EXPRESSION AND PURIFICATION OF RECOMBINANT LipL32 PROTEIN AND EVALUATION OF ITS USE IN ELISA FOR THE DIAGNOSIS OF LEPTOSPIROSIS

YUSZNIAHYATI BINTI YAAKOB

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YUSZNIAHYATI BINTI YAAKOB

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2016

DECLARATION

I hereby declare that the material in this thesis is my own except for quotations, excepts, equations, summaries and references, which have been duly acknowledged.

19 July 2016

Yuszniahyati Binti Yaakob

MZ1321011T

CERTIFICATION

NAME	:	**YUSZNIAHYATI BINTI YAAKOB**
MATRIC NO	:	**MZ1321011T**
TITLE	:	**EXPRESSION AND PURIFICATION OF RECOMBINANT LipL32 PROTEIN AND EVALUATION OF ITS USE IN ELISA FOR THE DIAGNOSIS OF LEPTOSPIROSIS**
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CERTIFIED BY;

1. SUPERVISOR (CHAIRPERSON)
Associate Professor Dr. Daisy Vanitha John Signature

2. COMMITTEE MEMBER 1
Assoc Prof. Dr. Kenneth Francis Rodrigues _____

3. COMMITTEE MEMBER 2
Mdm. Sophia Lau Tiek Ying _____

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Yuszniahyati Binti Yaakob

19 July 2016

ABSTRACT

Leptospirosis is a zoonotic disease caused by the spirochete, *Leptospira interrogans* (*L. interrogans*). Microscopic Agglutination Test (MAT) is a reference method for the diagnosis of Leptospirosis. However, this method is time consuming and laborious. ELISA using recombinant antigens has proven to be a good diagnostic test for Leptospirosis. *Leptospira* outer membrane protein (OMP), LipL32 is conserved in pathogenic species, and play an important role in immunopathogenicity and serodiagnosis. The objectives of this study were, to express and purify the synthetic *lipl32* gene, to determine the presence of Leptospira-specific antibodies in human serum samples using synthetic rLipL32 antigen in ELISA and to measure the level of regulatory cytokine, IL-10 for the determination of disease severity. In this study, *lipl32* gene was synthetically designed and expressed in *Escherichia coli* (*E. coli)* expression vector BL21 (DE3) and the protein was purified by immobilized metal affinity chromatography (IMAC). The purified rLipL32 was used as an antigen to detect Leptospira-specific IgM and IgG by ELISA. A total of 53 human serum samples (20 MAT positive samples, 20 MAT negative samples, 11 unknown serum samples and 2 RCPA serum samples) were tested. ELISA results showed that all MAT positive serum samples were positive for IgG and only 65 % (n=13) of these samples were positive for IgM. For the MAT negative serum samples, 25 % (n=5) and 45 % (n=9) of the samples were positive for IgM and IgG respectively by ELISA. The synthetic rLipL32 based ELISA scored 71 % in the RCPA programme out of the total participants in the survey. The MAT positive serum samples were tested for the presence of the regulatory cytokine, IL-10 to determine the disease severity. The concentration of IL-10 in all human serum samples ranged from 10.8 ± 0.04 pg/mL to 31.9 ± 1.38 pg/mL. When compared to the previous study, this level of IL-10 concentrations shows the mild and non-fatal outcome of Leptospirosis disease. These results demonstrates that ELISA based on synthetic rLipL32 antigen was able to detect Leptospira-specific IgM (sensitivity 65 % and specificity 75 %) and IgG (sensitivity 100 % and specificity 55 %) in the human serum samples. Synthetic rLipL32 based ELISA is able to detect Leptospira-specific antibodies in human serum samples and has the potential to serve as a rapid diagnostic test for Leptospirosis and for determination of seroprevalence in the community.

