UNIVERSITI PUTRA MALAYSIA

DETECTION OF Leptospira SPECIES TARGETING 16S rRNA, rpoB, AND lipL32 GENE FROM CLINICAL AND ENVIRONMENTAL SOURCES

NURUL FARHANA BINTI ZULKIFLI

FPSK(m) 2019 38

DETECTION OF *Leptospira* SPECIES TARGETING 16S rRNA, *rpoB*, AND *lipL32* GENE FROM CLINICAL AND ENVIRONMENTAL SOURCES

By

NURUL FARHANA BINTI ZULKIFLI

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Master of Science

January 2019

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the Master of Science

DETECTION OF *Leptospira* SPECIES TARGETING 16S rRNA, *rpoB,* AND *lipL32* GENE FROM CLINICAL AND ENVIRONMENTAL SOURCES

By

NURUL FARHANA BINTI ZULKIFLI

January 2019

Chair : Mohd Nasir Bin Mohd Desa, PhD
Faculty : Medicine and Health Sciences

Leptospirosis is currently emerging globally as reflected by the increase in the number of *Leptospira* infections. The scope of research on identification of individual *Leptospira* species through a rapid assay in local setting is still lacking. This study attempts to determine the *Leptospira* species distribution in clinical and environmental samples using molecular approach. The blood for clinical samples (n=64) were collected from patients whom have been suspected with leptospirosis, and the environmental samples (n=105) consisting of soils and waters were collected from areas with potential sources of *Leptospira* transmission. All samples were directly extracted for DNA and subjected to PCR using three sets of established primers; 16S rRNA, *rpoB*, and *lipL32.* Positive samples were further analysed on their DNA sequences and also through phylogenetic analysis. Six clinical samples were amplified for 16S rRNA gene and four clinical samples showed an amplification of *rpoB* gene that was highly matched to pathogenic *Leptospira* spp. in GenBank. Out of 13 positive results amplified for 16S rRNA from environmental samples, only five were detected to have been contaminated by *Leptospira* spp. while the others showed a higher number of contamination by different species of bacteria. The BLAST similarity results for clinical samples targeting *lipL32* showed that only one out of 64 samples was highly matched to pathogenic *Leptospira* spp. in GenBank. The phylogenetic trees constructed shows that the positive clinical samples were clustered into group with pathogenic *Leptospira* spp. while the environmental samples showed a clustering into uncultured *Leptospira*. Overall, this study showed the ability of the molecular approach in combination with PCR in determine *Leptospira* species directly on both clinical and environmental samples. Although the number of positive samples were low, but the tendency of the species to appear in limited positive samples may infer the species as the likely agents in causing leptospirosis at this study setting.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia
sebagai mematuhi keperluan untuk Ijazah Master Sains

PENGECAMAN SPESIS *Leptospira* MENYASARKAN GEN 16S rRNA, *rpoB,* DAN *lipL32* DARIPADA SUMBER KLINIK DAN ALAM SEKITAR

Oleh

NURUL FARHANA BINTI ZULKIFLI

Januari 2019

Pengerusi : Mohd Nasir Bin Mohd Desa, PhD
Fakulti : Perubatan dan Sains Kesihatan

Leptospirosis kini semakin merebak secara global seiring dengan peningkatan bilangan jangkitan *Leptospira*. Skop penyelidikan mengenai pengenalpastian spesies *Leptospira* secara individu melalui pengecaman segera di kawasan setempat masih kurang. Kajian ini cuba menentukan taburan spesies *Leptospira* dalam sampel klinikal dan alam sekitar menggunakan pendekatan molekul. Darah untuk sampel klinikal (n=64) dikumpulkan dari pesakit yang telah disyaki menghidap leptospirosis dan sampel alam sekitar (n=105) terdiri daripada tanah dan air dikumpulkan dari kawasan dengan sumber yang berpotensi mngandungi *Leptospira*. Semua sampel telah diekstrak secara langsung untuk mendapatkan DNA dan diguna pakai dalam PCR dengan menggunakan tiga set primer; 16S rRNA, *rpoB*, dan *lipL32*. Sampel yang positif kemudiannya dianalisis berdasarkan kepada penjujukan DNA mereka dan juga melalui analisis filogenetik. Enam sampel klinikal yang dapat mengamplifikasi gen 16S rRNA dan empat sampel klinikal yang menunjukkan pengamplifikasian gen *rpoB* menujukkan padanan yang hampir sama dengan *Leptospira* spp. patogenik dalam GenBank. Daripada 13 keputusan positif dari sampel alam sekitar yang telah mengamplifikasi gen 16S rRNA, hanya lima sampel yang dikesan telah tercemar oleh *Leptospira* spp. manakala yang lain menunjukkan pencemaran yang lebih tinggi oleh spesies bakteria yang berlainan jenis. Keputusan keserupaan BLAST untuk sampel klinikal yang menyasarkan gen *lipL32* menunjukkan hanya satu daripada 64 sampel mempunyai padanan yang paling hampir dengan *Leptospira* spp. patogenik dalam GenBank. Pokok filogenetik yang dibina menunjukkan sampel klinikal yang positif terkumpul ke dalam kumpulan *Leptospira* spp. patogenik manakala sampel alam sekitar menunjukkan pengelompokan ke dalam *Leptospira* yang tidak dikultur. Secara keseluruhan, kajian ini menunjukkan keupayaan pendekatan molekul dalam kombinasi dengan PCR untuk menentukan spesies *Leptospira* pada kedua-dua sampel klinikal dan alam sekitar secara langsung. Walaupun jumlah sampel yang positif adalah rendah, namun kecenderungan spesies itu muncul dalam sampel positif yang terhad ini mungkin menyimpulkan spesies itu sebagai agen yang menyebabkan leptospirosis dalam kajian dikawasan ini.

ACKNOWLEDGEMENTS

First and foremost I would like to express my sincere gratitude to my supervisor, Assoc. Prof Dr. Mohd Nasir Mohd Desa for the continuous support throughout my project and research with his knowledge, motivation and patience that allow me to finally completed my report for this research. My deepest appreciation and gratitude to my supervisor committee, Dr. Chee Hui Yee and Assoc. Prof Dr. Vasantha Kumari Neela as a co-supervisor from Department of Medical Microbiology and Parasitology for their advice, encouragement, and guidance throughout this study.

Besides that, I owe a great thanks to En. Sabri, my fellow lab mates in Applied Microbiology Lab, post-graduate students in Virology Lab, all staff of the Department of Biomedical Science and also members of MyLepto team for all their generosity to assist me for some parts of my project, offered much advice throughout my project, provide and shared some materials which is needed for my research, and also shared their experience in handling equipment and other apparatus in the laboratory. Their helpfulness has lead me to complete my research.

I finally would like to express my warmest appreciation and thanks to my family especially my beloved parents for their endless support, my sisters and brother for their eternal love, all my friends who always be by my side and provide moral support, and lastly to everyone that had contributed directly or indirectly in the completion of this research. Thank you very much.

This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the Master of Science. The members of the Supervisory Committee were as follows:

Mohd Nasir Bin Mohd Desa, PhD
Associate Professor
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Chairman)

Chee Hui Yee, PhD
Senior Lecturer
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Member)

Vasantha Kumari Neela, PhD
Associate Professor
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Member)

_____
ROBIAH BINTI YUNUS, PhD
Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

Date:

Declaration by graduate student

I hereby confirm that:

- this thesis is my original work;
- quotations, illustrations and citations have been duly referenced;
- this thesis has not been submitted previously or concurrently for any other degree at any other institutions;
- intellectual property from the thesis and copyright of thesis are fully-owned by Universiti Putra Malaysia, as according to the Universiti Putra Malaysia (Research) Rules 2012;
- written permission must be obtained from supervisor and the office of Deputy Vice-Chancellor (Research and Innovation) before thesis is published (in the form of written, printed or in electronic form) including books, journals, modules, proceedings, popular writings, seminar papers, manuscripts, posters, reports, lecture notes, learning modules or any other materials as stated in the Universiti Putra Malaysia (Research) Rules 2012;
- there is no plagiarism or data falsification/fabrication in the thesis, and scholarly integrity is upheld as according to the Universiti Putra Malaysia (Graduate Studies) Rules 2003 (Revision 2012-2013) and the Universiti Putra Malaysia (Research) Rules 2012. The thesis has undergone plagiarism detection software.

