UNIVERSITI PUTRA MALAYSIA

IDENTIFICATION OF SUITABLE BIOMARKERS FOR LEPTOSPIRAL MOLECULAR DIAGNOSIS AND GENE EXPRESSION IN Cavia porcellus Linnaeus MODEL

SHARMILAH KUMARI A/P KUMARAN

FPSK(p) 2021 18

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SHARMILAH KUMARI A/P KUMARAN

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

May 2021

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

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Chairman : Suresh Kumar Subbiah, PhD
Faculty : Medicine and Health Sciences

Leptospirosis is one of the reemerging and neglected infectious diseases, now has become major health concerns due to their global distribution and caused a high mortality rate among humans. The severity of the disease differs based on infecting *Leptospira* species/serovars, age groups, and the immune status of the patients. The major challenge with leptospirosis is the nonspecific clinical manifestation, which is often confused with other febrile illnesses like dengue, malaria, influenza, meningitis, and hepatitis. The detection of *Leptospira* among infected patients challenging due to the following factors: a tremendous number of leptospiral strains, biphasic nature of the illness, confusion in clinical presentation, and complicated manifestations of the laboratory examinations to detect the disease. The objectives of this study were to analyze suitable molecular markers for early detection of leptospirosis and to investigate the differential gene expression during infection in the guinea pig animal model. For identifying suitable molecular diagnostic markers, genes of interest like LipL32, OmpL1, Loa22, LenA, and LigB searched against on whole-genome sequences of sixteen *Leptospira* strains. In this section, a bioinformatics approach of *blastp* search, multiple sequence alignments, pairwise score identity, phylogenetic tree construction, primary protein structure analysis, signal peptide prediction, multiple sequence alignments of B-cell epitopes prediction, and structure modeling applied to examine the suitability of the gene as a marker for diagnosis. For the *in vivo* study, three groups of guinea pigs tested with three different types of pathogenic *Leptospira* strains, and uninfected animals were kept as a control group. Blood samples were collected on the third and tenth days of post-infection from the animals. Later, the blood samples were subjected to biochemical and RNA analysis. On the thirty days of post-infection, the animals were sacrificed, and major organs of lungs, heart, kidneys, spleen, brain, and liver investigated for organ damages using the histopathological approach. The results from the bioinformatics approach shown that five conserved regions of LipL32

(LipL32$_{12-37}$, LipL32$_{64-85}$, LipL32$_{87-128}$, LipL32$_{137-155}$, and LipL32$_{199-256}$), and four conserved regions of OmpL1 (OmpL1$_{118-135}$, OmpL1$_{137-151}$, OmpL1$_{194-210}$, and OmpL1$_{252-265}$) can be suitable molecular marker candidates. During the infection, the biochemical parameters like creatinine, total bilirubin, alanine transaminase, and aspartate transaminase level increased on the 10th day of post-infection, whereas hemoglobin and red blood cell inclined. For the RNA transcriptome analysis, the results revealed that beta-2-microglobulin (B2m), the gene responsible for tubulointerstitial nephritis, was highly expressed for animals infected with *Leptospira interrogans* on the 10th days of post-infection. The expression of B2m gene was well supported by renal histopathology as more lesions were identified in the infected animal kidneys. Thus, the studies suggested that the identified potential molecular markers and biomarker can use for the early detection of leptospirosis.

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

PENGENALPASTIAN BIOPENANDA YANG SESUAI UNTUK DIAGNOSIS MOLEKUL LEPTOSPIRA DAN EKSPRESI GEN DALAM MODEL *Cavia porcellus* Linnaeus

