UNIVERSITI PUTRA MALAYSIA

MOLECULAR DETECTION AND CHARACTERIZATION OF PATHOGENIC LEPTOSPIRA SPECIES IN ENVIRONMENTAL SAMPLES OF SELECTED DISTRICTS IN PERAK, MALAYSIA

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By

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Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Doctor of Philosophy

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MOLECULAR DETECTION AND CHARACTERIZATION OF PATHOGENIC *LEPTOSPIRA* SPECIES IN ENVIRONMENTAL SAMPLES OF SELECTED DISTRICTS IN PERAK, MALAYSIA

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Leptospirosis is an endemic zoonotic disease in Malaysia caused by pathogenic species of genus *Leptospira*. Most cases of human leptospirosis are resulted from environmental exposure to water and soils contaminated with bacteria shed in urine of infected carrier animals. Epidemiological information about leptospirosis and *Leptospira* in the Perak state is scarce despite high disease incidence and mortality rate. To assess public health risk for leptospirosis, this study aims to determine the cross-sectional prevalence of pathogenic *Leptospira* in recreational and residential public places, as well as to characterize the genetic diversity of pathogenic *Leptospira* isolated from environmental samples. A total of 228 environmental water and shore soils samples were collected from 20 amenity forests and wet markets, filtered, and subjected to cultivation of leptospires in enriched EMJH medium. Presence of pathogenic *Leptospira* was confirmed by specific amplification of *lipL32* gene by polymerase chain reaction (PCR). Results showed a high prevalence of pathogens (11 %, *n* = 25) throughout Perak, with highly varied localised prevalence among 13 positive sampling sites (6.7 - 41.7 %). Further distribution analysis implies a higher exposure risk in amenity forests than wet markets, through soil than water, as well as in the districts Kampar and Kinta than Batang Padang, Kuala Kangsar, Kerian, Larut, Matang & Selama. Unexpectedly the localised prevalence was not significantly associated with provision of waste management and site cleanliness. In addition to that, the total absence of pathogen at sites BF, GF, KW, LI, MM, PF, and SS in this present study has no relation to environmental parameters of samples, including temperature, pH, and water salinity. On another hand, seeking a sensitive molecular detection tool for accurate surveillance has driven this study to compare performance of other pathogen-specific diagnostic PCR assays on the positive samples. Unexpectedly a notably low and varied detection sensitivity (12 - 83 %) was determined among environmental isolates in relation to reference *Leptospira* strains sourced from human or animal hosts.

Among genetic markers studied, *lipL32* and *flaB* have been most prevalent, followed by *gyrB* and *lfb1*, whereas PCRs targeting *secY* and *ligB* showed high false-negativity. The absence of amplification was most likely attributed to mismatch in primer-annealing sites owing to high sequence polymorphisms. Phylogenetic analysis of partial 16S rRNA gene sequences revealed a subclade formed by all environmental isolates (except intermediate KF4) within the 'pathogens' clade, suggesting a fair distance from described host-associated *Leptospira* strains despite the low bootstrap values. A noticeable clustering of isolates sourced from similar sampling site, district, nor ecological niches was not observed. Through comparative polymorphic nucleotide analysis, ten pathogenic isolates were found closest to *L. kmetyi* and *L. alstonii* which have been prevailing in Malaysia, while the others probably represent novel species. In conclusion, genetically diverse pathogenic *Leptospira* spp. was widely distributed in Perak. Determining the virulence potential and whole-genome sequence for these atypical pathogenic isolates is important to validate the risk of leptospirosis, evolutionary relationship and reclassification of genus *Leptospira*.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia
sebagai memenuhi keperluan untuk Ijazah Doktor Falsafah

PENGESANAN MOLEKUL DAN PENCIRIAN SPESIS PATOGEN *LEPTOSPIRA* DARIPADA SAMPEL PERSEKITARAN DI DAERAH TERPILIH DI PERAK, MALAYSIA

