SEROLOGICAL AND MOLECULAR CHARACTERIZATION OF PATHOGENIC *LEPTOSPIRA* AMONG SMALL MAMMALS FROM SELANGOR WET MARKETS, MALAYSIA

By

NORLIZA BINTI BAHTIAR AFFENDY

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

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

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Chairman : Siti Norbaya binti Masri, MD, MPath
Faculty : Medicine and Health Sciences

In Malaysia, high number of leptospirosis cases observed in urban areas was concentrated with rat populations. The increasing trend of leptospirosis cases and death reports has called for an urgent need to study the *Leptospira* species in the animals. Thus, this study was conducted to investigate the serological and molecular epidemiology of *Leptospira* among small mammals in Selangor wet markets from December 2016 to February 2018. The serum of the captured animals was collected for the detection of leptospiral antibodies via Microscopic Agglutination Test (MAT). The rodent's kidneys were also harvested and subjected to *Leptospira* isolation by culture method and polymerase chain reaction (PCR) using *flaB* genes. Then, all positives samples were subjected to multi-locus sequence typing (MLST) Scheme 1 for genotypic characterization. A total of 89 small mammals captured were identified as: *Rattus norvegicus* (53.9%), *Rattus rattus* (23.6%) and *Suncus murinus* (22.5%). From 89 serum samples, 19.1% showed presence of leptospiral antibodies and reacted to three serovars; serovar Bataviae (n=14; 15.7%), serovar Javanica (n=2; 2.2%) and serovar Patoc (n=1; 1.1%) Whereas, for 89 culture samples, 16.9% (n=15) showed positive growth of spirochetes in which all of them were positive for pathogenic *Leptospira* via PCR of *flaB* gene. Polymerase chain reaction of 89 kidney samples showed 31.5% (n=28) positive for *flaB* gene. The phylogenetic analysis of *flaB* gene on 31 samples (15 culture isolates and 16 kidney samples without duplicate sample source) revealed 2 clusters of species with *L. interrogans* (n=28; 90.3%) being the predominant species and *L. borgpetersenii* (n=3; 9.7%). Genotyping by MLST was successfully performed on 27 samples and three clones namely *L. interrogans* serovar Bataviae ST 50 (n=19), *L. interrogans* ST205 (n=7) and *L. borgpetersenii* serovar Javanica ST 143 (n=1) were identified. In conclusion, a high detection rate of pathogenic *Leptospira* and its antibody in small mammals indicates wet market may pose a risk in spreading leptospirosis. The identified serovars in animals are also the common serovar found in infected human, indicating the inter-relationship of carriage and host to cause the disease.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

PERINCIAN SEROLOGI DAN MOLEKULAR *LEPROSPIRA* PATOGENIK DALAM MAMALIA KECIL DARI PASAR BORONG SELANGOR, MALAYSIA

Oleh

NORLIZA BINTI BAHTIAR AFFENDY

Julai 2020

Pengerusi : Siti Norbaya binti Masri, MD, MPath
Fakulti : Perubatan dan Sains Kesihatan

Di Malaysia, kes leptospirosis dicatatkan di kawasan bandar yang tinggi populasi tikus. Peningkatan kes dan laporan kematian akibat leptospirosis menggesa kepada keperluan kajian ke atas spesies *Leptospira* di dalam haiwan. Oleh itu, kajian ini bertujuan untuk menyiasat epidemiologi antibodi dan molekul *Leptospira* dalam mamalia kecil di pasar basah di Selangor dari Disember 2016 hingga Februari 2018. Sampel serum haiwan ditangkap telah diambil bagi pengesanan antibodi *Leptospira* melalui ujian penggalutinatan mikroskopik (MAT). Manakala ginjal roden juga diambil untuk pemencilan *Leptospira* melalui kaedah pengkulturan dan Tindak Balas Berantai Polymerase (PCR) menggunakan gen *flaB*. Setelah itu, kesemua sampel positif diunjurkan kepada Penjenisan Jujukan Multi-lokus (MLST) Skema 1 bagi tujuan penjenisan gen. Sejumlah 89 mamalia kecil ditangkap dan dikenal pasti sebagai: *Rattus norvegicus* (94.1%), *Rattus rattus* (23.6%) and *Suncus murinus* (22.5%). Daripada 89 sampel serum, 19.1% menunjukkan kehadiran antibodi *Leptospira* dan bertindak balas ke atas tiga serovars: serovar Bataviae (n=14; 15.7%), serovar Javanica (n=2; 2.2%) and serovar Patoc (n=1; 1.1%). Selain itu, daripada 89 sampel kultur, 16.9% (n=15) telah menunjukan pertumbuhan positif spiroket di mana ke semuanya positif bagi *Leptospira* patogenik melalui PCR gen *flaB*. Manakala, Tindak Balas Berantai bagi 89 sampel ginjal menunjukkan 31.5% (n=28) positif kepada gen *flaB*. Analisi pohon filogenetik gen *flaB* terhadap 31 sampel (15 sampel kultur dan 16 sampel ginjal tanpa sumber sampel pendua) menunjukkan dua kumpulan species di mana *L. interrogans* (n=28; 90.3%) menjadi spesies dominan dan *L. borgpetersenii* (n=3; 9.7%). Penjenisan gen melalui MLST telah berjaya dilakukan ke atas 27 sampel dan tiga klon: *L. interrogans* serovar Bataviae ST 50 (n=19), *L. interrogans* ST205 (n=7) and *L. borgpetersenii* serovar Javanica ST 143 (n=1) telah dikenal pasti. Secara ringkasnya, kadar pengesanan *Leptospira* patogenik dan antibodi yang tinggi di dalam mamalia kecil menunjukkan bahawa pasar basah berisiko dalam penyebaran leptospirosis. Serovar yang telah dikenal pasti pada haiwan adalah serovar sepunya yang ditemui pada manusia, menunjukkan saling perhubungan di antara pembawa dan perumah yang menyebabkan penyakit ini.

