Faculty of Medicine and Health Sciences

Genetic Diversity Based on LipL32, 16S rRNA and ColA Expression in *Leptospira* spp. Isolated From Rodent Hosts in Samarahan and Gedong Districts in Sarawak

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DECLARATION

I declare that the work in this thesis was carried out in accordance with the regulations of Universiti Malaysia Sarawak. Except where due acknowledgements have been made, the work is that of the author alone. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.

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ABSTRACT

Leptospirosis is a major zoonotic disease in the world that is caused by *Leptospira* spp., that may cause acute febrile illness in mammals with varied pathogenicity and clinical disease. Among factors that have been associated with pathogenicity of *Leptopsira* are *LipL32* and *ColA* gene. *LipL32* has been identified to play an important role in TLR-dependent pathway and inducing erythrocytes. *ColA* (LA0872), a gene coding for bacterial collagenase, a common invasive enzyme which facilitates the leptospires to quickly invade and spread into internal organs. Gene or protein that had been expressed by leptospires during infection or within specific host may provide glimpses into leptospiral pathogenic strategies. The study was conducted in Samarahan Division, Sarawak involving two locations; Kota Samarahan and Gedong. Traps were set at the study sites and 31 rodents were caught with 96.7% of them are from *Rattus rattus* species. The kidneys and blood samples were cultured into Ellinghausen-McCullough-Johnson-Harris (EMJH) media added with 5-fluorouracil. DNA and protein isolates from positive cultures were extracted and analysed. From the 31 kidney samples of rodents caught, 28 kidney tissue samples (90.3%) showed presence of leptospire-like organism in cultures. PCR analyses utilizing *16S* gene primers confirmed the presence of leptospires, in 90.3% of rodents caught in the study area. Almost half of the leptospires detected were saprophytic (45.2%) and pathogenic *Leptospira* spp. (38.7%). The highest number of species detected was pathogenic *Leptospira*, *Leptospira kmetyi* strain SS-S7 with 38.7%, from the whole isolates sequenced when compared to GenBank, followed by, intermediate *Leptospira*, *Leptospira wolfii* strain SS-S17 detected (12.9%), and saprophytic *Leptospira*, *Leptospira biflexa* strain Patoc_DB58 (19.4%), *Leptospira wolbachii* serovar Codice strain and *Leptospira idonii* strain Eri-1 16S ribosomal RNA (6.5% respectively) and *Leptospira terpestrae* serovar Hualin strain LT 11-33 (16.1%). There were no related DNA

sequences retrieved from *LipL32* primers after multiple passages of cultures. The DNA sequence degree of homology was varied from 80% to 99% which were clustered into 3 clusters (A1, A2 and A3). Most of the isolates were delineated into cluster A2 with 15 isolates (48.4%) which is close to NR.043043.1 sequence retrieved from NCBI database, followed by A1 cluster with 11 isolates (35.5%) (KY411405.1), and A3 cluster with 4 isolates (12.9%) (KY411411.1). All samples that shown presence of collagenase was belong to cluster A2. Interestingly, ColA protein was not detected in all intermediate and saphrophytic isolates and only detected in eight of the samples (25.8%) which only infected with pathogenic strain, *Leptospira kmetyi.* The findings indicated that a high proportion of rodents in the study area were infected with *Leptospira* spp. and about a third of them were harboured with pathogenic strain, *Leptospira kmetyi*. It is found that *ColA* gene was expressed only in pathogenic leptospires, and might indicate its importance in the pathogenesis of pathogenic *Leptospira* spp. in rodent hosts.