Oleh

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Leptospirosis adalah salah satu penyakit berjangkit yang diabaikan sekian lama dan telah muncul kembali, kini menjadi masalah kesihatan utama disebabkan oleh penyebaran global dan kadar kematian yang tinggi di kalangan manusia. Tahap keparahan penyakit berbeza berdasarkan jenis *Leptospira* yang dijangkiti, kumpulan umur, dan keupayaan imunologi pesakit. Kehadiran gejala kilinikal yang tidak spesifik merupakan cabaran utama dalam mengenalpasti penyakit ini, dimana penyakit ini disalahertikan dengan penyakit lain seperti demam denggi, malaria, influenza, radang meninges, dan hepatitis. Pengesanan bakteria *Leptospira* di kalangan pesakit mencabar kerana faktor-faktor berikut: sebilangan besar isolasi bakteria, sifat dwifasa penyakit, kekeliruan dalam gejala klinikal, dan diagnostik makmal perubatan yang rumit untuk mengesan penyakit. Objektif kajian ini adalah untuk menganalisis penanda molekul yang sesuai untuk pengesanan awal penyakit ini dan untuk menyelidiki perbezaan ekspresi gen di dalam model haiwan tikus belanda semasa jangkitan leptospirosis. Untuk mencari penanda diagnostik molekul yang sesuai, gen sasaran seperti LipL32, OmpL1, Loa22, LenA, dan LigB dikenalpasti pada enam belas genom bakteria *Leptospira*. Dalam bahagian ini, pendekatan bioinformatik seperti carian sasaran gen, penjajaran urutan pelbagai, skor identiti berpasangan, pembinaan pokok filogenetik, analisis struktur protein utama, ramalan isyarat peptida, penjajaran urutan pelbagai bagi epitop sel B, dan model struktur diterapkan untuk memeriksa kesesuaian gen sebagai penanda untuk diagnosik. Untuk kajian haiwan, tiga kumpulan tikus belanda diuji dengan tiga jenis isolasi patogenik *Leptospira*, dan kumpulan haiwan yang tidak dijangkiti disimpan sebagai kawalan. Sampel darah dari haiwan dikumpulkan pada hari ketiga dan kesepuluh selepas jangkitan. Kemudian, sampel darah dijalani analisis biokimia dan RNA. Pada hari ketiga puluh setelah jangkitan, haiwan dikorbankan dan organ dalaman utama seperti paru-paru, jantung, ginjal, limpa, otak, dan hati diselidiki untuk kerosakan organ menggunakan pendekatan histopatologi.

Pendekatan bioinformatik menunjukkan bahawa lima bahagian dari LipL32 ($LipL32_{12-37}$, $LipL32_{64-85}$, $LipL32_{87-128}$, $LipL32_{137-155}$, dan $LipL32_{199-256}$) dan empat bahagian dari OmpL1 ($OmpL1_{118-135}$, $OmpL1_{137-151}$, $OmpL1_{194-210}$, dan $OmpL1_{252-265}$) boleh menjadi calon penanda molekul yang sesuai. Semasa jangkitan, parameter biokimia seperti kreatinin, jumlah keseluruhan bilirubin, transaminase alanin, dan tahap transaminase aspartat meningkat pada hari ke-10 selepas jangkitan, sedangkan hemoglobin dan sel darah merah menurun. Untuk analisis transkrip RNA, hasilnya menunjukkan bahawa beta-2-microglobulin (B2m), gen yang bertanggungjawab untuk nefritis tubulointerstitial, sangat menonjol pada hari ke-10 selepas jangkitan bagi haiwan yang dijangkiti dengan isolasi *Leptospira interrogans*. Ekspresi gen B2m disokong oleh histopatologi ginjal kerana lebih banyak luka dikenalpasti pada ginjal haiwan yang dijangkiti. Oleh demikian, penanda molekul dan biomarker yang dikenalpasti menerusi kajian ini boleh digunakan untuk pengesanan awal penyakit leptospirosis.

ACKNOWLEDGEMENTS

The opportunity that I had by conducting this research in the Department of Medical Microbiology and Parasitology, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, was a great chance to improve my research skills. I consider myself a lucky individual as provided with an opportunity to be a part of this project granted by the Long Term Research Grant Scheme (LRGS).

First and foremost, I would like to show my gratitude to God that has always guided me to work on the right path of life, and without his blessing, this project could not become a reality. Next to Him are my parents and family members, who brought up love, support, and encouragement to finish my study.

Second, I wish to express my gratitude to my supervisor, and my co-supervisors, for giving me all the support and guidance. I am grateful and indebted to them for their expert, supervision and encouragement extended to complete this project. I like to thank Assoc. Prof Dr. Abdul Rahim Mutalib and Prof Dr. Mohd Zamri Saad from the Faculty of Veterinary Medicine for their contribution during the animal study.

