UNIVERSITI PUTRA MALAYSIA

ISOLATION AND CHARACTERIZATION OF PATHOGENIC LEPTOSPIRES FROM RODENTS AND SMALL MAMMALS CAPTURED IN HUMAN LEPTOSPIROSIS SUSPECTED AREAS IN SELANGOR, MALAYSIA

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By

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

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Chair : Assoc. Prof. Vasantha Kumari Neela, PhD
Faculty : Medicine and Health Sciences

Leptospirosis, previously known to be a neglected zoonotic disease in the tropical region is now re-emerging as a threat to public health in both urban and rural settings. It is known that rodents are the major carrier of pathogenic *Leptospira* sp. In Malaysia, the endemicity of leptospirosis in human is concentrated in areas where rats were highly populated such as in residential and recreational areas with improper trash management and poor sanitation. The statistics in Malaysia has shown an increasing trend of suspected leptospirosis cases and reported deaths since it has been gazetted as a notifiable disease in 2010. This makes an urgent call to study the carrier status of pathogenic leptospires infecting the human through the study of *Leptospira* sp. in rodents and small mammals in Malaysia. The objective of this study is to identify and characterize the predominant pathogenic *Leptospira* sp. circulating in the leptospirosis suspected areas in the Selangor state of Malaysia. The study was carried out from January 2016 to April 2017 in six suspected areas comprising of urban, semi-urban and recreational forest areas. The study sites were identified by the Selangor State Health Department as outbreak or hotspot areas. Rodents trapping was performed in all six study sites. The trapped rodents were dissected and kidneys harvested. The rodent kidneys were subjected to *Leptospira* isolation by culture and dark-field microscopy. The identification and pathogenic strain of the isolated leptospires were determined by PCR approach. The characterization included *secY* and *lipL32* PCR and multi-locus sequence typing (MLST). A total of 14 small mammals species were identified from the 266 captured small mammals with *Rattus norvegicus* (66%, n=100) being the dominant rat species in the urban area while

Maxomys whiteheadi (30%, n=23) dominated the recreational forest area. Among the 266 rodents captured, 217 kidney samples were cultured, while for the remaining 49 samples, DNA was directly extracted from the kidney. From 217 samples cultured 55 (25.3%) were positive for spirochetes examined under the dark-field microscope (DFM). Of the 55 culture and 49 DNA samples, 38/266 (14.3%) were identified to be positive for pathogenic *Leptospira* confirmed by *secY* and *lipL32* PCR. Phylogenetic analysis by *secY* PCR showed unique clusters for each species and all isolates clustered according to the respective species. *Leptospira interrogans* dominated all studied sites followed by *L. kirschneri* (n=5), *L. borgpetersenii* (n=2) and *L. weilii* (n=1). However, no significant association was shown between the infection rate in small mammals with three different sites category (urban, semi-urban and recreational forest) (chi-square, x2 0.5296; p= 0.767). From MLST analysis, three clones of *Leptospira* sp. were found to dominate the study sites; *L. interrogans* serovar Bataviae ST50, *L. kirschneri* serovar Grippotyphosa ST110 and *L. borgpetersenii* serovar Javanica ST143. While with the help of curator of MLST database, a new ST number (ST238, ST242 and ST243) representing new *L. interrogans* and *L. weilii* species was curated for samples isolated mostly from the recreational forest area sites. From the present study, *R. norvegicus* was identified as the common pathogenic *Leptospira* host dominating the urban area followed by *R. rattus*. However although *M. whiteheadi* dominated the recreational forest area, *S. muelleri* was the major carrier of the pathogenic *Leptospira.* In conclusion two species of rats (*R. norvegicus* and *S. muelleri*) were identified to play a vital role in environmental contamination in all study sites. This study also confirms for the first time, carriers of *Leptospira* locus ST110 *L. kirschneri,* ST242 *L. weilii,* ST238 *L. interrogans* and ST243 *L. interrogans* among small mammals in Malaysia. Thus further research and attention on the serovar status, of all isolates should be carried out as these species may potentially become highly pathogenic serovars contributing to increased risk of severe leptospirosis in Malaysia. In addition, these strains should be included into MAT panels as to improve the diagnosis in the future.