Signature: _____ Date: _____

Name and Matric No.: __Nurul Farhana binti Zulkifli, GS44515_____

Declaration by Members of Supervisory Committee

This is to confirm that:

- the research conducted and the writing of this thesis was under our supervision;
- supervision responsibilities as stated in the Universiti Putra Malaysia (Graduate Studies) Rules 2003 (Revision 2012-2013) are adhered to.

Signature: _____
Name of Chairman of Supervisory Committee: Associate Professor Dr. Mohd Nasir Bin Mohd Desa

Signature: _____
Name of Member of Supervisory Committee: Associate Professor Dr. Vasantha Kumari Neela

Signature: _____
Name of Member of Supervisory Committee: Dr. Chee Hui Yee

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

BLAST	Basic local alignment search tool
CSF	Cerebrospinal fluid
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EMJH	Ellinghausen, McCullough, Johnson, and Harris
IgG	Immunoglobulin G
IgM	Immunoglobulin M
LPS	Lipopolysaccharides
MAT	Microscopic agglutination test
OMP	Outer membrane protein
PCR	Polymerase chain reaction

CHAPTER 1

INTRODUCTION

1.1 Background

Leptospira is thin helicoidal bacteria, motile, and comprises of hooked ends which also assemble a distinctive question-mark shape when viewed under dark field microscope. The infection by this bacterium strain may cause a disease known as leptospirosis and the most severe disease known as Weil's disease that can take place both in human and animals. *Leptospira* has characteristics of gram negative bacteria due to the presence of lipopolysaccharides (LPS) and the double membrane structure. The expression of surface-exposed LPS structure form the source for characterization of *Leptospira* into 24 serogroups and about 250 serovars (Palaniappan et al., 2007).

Currently, *Leptospira* is clustered in three main subgroups which are pathogenic, intermediate and saprophytic or non-pathogenic species (Goarant, 2016). Saprophytic species are normally inhabitants and more abundant in the environment especially in water and soil (Vital-Brazil et al., 2010). *Leptospira* from this subgroup do not infect human to cause leptospirosis. The pathogenic species require a host to live and reproduce. Rodents such as rats serve as the main carriers of pathogenic *Leptospira* and these infected hosts may spread the bacteria through their urine. Water and soil that have been contaminated by *Leptospira* containing urine may expose human to the organisms. Indirect or direct contact with contaminated soil or water through cuts and wounds, also consumption of contaminated water and foods can cause the bacteria to invade the human body leading to infection and leptospirosis.

Leptospirosis is re-emerging zoonotic disease which is deadly and can be transmitted from infected animal to human. A reviewed by Garba et al., (2017) stated that the number of leptospirosis cases and deaths in Malaysia has gradually increased from year 2004 to 2014, with number of cases from 263 to 7806. However, the case showed a decrease of number in 2016 with 5284 cases ("PressReader.com - Connecting People Through News," n.d.). In year 2014, the number of deaths due to leptospirosis was the highest with 92 deaths (Garba et al., 2017). Most cases were related to tropical weather factors such as flooding that frequently occurred in Malaysia, and also the occupational exposure among those higher risk worker such as farm workers, miners, sewage workers, field agricultural workers, soldiers, veterinarians, and workers in fishing industry that come into contact with animals (Vke Mbbs, 2011). Population that work or involve in recreational activities is also at risk of exposure to water and soil contamination with the urine of reservoir animal without realizing the threat (Lau et al., 2010; Lim et al., 2011). The unhygienic environment that become the

breeding sites of the rodents cause the disease to spread rapidly. Food stores site, housing area, or markets area that reside the leftover food or rubbish are the example of places that may attract rats which later will contaminate the environment through their urine and can lead to the infection.

Leptospirosis is frequently misdiagnosed as a result of its non-specific presentation such as headache, nausea, vomiting that mostly resemble a regular symptoms of fever or other febrile diseases such as dengue. Thus, leptospirosis often difficult to be recognized unless it has reached the severe state known as Weil's disease. Bharti et al., (2003) stated that, specific recognition or diagnosis and classification of *Leptospira* is crucial to allow epidemiological research as well as for the early treatment, prevention and control of the disease. Some of the methods currently used for diagnosis *Leptospira* involves serology, culture and molecular technique. Microscopic agglutination test (MAT) is the most frequent serologic method used in diagnosing human leptospirosis. Agglutinating antibodies in serum can be detected through MAT which provides result with high sensitivity and specificity, and requires expertise to interpret the result. However, this method is time consuming and laborious since a panel of live *Leptospires* from different serovars need to be maintained and the antibodies can be detectable at most by the second week of after symptoms (P N Levett, 2001). The presence of *Leptospira* can also be tested by culturing technique. Ellinghausen, McCullough, Johnson, and Harris (EMJH) medium or modified Korthof medium are commonly used for culturing *Leptospira* and the apparent growth is viewed under dark-field microscopy (Sayyed Mousavi M. N. et al., 2017). However, culture of *Leptospira* is difficult and also time consuming as this technique requires a special media and the culture need to be maintained for up to several weeks of incubation with common problem such as contamination. Microscopic agglutination test (MAT) and culture technique are definitely not useful for an early diagnosis of *Leptospira*, thus the need of fast and specific identification of infectious agent has led to development of new molecular test. Polymerase chain reaction (PCR) has been developed as a major technique for the detection and identification of specific microbial gene. PCR diagnosis improves the accuracy of epidemiological evaluation of leptospirosis, allowing a better evaluation of the real incidence of the disease, PCR-based strategies for *Leptospira* detection help to improve the diagnosis of leptospirosis through their advantages of speed, sensitivity, and specificity.

The diversity of *Leptospira* genus comprised of hundreds of serovar and genetic types requires specific identification and classification. 16S rRNA primer derived from leptospiral ribosomal 16S RNA gene has been widely used to identify the fastidious organism including *Leptospira* species (Ahmed et al., 2012; Aviat et al., 2009; Ganoza et al., 2006; Merien et al., 1992). An analysis of RNA polymerase beta-subunit gene (*rpoB*) that possess the same key attributes as 16S rRNA gene has also been used as an alternative in species identification as well as initial screening and identification of *Leptospira* in environment and clinical samples (Scola et al., 2006). *RpoB* gene encodes for protein beta-subunit of RNA polymerase enzyme is crucial in DNA synthesis. Several researchers have marked that *rpoB* gene has greater discriminatory power and high resolution in identifying bacteria such as *Leptospira* spp., *Treponema* sp.,

Borellia sp., and *Corynebacterium* sp. (Scola et al., 2006; Renesto et al., 2000; Khamis, Raoult, & La Scola, 2004). *Leptospira* has been suggested to possess a unique virulence trait that is not present in other non-infectious *Leptospira* with the absence of virulence factor homologues among the protein of known function (Ghazaei, 2018). The major outer membrane lipoprotein, *lipL32* has been well known as the most abundant protein and a study showed that the gene was conserved in all pathogenic *Leptospira* (Haake et al., 2000; Krishna et al., 2013). The unique virulence factor of *Leptospira* has allowed a screening and identification of virulence potential among Leptospira species.

It is important to understand the distribution pattern of the species at local settings but data on the identification of individual *Leptospira* species of local strains from widely diverse area in Malaysia are still lacking. Rapid and simple assays for the identification of individual *Leptospira* species are currently not well established especially in Malaysia due to the fastidious and sensitive nature of the organisms which may not allowed for a wide surveillance study to be conducted on the organisms. Therefore the molecular approach was used in this study to identify *Leptospira* spp. from clinical and environmental sources obtained from two different hospitals and environmental samples from high risk area around Selangor state. All samples were directly extracted for DNA and used for PCR amplification by using published specific primes, omitting the culture steps and expensive serology approaches. A detection and nucleotide sequence analysis targeting 16S rRNA, *rpoB*, and *lipL32* genes used in this study may lead to the development of a genetic based-predictive tool for effective detection of *Leptospira* spp. and its virulence potential. This study further analysed the distribution of species, virulence-related genes, and phylogenetic relationship among local clinical and environmental sources. It is hypothesized that there is a significant association between the gene from the local strains and their distribution in providing a better understanding on the *Leptospira* population in Malaysia through molecular approach.

1.2 Objectives

1.2.1 General Objective

To determine the *Leptospira* spp. distribution in clinical and environmental samples using molecular method.

1.2.2 Specific Objectives

- To detect an availability of *Leptospira* DNA in clinical and environmental samples targeting 16S rRNA and *rpoB* genes using PCR method.
- To detect the presence of virulence genes targeting *lipL32* among the clinical and environmental samples.
- To determine the genetic relationship among the identified *Leptospira* spp. using phylogenetic tree.