Oleh

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Leptospirosis merupakan penyakit zoononis endemik yang disebabkan oleh spesis patogen genus *Leptospira*. Kebanyakan kes leptospirosis yang melibatkan manusia diakibatkan oleh pendedahan kepada air dan tanah yang dicemari oleh bakteria yang terdapat dalam air kencing binatang pembawa yang terjangkit. Maklumat epidemiologi mengenai leptospirosis dan *Leptospira* di negeri Perak amat kurang walaupun mempunyai kadar insiden penyakit dan kematian yang tinggi. Bagi menilai risiko kesihatan awam untuk leptospirosis, kajian ini bertujuan menentukan prevalens keratan lintang patogen *Leptospira* dari persekitaran di tempat-tempat awam di kawasan-kawasan rekreasi dan kediaman, serta untuk mencirikan kepelbagaian genetik patogen *Leptospira* yang terpencil daripada sampel persekitaran. Sejumlah 228 sampel air dan tanah pantai dikumpulkan daripada 20 hutan lipur dan pasar basah, disaring, dan *Leptospira* disuburkan dalam medium EMJH yang diperkayakan. Kehadiran patogen *Leptospira* disahkan oleh amplifikasi khusus gen *lipL32* melalui reaksi rantai polimerase (*polymerase chain reaction* atau PCR). Keputusan menunjukkan prevalens patogen yang tinggi (11 %, *n* = 25) di seluruh Perak, dan prevalens setempat berkepelbagaian tinggi dalam 13 buah kawasan pensampelan positif (6.7 - 41.7 %). Analisis taburan selanjutnya menunjukkan risiko pendedahan yang lebih tinggi di hutan-hutan lipur berbanding dengan pasar-pasar basah, melalui tanah berbanding dengan air, dan juga di daerah Kampar dan Kinta berbanding dengan Batang Padang, Kuala Kangsar, Kerian, Larut, Matang dan Selama. Tanpa dijangka, prevalens setempat tidak berkait secara signifikan dengan penyediaan pengurusan sisa buangan dan kebersihan tapak. Selain itu, ketiadaan patogen langsung di tapak-tapak BF, GF, KW, LI, MM, PF, dan SS dalam kajian ini tidak berkaitan dengan parameter persekitaran sampel, termasuk suhu, pH, dan tahap kemasinan air. Pada masa yang sama, pencarian alat pengesanan molekul sensitif untuk pengawasan yang tepat telah mendorong kajian ini untuk membandingkan prestasi cerakin PCR untuk diagnostik patogen khusus yang

lain terhadap sampel positif. Sensitiviti pengesanan yang pelbagai dan sangat rendah (12 - 83 %) dikenal pasti dalam *Leptospira* asingan persekitaran berkaitan dengan rujukan strain *Leptospira* yang diambil daripada hos manusia atau binatang. Antara penanda genetik yang dikaji, *lipL32* dan *flaB* didapati paling prevalen, diikuti oleh *gyrB* dan *lfb1*, manakala PCR yang mensasarkan *secY* dan *ligB* menunjukkan negatif-palsu yang tinggi. Ketiadaan amplifikasi kemungkinan besar disebabkan oleh ketakpadanan di kawasan-kawasan sepuh lindap primer (*primer-annealing*) kerana polimorfisme jujukan tinggi (*high sequence polymorphisms*). Analisis filogenetik jujukan genetik 16S rRNA separa menunjukkan subklad yang dibentuk oleh kesemua asingan persekitaran (kecuali KF4 pertengahan) dalam klad 'patogen', membuktikan jarak wajar daripada strain *Leptospira* berkaitan dengan perumah yang diterangkan walaupun mempunyai nilai sumber minimum yang rendah. Pengklusteran jelas asingan yang diambil daripada kawasan persampelan, daerah atau ceruk ekologi yang serupa tidak dicerap. Melalui analisis nukleotida polimorfik bandingan, sepuluh asingan patogen dijumpai paling hampir dengan *L. kmetyi* and *L. alstonii* yang banyak terdapat di Malaysia, manakala asingan lain berkemungkinan mewakili spesis yang baharu. Kesimpulannya, kepelbagaian genetik patogen *Leptospira* spp. tersebar secara meluas di Perak. Mengenal pasti potensi kevirulenan dan penjujukan seluruh genom (*whole-genome sequence*) untuk asingan patogen atipikal adalah penting untuk mengesahkan risiko leptospirosis, hubungan evolusi, dan klasifikasi semula genus *Leptospira*.

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I certify that a Thesis Examination Committee has met on 13 May 2019 to conduct the final examination of Yap May Ling on her thesis entitled "Molecular Detection and Characterization of Pathogenic *Leptospira* Species in Environmental Samples of Selected Districts in Perak, Malaysia" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Doctor of Philosophy.