Third, I took the opportunity to sincerely thanks all the staff members of UPM, especially the staff from the Microbiology laboratory, Medical Genetic Laboratory, Histopathology laboratory, my UPM teammates (Dr Narcisse Mary, Fatin Amirah, Sakinah, Amirah Peili, Lawal, Shesha, Dr Priya), bioinformatics team from Malaysia Genome Institute (Mohd Faizal Abu Bakar, Shuhaila Mat-Sharani, and Hirzahida Mohd-Padil), the veterinary team from Comparative Medicine and Technology Unit (COMeT) (Saiful Qushairi, and Rabiatul Adawiyah), Animal Research Facility (ARF) staffs for their kindness and contribution which were extremely valuable. Besides, I like to thank my scholarship funding from the Ministry of Higher Education, Malaysia (MyPHD).

Finally, I place my sense of gratitude to one and all that are directly and indirectly lend their helping hand in this project. I am also grateful for having a chance to meet as many experienced people and professionals who guide me through my PhD journey.

This thesis was submitted to the Senate of the 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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LIST OF ABBREVIATIONS

EIDs	Emerging infectious diseases
SARS	Severe acute respiratory syndrome
DFM	Dark-field microscopy
PCR	Polymerase chain reaction
LAMP	Loop-mediated isothermal amplification method
NASBA	Nucleic Acid Sequence-Based Amplification
MAT	Microscopic agglutination test
ELISA	Enzyme-linked immunosorbent assay
WHO	World Health Organization
LERG	Leptospirosis Epidemiology Reference Group
NGS	Next-generation sequencing
LPS	Lipopolysaccharide
CAAT	Cross agglutination absorption test
OMPs	Outer membrane proteins
Mb	Megabyte
cI	Chromosome I
cII	Chromosome II
p74	Third circular replicon
GC	Guanine-cytosine content
mol%	Mole percent
OM	Outer membrane
TM	Transmembrane protein
bacteria/ml	Bacteria per milliliter

%	Percentage
0C	Degree Celsius
PPE	personal protective equipment
GBD	Global burden of disease
DALYs	Disability Adjusted Life Years
US	United States
0F	Degree Fahrenheit
PUO	Pyrexia of unknown origin
HIV	Human immunodeficiency viruses
ARF	Acute renal failure
AKI	Acute kidney injury
ARDS	Acute respiratory distress syndrome
SPHS	Severe pulmonary hemorrhage syndrome
DPO	Days post-onset of the disease
leptospires/mL	Leptospires per milliliter
RBC	Red blood cell
EMJH	Ellinghausen McCullough Johnson and Harris medium
MLST	Multi Locus Sequence Typing
DNA	Deoxyribonucleic acid
qPCR	Real-time PCR
SYBR	Asymmetrical cyanine dye
IgG	Immunoglobulin G
IgM	Immunoglobulin M
RDTs	Rapid diagnostic tests

16S rRNA	16S ribosomal RNA subunit
secY	Preprotein translocase
flaB	Flagellin B
rrs	16S ribosomal RNA
rpoB	β subunit of RNA polymerase
gyrB	DNA gyrase subunit B
WGS	Whole-genome sequencing
TAE	Tris-acetate-EDTA buffer
PBS	Phosphate buffer saline
NCBI	National Center for Biotechnology Information
IACUC	Institutional Animal Care and Use
d.p.i	days post infection
RNA	Ribonucleic acid
RNA-seq	Ribonucleic acid-sequencing
RAST	Rapid Annotation using Subsystem Technology
ID	Identifier
blastp	Protein blast
MUSCLE	Multiple Sequence Comparison by Log- Expectation
signalP	Signal peptide
3D	three-dimensional
2D	two-dimensional
BLAST	Basic local alignment search tool
CDS	Coding sequence
bp	Base pair
aa	Amino acid

nr	Non-redundant
NJ	Neighbour Joining
ML	Maximum Likelihood
Mw	Molecular weight
pI	Theoretical isoelectric point
GRAVY	Grand average of hydropathicity
PDB	Protein Data Bank
SPF	Specific pathogen-free
GP	Guinea pig
ARF	Animal Research Facility
g	Gram
mL	Milliliter
IP	Intraperitoneal
0	Degree
mg/kg	Milligram per kilogram
EDTA	Ethylenediaminetetraacetic acid
µg	Microgram
cDNA	Complementary DNA
µl	Microliter
QC	Quality control
FPKM	Fragments Per Kilobase of transcript sequence per Millions
µm	Micrometre
H&E	Hematoxylin and Eosin
NADC	National Animal Disease Center
PEGs	Protein-encoding genes