REFERENCES

Adekambi, T., Drancourt, M., & Raoult, D. (2008). The rpoB gene as a tool for clinical microbiologist. *Trends in Microbiology*, *17*(1), 37–45.

Adler, B., & de la Peña Moctezuma, A. (2010). Leptospira and leptospirosis. *Veterinary Microbiology*, *140*(3), 287–296. https://doi.org/https://doi.org/10.1016/j.vetmic.2009.03.012

Ahmad, S. N., Shah, S., & Ahmad, F. M. (2005). Laboratory diagnosis of leptospirosis. *Journal of Postgraduate Medicine*, *51*(3), 195–200. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/16333192

Ahmed, N., Manjulata Devi, S., de los Á Valverde, M., Vijayachari, P., Machang'u, R. S., Ellis, W. A., & Hartskeerl, R. A. (2006). Multilocus sequence typing method for identification and genotypic classification of pathogenic Leptospira species. *Annals of Clinical Microbiology and Antimicrobials*, *5*. https://doi.org/10.1186/1476-0711-5-28

Ahmed, S. A., Sandai, D. A., Musa, S., Hock, H. C., Riadzi, M., Leong, L. K., & Hock, T. T. (2012). Rapid Diagnosis of Leptospirosis by Multiplex PCR. *Malaysian Journal of Medical Sciences*, *19*(3), 9–16.

Alexander, a D., Evans, L. B., Baker, M. F., Baker, H. J., Ellison, D., & Marriapan, M. (1975). Pathogenic leptospiras isolated from Malaysian surface waters. *Applied Microbiology*, *29*(1), 30–33.

Aviat, F., Blanchard, B., Michel, V., Blanchet, B., Branger, C., Hars, J., Mansotte, F., Brasme, L., De Champs, C., Bolut, P., Mondot, P., Faliu, J., Rochereau, S., Kodjo, A., & Andre-Fontaine, G. (2009). Leptospira exposure in the human environment in France: A survey in feral rodents and in fresh water. *Comparative Immunology, Microbiology and Infectious Diseases*, *32*(6), 463–476. https://doi.org/10.1016/j.cimid.2008.05.004

Azali, M. A., Yean Yean, C., Harun, A., Aminuddin Baki, N. N., & Ismail, N. (2016). Molecular Characterization of *Leptospira* spp. in Environmental Samples from North-Eastern Malaysia Revealed a Pathogenic Strain, *Leptospira alstonii*. *Journal of Tropical Medicine*, *2016*, 1–7. https://doi.org/10.1155/2016/2060241

Bahaman, A. R., & Ibrahim, A. L. (1988). A review of leptospirosis in Malaysia. *Veterinary Research Communications*. https://doi.org/10.1007/BF00362799

Bal, A. E., Gravekamp, C., Hartskeerl, R. A., De Meza-Brewster, J., Korver, H., & Terpstra, W. J. (1994). Detection of Leptospires in Urine by PCR for Early Diagnosis of Leptospirosis. *JOURNAL OF CLINICAL MICROBIOLOGY*, *32*(8), 1894–1898. Retrieved from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC263898/pdf/jcm00008-0072.pdf

Balamurugan, V., Gangadhar, N. L., Mohandoss, N., Thirumalesh, S. R. A., Dhar, M., Shome, R., Krishnamoorthy, P., Prabhudas, K., & Rahman, H.

(2013a). Characterization of leptospira isolates from animals and humans: phylogenetic analysis identifies the prevalence of intermediate species in India. *SpringerPlus*, *2*, 362. https://doi.org/10.1186/2193-1801-2-362

Balamurugan, V., Gangadhar, N. L., Mohandoss, N., Thirumalesh, S. R. A., Dhar, M., Shome, R., Krishnamoorthy, P., Prabhudas, K., & Rahman, H. (2013b). Characterization of leptospira isolates from animals and humans: Phylogenetic analysis identifies the prevalence of intermediate species in India. *SpringerPlus*, *2*(1), 1–9. https://doi.org/10.1186/2193-1801-2-362

Benacer, D., Woh, P. Y., Mohd Zain, S. N., Amran, F., & Thong, K. L. (2013). Pathogenic and Saprophytic Leptospira Species in Water and Soils from Selected Urban Sites in Peninsular Malaysia. *Microbes and Environments*, *28*(1), 135–140. https://doi.org/10.1264/jsme2.ME12154

Bharti, A. R., Nally, J. E., Ricaldi, J. N., Matthias, M. A., Diaz, M. M., Lovett, M. A., Levett, P. N., Gilman, R. H., Willig, M. R., Gotuzzo, E., & Vinetz, J. M. (2003). Leptospirosis: A zoonotic disease of global importance. *Lancet Infectious Diseases*. https://doi.org/10.1016/S1473-3099(03)00830-2

Brinkman, F. S. L., & Leipe, D. D. (2001). Phylogenetic analysis. In *Bioinformatics: A Practical Guide to the Analysis of Genes and Proteins* (pp. 323–358). https://doi.org/10.1006/viro.2002.1568

Brown, P. D., Gravekamps, C., Carringtony Van De Kemps, G. H., Hartskeerls, R. A., Edwards, C. N., Everard, C. O., Terpstra, W. J., & Levett, P. N. (1995). Evaluation of the polymerase chain reaction for early diagnosis of leptospirosis. *J Med Microbiol* (Vol. 43). Retrieved from www.microbiologyresearch.org

Cameron, C. E. (2015). Leptospiral Structure, Physiology, and Metabolism (pp. 21–41). Springer, Berlin, Heidelberg. https://doi.org/10.1007/978-3-662-45059-8_3

Case, R. J., Boucher, Y., Dahllöf, I., Holmström, C., Doolittle, W. F., & Kjelleberg, S. (2007). Use of 16S rRNA and rpoB genes as molecular markers for microbial ecology studies. *Applied and Environmental Microbiology*, *73*(1), 278–288. https://doi.org/10.1128/AEM.01177-06

Cerqueira, G. M., & Picardeau, M. (2009). A century of Leptospira strain typing. *Infection, Genetics and Evolution*, *9*(5), 760–768. https://doi.org/10.1016/J.MEEGID.2009.06.009

Chakravorty, S., Helb, D., Burday, M., & Connell, N. (2007). A detailed analysis of 16S ribosomal RNA gene segments for the diagnosis of pathogenic bacteria. *J Microbiol Methods*, *69*(2), 330–339. https://doi.org/10.1016/j.mimet.2007.02.005.A

Chandan, S., & Umesha S. (2014). Molecular Detection of Leptospira Using Polymerase Chain Reaction Based on LipL32 and rpoB Gene. *RRJoVST*, *3*(1), 16–23. Retrieved from www.stmjournals.com

Chandan Shivamallu, Sharanaiah Umesha, Govindaraju Shruthi, V. B. (2016). Use of rpoB gene analysis for detection and identification of *Leptospira*

species by direct sequencing. *European Journal of Biotechnology and Bioscience*, *4*(1), 34–43. Retrieved from https://www.researchgate.net/publication/303733744_Use_of_rpoB_gene_analysis_for_detection_and_identification_of_Leptospira_species_by_direct_sequencing

Chen, H. W., Zhang, Z., Halsey, E. S., Guevara, C., Canal, E., Hall, E., Maves, R., Tilley, D. H., Kochel, T. J., & Ching, W. M. (2013). Detection of Leptospira-specific antibodies using a recombinant antigen-based enzyme-linked immunosorbent assay. *American Journal of Tropical Medicine and Hygiene*, *89*(6), 1088–1094. https://doi.org/10.4269/ajtmh.13-0041

Chiani, Y., Jacob, P., Varni, V., Landolt, N., Schmeling, M. F., Pujato, N., Caimi, K., & Vanasco, B. (2016). Isolation and clinical sample typing of human leptospirosis cases in Argentina. *Infection, Genetics and Evolution*, *37*, 245–251. https://doi.org/10.1016/J.MEEGID.2015.11.033

Chiriboga, J., Barragan, V., Arroyo, G., Sosa, A., Birdsell, D. N., España, K., Mora, A., Espín, E., Mejía, M. E., Morales, M., Pinargote, C., Gonzalez, M., Hartskeerl, R., Keim, P. S., Bretas, G., Eisenberg, J. N. S., & Trueba, G. (2015). High Prevalence of Intermediate *Leptospira* spp. DNA in Febrile Humans from Urban and Rural Ecuador. *Emerging Infectious Diseases*, *21*(12), 2141–2147. https://doi.org/10.3201/eid2112.140659

Cinco, M. (2010). New insights into the pathogenicity of leptospires: Evasion of host defences. *New Microbiologica*, *33*(4), 283–292.

