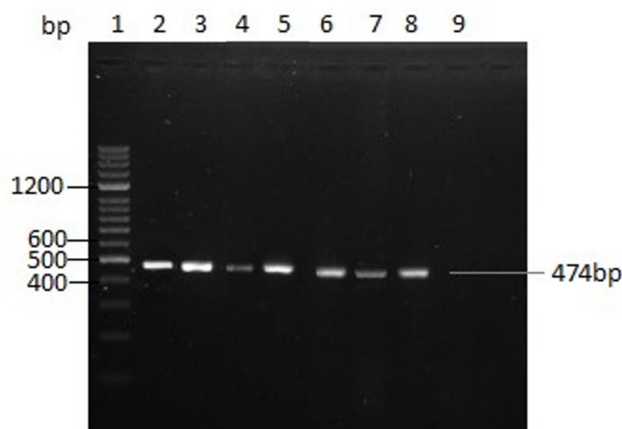

Figure 1: Representative gel of PCR for detection of lipl32. Lane 1: DNA marker (100 bp); Lane 2: Positive control; Lane 3-8: Samples; Lane 9: Negative control.

In the phylogenetic analysis, sequences of the detected *LipL32* represented by its accession number and selected *LipL32* sequences from GenBank which are of 96% to 100% similarity, were sorted into two major clusters (Figure 2). Cluster 1 was formed by *L. interrogans* of various serovars and *Leptospira noguchii* with 88% bootstrap value. Eight of the positive samples were included in Cluster 1, while 14 samples were grouped into Cluster 2 with *L. interrogans* and *L. borgpetersenii* at 96% bootstrap value.

DISCUSSION

LipL32 is a subsurface lipoprotein of *Leptospira* and reported as the most abundant protein with estimated 38,000 copies per cell (18) . The *LipL32* present in *Leptospira* species encodes for *LipL32* protein of 272 amino acids with 819 bp (19) . This protein was demonstrated to be expressed in vivo and a target of the humoral immune response (20, 21) . Presence of *LipL32* encoding *LipL32* protein is highly conserved in *Leptospira* pathogenic species but absent in saprophytic species. This enables the differentiation of pathogenic *Leptospira* in samples from the saprophytic group (21).
Human contracts leptospirosis through incidental contact with *Leptospira* contaminated environment. It is found that the transmission of leptospirosis is centred to the renal carrier excreting the pathogens into the environment and common in warm, moist climate, poor sanitation and poor rodent control leading to an environment favourable for *Leptospira* proliferation

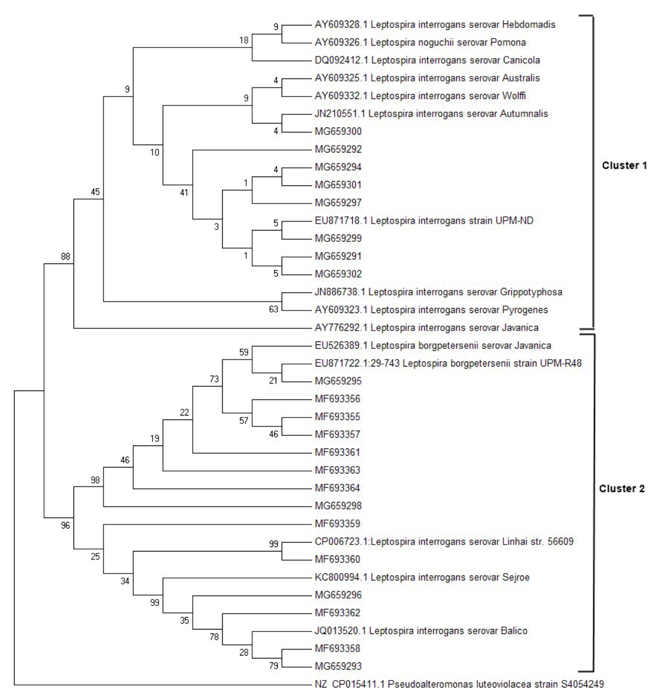

Figure 2: Phylogenetic tree of lipl32 constructed using MEGA 7.1v with 1000 bootstrap replicates. The analysis involved 22 nucleotide sequences of samples (MF693355 to MG659293) and 16 lipl32 sequences from GenBank. NZ_CP015411.1 serves as the control for outgroup. The lipl32 sequences were segregated into two major clusters against other lipl32 sequences from GenBank. Cluster 1 consisted of 8 of the positive samples grouped with *L. interrogans* and *L. noguchii*. Cluster 2 consisted of *L. interrogans, L. borgpetersenii* with 14 positive samples.

(22). Acute kidney injury is the main clinical symptoms of leptospirosis in human (23).