Keywords: Leptospirosis, *Leptospira* sp., rodents,

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ISOLASI DAN PENCIRIAN BAKTERIA *LEPTOSPIRA* DARI TIKUS DAN MAMALIA KECIL DI KAWASAN YANG DISYAKI PUNCA WABAK LEPTOSPIROSIS DI SELANGOR, MALAYSIA

Oleh

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Leptospirosis dahulunya merupakan penyakit zoonotik yang terabai di kawasan tropik di Malaysia dan muncul semula sebagai ancaman kepada kesihatan awam dalam persekitaran bandar dan luar bandar. Secara umumnya tikus adalah pembawa utama bakteria *Leptospira* yang merupakan punca penyakit leptospirosis. Di Malaysia, penyebaran wabak penyakit ini tular di kawasan persekitaran yang kotor terutamanya kawasan perumahan yang mempunyai jumlah penempatan tertinggi dan kawasan rekreasi yang sering dilawati. Statistik di Malaysia juga turut mendedahkan kenaikan kadar pesakit yang disyaki menghidap penyakit leptospirosis dan kadar kematian juga turut meningkat sejak tahun 2012. Justeru, kajian ini dijalankan bagi mengetahui spesies bakteria *Leptospira* yang manakah menjadi punca utama kepada penyakit leptospirosis yang dibawa dikalangan pelbagai spesies mamalia kecil di Malaysia. Objektif kajian ini dijalankan adalah untuk mengenalpasti spesies bakteria *Leptospira* yang menjadi punca utama kepada penularan wabak leptospirosis di kalangan manusia terutamanya di kawasan Selangor. Kajian ini telah dijalankan pada Januari 2016 hingga April 2017 di enam kawasan yang disyaki penularan wabak yang dilabel sebagai kawasan urban, semi-urban dan hutan rekreasi. Keenam-enam kawasan ini telah disyorkan sebagai kawasan disyaki wabak oleh Kementerian Kesihatan Malaysia dan Jabatan Kesihatan Negeri Selangor. Penangkapan sampel tikus dan mamalia kecil telah dilakukan di enam lokasi seperti yang telah disenaraikan. Isolasi bakteria hidup *Leptospira* telah dilakukan dengan menggunakan teknik pengkulturan dan pengimejan melalui mikroskop lapangan gelap (DFM). Identifikasi status pembawa penyakit telah dilakukan dengan menggunakan teknik PCR. Identifikasi PCR menggunakan beberapa primer telah dilakukan termasuklah *secY* and *lipL32* dan kombinasi primer dengan teknik MLST telah dijalankan. Sebanyak 14 spesies tikus dijumpai daripada 266 jumlah mamalia kecil yang telah disampelkan di mana spesies dari *Rattus norvegius* (66%, n=100) merupakan populasi terbanyak di kawasan urban manakala *Maxomys whiteheadi* (30%,