Clarridge, J. E. (2004). Impact of 16S rRNA gene sequence analysis for identification of bacteria on clinical microbiology and infectious diseases. *Clinical Microbiology Reviews*. https://doi.org/10.1128/CMR.17.4.840-862.2004

De Serres, G., Levesque, B., Higgins, R., Major, M., Laliberte, D., Boulianne, N., & Duval, B. (1995). Need for vaccination of sewer workers against leptospirosis and hepatitis A. *Occupational and Environmental Medicine*, *52*(8), 505–507. https://doi.org/10.1136/oem.52.8.505

Details - Public Health Image Library(PHIL). (n.d.). Retrieved May 4, 2018, from https://phil.cdc.gov/Details.aspx?pid=1220

Eshghi, A., Cullen, P. A., Cowen, L., Zuerner, R. L., & Cameron, C. E. (2009). Global proteome analysis of Leptospira interrogans. *Journal of Proteome Research*, *8*(10), 4564–4578. https://doi.org/10.1021/pr9004597

Evangelista, K. V, & Coburn, J. (2010). Leptospira as an emerging pathogen: a review of its biology, pathogenesis and host immune responses. *Future Microbiology*, *5*(9), 1413–1425. https://doi.org/10.2217/fmb.10.102

Faine, S., Adler, B., Bolin, C., & Perolat, P. (eds. (1999). Leptospira and Leptospirosis (2nd edition). *Book, MedSci, Armadale, Australia*, 293.

Fletcher, W. (1928). Recent work on leptospirosis, tsutsugamushi disease, and tropical typhus in the federated Malay States. *Transactions of the Royal*

Society of Tropical Medicine and Hygiene, *21*(4), 265-IN9. https://doi.org/10.1016/S0035-9203(28)90019-X

Florence, A., Victoriano, B., Smythe, L. D., Gloriani-Barzaga, N., Cavinta, L. L., Kasai, T., Limpakarnjanarat, K., Ong, B. L., Gongal, G., Hall, J., Coulombe, C. A., Yanagihara, Y., Yoshida, S., & Adler, B. (2009). Pacific Regional Office, World Health Organization. https://doi.org/10.1186/1471-2334-9-147

Gabor, E. M., De Vries, E. J., & Janssen, D. B. (2003). Efficient recovery of environmental DNA for expression cloning by indirect extraction methods. *FEMS Microbiology Ecology*, *44*(2), 153–163. https://doi.org/10.1016/S0168-6496(02)00462-2

Ganoza, C. A., Matthias, M. A., Collins-Richards, D., Brouwer, K. C., Cunningham, C. B., Segura, E. R., Gilman, R. H., Gotuzzo, E., & Vinetz, J. M. (2006). Determining risk for severe leptospirosis by molecular analysis of environmental surface waters for pathogenic Leptospira. *PLoS Medicine*, *3*(8), 1329–1340. https://doi.org/10.1371/journal.pmed.0030308

Garba, B., Bahaman, A. R., Khairani-Bejo, S., Zakaria, Z., & Mutalib, A. R. (2017). Retrospective Study of Leptospirosis in Malaysia. *EcoHealth*. https://doi.org/10.1007/s10393-017-1234-0

Gerke, P., & Rump, L. C. (2003). Leptospirosis--3 cases and a review. *Clinical Nephrology*, *60*(1), 42–8. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/12872857

Ghazaei, C. (2018). Pathogenic Leptospira: Advances in understanding the molecular pathogenesis and virulence. *Open Veterinary Journal*, *8*(1), 13–24. https://doi.org/10.4314/ovj.v8i1.4

Goarant, C. (2016). Leptospirosis: risk factors and management challenges in developing countries. *Research and Reports in Tropical Medicine*, *Volume 7*, 49–62. https://doi.org/10.2147/RRTM.S102543

Guerra, M. a. (2013). Leptospirosis: public health perspectives. *Biologicals : Journal of the International Association of Biological Standardization*, *41*(5), 295–7. https://doi.org/10.1016/j.biologicals.2013.06.010

Guerreiro, H., Croda, J., Flannery, B., Mazel, M., Matsunaga, J., Reis, M. G., Levett, P. N., & Haake, D. A. (2001). Leptospiral proteins recognized during the humoral immune response to Leptospirosis in humans. *Infection and Immunity*, *69*(8), 4958–4968. https://doi.org/10.1128/IAI.69.8.4958-4968.2001

Haake, D. A., Chao, G., Zuerner, R. L., Barnett, J. K., Barnett, D., Mazel, M., Matsunaga, J., Levett, P. N., & Bolin, C. A. (2000). The leptospiral major outer membrane protein LipL32 is a lipoprotein expressed during mammalian infection. *Infection and Immunity*, *68*(4), 2276–2285. https://doi.org/10.1128/IAI.68.4.2276-2285.2000

Haake, D. A., Walker, E. M., Blanco, D. R., Bolin, C. A., Miller, J. N., & Lovett, M. A. (1991). Changes in the surface of leptospira-interrogans serovar

grippotyphosa during invitro cultivation. *Infection and Immunity*, *59*((3)), 1131–1140.

Hartskeerl, R. A., Collares-Pereira, M., & Ellis, W. A. (2011). Emergence, control and re-emerging leptospirosis: dynamics of infection in the changing world. *Clinical Microbiology and Infection*, *17*(4), 494–501. https://doi.org/10.1111/J.1469-0691.2011.03474.X

Hookey, J. V, Bryden, J., & Gatehouse, L. (1993). The use of 16s rDNA sequence analysis to investigate the phylogeny of Leptosiraceae and related spirochaetes. *Journal of General Microbiology*, *139*, 2585–2590.

Iraola, G., Spangenberg, L., Lopes Bastos, B., Graña, M., Vasconcelos, L., Almeida, Á., … Naya, H. (2016). Transcriptome Sequencing Reveals Wide Expression Reprogramming of Basal and Unknown Genes in *Leptospira biflexa* Biofilms. *MSphere*, *1*(2). https://doi.org/10.1128/mSphere.00042-16

Ismail, S., Wahab, N. Z. A., Badya, N., Rahman, N. I. A., Yeo, C. C., Latif, A. Z. A., & Haque, M. (2014). A study on the presence of pathogenic leptospira spp. in environmental water samples obtained from selected recreational areas in terengganu, malaysia. *Research Journal of Pharmacy and Technology*, *7*(10), 1153–1157.

Khamis, A., Raoult, D., & La Scola, B. (2004). rpoB gene sequencing for identification of Corynebacterium species. *Journal of Clinical Microbiology*, *42*(9), 3925–3931. https://doi.org/10.1128/JCM.42.9.3925-3931.2004

Kim, M. J. (2013). Leptospirosis in the republic of Korea: historical perspectives, current status and future challenges. *Infection & Chemotherapy*, *45*(2), 137–44. https://doi.org/10.3947/ic.2013.45.2.137

Ko, A. I., Goarant, C., & Picardeau, M. (2009). Leptospira: the dawn of the molecular genetics era for an emerging zoonotic pathogen. *Nature Reviews. Microbiology*, *7*(10), 736–47. https://doi.org/10.1038/nrmicro2208

Krishna, S. V., Joseph, S., Ambily, R., Mini, M., Anto, L., & Mohan, S. G. (2013). Cloning and sequencing of the virulent gene LipL32 of Leptospira interrogans serovar Autumnalis. *Veterinary World*, *6*(4), 193–195. https://doi.org/10.5455/vetworld.2013.193-195

Latifah, I., Abdul Halim, A., Rahmat, M. S., Nadia, M. F., Ubil, Z. E., Asmah, H., Shafariatul, A. I., Picardeau, M., Siti Haslina, O., & Nasir, M. A. (2017). Isolation by culture and PCR identification of LipL32 gene of pathogenic Leptospira spp. in wild rats of Kuala Lumpur. *The Malaysian Journal of Pathology*, *39*(2), 161–166. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/28866698

Lau, C., Smythe, L., & Weinstein, P. (2010). Leptospirosis: An emerging disease in travellers. *Travel Medicine and Infectious Disease*, *8*(1), 33–39. https://doi.org/10.1016/J.TMAID.2009.12.002

Lee, S. H., Kim, B. J., Kim, J. H., Park, K. H., Kim, S. J., & Kook, Y. H. (2000). Differentiation of Borrelia burgdorferi sensu lato on the basis of RNA

polymerase gene (rpoB) sequences. *Journal of Clinical Microbiology*, *38*(7), 2557–2562.

