EXPRESSION OF LEPTOSPIRAL RECOMBINANT PROTEIN FOR SERODIAGNOSIS

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ABSTRACT

Currently, the diagnosis of leptospirosis is entirely dependent on laboratory assay. The microscopic agglutination test (MAT) is still the standard reference test, although it requires the maintenance of a leptospiral culture panel, which is not generally available in the routine diagnostic laboratory. Outer membrane lipoproteins are important antigens, based on their surface exposure and their accessibility to infection-related immune recognition. LipL32, LipL41, and Loa22 were selected for cloning and expression as antigen. PCR fragments derived from genes encoded LipL32, LipL41, and Loa22 were cloned into a pRSET-B vector having the six histidine fusion tag at the N-terminal, and expressed in the *E. coli* expression system. The recombinant proteins were purified via Nikle affinity column. The molecular weights of the purified rLipL32, rLipL41, and rLoa 22 proteins, were 35, 27, and 27 kDa, respectively. These three purified recombinant proteins were preliminarily reacted with MAT-positive sera in Western blot; only rLipL32 could react with leptospirosis sera, while rLipL41 and Loa22 could not. Mice were immunized with each recombinant protein, to induce antibody production. The mouse antiserum of each purified recombinant protein reacted with the whole-cell lysate of some selected leptospiral serovars at the expected molecular weight, determined in the available leptospiral genome. This implied the presence of a common epitope between natural and recombinant antigens. The recombinant proteins were investigated for their application in ELISA-based serodiagnosis, compared with the reference MAT assay, by detection of IgM and IgG in the sera of MAT-positive leptospirosis patients, in addition to MAT-negative suspected leptospirosis cases and a control group of normal individuals, patients with other febrile illnesses (e.g. scrub typhus, dengue fever, melioidosis). Recombinant LipL32 yielded the highest specificity (89%), compared with rLipL41 and rLoa22. It was able to differentiate MAT-positive cases from melioidosis patients, and a low percentage of positive cases were recognized in normal individuals. In suspected, MAT-negative leptospirosis cases, rLipL32 could detect serological positive, up to 43%.

KEY WORDS: LEPTOSPIROSIS/ RECOMBINANT OUTER MEMBRANE PROTEIN/ ELISA/ IgG/ IgM

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การผลิตโปรตีนของเชื้อเลปโตสไปราโดยเทคนิคทางอณูชีววิทยาเพื่อใช้ในการวินิจฉัยโรค
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บทคัดย่อ

กระบวนการตรวจวินิจฉัยโรคเลปโตสไปโรซีสต้องอาศัยวิธีการทางห้องปฏิบัติการ การตรวจโดยวิธี MAT เป็นวิธีมาตรฐาน แม้ว่าจะไม่สามารถตรวจได้ในห้องปฏิบัติการทั่วไป เนื่องจากต้องเพาะเลี้ยงเชื้อ โปรตีนบนผิวเซลล์ของเชื้อเลปโตสไปโรซีสมีความสามารถในการกระตุ้นระบบภูมิคุ้มกันของร่างกายได้ดี จึงเป็นแอนติเจนที่สำคัญเนื่องจากมีความสัมพันธ์กับการติดเชื้อ โปรตีน LipL32, LipL41 และ Loa22 เป็นแอนติเจนซึ่งถูกเลือกมาศึกษา และถูกโคลนเข้าสู่เวคเตอร์ pRSET-B รีคอมบิแนนท์โปรตีนที่ผลิตได้เพิ่มจำนวนในเชื้อ *E. coli* และทำให้บริสุทธ์โดยใช้ Nikle affinity column รีคอมบิแนนท์โปรตีน LipL32, LipL41 และ Loa 22 มีขนาด 35, 27 และ 27 kDa ตามลำดับ รีคอมบิแนนท์โปรตีนทั้งสามชนิดถูกนำมาทดลองเบื้องต้นโดยการทำปฏิกิริยากับซีรั่มที่มีผล MAT เป็นบวกโดยวิธี Western blot พบว่า รีคอมบิแนนท์โปรตีน LipL32 เท่านั้นที่มีปฏิกิริยา รีคอมบิแนนท์โปรตีนทั้งสามได้ถูกนำมาฉีดหนูเพื่อกระตุ้นการสร้างแอนติบอดี ซีรั่มหนูที่ได้สามารถทำปฏิกิริยากับเชื้อเลปโตสไปราบางสายพันธุ์ ซึ่งทำให้เห็นว่ารีคอมบิแนนท์โปรตีนทั้งสามชนิดมีคุณสมบัติเหมือนกับเชื้อเลปโตสไปรา รีคอมบิแนนท์โปรตีนถูกนำมาทดสอบในการตรวจหา IgM และ IgG ด้วยวิธี ELISA เพื่อเปรียบเทียบกับวิธีการ MAT โดยใช้ซีรั่มคนไข้ที่มีผล MAT เป็นบวก ซีรั่มที่จากคนที่สงสัยว่าเป็นเลปโตสไปโลซีสแต่ผล MAT เป็นลบ ซีรั่มคนปกติ และซีรั่มของคนไข้กลุ่มโรค febrile illness เช่น Scrub typhus, ไข้เลือดออก และ Melioidosis รีคอมบิแนนท์โปรตีน LipL32 เป็นโปรตีนที่มีความจำเพาะต่อโรคเลปโตสไปโรซีศสูงที่สุด คือ 89 เปอร์เซ็นต์และสามารถวินิจฉัยแยกโรคเลปโตสไปโรซีสจากโรค Melioidosis ในขณะที่มีเปอร์เซนต์ต่ำในกลุ่มซีรั่มคนปกติ ส่วนกลุ่มคนที่เป็นเลปโตสไปโลซีสแต่ผล MAT เป็นลบ รีคอมบิแนนท์โปรตีน LipL32 สามารถตรวจพบว่าเป็นโรคได้ถึง 43 เปอร์เซ็นต์

