DETERMINATION OF LIPL32 EPITOPE OF LEPTOSPIROSIS VIA *in silico* METHOD AND ELISA

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BIOTECHNOLOGY RESEARCH INSTITUTE
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DECLARATION

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

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ABSTRACT

Leptospirosis is a predominant zoonotic disease caused by a pathogenic strain of *L. interrogans*. Diagnosis of leptospirosis includes microscopic agglutination test (MAT assay) and enzyme-linked immunosorbent (ELISA) which utilizes 6 designated outer membrane protein (OMP). Of these LipL32 is found to be most useful. However current prediction of the LipL32 epitope has been largely depend on empirical rather than *in silico* methods. In addition, currently no known reports of LipL32 epitope prediction using more than one bioinformatic algorithm. In this study, full length LipL32 protein was analyzed using three bioinformatic algorithms, which were the Kyte and Doolittle hydropathy scale, the recognition factor and the Kolaskar and Tangaokar propensity scale. The application of these three algorithm overlaps resulted in the recognition of three antigenic regions namely the NrLipL32 (amino acid sequence Ala_{21}-Glu_{122}), the intermediate LipL32 (amino acid sequence Pro_{144}-Glu_{171}) and the CrLipL32 (amino acid sequence Thr_{200}-Ser_{261}) fragments. Investigation for sensitivity and specificity among the fragments via ELISA indicated that CrLipL32 has the highest sensitivity (IgM 73.3% and IgG 60%) indicating to be the immunodominant epitope of LipL32. In conclusion, this study demonstrated that CrLipL32 is the immunodominant fragment of LipL32.

ABSTRAK

Leptospirosis ialah merupakan penyakit zoonotik utama yang disebabkan oleh Leptospira strain patogenik. Diagnosa leptospirosis termasuklah teknik MAT dan ELISA dimana ia menggunakan 6 protien membran luar (PML) yang terpilih. Diantara ini, LipL32 telah terbukti sebagai sangat berguna. Walaubagaimanapun, pada masa ini ramalan terhadap epitop LipL32 telah banyak menggunakan kaedah empirikal berbanding in siliko. Tambahan lagi, hingga masa kini tiada laporan menunjukan meramal epitop LipL32 menggunakan lebih daripada satu algoritma bioinformatik. Dalam kajian ini, panjang penuh protein LipL32 telah dianalisa menggunakan tiga algoritma bioinformatik iaitu skala hydropati Kyte dan Doolittle, skala faktor rekognasi, dan skala faktor kecenderungan Kolaskar dan Tangaokar. Melalui hasil pertindihan aplikasi tiga algoritma ini, tiga bahagian antigen dapat dikenal pasti iaitu NrLipL32 NrLipL32 (urutan amino asid Ala_{21}-Glu_{122}*), pertengahan LipL32 (urutan amino asid* Pro_{144}-Glu_{171}*) dan CrLipL32 (urutan amino asid* Thr_{200}-Ser_{261}*). Kajian terhadap kesensitivitian dan kespesifisitian antara bahagian tadi melalui ELISA, menunjukkan CrLipL32 mempunyai sensitiviti tertinggi (IgM 73.3% dan IgG 60%) dan merupakan juga sebagai epitop immunodominan LipL32. Konklusinya, ini menunjukan CrLipL32 adalah merupakan bahagian immunodominan epitop LipL32.*

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CrLipL32 - Carboxyl terminal recombinant LipL32
Kg - kilogram
mg - Miligram
µg - Microgram
µL - Microlire
1st - First
2nd - Second
Bp - Base pair
G - Gram
G - Gravity
Hr - Hour
Hrs - Hours
kDa - Kilodalton
Kg - Kilogram
L - Litre
Mb - Megabase
mL - Mililllitre
NrLipL32 - Amino terminal recombinant LipL32
OD - Optical density
RCPA - Royal College of Pathologist of Australia
RE - Restriction enzyme
rLipL32 - Recombinant LipL32
Rpm - Rotation per minute
UV - Ultra violet