Levett, P. N. (n.d.). Systematics of Leptospiraceae. https://doi.org/10.1007/978-3-662-45059-8_2

Levett, P. N. (2001). Leptospirosis. *Clinical Microbiology*, *14*(2), 296–326. https://doi.org/10.1128/CMR.14.2.296

Lim, J. K., Vikneswaran A Murugaiyah, A. S. R., Rahman, H. A., Mohamed, N. S. F., Shamsudin, N. N., & Tan, J. C. (2011). A Case Study : Leptospirosis In Malaysia. *WebmedCentral*, 1–13. https://doi.org/10.9754/journal.wmc.2011.002764

Liu, J., Wu, W., Chen, C., Sun, F., & Chen, Y. (2011). Prokaryotic diversity, composition structure, and phylogenetic analysis of microbial communities in leachate sediment ecosystems. *Applied Microbiology and Biotechnology*, *91*(6), 1659–1675. https://doi.org/10.1007/s00253-011-3354-8

Maciel, E. A. P., de Carvalho, A. L. F., Nascimento, S. F., de Matos, R. B., Gouveia, E. L., Reis, M. G., & Ko, A. I. (2008). Household Transmission of Leptospira Infection in Urban Slum Communities. *PLoS Neglected Tropical Diseases*, *2*(1), e154. https://doi.org/10.1371/journal.pntd.0000154

Martínez Sánchez, R., Obregón Fuentes, A. M., Pérez Sierra, A., Baly Gil, A., Díaz González, M., Baró Suárez, M., Menéndez Capote, R., Ruiz Pérez, A., Sierra González, G., & López Chávez, A. U. (1998). [The reactogenicity and immunogenicity of the first Cuban vaccine against human leptospirosis]. *Revista Cubana de Medicina Tropical*, *50*(2), 159–66. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10349437

Martínez Sánchez, R., Pérez Sierra, A., Baró Suárez, M., Alvarez, A. M., Menéndez Hernández, J., Díaz González, M., Cruz de la Paz, R., de los Reyes, G., Montoya Batista, B., Sierra González, G., Armesto del Río, M., Saltarén Cobas, A., & Sabournin Ramos, O. (2000). [Evaluation of the effectiveness of a new vaccine against human leptospirosis in groups at risk]. *Revista Panamericana de Salud Publica = Pan American Journal of Public Health*, *8*(6), 385–92. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11209251

Matthias, M. A., Ricaldi, J. N., Cespedes, M., Diaz, M. M., Galloway, R. L., Saito, M., Steigerwalt, A. G., Patra, K. P., Ore, C. V., Gotuzzo, E., Gilman, R. H., Levett, P. N., & Vinetz, J. M. (2008). Human leptospirosis caused by a new, antigenically unique Leptospira associated with a Rattus species reservoir in the Peruvian Amazon. *PLoS Neglected Tropical Diseases*, *2*(4). https://doi.org/10.1371/journal.pntd.0000213

Merien, F., Amouriaux, P., Perolat, P., Baranton, G., & Girons, I. Saint. (1992). Polymerase Chain Reaction for Detection of Leptospira in Clinical Samples. *Journal of Clinical Microbiology, 30*(9), 2219–2224.

Mohamed El-Jalii, I. M. A. (2000). Epidemiology and Diagnosis of Human Leptospirosis in Malaysia. Retrieved from http://psasir.upm.edu.my/7950/

Mohd Ali, M. R., Mohamad Safiee, A. W., Yusof, N. Y., Fauzi, M. H., Yean Yean, C., & Ismail, N. (2017). Isolation of Leptospira kmetyi from residential areas of patients with leptospirosis in Kelantan, Malaysia. *Journal of Infection and Public Health*. https://doi.org/10.1016/j.jiph.2017.12.008

Morey, R. E., Galloway, R. L., Bragg, S. L., Steigerwalt, A. G., Mayer, L. W., & Levett, P. N. (2006). Species-specific identification of Leptospiraceae by 16S rRNA gene sequencing. *Journal of Clinical Microbiology*, *44*(10), 3510–3516. https://doi.org/10.1128/JCM.00670-06

Muñoz-Zanzi, C., Mason, M. R., Encina, C., Astroza, A., & Romero, A. (2014). Leptospira contamination in household and environmental water in rural communities in southern Chile. *International Journal of Environmental Research and Public Health*, *11*(7), 6666–80. https://doi.org/10.3390/ijerph110706666

Musso, D., & La Scola, B. (2013). Laboratory diagnosis of leptospirosis: A challenge. *Journal of Microbiology, Immunology and Infection*, *46*(4), 245–252. https://doi.org/10.1016/J.JMII.2013.03.001

Mwachui, M. A., Crump, L., Hartskeerl, R., Zinsstag, J., & Hattendorf, J. (2015). Environmental and Behavioural Determinants of Leptospirosis Transmission: A Systematic Review. *PLoS Neglected Tropical Diseases*, *9*(9). https://doi.org/10.1371/journal.pntd.0003843

Nally, J. E., Whitelegge, J. P., Bassilian, S., Blanco, D. R., & Lovett, M. A. (2007). Characterization of the outer membrane proteome of Leptospira interrogans expressed during acute lethal infection. *Infection and Immunity*, *75*(2), 766–773. https://doi.org/10.1128/IAI.00741-06

Natarajaseenivasan, K., Raja, V., & Narayanan, R. (2012). Rapid diagnosis of leptospirosis in patients with different clinical manifestations by 16S rRNA gene based nested PCR. *Saudi Journal of Biological Sciences*, *19*, 151–155. https://doi.org/10.1016/j.sjbs.2011.11.005

Oh, M.-D., & Lee, J.-K. (2012). Milestones in history of adult vaccination in Korea. *Clinical and Experimental Vaccine Research*, *1*(1), 9–17. https://doi.org/10.7774/cevr.2012.1.1.9

Page, R. D. M., & Holmes, E. C. (1998). Molecular evolution : a phylogenetic approach. *Blackwell Science*. Retrieved from https://books.google.com.my/books?hl=en&lr=&id=p2lWhjuK8m8C&oi=fnd&pg=PR3&dq=Molecular+Evolution:+A+Phylogenetic+Approach&ots=1driYna_qi&sig=EA5PmphEnPGl2KU4AIbAPdWL9XU#v=onepage&q=Molecular Evolution%3A A Phylogenetic Approach&f=false

Palaniappan, R. U. M., Ramanujam, S., & Chang, Y. F. (2007). Leptospirosis: Pathogenesis, immunity, and diagnosis. *Current Opinion in Infectious Diseases*. https://doi.org/10.1097/QCO.0b013e32814a5729

Paster, B. J., Dewhirst, F. E., Weisburg, W. G., Tordoff, L. A., Fraser, G. J., Hespell, R. B., Stanton, T. B., Zablen, L., Mandelco, L., & Woese, C. R. (1991). Phylogenetic analysis of the spirochetes. *Journal of Bacteriology*, *173*(19), 6101–6109. https://doi.org/10.1128/jb.173.19.6101-6109.1991

Picardeau, M. (2013a). Diagnosis and epidemiology of leptospirosis. *Medecine et Maladies Infectieuses*. https://doi.org/10.1016/j.medmal.2012.11.005

Picardeau, M. (2013b). Diagnosis and epidemiology of leptospirosis. *Médecine et Maladies Infectieuses*, *43*(1), 1–9. https://doi.org/10.1016/J.MEDMAL.2012.11.005

Picardeau, M., Bulach, D. M., Bouchier, C., Zuerner, R. L., Zidane, N., Wilson, P. J., Creno, S., Kuczek, E. S., Bommezzadri, S., Davis, J. C., McGrath, A., Johnson, M. J., Boursaux-Eude, C., Seemann, T., Rouy, Z., Coppel, R. L., Rood, J. I., Lajus, A., Davies, J. K., Médigue, C., & Adler, B. (2008). Genome sequence of the saprophyte Leptospira biflexa provides insights into the evolution of Leptospira and the pathogenesis of leptospirosis. *PLoS ONE*, *3*(2). https://doi.org/10.1371/journal.pone.0001607

Postic, D., Riquelme-Sertour, N., Merien, F., Perolat, P., & Baranton, G. (2000). Interest of partial 16S rDNA gene sequences to resolve heterogeneities between Leptospira collections: Application to L. meyeri. *Research in Microbiology*, *151*(5), 333–341. https://doi.org/10.1016/S0923-2508(00)00156-X