Keywords: Leptospirosis, rodent, collagenase, *LipL32*, *ColA*, Sarawak

Kepelbagaian Genetik Berdasarkan LipL32, 16S rRNA, rrs dan Ekspresi ColA dalam Leptospira spp. yang dipencilkan dari Hos Roden Di Daerah Samarahan Dan Gedong, Sarawak

ABSTRAK

Leptospirosis adalah penyakit zoonosis utama di dunia yang berpunca daripada Leptospira spp., yang menyebabkan penyakit seperti demam akut pada mamalia dengan patogenisiti dan penyakit klinikal yang pelbagai. Antara faktor yang dikaitkan dengan kepatogenikan Leptopsira patogenik adalah gen LipL32 dan ColA. LipL32 telah dikenal pasti memainkan peranan penting dalam tindak balas melibatkan TLR dan merangsang penghasilan eritrosit. ColA (LA0872), adalah gen yang mengekodkan kolagenase bakteria, iaitu enzim invasif yang memudahkan Leptospira spp. menyerang dengan cepat dan tersebar ke organ dalaman. Gen atau protein yang telah dieksperesi oleh Leptospira spp. semasa jangkitan atau di dalam hos-spesifik dapat memberikan sedikit gambaran mengenai strategi patogenik Leptospira spp. Oleh itu, kajian telah dilakukan di Bahagian Samarahan, Sarawak yang melibatkan dua lokasi; Kota Samarahan dan Gedong. Perangkap yang dipasang di kawasan kajian tersebut dan sebanyak 31 ekor roden telah ditangkap dengan 96.7% daripadanya adalah spesies Rattus rattus. Sampel ginjal dan darah telah dikultur menggunakan media Ellinghausen-McCullough-Johnson-Harris (EMJH) yang telah ditambah dengan larutan 5-fluorouracil. Pencilan DNA dan protein dari kultur positif, diekstrak dan kemudiannya dianalisa. Daripada 31 sampel tisu ginjal roden yang ditangkap, 28 sampel (90.3%) menunjukkan adanya organisma-seperti Leptospira spp. di dalam kultur. Analisis PCR yang menggunakan primer gen 16S mengesahkan adanya kehadiran Leptospira, pada 90.3% roden yang ditangkap di kawasan kajian. Hampir separuh daripada Leptospira yang dikesan adalah saprofit (45.2%) dan patogenik (38.7%). Bilangan spesies yang paling tinggi dikesan adalah Leptospira patogenik, Leptospira kmetyi strain SS-S7 dengan peratusan sebanyak

38.7% dari seluruh pencilan yang dilakukan jujukan gen dan dibandingkan dengan GenBank, diikuti dengan Leptospira *pertengahan,* Leptospira wolfii *strain SS-S17(12.9%) dan* Leptospira *saprofit,* Leptospira biflexa *strain Patoc_DB58* (19.4%), Leptospira wolbachii serovar Codice strain and *Leptospira idonii strain Eri-1 16S ribosomal RNA (masing-masing 6.5%) dan* Leptospira terpestrae *serovar Hualin strain LT 11-33 (16.1%). Tiada jujukan DNA yang dihasilkan dari primer* LipL32 *setelah dikultur beberapa kali. Tahap variasi homologi turutan DNA adalah dari 80% hingga 99% yang telah dikelompokkan kepada 3 kluster (kluster A1, A2 dan A3). Bilangan isolat tertinggi adalah pada kluster A2 dengan 15 pencilan(48.4%) yang hampir dengan turutan NR.043043.1 (sumber pangkalan data NCBI), diikuti oleh kluster A1 dengan 11 pencilan (35.5%) (KY411405.1), dan kluster A3 dengan 4 pencilan (12.9%) (KY411411.1). Semua sampel yang menunjukkan adanya kolagenase terdiri dari kluster A2. Menariknya, protein ColA tidak dikesan pada semua pencilan pertengahan dan saprofit, malah protein g ColA hanya dikesan di dalam lapan sampel (25.8%) yang dijangkiti oleh strain patogenik,* Leptospira kmetyi. *Hasil kajian menunjukkan bahawa sebilangan besar roden di kawasan kajian dijangkiti* Leptospira *spp. dan kira-kira satu pertiga dari roden tersebut dijangkiti strain patogenik, iaitu* Leptospira kmetyi. *Kolagenase A hanya dapat dikenal pasti di dalam* Leptospira *patogenik, yang berkemungkinan menunjukkan kepentingannya dalam patogenesis spesies tersebut pada hos roden.*