LIST OF SYMBOLS

% - percentage

^{o}C - Degree Celcius

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CHAPTER 1

INTRODUCTION

1.1 Background of study

Leptospirosis is a zoonotic disease caused by a pathogenic strain of the Leptospira bacterial spirochete, *L. interrogans* (Levett N., 2001). It is a predominant disease with incidence rates of 0.1-1/100 000 in temperate regions and >10/100 000 in tropical regions (WHO, 2013). Several countries have recently reported leptospirosis outbreaks such as in Sri Lanka, the Philippines and Austria (Agampodi *et. al.,* 2009 ; Koizumi *et. al.,* 2009 ; McCurry, 2009 ; Radl *et. al.,* 2011). In tropical countries such as Malaysia, leptospirosis is also one of the most prevalent zoonotic diseases with the highest number of fatalities reported in the year 2014 (92 fatalities) (Wahab, 2015). Both human and animals are found to be possible hosts for this disease. Leptospirosis is transmitted via ingesting contaminated food or drinks, or when exposed wounds or mucus membrane of the eye come into contact with contaminated water (Levitt N., 2001).

Diagnosis of leptospirosis suspected samples usually involve either culture of the microorganism on Ellinghausen-McCullough-Johnson-Harris media (EMJH) media, dark field microscopy (DFM), or by molecular diagnostics such as PCR and serology (Ahmad, 2005). In culture method, the isolated Leptopira will be culture on specialized media called EMJH media and observed under dark field microscopy (DFM). However this method is laborious, expensive, longer waiting time and it does not differentiate between pathogenic and non pathogenic Leptospira (Ahmad, 2005). Molecular method such as real time quantitative PCR, detect pathogenic Leptospira *lipl32* gene has been reported before which provide rapid result (Levett *et. al.,* 2005). However it is

expensive to be used as routine application (Saengjaruk *et. al.,* 2002). The gold standard method which is MAT assay used the whole cell as antigen and able to determine pathogenic Leptospira serovar that is important in epidemiological study (Pappas *et. al.,* 1985). However this method is laborious, hazardous and delay in getting result. Rapid serological test such as ELISA utilize pathogenic Leptospira recombinant outer membrane protein (OMP) such as Lig A, Lig B, OMPL1, LipL21, LipL32 and LipL41 to detect Leptospira specific antibodies (Shang *et. al.,* 1996 ; Flannery *et. al.,* 2001 ; Raghavan *et. al.,* 2002 ; Okuda *et. al.,* 2005 ; Sankar *et. al.,* 2010 ; Joseph *et. al.,* 2012). This method is safer and cost effective than MAT assay, culture and PCR , however it cannot be used in epidemiological study. Of these, LipL32 is reported to be the most suitable as it is highly conserved and are abundant on the *Leptospira* OMP profile (Haake *et al*., 2000 ; Flannery *et. al.,* 2001 ; Cullen *et al*., 2002 ; Haake *et al*., 2004 ; Tahiliani *et al*., 2005 ; Levett 2007 ; Hoke *et al*., 2008).

Epitope discovery is important as it plays a major role in improving serodiagnostic measures, as well as in the development of antigenic determinants for vaccine construction and in the production of specific monoclonal antibodies for immunotheraphy (Nardin *et. al.,* 2001 ; Shahhosseini *et. al.,* 2007 ; Coleman *et. al.,* 2011 ; Gonzalez *et. al.,* 2014). Before bioinformatics algorithm were developed, epitope determination depended on empirical methods such as nucleic magnetic resonance (NMR), X-ray crystallography, electron microscopy (EM), ELISPOT and surface plasmon resonance (Reineke & Schutkowski, 2009). Currently various bioinformatic algorithms are developed to determine the antigenic determinants of an antigen based on different principles; such as hydrophilicity, hydrophobicity, solvent accessibility, secondary structure, antigenicity, flexibility and others (Chou and Fasman, 1974 ; Garnier, *et. al.,* 1978 ; Hoop & Woods, 1981 ; Kyte and Doolittle, 1982 ; Eisenberg, *et. al.,* 1984 ; Emini *et. al.,* 1985 ; Welling *et. al.,* 1985 ; Karplus and Schulz, 1985 ; Jameson and Wolf, 1988 ; Pellequer, *et. al.,* 1993).