PressReader.com - Connecting People Through News. (n.d.). Retrieved April 26, 2018, from https://www.pressreader.com/malaysia/the-star-malaysia/20170218/282037621923497

Pui, C. F., Bilung, L. M., Apun, K., & Su'ut, L. (2017). Diversity of *Leptospira* spp. in Rats and Environment from Urban Areas of Sarawak, Malaysia. *Journal of Tropical Medicine*, *2017*, 1–8. https://doi.org/10.1155/2017/3760674

Rafizah, A. A. N., Aziah, B. D., Azwany, Y. N., Imran, M. K., Rusli, A. M., Nazri, S. M., Nikman, A. M., Nabilah, I., Zahiruddin, W. M., & Zaliha, I. (2013). Risk factors of leptospirosis among febrile hospital admissions in northeastern Malaysia. *Preventive Medicine*, *57*, S11–S13. https://doi.org/10.1016/j.ypmed.2012.12.017

Ramazan, R., Elaheh, F. S., Somayeh, Y., Elham, G., Asefe, K., & Nafiseh, A. (2015). Detection of Leptospira interrogans in plasma and urine samples by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). *African Journal of Microbiology Research*, *9*(2), 135–139. https://doi.org/10.5897/AJMR2014.7157

Renesto, P., Lorvellec-Guillon, K., Drancourt, M., & Raoult, D. (2000). rpoB gene analysis as a novel strategy for identification of spirochetes from the Genera borrelia, Treponema, and Leptospira. *Journal of Clinical Microbiology*, *38*(6), 2200–2203.

Report of the First Meeting of the Leptospirosis Burden Epidemiology Reference Group. (2010). http://apps.who.int/iris/bitstream/handle/10665/44382/9789241599894 _eng.pdf?sequence=1

Ridzlan, F. R., Bahaman, A. R., Khairani-Bejo, S., & Mutalib, A. R. (2010). Detection of pathogenic Leptospira from selected environment in Kelantan and Terengganu, Malaysia. *Tropical Biomedicine*, *27*(3), 632–638.

Ristow, P., Bourhy, P., Kerneis, S., Schmitt, C., Prevost, M.-C., Lilenbaum, W., & Picardeau, M. (2008). Biofilm formation by saprophytic and pathogenic leptospires. *Microbiology*, *154*(5), 1309–1317. https://doi.org/10.1099/mic.0.2007/014746-0

Saito, M., Villanueva, S. Y. A. M., Chakraborty, A., Miyahara, S., Segawa, T., Asoh, T., Ozuru, R., Gloriani, N. G., Yanagihara, Y., & Yoshida, S. (2013). Comparative Analysis of Leptospira Strains Isolated from Environmental Soil and Water in the Philippines and Japan. *Applied and Environmental Microbiology*, *79*(2), 601–609. https://doi.org/10.1128/AEM.02728-12

Sapian, M., Khairi, M. T., How, S. H., Rajalingam, R., Sahhir, K., Norazah, A., Khebir, V., & Jamalludin, A. R. (2012). Outbreak of melioidosis and leptospirosis co-infection following a rescue operation. *Medical Journal of Malaysia*, *67*(3), 293–297.

Sayyed Mousavi, M. N., Sadeghi, J., Aghazadeh, M., Asgharzadeh, M., & Samadi Kafil, H. (2017). Current advances in urban leptospirosis diagnosis. *Reviews in Medical Microbiology*, *28*(3), 119–123. https://doi.org/10.1097/MRM.0000000000000110

Schmid, G. P., Steere, A. C., Kornblatt, A. N., Kaufmann, A. F., Moss, C. W., Johnson, R. C., Hovind-Hougen, K., & Brenner, D. J. (1986). Newly recognized Leptospira species ("Leptospira inadai" serovar lyme) isolated from human skin. *Journal of Clinical Microbiology*, *24*(3), 484–6. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/3760144

Schneider, A. G., Casanovas-Massana, A., Hacker, K. P., Wunder, E. A., Begon, M., Reis, M. G., Childs, J. E., Costa, F., Lindow, J. C., & Ko, A. I. (2018). Quantification of pathogenic Leptospira in the soils of a Brazilian urban slum. *PLOS Neglected Tropical Diseases*, *12*(4), e0006415. https://doi.org/10.1371/journal.pntd.0006415

Scola, B. La, Bui, L. T. M., Baranton, G., Khamis, A., & Raoult, D. (2006). Partial rpoB gene sequencing for identification of Leptospira species. *FEMS Microbiology Letters*, *263*(2), 142–147. https://doi.org/10.1111/j.1574-6968.2006.00377.x

Sejvar, J., Bancroft, E., Winthrop, K., Bettinger, J., Bajani, M., Bragg, S., Shutt, K., Kaiser, R., Marano, N., Popovic, T., Tappero, J., Ashford, D., Mascola, L., Vugia, D., Perkins, B., Rosenstein, N., & Eco-Challenge Investigation Team. (2003). Leptospirosis in "Eco-Challenge" athletes, Malaysian Borneo, 2000. *Emerging Infectious Diseases*, *9*(6), 702–7. https://doi.org/10.3201/eid0906.020751

Sekhar, W. Y., Soo, E. H., Gopalakrishnan, V., & Devi, S. (2000). Comparative Evaluation of Two Rapid Commercial Diagnostic Kits Against the MAT Test for the Detection of Antibodies to Leptospira Interrogans. *Singapore Med J* (Vol. 41). Retrieved from http://www.smj.org.sg/sites/default/files/4108/4108a1.pdf

Setubal, J. C., Reis, M., Matsunaga, J., & Haake, D. A. (2006). Lipoprotein computational prediction in spirochaetal genomes. *Microbiology*, *152*(1),

113–121. https://doi.org/10.1099/mic.0.28317-0

Sharma, M., & Yadav, A. (n.d.). Leptospirosis: Epidemiology, Diagnosis, and Control, *25*(2). Retrieved from https://pdfs.semanticscholar.org/437b/3f3f2e4f7aaeda06115255d2618aafab23e9.pdf?_ga=2.152906942.686779273.1524896198-461906074.1524727238

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. https://doi.org/10.1099/ijs.0.64947-0

Smythe, L. D., Smith, I. L., Smith, G. A., Dohnt, M. F., Symonds, M. L., Barnett, L. J., & McKay, D. B. (2002). A quantitative PCR (TaqMan) assay for pathogenic Leptospira spp. *BMC Infectious Diseases*, *2*. https://doi.org/10.1186/1471-2334-2-13

Stoddard, R. A., Gee, J. E., Wilkins, P. P., McCaustland, K., & Hoffmaster, A. R. (2009). Detection of pathogenic Leptospira spp. through TaqMan polymerase chain reaction targeting the LipL32 gene. *Diagnostic Microbiology and Infectious Disease*, *64*(3), 247–255. https://doi.org/10.1016/j.diagmicrobio.2009.03.014

Tamura, K., & Nei, M. (n.d.). Estimation of the Number of Nucleotide Substitutions in the Control Region of Mitochondrial DNA in Humans and Chimpanzees ’. Retrieved from https://academic.oup.com/mbe/article-abstract/10/3/512/1016366

Thaipadungpanit, J., Wuthiekanun, V., Chantratita, N., Yimsamran, S., Amornchai, P., Boonsilp, S., Maneeboonyang, W., Tharnpoophasiam, P., Saiprom, N., Mahakunkijcharoen, Y., Day, N. P., Singhasivanon, P., Peacock, S. J., & Limmathurotsakul, D. (2013). Short report: Leptospira species in floodwater during the 2011 floods in the bangkok metropolitan region, Thailand. *American Journal of Tropical Medicine and Hygiene*, *89*(4), 794–796. https://doi.org/10.4269/ajtmh.13-0124

Thaipadunpanit, J., Chierakul, W., Wuthiekanun, V., Limmathurotsakul, D., Amornchai, P., Boonslip, S., Smythe, L. D., Limpaiboon, R., Hoffmaster, A. R., Day, N. P., & Peacock, S. J. (2011). Diagnostic Accuracy of Real-Time PCR Assays Targeting 16S rRNA and lipl32 Genes for Human Leptospirosis in Thailand: A Case-Control Study. *PLoS ONE*, *6*(1), e16236. https://doi.org/10.1371/journal.pone.0016236

Thayaparan, S., Robertson, I. D., Fairuz, A., Suut, L., & Abdullah, M. T. (2013). Leptospirosis, an emerging zoonotic disease in Malaysia. *Malaysian Journal of Pathology*, *35*(2), 123–132. https://doi.org/http://dx.doi.org/10.1108/17506200710779521