Kata kunci: ***Leptospirosis, roden, kolagenase, LipL32, ColA, Sarawak***

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LIST OF ABBREVIATIONS

Å	Angstrom
BSA	bovine serum albumin
CO_2	Carbon dioxide
°C	Celsius
DNA	Deoxyribonucleic acid
DST	IgM dot-ELISA dipstick test
ECM	extracellular matrix
ELISA	enzyme-linked immunosorbent assay
EMJH	Ellinghausen-McCullough-Johnson-Harris
FALGPA	N-(3-[2-Furyl]acryloyl)-Leu-Gly-Pro-Ala
HRP	Horseradish peroxidase
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IMR	Institute of Medical Research
JKNS	Jabatan Kesihatan Negeri Sarawak
km	Kilometer
kDa	kilodalton
LAMP-1	Lysosomal-associated membrane protein 1
LPS	Lipopolysaccharides
m	Metre
ml	Microlitre
mm	Milimetre
MAT	microscopic agglutination test

Mg	Miligram
MOH	Ministry of Health
nm	Nanometre
O.D	Optical density
OMPs	Outer membrane proteins
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
REA	Restriction Endonuclease Analysis
SD	Standard deviation
SEM	Scanning Electron Microscope
sq.km	Square kilometer
TEM	Transmission Electron Microscope
TLR	Toll-like receptor
UK	United Kingdom
UN	United Nation
UNIMAS	Universiti Malaysia Sarawak
USA	United State of America
WHO	World Health Organization
WWF	World Wildlife Fund

CHAPTER 1

INTRODUCTION

1.1 Study Background

Leptospirosis is a re-emerging zoonotic disease that is caused by *Leptospira* spp., a gram-negative microorganism that may cause acute, febrile illness occurring in humans or animals. The annual incidence is 10 times higher in humid tropics per year if compared to in temperate climates where the incidence is ranging from 0.1 to 1 per 100 000 per year (WHO, 2003). These figures however, are under-reported in many countries especially in countries that highly endemic due to the lack in diagnostic capabilities which resulting in poor surveillance and reporting of the disease (WHO, 2010).

A statistic on global morbidity and mortality of leptospirosis in 2015 has estimated about 1.03 million of cases reported worldwide which has caused more than 58 thousand deaths per year (Costa et. al., 2015). In Malaysia, the increasing number of cases and outbreaks since early 2000s have been observed throughout the country. From the period of 2004 to 2009, more than 5000 cases of leptospirosis were reported with 234 known fatalities. Perak recorded the highest case fatality rates (CFR) during this period which accounted for 6.81% and followed by Sarawak (6.42%) (Hakim, 2011). Due to the increasing number of reported cases, leptospirosis has been gazzetted as a notifiable disease on 9th December 2010 (Khebir et al., 2011; Garba et al., 2017). Since then, there is an increased in number of cases reported in the following years (2011 to 2012), especially in Sarawak. This may be attributed to mandatory reporting procedures implemented or increase in the awareness of the disease by health practicioners (Thaya et al., 2013).

Leptospirosis occur after a direct or indirect contact with the organism shed in the urine of reservoir animals into the environment. The reservoir animals, such as rodents can either be infected or as asymptomatic carriers and result in chronic renal carriage (Adler & de la Pena Moctezuma, 2010). The *Leptospira* spp. can survive in moist soil and water for a long period of time after being shed by infected animals through their urine (McBride et al., 2005; Adler & Moctezuma, 2010). After the leptospires infect the body through contaminated water and soil, the leptospires spread rapidly into the lungs, liver and kidneys and may cause tissue injury (McBride et al., 2005). It may be manifested clinically as high fever, myalgia, hemorrhage, jaundice, renal impairment and septic shock (Palaniappan et al., 2007).

The wide spectrum of symptoms from asymptomatic, mild to severe are dependent on several factors; amongst them are epidemiological conditions, host susceptibility and pathogen virulence. Leptospiral pathogens differs in their ability to cause disease: subjected to their virulence, motility and ability to survive within the host (McBride et al., 2005; Hartskeerl et al., 2011). *Leptospira* spp. are divided into pathogenic, intermediate and saprophytic types according to the molecular genetics diversity and pathogenic ability (Fouts et al., 2016; Lagadec et al., 2016).

