UNIVERSITI PUTRA MALAYSIA

ASSOCIATIONS BETWEEN IMMUNOLOGICALLY-RELATED GENETIC POLYMORPHISMS AND CYTOKINES EXPRESSION ON HUMAN LEPTOSPIROSIS SUSCEPTIBILITY AND SEVERITY

WAN SHAHRIMAN YUSHDIE B. WAN YUSOFF

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By

WAN SHAHRIMAN YUSHDIE B. WAN YUSOFF

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

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Chair : Professor Syafinaz Amin Nordin, MBChB, MPath, MHEd
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Leptospirosis is a zoonotic disease caused by spirochetes species of the genus *Leptospira.* Despite being the common causes of zoonotic morbidity worldwide, there is still a knowledge gap between leptospiral pathogenesis and human immune responses. This study aimed to explore the impact of selected immune parameters on leptospirosis susceptibility and severity. A prospective study was conducted among clinically-suspected leptospirosis cases admitted to hospitals in the south-central region of Malaysia (January 2016–December 2017). Of 132 clinically suspected leptospirosis patients samples collected, 94 were confirmed-leptospirosis. The study included 19 healthy controls. Gene polymorphisms for human leucocyte antigen (HLA) were determined using a sequence-specific oligonucleotide (SSO) probe for Malay subjects. Cytokine levels were quantified using a Simple Plex™. Immunophenotyping of T cells was performed using a flow cytometer. Single nucleotide polymorphism (SNP) studies for selected cytokines genes were performed using the BGISEQ sequencing platforms. When comparing fatal to non-fatal, laboratory investigation found that fatal cases had significantly higher blood urea nitrogen (BUN) and serum creatinine. Genotyping study of HLA revealed HLA-A*02, -A*23, -A*33, -B*15, -C*03, and -DRB1*16 alleles were significantly associated with leptospirosis susceptibility, while -DQB1*03 was significantly associated with less susceptibility to the disease. Furthermore, HLA-A*33 was significantly associated with severe leptospirosis, while HLA-C*03 was significantly associated with renal involvement. This study found a statistically significant association concerning severe cases for gene SNP IL-33 (rs1854709). In contrast, single nucleotide insertions and deletions (INDELs) in gene IL-18 (rs10642361) and IL-8 (rs2227541) were associated with mild disease. IL-6, IL-17A, IL-22, and IL-8 levels were significantly higher in fatal than non-fatal cases. These findings confirm that the host immune response influenced disease severity, indicating the significant associations between pro-inflammatory cytokines and chemokines in fatal cases. The importance of IL-8 was further supported by IL-

8 SNP (rs2227541) in mild cases. Th1 cells were significantly lower among leptospirosis patients than in healthy controls. Several HLA alleles were related to disease susceptibility and protection from leptospirosis. This study allowed a better understanding of the important role of the host immune-related biomarkers in the clinical outcome of leptospirosis infection. It may be useful in developing early prognostic and selective immunotherapy for the disease.

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

HUBUNGAN DIANTARA POLIMORPHISME GENETIK BERKAITAN-IMMUNOLOGI DAN PENGELUARAN SITOKIN DENGAN KECENDERUNGAN DIJANGKITI DAN TAHAP KEPARAHAN JANGKITAN LEPTOSPIROSIS PADA MANUSIA