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

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

BLAST	Basic local alignment search tool
CAAT	Cross-agglutination adsorption test
CDC	Centre for Disease Control and Prevention
CFR	Case-fatality rate
Ct	Cycle threshold
DFM	Dark-field microscopy
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
EMJH	Ellinghausen-McCullough-Johnson-Harris
5-FU	5-Fluorouracil
FDP	Forestry Department of Perak
gDNA	Genomic deoxyribonucleic acid
GE	Genomic equivalents
IMR	Institute of Medical Research
IR	Incidence rate
IS	Insertion sequence
LAMP	Loop-mediated isothermal amplification
LLOD	Lowest limit of detection
LPS	Lipopolysaccharide
MALDI-TOF	Matrix-assisted laser desorption/ionization time-of-flight
MAT	Microscopic agglutinating test
MLST	Multiple locus sequence typing
MLVA	Multiple locus variable-number tandem repeat analysis
MOH	Ministry of Health Malaysia
n	Number
NC	Negative control
NTC	Non-template control
NPHL	National Public Health Laboratory
OMP	Outer membrane proteins
PC	Positive control
PCR	Polymerase chain reaction
PFGE	Pulse-field gel electrophoresis

PRF	Permanent reserved forest
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
SNP	Single-nucleotide polymorphism
STAFF	Sulfamethoxazole-trimethoprim-amphotericin-fosfomycin-5-FU
Ta	Annealing temperature
UPM	Universiti Putra Malaysia
UTAR	Universiti Tunku Abdul Rahman
WHO	World Health Organization

CHAPTER 1

INTRODUCTION

1.1 Background

Leptospirosis is a zoonosis of global distribution with high endemicity and annual morbidity in tropical developing countries (Costa et al., 2015), including Malaysia. This disease is caused by spirochaete bacteria of genus *Leptospira* (Levett, 2001). To date, more than 300 serovars and 35 species of *Leptospira* are discovered and grouped into pathogens, saprophytes and intermediate species (Thibeaux et al., 2018a). Rodents and domestic animals (pigs, cattle, dogs) are common reservoirs that maintain pathogenic *Leptospira* in renal tubules and chronically excrete the agents in urine (Faine et al., 1999). Human get infected mostly via exposure to the urine-contaminated surface water and soils. Leptospires may enter host body through skin abrasion and mucosal membrane. In addition to the occupational and recreational risk factors, some events, like heavy rainfalls, flooding and poor sanitation, may put the entire community at risk of leptospirosis (Bharti et al., 2003).

1.2 Problem Statement

In spite of its high endemicity in South-East Asian countries, leptospirosis has been long neglected in Malaysia (Pappas et al., 2008). Concerning the drastic increase in incidence rate (IR) and reported fatality cases over the past decade, Ministry of Health Malaysia (MOH) has gazetted leptospirosis as notifiable disease in 2010 and provides the stakeholders with a comprehensive guideline on the diagnosis, management, prevention and control of leptospirosis (MOH, 2011). Regrettably, the statistics continued to rise with a peak IR in 2015 at 30.2 per 100,000 population (Abdul Wahab, 2015). The presence of pathogenic leptospires in environment has been well studied in several major states of leptospirosis (Kelantan, Terengganu and Sarawak) in high-risk areas (Ridzlan et al., 2010; Ismail et al., 2014; Pui et al., 2015, 2017; Azali et al., 2016; Mohammad Ali et al., 2017; Loong et al., 2018). The epidemiological data, however, is severely lacking in Perak despite its top ranked IR and case-fatality rate (CFR) from 2004 to 2012 (Benacer et al., 2016c).

In Malaysia, leptospirosis outbreaks and fatality cases are mostly related to water-associated activities in residential and recreational areas (Abdul Wahab, 2015). For a better environmental public health, it is important to identify places and to define an environmental condition that pose high risk in leptospirosis transmission. An evidence-based risk assessment is critical for relevant authorities to institute and implement effective preventive measures and mitigation strategies (World Health Organization [WHO], 2003; MOH, 2011). Therefore, this study intended to characterize the risk for environment-

mediated leptospirosis acquisition in public places, by determining the ubiquity of pathogenic *Leptospira* in water and soils.