Mbp	Millions of base pairs
A	Alanine
V	Valine
I	Isoleucine
L	Leucine
β	Beta
AST	Aspartate aminotransferase
Tbil	Total bilirubin
creat	Creatinine
ALT	Alanine transaminase
Hb	Hemoglobin
WBC	White blood cell
U/L	Units Per liter
umol/L	Micromole per liter
G/L	Gram per liter
V	Volt
RIN	RNA Integrity Number
$A_{260/280}$	Absorbance at 260 and 280 nm
$A_{260/230}$	Absorbance at 260 and 230 nm
ng/µl	Nanogram per microliter
N	Undefined bases
G	Giga
mRNA	Messenger RNA
TM	Text mining
kDa	Kilo Dalton

CHAPTER 1

INTRODUCTION

1.1 Background

Zoonosis consists of associate degree array of infections, various known throughout human history, while some of the infections recently identified to cause disease to humans or animals. The occurrence of new infectious diseases known as emerging infectious diseases (EIDs) and appear of well-known past maladies is called reemerging EIDs. Some of the EIDs and reemerging EIDs include leptospirosis, severe acute respiratory syndrome (SARS), dengue, malaria, rabies, and typhoid. Bacterial zoonosis spread via one amongst listed processes: direct transmission of infected animals or their tissues; animal bites or scratches; mechanical passage by invertebrate vectors or bites; and intake of contaminated foods or drinks (Chikeka & Dumler, 2015). Based on the route of transmission, zoonotic diseases classify as vector-borne, foodborne, waterborne, or airborne diseases.

Leptospirosis has gained attention worldwide due to its reemergence and recent epidemic cases in both humans and animals (Fernandes et al., 2016; Rajapakse, 2014). The term "neglected infectious disease" lately used to refer to this tropical disease, which are misdiagnosed and reported besides the absence of prevalence data or incidence (Karpagam & Ganesh, 2020). The disease was first discovered by Adolph Weil in 1886 and subsequently named after him, as Weils's disease (Adler, 2015). When the infection identifies first, the origin of the illness was unknown, but by nature, it seemed to be infectious and mostly related to outdoor occupations involving water. In the past, the case incidence rate was higher among coal miners, rice-field workers, and sewer workers (Atil et al., 2020). Although the disease had existed for more than 100 years and play a significant role in the global scene, the details on the molecular mechanisms, the pathogenesis of the disease, and the progress of virulence genes or virulence-related factors remain restricted (Ghazaei, 2018).

Leptospirosis is an enzootic disease caused by pathogenic spirochetes, *Leptospira*. The leptospires appear long, thin, highly motile, spiral-shaped bacteria with the capability of infecting a wide range of hosts. The general structure of *Leptospira* resembles Gram-negative bacteria with two membrane layers (outer and inner membranes), with peptidoglycan-containing periplasmic space (Raddi et al., 2012). Unless like visualizing other bacteria using light microscopy, leptospires were best visualized using dark-field microscopy (DFM) due to its capability of fast-moving and thin morphology. Besides, they can adapt to different environments like water source and mammalian host system. Due to the broad range of adaptation for different environmental conditions, leptospires acquired a large genome that is unique among other bacteria like *Treponema* or *Borrelia* from the same genus (Haake & Matsunaga, 2010).

Moreover, leptospirosis is recognized as an infectious disease affecting almost all mammals. Among mammals, rodents carry a vast number of zoonotic pathogens, approximately 10.7% out of 2220 species being hosts for 85 unique zoonotic diseases (Han et al., 2015). Rodents become host to a broad range of infectious disease due to its rapid life cycle, frequent reproducing ability in comparison with other mammal species, and its abundance in worldwide (Han, Kramer, & Drake, 2016). For leptospirosis, rats act as asymptomatic renal carriers, which be healthier in behavior but continuously secrete the leptospires via urine throughout their lifespan. The disease also reported in domestic animals like dogs, cattle, swine, horses, and sheep. With an increasing number of rodents, the infection successfully transferred to domestic animals, which later on become a source of human leptospirosis.