In this study, the overall distribution of *LipL32* found in the kidney of the rats was 37% (22/60). This may suggest that 37% of the rats captured were potentially infected with pathogenic species of *Leptospira* in the studied areas. High bootstrap values of more than 80% separating sub-clades in Cluster 1 and sub-clades in Cluster 2 suggest homogenous orthologs of *LipL32* among species in the clusters. This suggests the suitability of *LipL32* as a convenient detection in rats of various site origins. In Cluster 1, MG659300 and MG659299 were clustered into monophyletic groups with individual strains of *L. interrogans* serovar Autumnalis and *L. interrogans* strain UPM-HD respectively, suggesting these individual strains are the close phylogenetic identity of the sample. There were *L. interrogans* strains clustered together with *L. borgpetersenii* strains in Cluster 2 with high bootstrap value of 96% instead of being grouped into Cluster 1, contrasting to other studies which clustered *L. interrogans* with L. noguchii and L. kirschneri only (24, 25) . Studies found that *LipL32* sequence of *L. interrogans* strain is highly similar to that of *L. borgpetersenii* (26). Although contrasting to phylogeny obtained in other studies, *L. borgpetersenii* strains still formed different sub-clade from the *L. interrogans* strains

within Cluster 2. MG659295, MF693356, MF693355, MF693357, MF693361, MF693363, MF693364 and MG659298 were grouped into a sub-clade with *L. borgpetersenii* having a high bootstrap value of 98%, suggesting a close relatedness of *LipL32* of the samples to this pathogenic species. A few *L. interrogans* strains were grouped together into sub-clades with MF693359, MF693360, MG659296, MF693362, MF693358 and MG659293, whereby MF693360 was separated into a monophyletic group with *L. interrogans* serovar Linhai with 99% bootstrap values. Based on BLASTN results and phylogenetic analysis, L. interogans is the dominant *Leptospira* species circulating in two public markets involved in this study, while two samples only shared 99% similarity to *LipL32* sequences of *L. borgpetersenii*. In previous studies, *L. borgpetersenii* serovar Javanica and *L. interrogans* serovar Bataviae were found as the dominant serovars in urban rats' population in Kuala Lumpur, while *L. noguchii* was the dominant *Leptospira* in urban areas of Sarawak (12, 27).

Although specific serovars of the dominant *Leptospira* species cannot be determined, the presence of *LipL32* closely related to *L. interrogans* and *L. borgpetersenii* may infer risk of rats carrying the pathogen circulating in the studied areas. Specific serovars of the *Leptospira* species could only be determined through microscopic agglutination test (MAT) using blood sera of rats and verified through PCR amplification using DNA from a pure culture. Based on our results, the collected 60 samples may not fully represent a probable large population of rats in the two sub-districts of Selangor; however, the presence of pathogenic *Leptospira* in public markets located in the vicinity of populated residential areas might raise the concern of health risks (27). Risks of contracting leptospirosis are greater especially where the density of reservoir population is high that could increase chances of humans contracting the infection (3). As the rats are mobile, they may further transmit the bacteria around.

CONCLUSION

In summary, this study provides an important insight that rats are the potential carriers of pathogenic *Leptospira* circulating in public markets located in the vicinity of residential areas and therefore are public health importance. Meanwhile, multi-gene detection is highly recommended for identifying serovar, species and pathogenic potential of the bacteria for a complete surveillance coverage. Nevertheless, limited resources and facilities may not suit the fastidious nature of *Leptospira* to be grown in common laboratories. A single *LipL32* direct PCR detection alone may provide some hope for surveying the pathogenic strains due to the *LipL32* gene homogeneity and PCR sensitivity. This may facilitate the detection of leptospirosis from rats using *LipL32* gene as the molecular marker of infection.

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REFERENCES

1. Picardeau M. Virulence of the zoonotic agent of leptospirosis: still terra incognita? Nat Rev Microbiol. 2017;15(5):297-307.
2. Barragan V, Nieto N, Keim P, Pearson T. Meta-analysis to estimate the load of *Leptospira* excreted in urine: beyond rats as important sources of transmission in low-income rural communities. BMC Res Notes. 2017;10(1):71.
3. Haake DA, Levett PN. Leptospirosis in humans. Curr Top Microbiol Immunol. 2015;387:65-97.
4. Ahmed N, Devi SM, Valverde Mde L, Vijayachari P, Machang'u RS, Ellis WA, et al. Multilocus sequence typing method for identification and genotypic classification of pathogenic *Leptospira* species. Ann Clin Microbiol Antimicrob. 2006;5:28.
5. Adler B, Lo M, Seemann T, Murray GL. Pathogenesis of leptospirosis: the influence of genomics. Vet Microbiol. 2011;153(1-2):73-81.
6. Hoke DE, Egan S, Cullen PA, Adler B. *LipL32* is an extracellular matrix-interacting protein of *Leptospira spp*. and *Pseudoalteromonas tunicata*. Infect Immun. 2008;76(5):2063-9.
7. Vieira ML, Atzingen MV, Oliveira TR, Oliveira R, Andrade DM, Vasconcellos SA, et al. In vitro identification of novel plasminogen-binding receptors of the pathogen *Leptospira interrogans*. PLoS One. 2010;5(6):e11259.
8. Ridzlan FR, Bahaman AR, Khairani-Bejo S, Mutalib AR. Detection of pathogenic *Leptospira* from selected environment in Kelantan and Terengganu, Malaysia. Trop Biomed. 2010;27(3):632-8.
9. Samsudin S, Masri SN, Jamaluddin TZMT, Siti Nor Sakinah Saudi SN, Ariffin UMK, Amran F, et al. Seroprevalence of *Leptospira*l antibodies among healthy municipal service workers in Selangor. Hindawi Publishing Corporation. 2015;2015:6.
10. Faulde M, Freise J. [Public health pests. Arthropods and rodents as causative disease agents as well as reservoirs and vectors of pathogens]. Bundesgesundheitsblatt Gesundheitsforschung Gesundheitsschutz. 2014;57(5):495-503.
11. Bahaman AR, Ibrahim AL. A review of leptospirosis in Malaysia. Vet Res Commun. 1988;12(2-3):179-89.
12. Benacer D, Mohd Zain SN, Amran F, Galloway RL, Thong KL. Isolation and molecular characterization of *Leptospira interrogans* and *Leptospira borgpetersenii* isolates from the urban rat populations of Kuala Lumpur, Malaysia. Am J Trop Med Hyg. 2013;88(4):704-9.