Oleh

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Leptospirosis adalah penyakit zoonotik yang disebabkan oleh spesies *spirochets* dari genus *Leptospira*. Walaupun penyakit ini menjadi salah satu penyebab kepada jumlah morbiditi disebabkan penyakit zoonosis di serata dunia, masih terdapat jurang pengetahuan di dalam patogenesis dan kaitannya dengan tindakbalas sistem imun manusia yang dijangkiti bakteria leptospiral. Tujuan kajian ini dijalankan adalah untuk meneroka serta mengenal pasti kesan beberapa parameter sistem imun terhadap tahap risiko seseorang untuk dijangkiti dan tahap keparahan jangkitan leptospirosis. Kajian ini tertumpu kepada hospital-hospital di wilayah tengah selatan Malaysia (Januari 2016 – Disember, 2017). Kes leptospirosis yang dimasukkan ke hospital-hospital tersebut adalah dipilih berdasarkan gejala klinikal pesakit yang berkait rapat dengan penyakit leptospirosis. Sebanyak 132 sampel telah diambil dari pesakit yang disyaki mengidap leptospirosis, dan dari jumlah tersebut 94 disahkan leptospirosis. Kajian ini turut menggunakan 19 sampel dari penderma sihat sebagai rujukan normal. Bagi polimorfisme genetic untuk *human leucocyte antigen* (HLA) kelas I dan kelas II, hanya subjek berbangsa Melayu dipilih. Ia telah ditentukan dengan menggunakan teknik *sequence-specific oligonucleotide* (SSO). Paras sitokin diukur dengan menggunakan *Simple Plex*^{Tm}. Fenotip sel T penolong diukur dengan menggunakan sitometer. Kajian polimorfisme nukleotida khusus untuk gen sitokin dijalankan dengan menggunakan *BGISEQ sequencing platforms*. Perbandingan antara keputusan makmal untuk kes kematian dan juga kes bukan kematian leptospirosis menunjukkan kes kematian adalah sangat berkait rapat dengan peningkatan paras urea nitrogen darah (BUN) dan kreatinin di dalam serum pesakit. Kajian genotip HLA menunjukkan alel HLA-A*02, -A*23, -A*33, -B*15, -C*03, dan -DRB1*16, adalah sangat berkaitan dengan kebarangkalian tinggi untuk mengidap leptospirosis, manakala -DQB1*03 berkaitan dengan kurangnya kebarangkalian untuk mengidap penyakit tersebut. Selanjutnya, HLA-A*33 adalah sangat berkait rapat dengan

leptospirosis tahap parah, sementara HLA-C*03 adalah berkait rapat dengan penyakit buah pinggang di kalangan pesakit leptospirosis. Manakala, alel HLA kelas II HLA-DQB1*03 adalah berkait rapat dengan kebarangkalian yang lebih rendah untuk mengidap penyakit leptospirosis. Kajian ini juga mendapati hubungan yang ketara secara statistik di antara gen tunggal sitokin nucleotide polymorphisms (SNP) IL-33 (rs1854709), sementara itu sisipan dan penghapusan (INDELs) pada gen sitokin IL-18 (rs10642361) dan chemokine IL-8 (rs2227541) adalah berkait dengan penyakit leptospirosis yang ringan. Untuk sitokin, kajian ini mendapati paras IL-6, IL-17A, IL-22 dan IL-8 adalah jauh lebih tinggi pada kes melibatkan kematian berbanding dengan kes yang tidak membawa maut. Untuk ujian imunofenotip sel T penolong, kajian menunjukkan bahawa sel Th1 adalah jauh lebih rendah di kalangan pesakit leptospirosis berbanding dengan kumpulan sebagai rujukan sihat. Penemuan menunjukkan tindak balas imun mempengaruhi tahap keparahan leptospirosis, berdasarkan hubungan signifikan antara sitokin pro-radang dan kemokin dalam kes-kes yang membawa maut. Secara keseluruhan, hasil keputusan kajian ini mengenalpasti beberapa gen polimorfisme yang khusus pada HLA dan gen sitokin adalah berkait rapat dengan kebarangkalian dan risiko mengalami jangkitan dan hubungan antara sitokin pro-radang dengan penyakit leptospirosis. Kepentingan IL-8 disokong dengan kehadiran SNP untuk IL-8 (rs2227541) di dalam kes penyakit ringan. Hasil kajian ini memberi pemahaman yang lebih baik berkaitan kepentingan peranan system imun kepada tahap keparahan penyakit leptospirosis. Penemuan ini adalah berguna dalam usaha penghasilan ujian pronostik awal dan pemilihan imunoterapi selektif untuk 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 Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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This is to confirm that:

- the research and the writing of this thesis were done under our supervision;
- supervisory responsibilities as stated in the Universiti Putra Malaysia (Graduate Studies) Rules 2003 (Revision 2015-2016) are adhered to.

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A	Adenine
ALT	Alanine aminotransferase
APC	Allophycocyanin
APCs	Antigen-presenting cells
AST	Aspartate aminotransferase
BUN	Blood urea nitrogen
C	Cytosine
CBMC	Cord blood mononuclear cells
CD	Cluster of differentiation
CFR	Case fatality rate
CISH	Multiple cytokines inducible SH2-containing protein
CM-CSF	Granulocyte-macrophage colony-stimulating factor
CMI	Cell mediated immunity
CWD	Common and well-documented
DC	Dendritic cells
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DSS	Dengue shock syndrome
EDTA	Ethylenediaminetetraacetic acid
FecA	Ferric citrate transporter A
FITC	Fluorescein isothiocyanate
G	Guanine
GLP	Glycolipoproteins
hap-1	Haemolysis associated protein-1