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LIST OF ABBREVIATIONS

Abbreviation or symbol	Term
/	Per
%	Percentage
α	Alpha
β	Beta
°C	Degree celsius
ATP	Adenosine tri phosphate
BSA	Bovine serum albumin
CDC	Centers of disease control
CF	Complement fixation
CLIA	Chemiluminescent immunoassay
CSF	Cerebrospinal fluid
DNA	Deoxyribonucleic acid
DHF	Dengue hemorrhagic fever
DW	Distilled water
e.g	Example gratia
ELISA	Enzyme-linked immunosorbent assay
EMJH	Ellinghausen, McCullough, Johnson, Harris
et al	Et all
etc.	Et cetera
g	Gram(s)
g/mol	Gram(s) per mole
hr	Hour(s)
HRP	Horseradish peroxidase
IFA	Indirect fluorescent antibody
IgG	Immunoglobulin G
IgM	Immunoglobulin M

LIST OF ABBREVIATIONS (Cont.)

Abbreviation or symbol	Term
IHA	Indirect hemagglutination assay
IPTG	Isopropyl-β-D-thiogalactopyranoside
Kb	kilobase
kDa	Kilo Dalton
LA	Latex agglutination test
LB	Luria bertani
LEW	Lysis-equilibration-wash buffer
Lig	Immunoglobulin-link protein
LPS	Lipopolysaccharide
M	Molar(s)
mA	Milli Ampere(s)
MAb	Monoclonal antibody
MAT	Microscopic agglutination test
µg	Microgram(s)
µl	Microliter(s)
mg	Milligram(s)
ml	Milliliter(s)
mM	Millimolar(s)
MW	Molecular weight
ng/ml	Nanogram(s) per milliliter
nm	Nanometer(s)
NSS	Normal saline solution
OD	Optical density
OR	Odds ratio
OMP	Outer membrane protein
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffer saline

LIST OF ABBREVIATIONS (Cont.)

Abbreviation or symbol	Term
PCR	Polymerase chain reaction
pH	Negative logarithm of hydrogen ion activity
PVDF	Polyvinylidene fluoride
Rpm	Round per minute
RNA	Ribonucleic acid
rRNA	Ribosonal ribonucleic acid
SD	Standard deviation
SDS	Sodium dodecylsulfate
spp.	Species
TAE	Tris-acetate electrophoresis buffer
TBS	Tris buffer saline
TBS-T	TBS containing Tween-20
TLR	Toll-like receptor
TEMED	N, N, N, N –tetramethylethylenediamine
TN	True negative
TP	True positive
Tris	Tris (hydroxymethyl aminomethane)
UDW	Ultrapure distilled water
V	Volt(s)
v/v	Volume by volume
w/v	Weight by volume
WHO	World Health Organization

