UNIVERSITI PUTRA MALAYSIA

PREVALENCE OF LEPTOSPIRA SPECIES IN WATER AND SOIL IN SIX STATES OF PENINSULAR MALAYSIA AND THE PATHOGENICITY OF L. HEBDOMADIS

FAIRUZ RIDZLAN BIN A. RASHID

FPV 2011 9

Dedicated to my twin brother and younger sister

“NUNC AUT NUNQUAM”

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By

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

PREVALENCE OF *LEPTOSPIRA* SPECIES IN WATER AND SOIL IN SIX STATES OF PENINSULAR MALAYSIA AND THE PATHOGENICITY OF *L. HEBDOMADIS*

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Chairman: Prof. Dato' Abdul Rani Bahaman, PhD

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Leptospirosis is recognized as one of the important zoonotic diseases in the world including Malaysia. The tropical condition and rainy season in Malaysia favour the growth of leptospires and have potentially cause leptospirosis outbreak. Overall, a total of 902 water samples and 231 soil samples were collected from selected places in Peninsular Malaysia. The water and soil samples were filtered and inoculated into semisolid Johnson-Seiter (JS) medium, incubated at room temperature and in dark condition for 2 months. The cultures were examined under the dark-field microscope for growth of leptospires. A series of characterization such as 8-Azaguanine Inhibition Test, Polymerase Chain Reaction (PCR) assay and serogrouping by Microscopic Agglutination Test (MAT) were done to the isolates to identify whether they are

pathogenic leptospires. Among the leptospiral isolates, one isolate was selected to study its pathogenicity in hamster model. Giemsa stain, bacterial culture, serological examination, PCR assay and histopathology of target organ were done to determine the pathogenicity of the isolate to the animal model.

A total of forty-three cultures (3.8%) exhibited positive growths which were seen under dark field microscope. The positive cultures were from 39 water and 4 soil samples. Among leptospiral isolates, only 21% (9/43) were confirmed as pathogenic *spp.* based on 8-Azaguanine Test and PCR. Serogrouping of the isolates with MAT showed that *hebdomadis* was the dominant serovar in 4 isolates. In the experimental animal study, growth of leptospires was not seen in all bacterial cultures. However, through PCR assay, leptospires were detected in blood and kidney samples at Day 5 post inoculation. Besides that, the antibody titre produced against the isolate was at 1:160. Histologically, selected isolates produced hemorrhagic glomerulitis, tubulointerstitial nephristis, and necrosis in hepatic and splenetic cell.

In conclusion, the results demonstrated that pathogenic leptospires can be detected in Malaysian environment and an isolate obtained can cause leptospiral infection to hamsters. Understanding the prevalence of leptospirosis is important to target the sources of contamination and risk activities associated at reside places can be prevented.

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

PREVALENS SPESIS *LEPTOSPIRA* DARI AIR DAN TANAH DI ENAM NEGERI DI SEMENANJUNG MALAYSIA DAN PATOGENISITI OLEH *L. HEBDOMADIS*

Oleh

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Leptospirosis diiktiraf sebagai salah satu daripada penyakit zoonotik penting di dunia termasuk Malaysia. Keaadan tropika dan musim hujan di Malaysia memihak kepada pertumbuhan leptospires dan mempunyai potensi untuk menyebabkan wabak leptospirosis. Secara keseluruhan, sebanyak 902 sampel air dan 231 sampel tanah telah diperoleh dari tempat-tempat terpilih di Semenanjung Malaysia. Sampel air dan tanah telah ditapis dan dikultur pada media separa pepejal Johnson-Seiter (JS), dieram pada suhu bilik dalam keadaan gelap selama 2 bulan. Kultur diperiksa di bawah mikroskop medan gelap untuk melihat pertumbuhan leptospira. Suatu siri ujian pencirian seperti Ujian Perencatan 8-Azaguanine, Jujukan Berantai Polymerase (PCR) dan serogrouping oleh Ujian Penggumpalan Mikroskopik (MAT) telah dilakukan ke atas isolat bagi

mengenal pasti leptospira patogen. Antara kesemua isolat leptospira, satu isolat telah dipilih untuk mengkaji tahap patogen dengan menggunakan hamster. Giemsa stain, kultur bakteria, pemeriksaan serologi, PCR dan histopatologi telah dikaji pada organ sasaran untuk menentukan tahap isolat pada hamster.