HIV	Human immunodeficiency virus
HLA	Human Leukocyte Antigen
IFN-γ	Interferon gamma
Ig	Immunoglobulins
IL	Interleukin
ILC2s	Group 2 innate lymphoid
IMR	Institute of Medical Research
INDELs	Single nucleotide insertions and deletions
JNK	c-Jun N-terminal kinase
KDO	2-keto-3-deoxyoctonoic acid
kg	Kilogram
L.	*Leptospira*
LERG	Leptospirosis Burden Epidemiology Reference Group
LigA	Leptospiral immunoglobulin like protein A
LipL41	Leptospiral lipoprotein 41
LipL21	Leptospiral lipoprotein 21
LipL32	Leptospiral lipoprotein 32
Loa22	Leptospiral lipoprotein 22
LPS	Lipopolysaccharides
mAbs	Monoclonal antibodies
MAT	Microscopic agglutination test
Mbp	Mega base pairs
MFI	Median fluorescence intensity
MHC	Major histocompatibility complex
mL	Milliliter
MoDCs	Human monocyte-derived dendritic cells

MOH	Ministry of Health
mRNA	Messenger ribonucleic acid
MSCR	Malaysian Stem Cell Registry
NF-κB	Nuclear factor kappa B
NK	Natural killer cells
NMRR	National Medical Research Register
ºC	Celcius
OmpL1	Outer membrane protein L1
OMPs	Outer membrane proteins
OR	Odds ratio
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PE	Phycoerythrin
PF	Periplasmic flagella
pH	Potential hydrogen
PRRs	Pattern recognition receptors
RA	Rheumatoid arthritis
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
Rs	Reference SNP cluster ID
RTB	Pulmonary tuberculosis
SA-PE	Streptavidin-phycoerythrin
SNP	Single nucleotide polymorphism
SphH	Sphingomyelinase H

SPHS	Severe pulmonary haemorrhagic syndrome
Spp.	Species
SSO	Sequence-specific oligonucleotide
sST2	Plasma soluble ST2
ST2	Suppression of tumorigenicity 2
T	Thymine
TAP	Associated with antigen processing
TGF-1	Transforming growth factor-1
Th	T helper
THP-1	Human acute monocytic leukemia cell line
TLR	Toll-like receptor
TNF-α	Tumour necrosis factor alpha
TolC	Outer membrane efflux protein channel
Treg	T regulatory cells
UPM	Universiti Putra Malaysia
WHO	World Health Organization
γδ T	Gamma-delta T cells

CHAPTER 1

INTRODUCTION

Leptospirosis is a globally re-emerging zoonotic disease caused by the bacterium *Leptospira* (Adler & Moctezuma, 2010; Costa et al., 2015). It is estimated that the overall global annual leptospirosis incidence at one million cases, with approximately 60,000 deaths per year (CDC, 2018). Worldwide, increasing incidence rates of leptospirosis have been reported, especially in tropical regions. Various factors contribute to the disease's re-emergence. Many studies have reported multiple outbreaks of leptospirosis in various countries, especially after monsoon seasons and floods (Gracie et al., 2014; Smith et al., 2013). Global climate change contributes to the increasing occurrence of these extreme weather events worldwide (Lau et al., 2010). The emergence of new urban slums triggered by rapid development worldwide contributed to the leptospirosis epidemic. Urban slums are ideal for rat-borne transmission caused by overpopulated areas with inadequate sewage and waste management (Amilasan et al., 2012; Felzemburgh et al., 2014; Lau et al., 2010). The growing popularity of global tourism and nature-based recreational activities leads to the increasing incidence of leptospirosis worldwide (Monahan et al., 2009).

Many mammals function as the primary vectors for leptospirosis, especially rodents. Humans become infected through direct contact with infected animal reservoirs' urine or indirect exposure to urine-contaminated environments (Bharti et al., 2003). Leptospirae enter the body through scraped wounds, mucosa, or ingesting contaminated water. The pathogens then spread from the blood onto the whole body (E. J. Goldstein, 1991). Activities involving handling infected animals or repeated exposure to contaminated water and soil are significant risk factors for leptospirosis. These include occupational exposure, leisure activities, and living environments where animal carriers are in close contact (Bharti et al., 2003; Haake & Levett, 2015). Leptospirosis can be found worldwide, and it is more common in humid and warm climates (Evangelista & Coburn, 2010).