Epitope determination of *Leptospira* OMP such as LigA, LipL21, LipL41 and OMPL1 have been reported previously using bioinformatics tools such as ANTIGENIC or MHCPred which were then evaluated in ELISA for possible *Leptospira* specific antibodies detection (Xu *et. al.,* 2005 ; Wiwannitkit *et. al.,* 2007 ; Lin *et. al.,* 2008 ; Lin *et. al.,* 2010). Monoclonal antibodies derived from the LipL32 epitope has been reported to be able to neutralize leptospirosis infection in mouse models (Manewatch *et. al.,* 2013). However current methodologies on LipL32 epitopes investigations predominantly involve empirical studies such as immunoblotting, peptide array, truncated LipL32 recombinant protein based-immunoassay and phage consensus mimotope identification (Hauk *et. al.,* 2008 ; Lottersburg *et. al.,* 2009 ; Manewatch *et. al.,* 2013) while *in silico* investigations are still lacking (Lin *et. al.,* 2008 ; Lin *et. al.,* 2010). Importantly, *in silico* analysis have been mainly carried out using a single bioinformatic algorithm and there is currently no known study which involves the application of more than one algorithm for LipL32 epitope determination.

1.2 Problem statement

Current methodology of LipL32 epitope determination has focus on experimental method rather than *in silico* method and there is no known research reported which involve application of more than one bioinformatic algorithm.

1.3 Hypothesis

This study hypothesizes that the comparative analysis of 3 bioinformatic algorithms can identify an immunodominant LipL32 epitope of higher specificity and sensitivity for detection of *Leptospira* specific IgM and IgG antibodies in ELISA.

1.4 Objectives

The objectives of this study are :

1. To determine the possible antigenic determinants of LipL32 antigen via the utilization of 3 bioinformatic algorithms; the Kyte and Doolittle hydropathy scale, the recognition factor scale and the Kolaskar and Tangaokar propensity scale.
2. To determine fragments derived from the algorithm combinations for possible *Leptospira* specific antibodies with higher sensitivity and specificity for ELISA detection.

CHAPTER 2

LITERATURE REVIEW

2.1 History of Leptospirosis

Leptospirosis is a disease known by various names such as cane-cutter disease, 'Schlammfieber (mud fever)' and swine herd's disease. Ancient Chinese text has described it as 'rice field jaundice' while in Japan it has been known as 'autumn fever' or 'seven day fever' (Adler, 2015). The earliest recorded cases of leptospirosis dated back to 1886 by Adolph Weil, where he described the clinical features of severe Leptospirosis which are jaundice, splenomegaly, renal dysfunction, conjunctivitis and skin rashes, subsequently known as Weil's disease (Weil, 1886). It appeared to be infectious in nature and often associated with outdoor occupations in which a person came in contact with water. Epidemics were common among sewer workers, rice-field workers and coal miners (Landouzy, 1883). The first scientific demonstration of leptospires was conducted by Stimson in 1907 where he was able to observe the spirochetes using Levaditi silver staining technique in kidney tissue sections of a patient who suspected died of yellow fever. The patient was suffering from Weil's disease as spirochetes were observed only in the kidney but not in any other vital organs. The spirochetes were named *Leptospira interrogans* by Stimson, as it is known today, because it had hooked ends and resembled a question mark (Stimson, 1907). Isolation of the etiologic agent of Leptospirosis occurred almost simultaneously and independently in Japan and in Europe but Inada and Ido were the first ones to successfully isolate the pathogenic Leptospira, followed by two groups of German physicians that studied German soldiers afflicted with "French disease". (Uhlenhuth and Fromme, 1915 ; Hubener and Reiter, 1915 ; Inada *et. al.,* 1916).