The known pathogenic *Leptospira* spp. are *Leptospira alexanderi, Leptospira alstonii, Leptospira borgpetersenii, Leptospira interrogans, Leptospira kirschneri, Leptospira kmetyi, Leptospira mayottensis, Leptospira noguchii, Leptospira santarosai* and *Leptospira weilii.* The intermediate types consist of *Leptospira broomii, Leptospira fainei, Leptospira inadai, Leptospira licerasiae* and *Leptospira wolffii* that occasionally cause disease to human and animal. The last group, saprophytic type includes *Leptospira biflexa,*

Leptospira meyeri, Leptospira vanthielii, Leptospira wolbachii and *Leptospira yanagawae,* and are found in natural water bodies and do not cause disease (Sun et al., 2020).

1.2 Problem Statement

In Malaysia, rodents are the major reservoir for leptospires and has become the source of infection to human and animals (Levett, 2001; Loan et al., 2015). A previous study on identification of circulating leptospires in Kuala Lumpur had shown, from the 300 rats trapped, 20 positive cultures isolated are highly pathogenic (Benacer et al., 2013). In Sarawak, a study on diversity of pathogenic, intermediate and saprophytic *Leptospira* spp. in rats, soil and water have been conducted. The most pathogenic samples were detected in soil samples (11.6%), while only 5.6% of pathogenic samples detected in rats. From this study, 76 *Leptospira* spp. were isolated. DNA sequencing analysis had shown the dominant pathogenic *Leptospira* spp. circulating in Sarawak are *Leptospira* noguchii. Therefore, prevalence studies of *Leptospira* spp. from animals and the environment are important as the presence of leptospires especially, the pathogenic type could pose health risk to human.

The invasiveness of pathogenic *Leptospira* spp. is closely related to the role of the enzyme collagenase in the spirochete. Collagenase A enzyme, (colA), produced by *L. interrogans* serovar Lai strain Lai, has the ability to hydrolyze COL1/3/4 with high hydrolytic activity in vitro. The colA gene knockout mutant demonstrated an attenuated transcytosis through the monolayers of HUVEC and HEK293 cells. Besides that, there was a significant decrease in leptospire loading in tissues and discharge from urine in hamsters (Kassegne et al., 2014). To date, most research on colA gene expression in organs are conducted in laboratory animals infected with pathogenic *Leptospira* spp., specifically *L.*

interrogans. The expression of this gene in other *Leptospira* spp. infected animals in the wild is not widely documented.

Therefore, this study aims to identify the current circulating *Leptospira* spp. infecting wild rodents in Samarahan division, and also to quantify the collagenase A enzyme in infected wild rodents by different types of *Leptospirosis* spp. in their natural habitats.

1.3 Objectives

i. To determine the occurence of *Leptospira* spp. in Samarahan Division isolated from rats based on *Lipl32*, *16S rRNA* and *rrs* genes.

ii. To correlate the diversity of *Leptospirosis* spp. isolated from rats based on *Lipl32*, *16S rRNA* and rrs genes sequencing with the rodent host.

iii. To investigate the level of ColA protein expression in infected rodents.

iv. To correlate the expression of ColA protein with *Leptospira* spp. isolated from rodent host.

CHAPTER 2

LITERATURE REVIEW

2.1 *Leptospira* spp.

2.1.1 Taxonomy and classification

The genus *Leptospira* belongs to the family Leptospiraceae under the order of Spirochaetales (Figure 2.1). Classification of *Leptospira* spp. is based on the surface-exposed epitopes in the mosaic of the lipopolysaccharides (LPS) antigens, where the speicificty of epitopes depends on their sugar composition and orientation. The species and subspecies of *Leptospira* are called serogroups and serovars, usually associated with the natural host (Bharti et al., 2003).

In 1979, the family *Leptospiraceae* was defined to consist both genera, *Leptospira* and *Leptonema* (Hovind Hougen, 1979). Later it become three genera that consists of *Leptospira, Leptonema* and *Turneriella* (Levett et al., 2005). The genera are categorized based on DNA-DNA relatedness, 16S rRNA gene sequences and differences in G+C contents. The differences of the G+C contents among the genera are *Leptospira* (33-43%) *Leptonema* (54%) and *Turneriella* (53.6%) (Stackebrandt et al., 2013; Yasuda et al., 1987).