ABSTRAK

EXPRESI DAN PENULENAN PROTEIN REKOMBINAN LipL32 DAN PENGGUNAANNYA DALAM ELISA BAGI DIAGNOSIS PENYAKIT LEPTOSPIROSIS

Leptospirosis adalah penyakit zoonosis yang disebabkan oleh sejenis spirochete, Leptospira interrogans (L. interrogans). Ujian Pengumpalan Mikroskopik (MAT) adalah satu kaedah rujukan bagi diagnosis penyakit Leptospirosis. Walau bagaimanapun, kaedah ini memakan masa dan agak sukar. ELISA menggunakan protein rekombinan sebagai antigen telah terbukti sebagai ujian diagnostik yang baik untuk Leptospirosis. Protein membran luar (OMP) Leptospira, LipL32 dipulihara dalam spesies patogenik, dan memainkan peranan penting dalam 'immunopathogenicity' dan serodiagnosis. Objektif bagi kajian ini adalah mengekspres dan menulenkan gen sintetik lipl32, menentukan kehadiran antibodi Leptospira-khusus dalam sampel serum manusia menggunakan sintetik protein rLipL32 sebagai antigen dalam ELISA dan mengukur tahap cytokine kawal selia, IL-10 bagi menentukan keterukan penyakit Leptospirosis. Dalam kajian ini, gen lipl32 direka secara sintetik dan diekpres dalam Escherichia coli (E. coli) BL21 vektor (DE3) dan protein ditulenkan menggunakan 'immobilized metal affinity chromatography' (IMAC). Protein rLipL32 yang ditulenkan, telah digunakan sebagai antigen untuk mengesan antibodi Leptospira-khusus oleh ELISA. Sebanyak 53 sampel serum manusia (20 MAT sampel positif, 20 MAT negatif sampel, 11 serum sampel yang tidak diketahui dan 2 serum sampel dari RCPA) telah diuji. Keputusan ELISA menunjukkan bahawa semua sampel serum positif MAT adalah positif untuk IgG dan hanya 65 % (n = 13) sampel ini adalah positif bagi IgM. Bagi sampel serum negatif MAT, 25 % (n = 5) dan 45 % (n = 9) sampel adalah positif untuk IgM dan IgG masing-masing dalam ELISA. Bagi program RCPA, ELISA berdasarkan protein rLipL32 sintetik menunjukkan keputusan sebanyak 71 % daripada jumlah peserta yang menyertai program tersebut. Sampel serum positif MAT telah diuji bagi kehadiran cytokine kawal-selia, IL-10 bagi menentukan keterukan penyakit Leptospirosis. Kepekatan IL-10 dalam semua sampel serum manusia adalah di antara 10.8 ± 0.04 pg/mL kepada 31.9 ± 1.38 pg/mL. Berdasarkan perbandingan dengan kajian sebelumnya, tahap kepekatan IL-10 dalam kajian ini menunjukkan penyakit Leptospirosis yang tidak membawa kepada maut. Keputusan menunjukkan bahawa ELISA berdasarkan antigen rLipL32 sintetik dapat mengesan Leptospira-khusus IgM (sensitiviti 65 % dan spesifisiti 75 %) dan IgG (sensitiviti 100 % dan spesifisiti 55 %) dalam sampel serum manusia. ELISA berdasarkan rLipL32 mampu mengesan antibodi Leptospira-khusus dalam sampel serum manusia dan mempunyai potensi sebagai ujian diagnostik pantas bagi Leptospirosis dan menentukan 'seroprevalence' dalam kalangan masyarakat.

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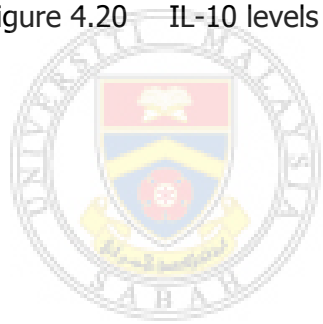

LIST OF ABBREVIATIONS

µg	-	Microgram
µL	-	Microliter
1st	-	First
2nd	-	Second
Bp	-	Base pair
g	-	Gram
g	-	Gravity
hr	-	Hour
hrs	-	Hours
kDA	-	Kilo Dalton
kg	-	Kilogram
L	-	Litre
Mb	-	Mega base
mg	-	Milligram
mL	-	Millilitre
OD	-	Optical density
RCPA	-	Royal College of Pathologists of Australasia
RE	-	Restriction Enzyme
rLipL32	-	Recombinant LipL32
rpm	-	Rotation per minute
UV	-	Ultra violet

LIST OF SYMBOLS

% - Percentage

°C - Degree Celsius

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CHAPTER 1

INTRODUCTION

1.1 Preface

Leptospirosis, caused by infection of pathogenic *Leptospira interrogans* (*L. interrogans*) is a zoonotic disease affecting both human and animals. Based on data by the World Health Organization's Leptospirosis Burden Epidemiology References (LERG), there is an estimated 873,000 cases of human Leptospirosis reported annually with 48,000 fatalities (Bandara *et al.*, 2014). Countries with the highest reported incidence are located in the Caribbean, Latin America, Indian subcontinents, Southeast Asia, Oceania and Eastern Europe (Pappas *et al.*, 2008; Shafei *et al.*, 2012).