Sebanyak 43 sampel (3.8%) menunjukkan pertumbuhan positif yang dilihat di bawah mikroskop medan gelap. Kultur positif adalah daripada 39 sampel air dan 4 sampel tanah. Antara isolat leptospira, hanya 21% (9/43) telah disahkan sebagai patogen berdasarkan Ujian 8-Azaguanine dan PCR. Serogrouping daripada isolat dengan MAT menunjukkan bahawa *hebdomadis* adalah serovar yang dominan pada dalam empat isolat. Dalam kajian haiwan uji kaji, pertumbuhan leptospira tidak dilihat untuk kesemua kultur bakteria. Walau bagaimanapun, melalui PCR, leptospira dikesan dalam sampel darah dan buah pinggang pada Hari ke-5 pos inokulasi. Selain itu, titer antibodi yang dihasilkan oleh isolat ialah 1:160. Mengikut pemeriksaan histologi, isolat terpilih itu menghasilkan glomerulitis berdarah, nephristis tubulointerstitial, dan nekrosis di hepatik dan sel limpa.

Kesimpulannya, leptospira patogen boleh dikesan di persekitaran Malaysia dan isolat yg diperoleh boleh menyebabkan jangkitan leptospiral kepada hamster. Pemahaman terhadap prevalens leptospirosis adalah penting untuk mengenal pasti punca kontaminasi dan bahaya aktiviti di persekitaran yang terlibat dapat dicegah.

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APPROVAL

I certify that a Thesis Examination Committee has met on 29th December 2011 to conduct the final examination of Fairuz Ridzlan bin A. Rashid on his thesis entitled "PREVALENCE OF *LEPTOSPIRA* SPECIES IN WATER AND SOIL IN SIX STATES OF PENINSULAR MALAYSIA AND THE PATHOGENICITY OF *L. HEBDOMADIS*" 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 Master of Science.

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DECLARATION

I declare that the thesis is my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously, and is not concurrently submitted for any other degree at Universiti Putra Malaysia or at any other institutions.

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FAIRUZ RIDZLAN BIN A. RASHID

Date: 29th December 2011

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

bp	base pair
ºC	degree Centigrade
dH_2O	distilled water
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
e.g.	for example
EDTA	ethylenediamine tetraacetic acid
EtBr	ethidium bromide
g	gram
H_2O	water
HCl	hydrochloric acid
kb	kilobase pair (number of bases in thousands)
M	molar, or molarity, moles of solute per liter of solution
$MgCl_2$	magnesium chloride
Min	minutes
ml	milliliter
mm	millimeter
mM	millimolar
µg	microgram
µl	microliter
mol	mole
NaCl	sodium chloride
NaOH	sodium hydroxide
ng	nanogram
%	percent
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolution per minute
Taq	Thermus aquaticus DNA (polymerase)

TBE	Tris borate EDTA electrophoresis buffer
UV	ultraviolet
V	volts

CHAPTER 1

INTRODUCTION

Leptospira can be divided into pathogenic and saprophytic species by pathogenicity and serological characterization. Under pathogenic leptospires cluster, it is known as *Leptospira interrogans* while *Leptospira biflexa* is referred as saprophytic leptospires. The leptospires species are further divided into serogroups and followed by serovars. Currently about 24 serogroups and almost 250 serovars of *L. interrogans* have been arbitrarily characterized (Palaniappan *et al*., 2007). Serovars that enclosed with overlapping antigenic determinant are clustered into a larger serogroups. Currently, in correspond with development of advanced genetic tool, for examples phylogenetic analyses of 16S rRNA genes, the leptospires species are categorized into three groups designated as pathogenic, saprophytic and intermediate (Evangelista and Coburn, 2010). Leptospirosis is now recognized as remerging global health problem and plays an important role as a zoonotic disease in the world (Vijayachari *et al*., 2008).