Leptospirosis remains a public health issue in tropical nations such as Malaysia due to the nation's all-year-round tropical climate, frequent heavy rainfall, monsoon season floods, and vast populations of animal reservoirs for *Leptospira* spp. Leptospirosis in Malaysia has been a reportable disease since 2010, with an increasing incidence and fatality rate every year (Benacer et al., 2016). The economy, livelihood, and well-being of people in Malaysia have been significantly affected by leptospirosis (Kit, 2002). From 2005 to 2014, Malaysia's incident rate was 1.5 to 25.9 per 100,000, with the reported case fatality rate (CFR) ranging from 1.2 to 5.3% (Wahab, 2015). Many researchers proposed that the substantial morbidity and mortality rate be higher than reported due to misdiagnosed and underdiagnosed cases.

Leptospiral infection can cause a broad spectrum of clinical symptoms and manifestations in humans. The disease may be asymptomatic or very mild flu-like illness, moderate systemic self-limited type, or life-threatening severe multiple organ damage resulting in severe health complications and even death (Ashford et al., 2000; Levett, 2001). Weil's disease is an icteric form of severe leptospirosis that occurs in around 10% of patients, characterised by haemorrhagic tendency with multisystem damage involving impaired hepatic and renal function and acute pulmonary injury (Adler & Moctezuma, 2010; Chaikajornwat et al., 2020). Acute renal failure is one of the causes of mortality in leptospirosis (Costa et al., 2001). About 44-67% of leptospirosis cases involve acute renal failure (Sitprija et al., 2003), with a 17-36% mortality rate (Leblebicioglu et al., 1996; Nicholson et al., 1989). Renal damage is a common complication of severe leptospirosis disease characterised by interstitial and tubular impairment (Cerqueira et al., 2008). In Weil's disease, the presence of jaundice is a prominent characteristic. Liver involvement is represented by increased blood conjugate bilirubin, transaminases, and alkaline phosphatase levels (Brito et al., 2018). Liver involvement in severe leptospirosis involves nonspecific hepatocellular damage and increased tissue haemorrhages (Gancheva, 2016; Vijayachari et al., 2008). However, liver involvement has rarely been the cause of death, with most patients usually regaining their normal liver function after recovery (Wysocki et al., 2014). In various studies, pulmonary involvement was documented in about 20-70% of leptospirosis, with a fatality rate of over 50% recorded in cases with the severe pulmonary haemorrhagic syndrome (SPHS) (Carvalho & Bethlem, 2002; McBride et al., 2005; Papa et al., 2009).

Many studies proposed that the host immune response plays a significant role in developing severe leptospirosis (Chin et al., 2018; Li et al., 2018). Only a few studies have examined the connection between host genetic polymorphisms and leptospirosis disease incidence and susceptibility. Human leukocyte antigen (HLA)-DQ*06 was associated with a greater risk of leptospirosis among Caucasian triathletes who swallowed water while swimming in a contaminated lake (Lingappa et al., 2004). Patients with a history of leptospirosis had significantly higher frequencies of HLA class I HLA-A*24, HLA-A*31, and HLA-B*08 alleles, according to a report conducted on the Terceira Island population in the Azores, Portugal (Fialho et al., 2009). In contrast, a study on participants with a clinical history of leptospirosis on the neighbouring Azores Island of Sao Miguel found no connection between HLA Class I (-A and -B) with susceptibility to the disease. Nonetheless, the study indicated a potential correlation between HLA-A*26, although not statistically significant (Esteves et al., 2014).

Concerning the determination of possible variants by SNPs genotyping, the study by Estevas et al. found a significant correlation between -511GG in IL1b, +1196CG in IL12RB1, and -292TA and +3415CG in CISH (multiple cytokines inducible SH2-containing protein) with susceptibility to leptospirosis (Esteves et al., 2014). Another study on Argentine found that TLR (Toll-like receptor)2 Arg753Gln and TLR1 I1e602Ser SNPs were significantly associated with the risk of developing leptospirosis and its severity (Cedola et al., 2015).

Few studies have shown that cytokines play a role in the severity of leptospirosis. Pro-inflammatory cytokine tumour necrosis factor-alpha (TNF-α) was significantly higher in fatal than survived leptospirosis cases (Kyriakidis et al., 2011; Reis et al., 2013). Interleukin 10 (IL-10), an important anti-inflammatory cytokine produced by T helper type 2 (Th2) cells, was significantly higher in severe and fatal leptospirosis (Chirathaworn et al., 2016; Kyriakidis et al., 2011; Reis et al., 2013). Previous studies have shown that IL-6 in severe cases is significantly higher than in mild leptospirosis cases (Chirathaworn et al., 2016; Papa & Kotrotsiou, 2015; Reis et al., 2013). CXCL8 (IL-8) chemokine levels were higher in fatal than non-fatal leptospirosis cases in several studies (Reis et al., 2013; Wagenaar et al., 2009).