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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:

Siti Norbaya binti Masri, MD, MPath
Associate Professor (Medical)
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Chairman)

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

Fairuz binti Amran, MPath
Unit Head of Bacteriology
Infectious Diseases Research Centre (IDRC)
Insittute for Medical Medical Research
(Member)

_____
ZALILAH MOHD SHARIFF, PhD
Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

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Name of Chairman of Supervisory Committee: Assoc. Prof. Dr. Siti Norbaya binti Masri

Signature: _____
Name of Member of Supervisory Committee: Assoc. Prof. Dr. Mohd Nasir bin Mohd Desa

Signature: _____
Name of Member of Supervisory Committee: Dr. Fairuz binti Amran

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

bp	Base pair
cm	centimeter
DNA	Deoxyribonucleic acid
EMJH Media	Ellighausen, Mccullough, Johnson, Harris Media
flaB gene	Flagellin B gene
g	Gram
IMR	Institute for Medical Research
L	Litre
LPS	Lipopolysaccharide
MAT	Microscopic Agglutination Test
mg	Milligram
mg/ml	Miligram per millilitre
ml	Millilitre
MLST	Multi Locus Sequence Typing
NCBI	National Centre of Biotechnology
ng	Nanogram
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
pH	Potential of Hydrogen
rRNA	Ribosomal ribonucleic aicd
SPSS	Statistical Package for the Social Sciences
ST	Sequence Type
TBE	Tris-Borate-EDTA

UV	ultravoilet
WHO	World Health Organization
μL	Microliter
5-FU	5-Fluorouracil

CHAPTER 1

INTRODUCTION

1.1 Background of the Study

Leptospira is a spirochaete bacteria that belongs to the family of *Leptospiraceae*, which is further categorized into three major groups; pathogenic, intermediate and saprophytic groups (Faine 1994; Levett 2001). The pathogenic species of *Leptospira* cause a worldwide disease known as leptospirosis (World Health Organization, 2010), also known as a rat-urine disease in Malaysia (Fong, 2018). It was first described as the causative agent of severe human syndrome Weil's disease by Adolph Weil in 1886 (Adler and de la Peña Moctezuma, 2010; Karpagam and Ganesh, 2020). Subsequently, a study done in Japan indicates that *Leptospira* can also be found in animals (Ido et al., 1917; Adler and de la Peña Moctezuma, 2010). Currently, more than 20 serogroups with more than 350 serovars comprises both mlpathogenic and saprophytic *Leptospira* strains has been identified worldwide (Trott et al., 2018; Karpagam and Ganesh, 2020) and in Malaysia approximately 37 serovars had been found from human and animal samples (Ab Rahman et al., 2018). The antigenic variation among serovars differentiates between *Leptospira* species (Adler 2015; Levett, 2001).

Leptospirosis has emerged as globally important infectious disease that not only occurs in rural areas but also urban environments of industrialized and developing countries worldwide, with tropical regions recorded the highest number of incidence (Bharti et al., 2003; Brockmann et al., 2010). Every year, an estimated of 1.03 million cases and 58,900 deaths recoded around the world (World Health Organization, 2010) indicating major threat to public health sector and in Malaysia there were approximately 41, 736 probable and laboratory confirmed leptospirosis cases with 502 deaths were recorded (data from Ministry of Health, Malaysia). The major challenge in clinical diagnosis of leptospirosis is non-specific and varies disease manifestations ranging from asymptomatic to potentially fatal (Levett, 2004; Adler, 2015; Garba et al., 2017). It is often misdiagnosed with commonly found tropical diseases such as malaria, dengue, typhoid as well as viral infections (Ridzuan et al., 2016).