A warm, humid and shady place, and a fresh water with pH between 6.7 and 7.3 and low in salinity allow durable survival of shed leptospires outside of host body (Khairani-Bejo, 2004a; Trueba, 2004). However, these findings were merely made through controlled experiments in laboratory. Given that the effect of several relevant environmental parameters on prevalence of pathogenic *Leptospira* in the context of natural environment would be investigated in present study. Another main interest of this study lies in finding if environmental cleanliness would influence the presence and ubiquity of pathogenic *Leptospira* in a place, knowing that many human leptospirosis outbreaks occurred in association with poor cleanliness and inadequate waste disposal facility that attract rats and strays (Reis et al, 2008).

Numerous diagnostic polymerase chain reaction (PCR) assays, targeting different leptospiral genes, have been described for detecting pathogenic leptospires (Guernier et al., 2017). Nonetheless, PCR has not yet been developed to an extent where it is universally accepted or routinely applied in environmental screening (Wynwood et al., 2014). As demonstrated in a number of pilot studies, the choice of PCR primers would considerably affect the resulted environmental prevalence (Lai, 2014; Tan, 2014; Eee, 2014; Goh, 2014; Thachaini Thevi, 2014; Chen, 2015; Yew, 2015; Wang, 2015; Chong, 2015). An accurate detection is of great importance especially during outbreak investigation where a public health investigator would trace the source of infection in environment for evidence (MOH, 2011). Given that, this study pursues to compare a range of routine PCR assays to seek for ones that provide highest sensitivity by detecting all *lipL32*-positive environmental samples.

Historically, pathogenic species of *Leptospira* are coherently recovered from infected human and animals whilst *Leptospira* in soils and water are free-living saprophytes (Henry and Johnson, 1978). Yet, novel pathogenic species were rather recovered from soil recently (Slack et al., 2009; Thibeaux et al., 2018a). Not only having distinct accessory gene patterns, these atypical pathogenic species were also incapable to establish acute infection or renal colonization in these animal models (Thibeaux et al., 2018b). As a result, it is interested to elaborate the divergence between pathogenic strains sourced from hosts and environment in order to gain insights on molecular diagnostics and classification scheme. Another evolution-related subjects worth exploring on would be the genetic relatedness and biodiversity among isolates recovered from different geographic locations and conditions, which has not yet been reported in any region in Malaysia. Revealing the evolutionary relationship among members of the highly heterogeneous genus *Leptospira* will help researchers to revisit current taxonomy and evolution in pathogenomics.

1.3 Objectives of Study

In general, this study aimed to investigate the distribution in high-risk public places in Perak of pathogenic species of *Leptospira*, and to characterize their genetic properties in terms molecular PCR diagnostics and phylogenetic relationship.

In specific, the objectives of this study comprise the followings:

1. To determine public health risk for leptospirosis in selected recreational and residential places by performing molecular analysis of environmental waters and soils for pathogen-specific leptospiral *lipL32* gene
2. To relate the distribution and prevalence of pathogenic *Leptospira* species with waste management and environmental parameters
3. To compare the sensitivity of current diagnostic PCR assays in detecting pathogenic *Leptospira* species recovered from environmental samples
4. To identify and to characterize genetic diversity and relatedness among the environmental *Leptospira* isolates through phylogeny