Very few studies are available on the role of T cells in leptospirosis infections. Most of the current studies focused on *in-vitro* research, with limited data available for the host in-vivo immune response to leptospirosis. Previous *in-vitro* studies have documented that pathogenic *Leptospira* antigen can stimulate T lymphocyte cells to produce cytokines. A study on pathogenic *L. interrogans* serovar copenhageni glycolipoproteins (GLP) stimulation of peripheral blood mononuclear cells (PBMC) found expressions of both T helper cells-related cytokines TNF-α, IL-10, and upregulation of CD69 and HLA-DR were significantly increased. When nonpathogenic *L. Biflixa* serovar patoc was used, no cellular activation was observed (Diament et al., 2002). An *in-vitro* investigation on whether leptospirae could stimulate the proliferation of immune cells and the production of cytokines from human PBMC found that pathogenic *L.interrogans* serovar copenhageni could induce the expansion of γδ T cells and the synthesis of Th1-related cytokines IFN-γ, TNF-α, and IL-12 from PBMCs of healthy volunteers (Klimpel et al., 2003). Another research on whole blood found that *in-vitro* stimulation with heat-killed *L. Interrogans* is associated with Th1-associated cytokine production, including TNF-α and IL-12 (Fost et al., 2003). A study by Goris et al. also showed that viable pathogenic strains of *Leptospira* induced the secretion of TNF-α and IL-6 pro-inflammatory cytokines in the whole blood of healthy subjects. The study indicated the synergistic cooperation of TLR2, TLR4, and TLR5 induced cytokine productions. The study's findings showed that the single inhibition of either TLR2, TLR4, or TLR5 prevented the development of TNF-α by virulent pathogenic strains of *Leptospira*. In contrast, the combined blockade of TLR2/4/5 did not reduce cytokines production (Goris et al., 2011). An *in-vitro* study for the cellular immune response from clinical samples of leptospirosis patients to leptospiral antigens found that the output of Th1-related TNF-α cytokine by CD4+ T cells in severe leptospirosis was significantly higher than mild patients and healthy controls (Volz et al., 2015).

The host immune response mechanisms in leptospirosis patients with different clinical manifestations are still not well understood. Also, scarce information is available on the early diagnosis method for the disease. This study will analyse the data together with the clinical profiles of the patients. Findings of immunologically related genotyping, cellular, and protein expression could provide hindsight for the disease immune 'molecular signatures'. It will help to accelerate the effort to establish early prognostic markers for leptospirosis.

1.1 Problem Statement

Leptospirosis clinical manifestations range from mild febrile fever to severe clinical manifestations accompanied by fatal organ damage. The diverse clinical manifestations of leptospirosis lead to difficulties in assessing the disease's outcome. A deeper understanding of the disease's immunopathogenesis is required to enhance its prognosis, thus improving the treatment of patients. The role of the host immune response in the severity of leptospirosis disease and its mechanisms is still unclear. The information on the effect of genetic polymorphism, cytokines, and T cell subsets on the disease's nature is still limited. To determine the risk of the disease developing into a severe form of leptospirosis, knowledge of disease progression and reliable prognostic markers is critically required. Numerous studies have demonstrated the application of cytokines as prognostic markers in infectious diseases (Ge et al., 2020; Takahashi et al., 2016). Therefore, current research has been conducted in patients with leptospirosis to correlate disease severity with Class I and Class II HLA polymorphisms, selected cytokine genetic polymorphisms, cytokines, and T-helper cell subsets.

1.2 Hypothesis

Differences in MHC class I and class II genes polymorphisms, expression of cytokines, T helper phenotypes, and cytokine genes polymorphisms may affect disease susceptibility and severity in leptospirosis.

1.3 General Objective

To investigate the role of selected immune parameters on disease susceptibility and severity in leptospirosis.

1.4 Specific Objectives

1. To identify the association between HLA genotype and leptospirosis susceptibility.
2. To determine levels of selected cytokines in leptospirosis patients and healthy populations.
3. To categorise T-helper cell subsets in leptospirosis patients and healthy populations.

4. To determine the relationship between cytokine gene polymorphisms and the severity of leptospirosis.

5. To investigate the relationship between cytokines, T-helper cell subsets, gene polymorphisms, and disease severity.

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