The symptoms vary for humans and animals. For humans, the severity of the disease vary according to the infecting *Leptospira* serovars, age group, and immunological capacity of the patients (Fraga et al., 2014). The spectrum of the disease in human vary from mild febrile illness like to life threatening infection accompanied by major organs failure, which may lead to death. Pathological reports showed damages to major organs like liver (Miyahara et al., 2014; Merien et al., 1998), lung (Nally et al., 2004; Pereira et al., 2002), and kidney (Herath et al., 2014; Araujo et al., 2010). However, animal leptospirosis diverse from human leptospirosis as the knowledge intensively prejudices towards the economic impacts of livestock and food industries (Pereira et al., 2017). Premature birth, stillbirth, miscarriage, the natality of sick young baby animals, and the decline in birth weight are some of the leading problems in livestock industries involving cattle, sheep, and buffalo.

There are several available diagnosis methods for human leptospirosis. All existing diagnosis techniques are categorized into molecular, serological, and genomics study methods (Jose & Sumana, 2013). The conventional polymerase chain reaction (PCR), quantitative PCR assays, loop-mediated isothermal amplification method (LAMP), Nucleic Acid Sequence-Based Amplification (NASBA) (Ahmed et al., 2012) are few methods from molecular diagnosis. Serological diagnosis methods consist of microscopic agglutination test (MAT) (Murray et al., 2011; Smythe et al., 2009) and enzyme-linked immunosorbent assay (ELISA) (Signorini et al., 2013). The selection of the diagnostic methods relies on some points like the accuracy of the diagnostic, financial and technical feasibility, and the requirements for an early or fast result (Picardeau et al., 2014).

Due to the misdiagnosis of leptospirosis cases with malaria and dengue, most of the cases are under-reported. Thus, morbidity and mortality rates of the disease miscalculate and strengthen down its status as a neglected disease. The absence of the proper disease records prevents attempts made to solve the problems by identifying principal barriers in terms of diagnostics methods and competent prevention and control steps (Costa et al., 2015). In 2010, the World Health Organization (WHO) appointed the Leptospirosis Epidemiology Reference Group (LERG) to keep tracing arising leptospirosis cases with One Health approaches.

The LERG responsibility including (1) gives an estimation of human leptospirosis cases based on age and sex in WHO locality; (2) support individual countries on their disease burden studies, records and outline their outcomes; (3) support respective countries to utilize disease burden for cost-effectiveness investigation of mediation and control measures and (4) rise up the attention of states' commitment to the execution of leptospirosis avoidance, control and mediation (Abela-Ridder, Sikkema, & Hartskeerl, 2010). Based on LERG approaches, the yearly morbidity of cases was computed tremendous in countries rely on South and Southeast Asia with huge populations like India and Indonesia (Costa et al., 2015). In Malaysia, 7806 cases recorded in 2014 with 92 death cases, alarming the disease endemic in the country (Garba et al., 2017).

Besides antibiotics therapy, no other therapeutics available for acute leptospirosis at present (Rajapakse, 2014). Nearly all leptospirosis cases are mild and can be cured immediately with early administration of antibiotics. For mild leptospirosis patients, anti-microbial therapy like penicillin, ampicillin, ceftriaxone, cefotaxime, doxycycline, azithromycin, amoxicillin used in hospitalized treatments (David, 2015; Brett-Major & Coldren, 2012; Suputtamongkol et al., 2010).

1.2 Problem statement

Leptospirosis, one of the most significant re-emerging bacterial zoonotic diseases with global distribution except in the Antarctica region (Adler, 2010). Most of the incidents have been rumored in diverse environments, from metropolitan after floods to isolated rural areas with restricted access to hygienic drinking water and proper sanitation system. Leptospirosis is known as a disease of poverty other than being an occupationally related disease (Schneider et al., 2013). In the present years, the leptospirosis cases gained worldwide attention as it linked to water-related recreational activities and military expeditions (Schneider et al., 2013; Sejvar et al., 2003). The detection of *Leptospira* among infected patients was challenging due to the following factors: biphasic nature of *Leptospira* itself, confusion in clinical presentation, a tremendous number of leptospiral strains (Marquez et al., 2017), and complicated manifestations of the laboratory examinations to detect the disease.