In recent years, outbreaks of human Leptospirosis has been observed to be related to recreational activities and consequently emerged as a strong risk-factor for Leptospirosis transmission (Bandara *et al.*, 2014). Moreover, human Leptospirosis cases among international travellers have increased in the past twenty years, thus causing major public health concern worldwide (Ricaldi and Vinetz, 2006).

Up to the present day, diagnosis of Leptospirosis remains a challenge. Though microscopic agglutination test (MAT) assay is the gold standard for diagnosis of *Leptospira* infection, this assay is not effective in detection at the early phase of the disease. Therefore, researchers have attempted to develop different enzyme-linked immunosorbent assays (ELISAs) for early detection of *Leptospira*

infections using recombinant *Leptospira* outer membrane proteins (OMP) as antigens (Dey *et al.,* 2004; Tomich *et al*., 2007; Chen *et al*., 2013; Alizadeh *et al.,* 2014).

Most of these assays focused on detection of the LipL32 protein, which is found abundantly in *Leptospira* total protein profile and one of the most important OMPs expressed during human infection of Leptospirosis in addition to being highly conserved in pathogenic *Leptospira* species (Khodaverdi *et al*., 2013; Haake *et al.,* 2000). *Leptospira* OMP was suggested to have a role in the mechanism of evasion of the immune response as well as the persistence in the host (Patricia *et al*., 2014). LipL32 has been identified as an extracellular matrix (ECM) binding protein which specifically binds to fibronectin, collagen I and laminin (Patricia *et al.,* 2014). A guinea pig model of Leptospirosis showed that LipL32 protein is expressed during leptospiral infection (Hoke *et al*., 2008) and also shown to be capable of inducing tubulointerstitial nephritis in mouse proximal tubule cells (Yang *et al.,* 2002; Haake *et al.,* 2000).

Various ELISAs using rLipL32 have been developed for detection of Leptospirosis in humans (Flannery *et al*., 2001; Chen *et al*., 2013), canines (Dey *et al*., 2004), bovines (Tomich *et al*., 2007) and swine (Hartleben *et al*., 2013). In these studies, the *lipl32* gene was isolated by using a specific set of primers, then the PCR-amplified gene was cloned into an expression vector and transformed into a bacterial host for production of the rLipL32. The purified protein then served as antigen in each ELISA developed. Varying levels of performance were also noted among these studies. The rLipL32 ELISA which was developed by Flannery *et al*. (2001), has the sensitivity and specificity of 56 % and 95 % respectively. On the other hand, Chen *et al.* (2013) has demonstrated 65 % sensitivity. The sensitivity and specificity in bovine rLipL32 ELISA (Tomich *et al*., 2007) was 99.3 % and 86.33 % respectively. Meanwhile, the rLipL32 ELISA in swine (Hartleben *et al*., 2013) has the sensitivity of 100 % and 85.1 % of specificity. On the contrary, the rLipL32 ELISA in the current study was developed by using the codon optimization method.

Limited study use codon optimized gene for the diagnosis of human Leptospirosis. Essentially, the current study shows the performance of the rLipL32 ELISA developed from the codon optimized gene.

Organ damages have been observed in severe Leptospirosis (Weil's disease) which involves immune response of the host towards *Leptospira* infection. Several mediators have been suggested to play a role in Leptospirosis pathogenesis, including tumour necrosis factor alpha (TNF-α), interleukin-10 (IL-10), interleukin-8 (IL-8), interleukin-6 (IL-6), and interleukin-1 beta (IL-1β). Thus, assessing the levels of these cytokines in patients would provide an insight to the degree of severity of the Leptospirosis (Kyriakidis *et al.*, 2011; Reis *et al.,* 2013; Chirathaworn and Kongpan, 2013).

In this study, the synthetic gene encoding LipL32 protein was designed and followed by expression in *Escherichia coli*. The expressed protein was purified and subsequently used as antigens in ELISA for detection of Leptospira-specific antibodies in human serum samples. IL-10, a regulatory cytokine was measured in positive serum samples.

1.2 Objectives

The objectives of this research can be divided into three parts:

1. To express and purify the synthetic recombinant LipL32 protein in *E.coli*.
2. To determine the presence of Leptospira-specific antibodies in human serum samples using synthetic recombinant LipL32 antigen in ELISA.
3. To measure the level of regulatory cytokine, IL-10 for the determination of disease severity.