2.2 *Leptospira interrogans* the etiological agent

Leptospirosis is a worldwide zoonotic disease that is caused by a pathogenic strain of leptospira called *Leptospira interrogans* (Levett, 2001). They belong to the order of Spirochaetales, family of Leptospiraceae, and the genus Leptospira. These spirochetes are both catalase and oxidase positive and grow in a simple media that contain vitamin B, ammonium salts and long chain fatty acids which they utilize as a sole carbon source (Faine *et. al*., 1999). *L. interrogans* are long, thin, helical, highly motile spirochetes with two periplasmic flagella (endoflagella) and their cell envelope consist of a double layer structure in which their cytoplasmic membrane are closely associated with the peptidoglycan cell wall and overlaid by an outer membrane (Levett, 2001 ; Charon and Goldstein, 2002 ; Cullen *et. al.,* 2004). Their cell membrane share both properties of Gram-negative and Gram-positive bacteria but are found to be low in toxicity (Faine *et. al.,* 1999 ; Haake, 2000). Leptospires have a corkscrew-shape that differentiates them from other spirochetes by the presence of end hooks. They are about 0.1 µm in diameter and by 6-20 µm in length and require the use of dark field microscopy or phase contrast for observation (Faine *et al*., 1999). They are also obligate aerobes with an optimum growth temperature ranging from 28^{o}C to 30^{o}C (Smilbert, 1977 ; Johnson & Faine., 1984; Haake, 2000). They are not resistant to drought or hypertonicity, however they are supported in alkalinization to pH 7.8 (Mohammed *et al*., 2011). The *Leptospira* genome consists of two circular chromosomes and complete genomic sequence of it has been established (Boursaux *et. al.,* 1998 ; Ren *et. al.,* 2003). Their genomes are larger than other spirochetes such as *Treponema* and *Borellia* which explains their ability to survive in varied environments such as animal host and freely in the harsh environment (Fraser *et. al.,* 1997 ; Fraser *et. al.,* 1998).

Serological classification of *Leptospira* consists of the pathogenic species, *Leptospira interrogans* and the environmental saprophytic strains *Leptospira biflexa* (Dikken & Kmety, 1978). Classification system depends on the antigenicity of their outer envelope (OMP) and they are further divided into various serovars after cross

absorption with available homologous antigen (Johnson & Faine., 1984 ; Kmety and Dikken, 1993). Antigenically related serovar are grouped into serogroups and currently *Leptospira interrogans* comprise 24 serogroups with over 200 serovars such as Canicola, Pomona, Hardjo, Grippotyphosa and others (Kmety and Dikken, 1993)**.** Among the serovars encountered, Icterohaemorrhagiae has been reported to be associated with disease severity and seen in many countries (Storck *et al*., 2010).

Genotypic classification based on DNA relatedness, reveal that Leptospira consist of several genome species such *L. alexanderi, L. alstonii, L. borgpetersenii, L. inadai, L. interrogans, L. fainei, L. kirschneri, L. licerasiae, L. noguchi, L. santarosai, L. terpstrae, L. weilii, L. wolffii, L. biflexa, L. meyeri, L. yanagawae, L. kmetyi, L. vanthielii, and L. wolbachii,* comprising both pathogenic and saprophytic species (Ramadass *et. al*., 1992). Genomic classification provides useful taxonomic data, however pathogenic and nonpathogenic serovar usually occur in the same species (Yasuda *et al*., 1987; Brenner *et. al.,* 1999). Thus, serological classification is used commonly, although this classification system does not have any taxonomic standing but they are useful for epidemiological purpose. Other than serological tests, conventional methods can also be used to differentiate between pathogenic and saprophytic strains. The ability of the pathogenic species to grow at low temperature in the presence of 8-azaguanine or formation of spherical cells in 1M NaCl distinguishes them from the saprophytic species (Kmety *et. al.,* 1966)