n=23) merupakan spesies terbanyak di kawasan hutan rekreasi. Daripada 266 sampel mamalia kecil yang ditangkap, 217 sampel telah diproses bagi isolasi bakteria melalui teknik kultur, manakala pengekstrakan DNA bakteria *Leptospira* daripada baki 49 sampel lagi telah dilakukan dan tiada kultur dilakukan bagi sampel ini. Daripada 217 sampel organ yang dikultur, hanya 55/217 (25.3%) sahaja yang menunujukkan penampakan bakteria *Leptospira* di bawah DFM, manakala keputusan PCR yang telah dijalankan ke atas 55 sampel positif kultur dan 49 sampel DNA menunjukkan sebanyak 38/266 (14.3%) sampel positif terhadap *Leptospira* bakteria bahaya penyebab leptospirosis. Analisis filogenetik dengan penggunaan primer *secY* menunjukkan keunikan dan kepelbagaian di dalam genetik sampel yang terisolat dan majoriti spesies *Leptospira* dari *L. interrogans* (n=30) mendominasi semua kawasan kajian diikuti oleh *L. kirschneri* (n=5), *L. borgpetersenii* (n=2) dan *L. weilii* (n=1). Walaubagaimanapun, tiada hubung kait ditunjukkan daripada analisa statistik di antara haiwan pembawa bakteria *Leptospira* di tiga kategori kawasan persampelan (urban, semi-urban dan hutan rekreasi) (chi-square, x^2 0.5296; p= 0.767). Daripada analisa MLST, tiga serovar bakteria *Leptospira* yang mendominasi kawasan kajian dikenali sebagai *L. interrogans* serovar Bataviae (ST50), *L. kirschneri* serovar Grippotyphosa (ST110) dan *L. borgpetersenii* serovar Javanica (ST143). Manakala id (ST) baru (ST238, ST242 dan ST243) telah diberikan kepada isolat bakteia *L. interrogans* dan *L. weilii* yang tidak didapati di dalam pangkalan data MLST. Kesimpulan daripada kajian ini mendapati, spesies mamalia kecil dari tikus yang dikenali sebagai *R. norvegicus* merupakan spesies yang utama pembawa bakteria bahaya *Leptospira* di kawasan urban diikuti dengan *R. rattus*. Manakala, spesies tikus dari *S. muelleri* merupakan pembawa utama bakteria bahaya *Leptospira* di kawasan hutan rekreasi walaupun populasi terbanyak yang mendominasi kawasan tersebut adalah dari spesies *M. whiteheadi*. Justeru daripada pendapatan kajian ini, dua species tikus ini merupakan spesies pembawa utama yang menjadi punca dalam penularan wabak leptospirosis. Kajian ini adalah yang pertama menjumpai isolat ST110 *L. kirschneri* dan ST242 *L. weilii*, ST238 *L. interrogans* dan ST243 *L. interrogans* di kalangan haiwan mamalia kecil di Malaysia. Justeru, pengesahan kajian selanjutnya dan perhatian mengenai status serovar bagi setiap isolat haruslah dijalankan kerana serovar dari isolat ini mungkin boleh menjadi punca penyebab jangkitan *Leptospira* yang serius di Malaysia. Selain itu, kepentingan menambah senarai panel serovar terutamanya isolat dari Malaysia bagi meningkatkan keberkesanan diagnosis penyakit leptospirosis di masa hadapan.

Kata kunci: Leptospirosis, spesies *Leptospira,* tikus

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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 Master of Science. The members of the Supervisory Committee were as follows:

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LIST OF ABBREVIATIONS

bp	Basepair
BSA	Bovine Serum Albumin
Buffer AL	DNA Binding Buffer
Buffer ATL	Tissue Lysis Buffer
Buffer W1	Wash Buffer
Buffer W2	Desalting Buffer
Buffer AE	Elution Buffer
CAAT	Cross Agglutination Absorption Test
D	Simpson's Diversity Index,
DFM	Dark-Field Microscope
dH_2O	Distilled water
DNA	Deoxyribonucleic acid
DMSO	Dimethylsulfoxide
EMJH	Liquid Ellinghausen, Mccullough, Johnson, Harris
g	Gram
gyrB	DNA *gyrase* subunit B
L	Litre
LPS	Lipopolysaccharide
M	Molar
MAT	Microscopic Agglutination Test
mg	Miligram
mg/ml	Milligram per mililitre
min	Minute
mL	Mililitre