1.3 Research Approach

There are three approaches towards achieving the objectives. The first approach is to design a synthetic gene encoding the LipL32 protein. The LipL32 amino acid sequence for protein (GenBank accession number ACZ73827.1) was used for generation of the synthetic gene. The synthetic gene was codon-optimized for expression in *E.coli* BL21 (DE3). The DNA sequence generated after reverse translation of the optimized codons. The optimized codon or synthetic gene was custom cloned into pET22b vector and transformed into *E.coli* BL21 (DE3) for protein expression and protein production.

The second approach uses the immobilized metal affinity chromatography (IMAC) to purify the expressed rLipL32 protein. HisTrap FF Crude column from GE Healthcare is used to purify the protein through AKTA Pure System. The purified rLipL32 later used as antigen in ELISA for detection of Leptospira-specific antibodies in human serum samples.

The third approach uses Human IL-10 ELISA kit from BD, Bioscience to assess the level of IL-10 presence in MAT positive serum samples. The level of the IL-10 in the serum samples were compared to the previous studies for the determination of the degree of disease severity.

CHAPTER 2

LITERATURE REVIEW

2.1 Overview of Leptospirosis

Leptospirosis is a zoonotic disease occurs worldwide. It is endemic in tropical and subtropical countries in South-East Asia (Shafei *et al.,* 2012). An increase in incidents has been seen in recent years due to flooding and outdoor recreational activities (Lim, 2011). Accurate clinical diagnosis can be difficult without laboratory confirmation and the disease burden is generally under-reported. World Health Organization (WHO) has formed Leptospirosis Burden Epidemiology Reference Group (LERG) to establish correct estimations of the disease burden for better adequate intervention, control and prevention (WHO, 2010). The incubation period for Leptospirosis is 2 days to 4 weeks and the illness begins abruptly with fever.

Leptospirosis may occur in two phases. In the first phase, the symptoms which can be observed are fever, chills, severe headache, nausea, vomiting, myalgia and diarrhoea. Many of these symptoms can be mistaken for other diseases. The patient usually recovers for a time but become ill again. The second phase is called Weil's disease and is more severe and the symptoms include sepsis with multiple organ dysfunction, jaundice and haemorrhage which turn to circulatory shock and eventually death (Wagenaar *et al.,* 2009). The illness lasts from few days to 3 weeks and without treatment, recovery may take several months (Musso and Scola, 2013).

2.2 *Leptospira,* The Organism

Leptospires are spirochetes, which are thin, highly motile with an approximate size of 0.1µm in diameter and 6-20 µm in length with a distinctive hook or question-mark shape (Adler and Moctezuma, 2010) (Figure 2.1). The genus *Leptospira* can be divided into pathogenic and saprophytic strains, which belongs to the family Leptospiraceae and order Spirochaetales. Currently, there are 23 species, 25 serogroups and 250 serovars of pathogenic leptospires have been described and usually associated with natural host (Musso and Scola, 2013; Levett, 2015) (Table 2.1). Table 2.2 shows 23 *Leptospira* species that have been identified so far (Levett, 2015). The serovar classification of *Leptospira* is based on the expression of the surface-exposed epitopes in a mosaic of the lipopolysacharide (LPS) antigens and the specificity of the epitopes depends on their sugar composition and orientation (Adler and Moctezuma, 2010).

Leptospires are slow-growing obligate aerobes with an optimal growth temperature of 28°C-30°C (Faine *et al.,* 1999). Leptospires can grow in simple media enriched with vitamin B1 and B2, ammonium salts and long-chain of fatty acids (Faine *et al.,* 1999). The most widely used media to culture leptospires is the Ellinghausen-McCullogh-Johnson-Harris (EMJH) medium which contains oleic acid, bovine serum albumin (BSA), and polysorbate (Tween) (Adler and Moctezuma, 2010).

The genome size of *Leptospira* is approximately 3.9-4.6 Mb (megabase) depending on the species. Six genome sequences of *Leptospira* have been published including two isolates of *L. interrogans* (serovar Lai and Copenhageni), two isolates of *L. borgpetersenii* (serovar Hardjo) and two isolate of *L. biflexa* (serovar Patoc) (Bulach *et al.,* 2006; Picardeau *et al.,* 2008; Adler and Moctezuma, 2010). The features of these genomes are summarized in Table 2.3.