Thevanesam, V., Burns, M. A., Agampodi, S. B., Palihawadana, P., Thaipadungpanit, J., Perera, S., Nugegoda, D.B., Smythe, L., Craig, S. B.,

Dohnt, M., Boonsilp, S., Senaratne, T., Kumara, A., Peacock, S. J., & Vinetz, J. M. (2011). Leptospirosis Outbreak in Sri Lanka in 2008: Lessons for Assessing the Global Burden of Disease. *The American Journal of Tropical Medicine and Hygiene*, *85*(3), 471–478. https://doi.org/10.4269/ajtmh.2011.11-0276

Thibeaux, R., Geroult, S., Benezech, C., Chabaud, S., Soupé-Gilbert, M.-E., Girault, D., Bierque, E., & Goarant, C. (2017). Seeking the environmental source of Leptospirosis reveals durable bacterial viability in river soils. *PLoS Neglected Tropical Diseases*, *11*(2), e0005414. https://doi.org/10.1371/journal.pntd.0005414

Trueba, G., Zapata, S., Madrid, K., Cullen, P., & Haake, D. (2004). Cell aggregation: a mechanism of pathogenic Leptospira to survive in fresh water. *International Microbiology : The Official Journal of the Spanish Society for Microbiology*, *7*(1), 35–40. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/15179605

Vinod Kumar, K., Lall, C., Raj, R. V., Vedhagiri, K., & Vijayachari, P. (2015). Coexistence and survival of pathogenic leptospires by formation of biofilm with *Azospirillum*. *FEMS Microbiology Ecology*, *91*(6), fiv051. https://doi.org/10.1093/femsec/fiv051

Viratyosin, W., Ingsriswang, S., Pacharawongsakda, E., & Palittapongarnpim, P. (2008). Genome-wide subcellular localization of putative outer membrane and extracellular proteins in Leptospira interrogans serovar Lai genome using bioinformatics approaches. *BMC Genomics*, *9*. https://doi.org/10.1186/1471-2164-9-181

Vital-Brazil, J. M., Balassiano, I. T., de Oliveira, F. S., Costa, A. D. de S., Hillen, L., & Pereira, M. M. (2010). Multiplex PCR-based detection of Leptospira in environmental water samples obtained from a slum settlement. *Memorias Do Instituto Oswaldo Cruz*, *105*(3), 353–355. https://doi.org/10.1590/S0074-02762010000300020

Vke Mbbs, L. (2011). Leptospirosis: a re-emerging infection. *Malaysian J Pathol*, *33*(1), 1–5. Retrieved from http://www.mjpath.org.my/2011.1/Leptospirosis.pdf

Vos, M., Quince, C., Pijl, A. S., de Hollander, M., & Kowalchuk, G. A. (2012). A comparison of rpoB and 16S rRNA as markers in pyrosequencing studies of bacterial diversity. *PLoS ONE*, *7*(2). https://doi.org/10.1371/journal.pone.0030600

Wagenaar, J. F. P., Goris, M. G. A., Gasem, M. H., Isbandrio, B., Moalli, F., Mantovani, A., Boer, K. R., Hartskeerl, R. A., Garlanda, C., & van Gorp, E. C. M. (2009). Long pentraxin PTX3 is associated with mortality and disease severity in severe Leptospirosis. *Journal of Infection*, *58*(6), 425–432. https://doi.org/10.1016/j.jinf.2009.04.004

Wang, Z., Jin, L., & Węgrzyn, A. (2007). Microbial Cell Factories Leptospirosis vaccines. https://doi.org/10.1186/1475-2859-6-39

Wójcik-Fatla, A., Zając, V., Wasiński, B., Sroka, J., Cisak, E., Sawczyn, A., &

Dutkiewicz, J. (2014). Occurrence of Leptospira DNA in water and soil samples collected in eastern Poland. *Annals of Agricultural and Environmental Medicine*, *21*(4), 730–732. https://doi.org/10.5604/12321966.1129924

Woo, T. H. ., Smythe, L. D., Symonds, M. L., Norris, M. A., Dohnt, M. F., & Patel, B. K. . (2006). Rapid distinction between Leptospira interrogans and Leptospira biflexa by PCR amplification of 23S ribosomal DNA. *FEMS Microbiology Letters*, *150*(1), 9–18. https://doi.org/10.1111/j.1574-6968.1997.tb10343.x

Woods, K., Nic-Fhogartaigh, C., Arnold, C., Boutthasavong, L., Phuklia, W., Lim, C., Chanthongthip, A., Tulsiani, S. M., Craig, S. B., Burns, M. A., Weier, S. L., Davong, V., Sihalath, S., Limmathurotsakul, D., Dance, D. A. B., Shetty, N., Zambon, M., Newton, P. N., & Dittrich, S. (2018). A comparison of two molecular methods for diagnosing leptospirosis from three different sample types in patients presenting with fever in Laos. *Clinical Microbiology and Infection*, *24*(9), 1017.e1-1017.e7. https://doi.org/10.1016/J.CMI.2017.10.017

Wuthiekanun, V., Sirisukkarn, N., Daengsupa, P., Sakaraserane, P., Sangkakam, A., Chierakul, W., Smythe, L. D., Symonds, M. L., Dohnt, M. F., Slack, A. T., Day, N. P., & Peacock, S. J. (2007). Clinical diagnosis and geographic distribution of leptospirosis, Thailand. *Emerging Infectious Diseases*, *13*(1), 124–6. https://doi.org/10.3201/eid1301.060718

Yaakob, Y., Rodrigues, K. F., & John, D. V. (2015). Leptospirosis: Recent incidents and available diagnostics– A review. *Medical Journal of Malaysia*.

Yanagihara, Y., Villanueva, S. Y. A. M., Yoshida, S., Okamoto, Y., & Masuzawa, T. (2007). Current status of leptospirosis in Japan and Philippines. *Comparative Immunology, Microbiology and Infectious Diseases*, *30*(5–6), 399–413. https://doi.org/10.1016/J.CIMID.2007.05.003

Yasouri, S. R., & Ghane, M. (2014). A comparison between culture and PCR technique order to isolate and identify Leptospira spp . from environmental samples in the northern part of Iran Pelagia Research Library. *European Journal of Experimental Biology*, *4*(3), 606–611.

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*, *10*(2), 273–277. https://doi.org/10.1016/J.MEEGID.2010.01.001

Zhang, Y., Li, S., & Dai, B. (1993). [Amplified 23S rRNA gene of 52 strains of Leptospira and detection of leptospiral DNA in 55 patients by PCR]. *Hua Xi Yi Ke Da Xue Xue Bao = Journal of West China University of Medical Sciences = Huaxi Yike Daxue Xuebao*, *24*(3), 262–7. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8288193

BIODATA OF STUDENT

My name is Nurul Farhana binti Zulkifli. I was born on January 26th, 1992 at Hospital Besar Kuala Terengganu. I am the second child in my family. My primary school is Sekolah Kebangsaan Bukit Losong, Kuala Terengganu and my secondary school is Sekolah Menengah Kebangsaan Sultan Sulaiman, Kuala Terengganu. After I finished off my school years, I entered Pahang Matriculation College and studied there for 1 year .Then, I further my study in Bachelor of Science (Cell Biology and Molecule) at Universiti Putra Malaysia for 4 years.

LIST OF PUBLICATION

1. Research paper

Zulkifli NF, Wan, SJ, Neela VK, Yee CH, Masri SN, Al-Obaidi MMJ, Desa MNM. Detection of *leptospira* species in environmental samples by amplification of 16S rRNA and *rpoβ* genes. *Sains Malaysiana* 2018, 47(8): 1793-1798. DOI: 10.17576/jsm.2018-4708-18.