The biphasic of leptospirosis started with the bacteremia phase, followed by the immune stage (Brito, 2018), which creates a more complex clinical presentation. The symptoms of the disease vary from a mild influenza-like illness to an acute fatal form with multi-organ failure (Priyadarsini, 2018; Raja & Natarajaseenivasan, 2013). The most common symptoms in the first phase are fever, chills, headaches, myalgia, vomiting, and sometimes jaundice also presence. In the acute stage, multi-organ dysfunction leads to death. Based on initial clinical symptoms, it's tough to make a prediction in which patients may evolve with the severe leptospirosis. Even though little clinical evidence has shown alcohol consumption, leukocytosis, thrombocytopenia, and arrhythmias have a connection with acute leptospirosis, yet no reliable data set or scoring systems available to indicate patients mostly evolved

with severe leptospirosis (Rajapakse, 2014). The severity of the organ damages relied on the virulence of infecting leptospiral bacteria and host susceptibility (Wynwood et al., 2014).

Furthermore, an increased number of leptospiral strains number from year to year also caused a problem in identifying the disease among infected patients. Prior in the year 1989, there are only two *Leptospira* species were available, in which all pathogenic strains grouped to *L. interrogans* while all non-pathogenic strains under *L. biflexa* (Musso & La Scola, 2013). In recent year, the *Leptospira* genus reclassified using genetic techniques, which resulting 22 different species consist of pathogenic, intermediate and non-pathogenic species (Marquez et al., 2017) and further categorized into 250 pathogenic (Lata et al., 2018) and 60 non-pathogenic serovars (Adler, 2010). The list of currently available leptospira strains (as of July 2019) recorded in Appendix 1. The increase of pathogenic serovars and strains relatively linked to changes occur in a mammalian host, where the strains mostly adapted to urban rodents (Haake et al., 2015).

As a result of non-definite and vast clinical presentation in the early phase and acute leptospirosis, the diagnosis of leptospirosis relies on particular laboratory tests (Toyokawa et al., 2011). The laboratory examination is arduous as the diagnostics tests needed specific equipment and well-trained staff, which found at the reference laboratories only. On the other hand, the available diagnostic tests are not always serovar-specific due to cross-reactivity against different serovars that may occur between organisms in the same serogroup (Khodaverdi et al., 2013). Although several diagnostics methods and techniques available for leptospirosis, a new approach need to be designed because currently, available diagnosis methods not very efficient. With the growing advance of genomic sequencing, the enormous data and facts on *Leptospira* genomes accessible in public databases. The utilization of Next-generation sequencing (NGS) and bioinformatics analysis on *Leptospira* genomes might improve the health care industry in both clinical microbiology and epidemiological studies. The advancement might lead to the selection of target genes present in almost all leptospiral strains, which later on can use as target antigen in diagnosis. Besides, the *Cavia porcellus* (guinea pig) model used to mimic human leptospirosis to identify gene expression using transcriptome study, and the blood profiles and organ damages also investigated further insight into the disease. The *Cavia porcellus* shared similar immunological genes to humans compared to the mouse gene (Schuller et al., 2015), so the model used in the current study. The outcome of this research is essential for both patient care and the efficient implementation of public health measures.

1.3 Research questions

1. What are the available *Leptospira* whole-genome sequences in public databases and outer membrane proteins reported for *Leptospira* species?
2. Which target genes suitable for leptospirosis molecular diagnosis?
3. Which genes highly expressed during leptospirosis infection in an animal model?
4. What are the changes that can observe from the blood profile and organs of the pathogenic *Leptospira* infected animal model?

1.4 Objectives

1.4.1 General objective

To analyze suitable markers used for leptospirosis molecular diagnosis and to investigate the gene expression in animal model during infection

1.4.2 Specific objectives

i. To determine the list of *Leptospira* whole-genome sequences from the NCBI database and reported leptospiral outer membrane proteins from the literature search
ii. To identify the potential target genes from outer membrane proteins that can use for molecular diagnosis of leptospirosis
iii. To identify highly expressed genes during leptospirosis using *Cavia porcellus* as an animal model
iv. To observe the variation of biochemical parameters in the infected blood of the animal and the structural damage of the organs during *Leptospira* infection

1.5 Hypotheses

i. There are *Leptospira* whole-genome sequences and leptospiral outer membrane proteins accessible through the NCBI database and past literature.
ii. There are few potential target genes from outer membrane proteins that can use for leptospiral molecular diagnosis.
iii. There are several genes highly expressed in the *Cavia porcellus* during leptospirosis.
iv. There is an alteration in *Cavia porcellus* blood biochemical parameters, and organ damages detect in *Cavia porcellus* during leptospirosis.

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