MLST	Multi Locus Sequence Typing
mM	Milimolar
NCBI	National Center of Biotechnology Information
ng	Nanogram
PBS	Phosphate Buffered Saline
PCR	Polymerase chain reaction
PFGE	Pulse Field Gel Electrophoresis
pH	Potential of hydrogen
rpoB	RNA polymerase-β-subunit
rRNA	Ribosomal ribonucleic acid
secY	Preprotein translocase subunit
rrs	16S Ribosomal ribonucleic acid
SPSS	Statistical Package for the Social Sciences
ST	Sequence Type
TBE	Tris-Borate-EDTA
UV	Ultraviolet
WHO	World Health Organization
µg	Microgram
µg/mL	Microgram per mililitre
µL	Microlitre
5-FU	5-Fluorouracil

CHAPTER 1

INTRODUCTION

1.1 Study Background

Leptospirosis locally known as rat urine disease is a bacterial infection caused by the corkscrew-shaped spirochete *Leptospira* (Bharti et al., 2003). Rodents serve as the main reservoir for *Leptospira* although all mammals do carry the bacteria in their kidney. *Leptospira* replicates in the renal tubules of rodents, domestic and wild animals, which are then shed in the environment through their urine (Sumanta et al., 2015). In rodents, *Leptopsira* has the ability to escape from the host immune system and then establish persistent renal colonization by forming an amorphous, biofilm-like structure in the renal tubules (Agudelo-Flórez et al., 2013). Human gets infected either through contact with contaminated animal urine or body fluids or from water and soil contaminated with leptospiral spp. (Levett, 2001; Matthias et al., 2008). Human leptospirosis is reported to be concentrated in areas where rats are highly populated due to improper trash management and poor sanitation (Barcellos & Sabroza, 2001; Bharti et al., 2003). The spirochete *Leptospira* enter the human body through the skin (cuts or wound) or mucous membranes (eyes, nose, or mouth). The disease is usually vulnerable among poverty in middle and low-income countries and considered as occupational disease, and this includes people who work in a paddy field, sewer, gold mining, food markets and farmland (Schneider et al., 2013; WHO, 2003a).

Leptospirosis is known to be highly endemic in tropical and subtropical regions (Bharti et al., 2003), nevertheless a wide geographical distribution with global annual cases of 1.03 million and 58,999 deaths are reported (Costa et al., 2015). A systematic review revealed Oceania region (Australia, New Zealand and pacific island countries and territories) to be the most affected by leptospirosis with morbidity (150.68 cases/100,000 populations per year) and mortality (9.61 deaths/ 100,000 populations per year) (Costa et al., 2015). Recreational activities and ecotourism served as the leading cause of leptospirosis in the younger generation (Mayer-Scholl et al., 2014; Sejvar et al., 2003; Van Crevel et al., 1994). Few outbreaks in Malaysia that is associated with recreational activities include: outbreak associated with swimming in Beaufort (Koay et al., 2004), outbreak of melioidosis co-infection with leptospirosis (Sapian et al., 2012), and a death case of a national service trainee died of suspected leptospirosis (Thayaparan et al., 2013). Then followed by a few recent sporadic cases reported in national daily news and the press release by the officials of the ministry of health include: two death cases of the students swimming in contaminated rivers in Jeram and waterfall in Gunung Pulai in the year 2017 respectively and another case involving student infected with leptospirosis upon visitation to the recreational park in Jelebu (Garba et al., 2017).

Leptospira which are classified into pathogens, intermediates and saprophytes are distributed to 23 species to date comprising ten pathogenic *(Leptospira interrogans, L. kirschneri, L. borgpetersenii, L. santarosai, L. noguchii, L. weilii, L. alexanderi, L. kmetyi, L. alstonii* and *L. mayottensis*), six intermediates (*L. inadai, L. broomii, L. fainei, L. wolffii, L. licerasiae* and *L. venezuelensis*) and seven saprophytes (*L. biflexa, L. wolbachii, L. meyeri, L. vanthielii, L. terpstrae, L. idonii* and *L. yanagawae*) (Bourhy et al., 2014). Atleast 20 serogroups and more than 300 serovars have been described (Mayer-Scholl et al., 2014; Picardeau, 2013). Pathogenic species are usually carried by rodents, while intermediates and saprophytes are commonly seen in the environment (water or soil). To date based on serological or bacteriological diagnosis, at least thirty-seven serovars have been isolated and characterized with majority of them carried by rodents (Benacer et al., 2013).