13. Hasoun GA, Bahaman AR, Bejo SK, Zakaria Z, Garba B. Serological and molecular prevalence of *Leptospira* infection in rat populations in Kuala Lumpur. Aust J Basic & Appl Sci. 2017;11(1):62-72.
14. Mohamed-Hassan SN, Bahaman AR, Mutalib SKB. Prevalence of pathogenic leptospires in rats from selected locations in Peninsular Malaysia. Research Journal of Animal Sciences. 2012;6(1):12-25.
15. Dzulaikha K NYM, Maizatulriah JJ, Marfiah AW editor Association of rainfall and the occurrence of pathogenic *Leptospira* spp. in recreational stream water, Hulu Langat, Selangor. 2nd International Conference for Innovation in Biomedical Engineering and Life Sciences: ICIBEL 2017 (in conjunction with APCMBE 2017),10 - 13 December 2017; 2018; Penang Malaysia: Springer Singapore.
16. Khairani-Bejo S, Oii SS, Bahaman AR. Rats: Leptospirosis reservoir in Serdang Selangor residential area. Journal of Animal and Veterinary Advances. 2004;3(2):66-9.
17. Garba B, Bahaman AR, Khairani-Bejo S, Zakaria Z, Mutalib AR. Retrospective Study of Leptospirosis in Malaysia. Ecohealth. 2017;14(2):389-98.
18. Malmstrom J, Beck M, Schmidt A, Lange V, Deutsch EW, Aebersold R. Proteome-wide cellular protein concentrations of the human pathogen *Leptospira interrogans*. Nature. 2009;460(7256):762-5.
19. Kumaran SK, Bakar MFA, Mohd-Padil H, Mat-Sharani S, Sakinah S, Poorani K, et al. 3D modelling of the pathogenic *Leptospira* protein *LipL32*: A bioinformatics approach. Acta Trop. 2017;176:433-9.
20. Lin X, Zhao J, Qian J, Mao Y, Pan J, Li L, et al. Identification of immunodominant B- and T-cell combined epitopes in outer membrane lipoproteins *LipL32* and LipL21 of *Leptospira interrogans*. Clin Vaccine Immunol. 2010;17(5):778-83.
21. Murray GL. The lipoprotein *LipL32*, an enigma of *Leptospira*l biology. Vet Microbiol. 2013;162(2-4):305-14.
22. Ellis WA. Animal leptospirosis. Curr Top Microbiol Immunol. 2015;387:99-137.
23. Kamath N, Iyengar A. Infections and the kidney: a tale from the tropics. Pediatr Nephrol. 2018;33(8):1317-26.
24. Haake DA, Suchard MA, Kelley MM, Dundoo M, Alt DP, Zuerner RL. Molecular evolution and mosaicism of *Leptospira*l outer membrane proteins involves horizontal DNA transfer. J Bacteriol. 2004;186(9):2818-28.
25. Foronda P, Martin-Alonso A, Del Castillo-Figueruelo B, Feliu C, Gil H, Valladares B. Pathogenic *Leptospira* spp. in wild rodents, Canary Islands, Spain. Emerg Infect Dis. 2011;17(9):1781-2.
26. Pui CF, Bilung LM, Apun K, Su'ut L. Diversity of *Leptospira* spp. in Rats and Environment from Urban Areas of Sarawak, Malaysia. J Trop Med. 2017;2017:3760674.
27. Mohamad-Ikbal NH, Bhassu S, Simarani K, Uni S, Chan CC, Omar H. Prevalence and genetic divergence of *Leptospira interrogans* and *Leptospira borgpetersenii* in house rats (Rattus rattus) from Peninsular Malaysia. Asian Pac J Trop Med. 2019;12(10):463-71.