2. Abstract submission for Malaysian Society for Microbiology Postgraduate Symposium 2016

Zulkifli NF, Wan, SJ, Neela VK, Yee CH, Desa MNM. DNA-Based Detection and Distribution of Liptospira Species by 16S rRNA and rpoB Genes from Environmental Samples. *Malaysian Society for Microbiology Postgraduate Seminar.* 2016

Sains Malaysiana 47(8)(2018): 1795–1800
http://dx.doi.org/10.17576/jsm-2018-4708-18

Detection of *Leptospira* Species in Environmental Samples by Amplification of 16S *rRNA* and *rpoβ* Genes

(Pengesanan Spesies *Leptospira* dalam Sampel Alam Sekitar melalui 16S *rRNA* dan Gen *rpoβ* Diperkuat)

NURUL FARHANA ZULKIFLI, STEFFI JULAN WAN, VASANTHA KUMARI NEELA, HUI YEE CHEE, SITI NORBAYA MASRI, MAZEN M. JAMIL AL-OBAIDI & MOHD NASIR MOHD DESA*

ABSTRACT

This study attempted to identify and determine distribution of Leptospira *spp. in environmental samples using 16S* rRNA *and* rpoβ *genes amplification. The samples were collected from high risk areas in Selangor, Malaysia. A total of 105 environmental samples consisting of soil and water were subjected to direct DNA extraction and PCR reaction. PCR products were analysed using gel electrophoresis and subjected to sequence analysis. Thirteen out of 105 (12.38%) samples were amplified for 16S* rRNA *with an expected amplicon size of 330 bp, while 50 out of 105 (47.62%) samples showed amplification using* rpoβ *primers, but were not of expected size. Of the 13 16S* rRNA *amplified samples, only 5 were identified as* Leptospira *in the gene sequence analysis and clustered under uncultured group via phylogenetic tree. This study showed the DNA-based approach using PCR and sequence analysis is able to detect the presence of* Leptospira*, although environmental samples may contain diverse microbial populations that may complicate the detection. Overall, the study suggested the importance of surveillance for* Leptospira *from environmental samples.*

Keywords: Environment; Leptospira*; rpoβ; Selangor; 16S* rRNA

ABSTRAK

Kajian ini dijalankan untuk mengenal pasti dan menentukan penyebaran Leptospira *spp. dalam sampel alam sekitar menggunakan 16S* rRNA *dan gen rpoβ diperkuat. Sampel diperoleh daripada kawasan yang mempunyai risiko tinggi di Selangor, Malaysia. Sampel keseluruhan adalah 105 yang terdiri daripada tanah dan air yang tertakluk secara langsung kepada pengekstrakan DNA dan reaksi PCR. Produk PCR dianalisis menggunakan elektroforesis gel dan tertakluk kepada analisis urutan. Tiga belas sampel daripada 105 (12.38%) menunjukkan amplifikasi untuk 16S* rRNA *dengan saiz amplikon terjangka 330 bp, manakala 50 sampel daripada 105 (47.62%) menunjukkan sampel amplikasi menggunakan pencetus rpoβ, tetapi tidak mempunyai saiz yang dijangka. Daripada 13 16S* rRNA *sampel diperkuat, hanya 5 dikenal pasti sebagai* Leptospira *dalam urutan gen analisis dan berkelompok di bawah kumpulan tidak dibiak melalui pohon filogenetik. Kajian ini menunjukkan pendekatan berasaskan DNA menggunakan PCR dan analisis urutan dapat mengesan kehadiran* Leptospira*, walaupun sampel alam sekitar berkemungkinan mengandungi pelbagai populasi mikrob yang boleh merumitkan pengesanan. Secara keseluruhan, kajian ini mencadangkan kepentingan pengawasan* Leptospira *daripada sampel alam sekitar.*

Kata kunci: Alam sekitar; Leptospira*; rpoβ; Selangor; 16S* rRNA

INTRODUCTION

Leptospirosis is a fatal zoonosis that is widely spread in numerous tropical regions especially in frequently flooding districts, causing large epidemics (Haake & Levett 2015). Leptospirosis cases in Malaysia recently shows an increasing pattern from 263 cases in year 2004 to 5370 cases in 2015 (Garba et al. 2017). *Leptospira* spp. are an aerobic spirochete, motile and a Gram-negative bacterium which can be divided into 2 groups; non-pathogenic (saprophytic) and pathogenic strains. Saprophytic leptospires do not cause infections to humans and are more abundant in the environment due to their rapid growth (Ismail et al. 2014). Meanwhile, pathogenic leptospires need a host for reproduction and surviving. Generally, rodents and wild animals such as rats, cattle, dogs and pigs are the main sources of pathogenic leptospires, as they may excrete leptospires that can survive in urine for months or even years (Lim et al. 2011). Unfortunately, data on identification and distribution of individual *Leptospira* spp. (local strains) from various areas in Malaysia are still lacking. Rapid and simple assays for the identification of individual *Leptospira* spp. are currently not well established especially in Malaysia, due to the fastidious and sensitive nature of the microorganism that hinders a wide surveillance study to be feasibly conducted on the distribution of the microorganism. 16S *rRNA* and *rpoβ* genes have been earlier demonstrated as suitable targets for identifying and discriminating bacterial spp. (Case et al. 2007). Therefore, this study attempted to detect the presence and investigate the distribution of

Malaysian Society for Microbiology Postgraduate Seminar (MSMPS2016)
24 August 2016 Faculty of Science and Technology, Universiti Kebangsaan Malaysia, Bangi, Selangor, Malaysia

Poster **MM13**

DNA-BASED DETECTION AND DISTRIBUTION OF *Leptospira* SPECIES BY 16S rRNA AND *rpoB* GENES FROM ENVIRONMENTAL SAMPLES

Nurul Farhana Z.[1], Steffi Julan W.[1], Vasantha Kumari N.[2], Chee H. Y.[2] and Mohd Nasir M. D.[1*]

[1]*Department of Biomedical Science;* [2]*Department of Medical Microbiology and Parasitology, Faculty of Medicine and Health Sciences, UPM Serdang.*

*Corresponding author: mnasir@upm.edu.my

Leptospirosis is currently emerging globally as reflected by the increase in the number of *Leptospira* infections. Transmission of the bacteria takes place between environment, animals, and human. Infection may cause a mild to severe complication. PCR diagnosis improves the accuracy of epidemiological evaluation of leptospirosis, allowing a better evaluation on incidence of the disease. The most commonly used genes for identifying microbial community involves 16S rRNA gene and also rpoB gene (RNA polymerase β-subunit). This study attempts to identify and determine the distribution of *Leptospira* spp. using 16S rRNA and rpoB genes from environmental samples in high risk areas around Selangor, Malaysia. A total of 60 environmental samples consisting of soils and waters were extracted for DNA and subjected to PCR using 2 sets of primers, targeting 16S rRNA and rpoB genes of *Leptospira*. PCR products were analysed through gel electrophoresis and positive results were subjected to sequence analysis. Ten out of 60 (16.67%) samples were amplified for 16S rRNA gene with expected amplicon size (330bp), while 22 out of 60 (36.67%) samples were amplified using the rpoB gene's primers but were not of the expected amplicon size. Out of the 10 positive samples, only 5 were detected to have been contaminated by *Leptospira* spp. based on sequencing results of the 16S rRNA gene. This study showed that the DNA-based approach by using PCR was able to detect *Leptospira* spp. although environmental samples may contain diverse microbial populations that complicate detection. The use of rpoB requires further validation but the presence of *Leptospira* spp. in the environment in the high risk areas around Selangor points out the importance of molecular screening. Further study on the distribution of the species in the environment is needed to reduce the posibility of *Leptospira* infection.

UNIVERSITI PUTRA MALAYSIA

STATUS CONFIRMATION FOR THESIS / PROJECT REPORT AND COPYRIGHT

ACADEMIC SESSION : Second Semester 2018/2019

TITLE OF THESIS / PROJECT REPORT :

DETECTION OF *Leptospira* SPECIES TARGETING 16S rRNA, *rpoB*, AND *lipL32* GENE FROM CLINICAL AND ENVIRONMENTAL SOURCES

NAME OF STUDENT: NURUL FARHANA BINTI ZULKIFLI

I acknowledge that the copyright and other intellectual property in the thesis/project report belonged to Universiti Putra Malaysia and I agree to allow this thesis/project report to be placed at the library under the following terms:

1. This thesis/project report is the property of Universiti Putra Malaysia.
2. The library of Universiti Putra Malaysia has the right to make copies for educational purposes only.
3. The library of Universiti Putra Malaysia is allowed to make copies of this thesis for academic exchange.

I declare that this thesis is classified as :

*Please tick (√)

☐	**CONFIDENTIAL**	(Contain confidential information under Official Secret Act 1972).
☐	**RESTRICTED**	(Contains restricted information as specified by the organization/institution where research was done).
☐	**OPEN ACCESS**	I agree that my thesis/project report to be published as hard copy or online open access.

This thesis is submitted for :

☐ **PATENT** Embargo from _____ (date) until _____ (date)

Approved by:

_____	_____
(Signature of Student)	(Signature of Chairman of Supervisory Committee)
New IC No/ Passport No.:	Name:
Date :	Date :

[Note : If the thesis is CONFIDENTIAL or RESTRICTED, please attach with the letter from the organization/institution with period and reasons for confidentially or restricted.]