Pathogenic *Leptospira* have the ability to replicate in renal tubules of animals, making the animals as the carrier as they shed *Leptospira* into environments through their urine (Levett, 2001; Ellis, 2015). Previous studies reported that most mammals' especially domestic and wild animals are natural reservoirs for leptospires, which they infrequently act as maintenance and accidental host (Levett, 2001; Garba et al., 2017). Meanwhile, rodents are found to be served as the main reservoir for *Leptospira* and important source of infection for human and other susceptible animals (Levett 2001; Adler, 2015; Garba et al., 2017). The finding of rats as the renal and asymptomatic carrier for pathogenic *Leptospira* was proven by Inada et al., in 1917 through the observation and investigation on wide range of rodents including house and wild rats (Noguchi, 1918; Levett, 2001;

Adler, 2015). The ability of *Leptospira* infecting rodents without compromising the animal immune response remains unknown.

Human is the accidental host as the infection is acquired from direct contact with the urine of the infected animals and indirect contact with contaminated soil or water (Levett, 2001; Adler and de la Peña Moctezuma, 2010). The transmission is often related to individual occupational, recreational or avocational activities (Adler, 2015; Garba et al., 2017). *Leptospira* may enter the body through cuts and abrasions of skin or an intact mucous membrane such as the conjunctival or oral surfaces (Faine, 1994; Levett 2001; Adler 2015). Different incubation period of the bacteria in an individual depending on the immune response cause the detection of the disease more difficult (Musso and La, 2013; Adler 2015). To date, laboratory diagnostic tests for leptospirosis comprise of serology, culture and polymerase chain reaction (PCR) detection (Levett, 200; Musso and La, 2013; Adler, 2015). The sensitivity of the tests depends on the time of sample collection and duration of the symptoms (Cumberland et al., 1999).

Leptospirosis has been considered to be primarily an occupational disease that associated with work-related activities such as farming, butchering, sewer maintenance and military maneuver (Bahaman and Ibrahim, 1988; Rafizah et al., 2013; Garba et al., 2017). Market is a place that plays an important role in society globally and contributes to income generation in the Asia region specifically with various type of markets available such as night market and wet market (Feng and Wu, 2016; Sarnobat et al., 2019). In Malaysia, wet market is a place for local to find daily basis need due to its reasonable price (Azman et al., 2012; Feng and Wu, 2016). Despite a wide range of option, the level of cleanliness causes major concern and can be questionable (Webster 2004; Ab Rahman et al., 2018). Waste produced from the market does not being managed properly, thus the area becoming infestation area for the pests such as rats (Samsudin et al., 2018). This situation leads the wet markets to become one of the potential areas for disease transmission causing the visitors and market workers as susceptible groups to acquire the disease from the contaminated environments or direct contact with infected animals (Webster, 2004; Woo et al., 2006).

1.2 Problem Statement

The cycle of leptospirosis transmission involves a complex interaction between human, environments as well as the animal reservoirs as they coexist (Lau, 2016). It is crucial to build comprehensive data on the circulating strains not only to understand the potential zoonosis but also developing useful tools for a diagnostic test. However, in Malaysia, there is limited information on the antibody and genotype of *Leptospira* in animals. Previous study had reported on the finding of the World Health Organization (WHO) and local serovars in human. It is important to identify the strains or serovars of Leptospira in animals as the disease transmission is due to close contact between human and animals. One of the factors that contribute to the low serological detection of *Leptospira* in human in Malaysia is due to the list of serovars in the MAT panel are mainly from WHO serovars, in which it might be different with the serovars circulating in Malaysia. So, the newly identified local strains or serovars in animals from culture can

be used as an antigen in MAT. Thus, the sensitivity of the test can be increased in the future. Thus, there is a need to study the serological and genotype of *Leptospira* in animals in order to provide more data on the *Leptospira* and its relationships with the animals.

1.3 Objectives

1.3.1 General Objective

To study the serological and molecular epidemiology of *Leptospira* in small mammals captured from Selangor wet markets.

1.3.2 Specific Objectives

1. To determine the seropositivity of leptospiral antibody and serovar distribution in small mammals via MAT.
2. To detect *Leptospira* species in small mammals by culture and PCR techniques.
3. To identify and characterize pathogenic *Leptospira* via *flaB* gene amplification and MLST.

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