The pioneer study on leptospirosis in Malaysia was started by Fletcher in 1925. He reported the first fatal case of human leptospirosis and successfully detected *Leptospira icterohaemorhagiae* (Fletcher, 1928). Extensive work done by Alexander and workers (1975) from 1953 to 1955 had isolated and identified 30 pathogenic leptospiral serovars from civilian and army personnel. According to Gordon-Smith *et al*. (1961b), the predominant maintenance host of leptospirosis in Malaysia were rats. Other animals

such as dogs, pigs and cattle can probably contribute to occurrence of leptospirosis. Baker and Baker (1970) had developed a screening method for the isolation of waterborne leptospires which involved inoculation of the organisms into Golden Syrian hamsters. From 1928 until the present day, 38 leptospiral serovars from 17 serogroups have been successfully identified in animals and humans (Evangelista and Coburn, 2010). In 1967, leptospirosis was considered endemic with a high incidence of antibodies to leptospires observed among various occupational groups throughout the country (Ungku Omar, 1967).

In an area, whenever favourable conditions for leptospires to thrive are fulfilled, the disease can occur (Bharti *et al*., 2003). Several cases or outbreaks have been reported in various places in the past few years and Southeast Asia has been affirmed as highly endemic to human leptospirosis (Sugunan *et al*., 2009). The reason leptospirosis occurred in these areas were mainly attributed to natural disasters such as floods and cyclone. Other contributions related to the occurrence of the disease were low economic regions, poor sanitation management and agricultural activities (Evangelista and Coburn, 2010). The first isolation of leptospires was demonstrated by Inada *et al*. in1916; they investigated the transmission of this disease by challenging an animal model with infected patient blood. Two years later, the same study was done by a German researcher who detected spirochetes in the blood of guinea pigs after inoculation with the blood of infected soldiers (Faine *et al*., 1999).

Leptospires are ubiquitous and the primary source of the organism comes from reservoir of animals such as rodents (Bahaman and Ibrahim, 1988). Animal renal tubules which

have been colonized by leptospires will continuously contaminate the environment through the infected urine. Contamination of environment with pathogenic leptospires is considered as the source of leptospirosis and act as vital part in the widespread of the disease. There are two modes of transmission which can be either direct or indirect. In addition, direct transmission occurs when infected animal come in contact with a new host through body fluids, tissues or urine of acute infected animals or asymptomatic carriers. Direct transmission among animals can be initiated by transplacental, sexual contact or suckling milk from infected mother (Faine *et al*., 1999). Humans that handle animals or animal tissues are susceptible to leptospires. Veterinarians, butchers, rodent control workers, cattle and pig farmers may be exposed to leptospirosis as an occupational infection (Ellis, 1997).

On the other hand, indirect transmission ensues when an animal acquires leptospires from an environment that had been contaminated with urine of carrier animals. The possible route of indirect transmission is through conjunctiva, scratches, cuts or abrasions on skin surfaces (Faine *et al*., 1999). Water sports or other recreational activities may increase the possibilities for people to be infected by leptospires via contaminated waters. Outbreaks associated with recreational exposure to water have been reported from several countries including Malaysia. Sejvar *et al*. (2003) reported that some athletes, who participated in the eco-challenge Sabah 2000, a multi-sport expedition race, had developed febrile illness and were found to be serologically positive to leptospiral infection.

To date, prevalence of pathogenic leptospires in Malaysian environment is not extensively studied. The wet and warm climates of Southeast Asia especially in Malaysia should provide a suitable condition for leptospires to grow. Leptospires can survive for long period of time in the environment and certain favourable conditions are required for them to multiply such as pH, temperature and moisture (Smith and Self, 1955; Baker and Baker, 1970). Isolation of *L. interrogans* in captured rats proved a significant degree of environmental contamination through the reservoir excretions contributes the spread of the disease (de Faria *et al.,* 2008). In this study, it is hypothesized that pathogenic leptospires can be isolated and identified in water and soil samples in Peninsular Malaysia. Besides that, the pathogenicity of the isolate can be observed in experimental animal. Therefore, the objectives were:

1. To determine the prevalence of *Leptospira* in selected environment in Peninsular Malaysia,
2. To characterize the isolates by using phenotypic and molecular techniques,
3. To determine the pathogenicity of the leptospiral isolate in hamsters.

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