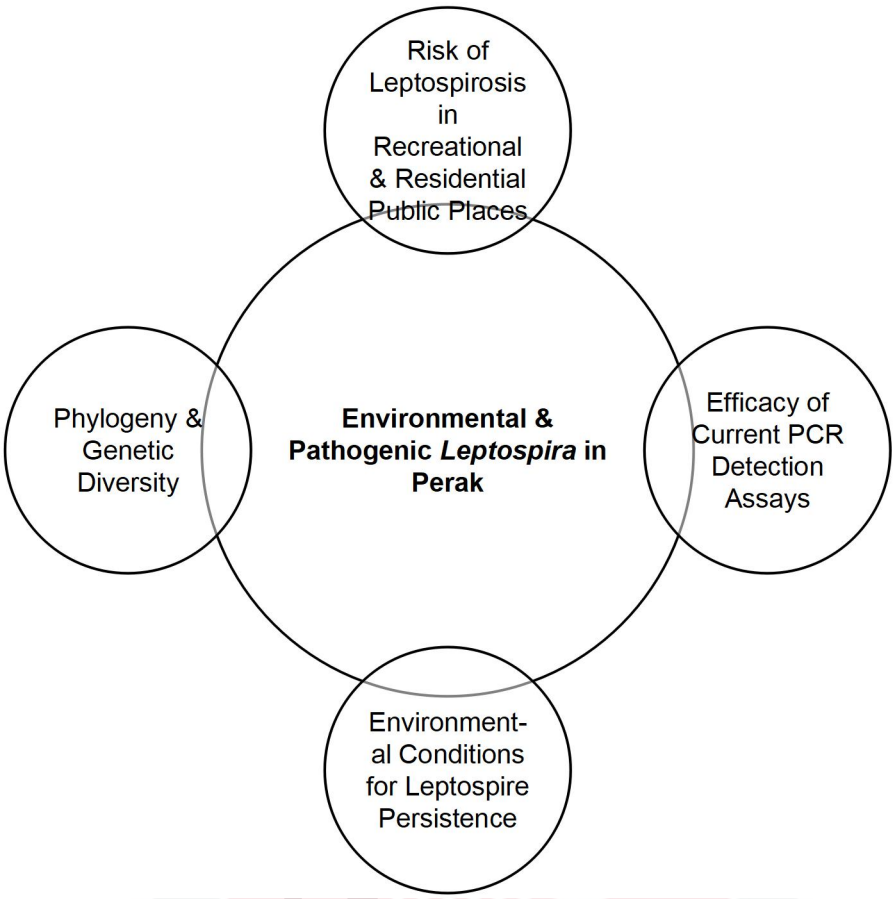

Figure 1.1: An overview of research design of this present study

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BIODATA OF STUDENT

The student, Yap May Ling, was born in November 1976 in Ipoh, Perak. Being the eldest child in a modest family of five, she was brought up in a remote small village surrounded with rubber estate in northern region of Perak. In 1982, she commenced her primary education in S.R.J.K.(C) Air Kala which is a Chinese vernacular school in the Air Kala New Village, while helping the family out in running a noodle stall in wet market and rubber tapping since 10 years old. She continued with secondary education firstly in the Sekolah Menengah Dato Ahmad, Lenggong. Upon getting a distinguished result in Lower Certification of Education examination in 1992 and hoping for a better prospect, her family decided to transfer her to S.M.J.K.(C) Tsung Wah located in Kuala Kangsar. Although she didn't attend tuition class like most other classmates do, she is proud to get third place in the school prior to SPM examination.

In 1995, unexpectedly with good luck she was offered to study in Diploma in Forestry in the Faculty of Forestry, Universiti Putra Malaysia (UPM) (formerly known as Universiti Pertanian Malaysia) in Selangor. On the following year, she was given a chance to promote to Bachelor of Science (Hons.) majoring in Microbiology in the Faculty of Science and Environmental Studies. Upon completion of study in 1999 with an exceptional grade in final-year project, she decided to pursue in Master of Science (Virology) in similar university. Her postgraduate research in the Faculty of Veterinary Medicine aimed to develop recombinant subunit protein vaccine against a local nephropathogenic strain of avian infectious bronchitis virus.

Not forgetting her dream since young, the student started her career in teaching in 2005 by firstly worked as a school teacher in Sekolah Seri Suria, a private school located in Selangor. Still having strong desire and enthusiasm to working in tertiary education, she became a lecturer in INTI International University in Nilai, Negeri Sembilan for period of 4 years, during which she gave birth to a beautiful baby girl. Being exhausted with the long-distance driving to work place everyday between two states, she decided to move out from the urban city. In year end of 2010, she came to Kampar, a serene and peaceful town in Perak, where she continued her lecturing job in the Universiti Tunku Abdul Rahman (UTAR). Looking for a better infrastructure and living convenience, her family eventually settled down in Ipoh city and back to the place where she was born.

PUBLICATIONS

Yap, M.L., Sekawi, Z., Chee, H.Y., Ong, A.H.K. and Neela, V.K. 2019. Comparative analysis among current diagnostic PCR assays in detecting pathogenic *Leptospira* isolates from environmental samples. *Asian Pacific Journal of Tropical Medicine* 12(10):

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