In Malaysia, leptospirosis is an emerging public health problem affecting both urban and rural population hence has been gazetted as a notifiable disease since 2010 by the Ministry of Health, Malaysia. According to them, the incidence of leptospirosis showed an increasing trend from 263 in 2004 to 5370 in 2015 with mortality of 20 to 96 cases per annum. Eventhough the reported number of clinical leptospirosis cases in Malaysia is high, the number of laboratory-confirmed cases is low. One of the contributing factors could be the information on the circulating serovars or infecting strains are limited. Updating the serovar panel is utmost important in diagnostic assays for leptospirosis. The epidemiology of the leptospirosis is not clear in Malaysia despite rodents were identified as the major reservoirs. Hence, the present study is undertaken to perform a systemic investigation on the *Leptospira* strains circulating among small mammals with primary focus on rodents in the urban, semi-urban and recreational forest sites where human leptospirosis are frequently reported in the Selangor state of Malaysia. The study was aim to identify the circulating pathogenic species and their associated animal reservoirs. This will guide public health professionals in further epidemiological investigations to determine the risk factors associated with leptospirosis in Malaysia and also to improve the diagnostic panel with more pathogenic species.

1.2 Problem Statement

In Malaysia, leptospirosis is an emerging public health problem affecting both urban and rural population. The listing of leptospirosis as a notifiable disease since 2010 has shown increasing trends of infection incidence and mortality rates. Rodents especially rats serve as the main reservoir for supplying pathogenic leptospiral strains for human infection in Malaysia as elsewhere. In Malaysia, a majority of the cases are either due to vacationing at recreational forest waterfalls or from poorly maintained non-hygienic residential premises. Although most clinical symptoms and laboratory indications suspected for human leptospirosis, most cases do not achieve a confirmed diagnosis. One of the main reasons is the lack of comprehensive data on the circulating strains, which is not only essential to understand the potential zoonosis, but also very crucial to be included in the diagnostic panel especially the gold standard microscopic

agglutination test (MAT). Secondly, for any public health measures (awareness, control, and prevention) to be taken, the knowledge on the ecological niche of the reservoir animals or maintenance host, the prevalence, and type of the leptospiral species and characterization of strains is utmost important. Hence, the present study is exceptionally important to obtain the baseline data on circulating and prevailing strains, reservoir host and their ecological niche, and most importantly live strains to be included in MAT panel. This study may also help unravel the mystery behind the suspected leptospirosis cases frequently reported from these geographical niches.

1.3 Objectives

1.3.1 General Objective

To obtain a comprehensive understanding of the leptospiral species circulating among rodents and small mammals inhabiting selected residential and recreational sites suspected of human leptospirosis in the Selangor state of Malaysia.

1.3.2 Specific Objectives

1. To determine leptospires carrying small mammals and to determine the reservoir animal species in the study population.

2. To identify the prevailing pathogenic *Leptospira* species.

3. To determine the genetic relationship among the *Leptospira* strains and identify the circulating genotypes.

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LIST OF PUBLICATIONS

Azhari, N. N., Ramli, S. N. A., Joseph, N., Philip, N., Mustapha, N. F., Ishak, S. N., Mohd-Taib, F. S., Md Nor, S., et al. (2018). Molecular characterization of pathogenic Leptospira sp. in small mammals captured from the human leptospirosis suspected areas of Selangor state, Malaysia. *Acta tropica*, *188*, 68-77. (Published)

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