CHAPTER I
INTRODUCTION

Thailand is agricultural country. Many people work in farms and most of them caught up infection from their work by contacting to animal and environment. Leptospirosis is a zoonotic infection with a worldwide distribution affecting wild and domestic vertebrates, the incidence of the disease being higher in tropical climates (Plank and Dean, 2000). The causative bacteria, pathogenic *Leptospira* species, are transmitted to human most commonly by indirect contact with contaminated fresh water (Marien *et al.*, 2005). Infection can occur in many mammalian species and some reptiles. Infected animals often become carriers and leptospires are maintained in their kidneys or genital organs. There is no relationship between the severity of the infection and the subsequent carrier status. Animals that do not develop clinical disease but become chronic carriers may be described as maintenance hosts whilst those that develop clinical leptospirosis and carry infection for a short term may be described as accidental hosts. Leptospiral organisms can be found in animals including rodents, cattle, pigs, horses, and dogs.

Most human infections occur through contact with urine excreted by infected mammals primarily through skin abrasions, open wounds or mucous membranes, and occasionally through ingestion or inhalation. Leptospirosis is normally not transmitted person to person, and the incubation period is usually between 4-19 days. Patients with leptospirosis commonly present with a flu-like illness with high fever, headache, muscle pains, red eyes, sore throat and rash. In some cases, the disease can cause anemia and affect the liver, kidneys, lungs, and other internal organs. Symptoms of leptospirosis include high fever, severe headache, chills, hemorrhage, muscle aches, vomiting and may include jaundice, red eyes, abdominal pain, diarrhea or a rash, which are characteristic of a typical protean clinical presentation. If the disease is not treated in time, patients may develop renal damage, meningitis, liver failure, and

respiratory distress; in rare cases death can occur (Rathinam, 2002). The clinical presentation of leptospirosis in humans is variable, and can range from a mild flu-like illness to a severe disease with pulmonary hemorrhage, renal failure, and occasionally death (Levtt *et al*., 2001). This disease remains underdiagnosed largely due to the broad spectrum of signs and symptoms attributable to this spirochetal pathogen. Consequently, leptospirosis is easily mistaken for other febrile illnesses including influenza, dengue fever, meningitis, or hepatitis. Therefore, rapid and appropriate laboratory diagnostic tests are needed to aid clinical case identification and to facilitate the implementation of rapid outbreak investigations for optimal treatment and patient management (Tansuphasiri *et al*., 2005).

The majority of leptospirosis cases are diagnosed by serology. The reference standard assay is the microscopic agglutination test (MAT). Locally isolated strains, which often increase the sensitivity of the test compared with reference strains, can also be included in the battery of antigens. However, the range of serovars should not be limited to local strains in case the infection is due to a rare serovar or perhaps to a strain that is currently unknown in the region concerned. For this reason, a saprophytic strain is included (*L. biflexa* strain Patoc I) which cross-reacts with human antibodies generated by a number of pathogenic serovars (Bharti *et al*., 2003). Other serologic tests have been performed to diagnose leptospirosis (Appassakij *et al*., 1995; Gussenhoven *et al*., 1997). To date, only a few leptospiral outer membrane proteins have been characterized in detail, including a porin, OmpL1 (Haake *et al*., 1993) and two lipoproteins, LipL36 and LipL41 (Shang *et al*., 1996). Development of a single specific antigenic reagent suitable for serological detection of infections with all serovars remains a great challenge. Recently, recombinant antigens have been produced using porin transmembrane protein (OmpL1), lipoproteins (LipL32, LipL36 and LipL41) and a heat-shock protein (Hsp58), all of which have been used in ELISA for serodiagnosis of human leptospirosis (Flannery *et al*., 2001).

The LipL32 is a major leptospiral outer membrane protein whose expression is restricted to pathogenic *Leptospira* species (Haake *et al*., 2000). The alignment also indicated that the outer membrane proteins were polymorphic (Cullen *et al*., 2002).

LipL32 is highly conserved in pathogenic *Leptospira* across the globe, as evidenced by the LipL32 sequences submitted from various parts of the world (Tahiliani *et al*., 2005). LipL32 is known to be the most abundant outer membrane protein, which is expressed during all mammalian infections and reacts with patient serum from all phases of infection further pointing to its usefulness as an antigen for specific detection of leptospirosis (Flannery *et al*., 2001; Zuerner *et al*., 1991).

Loa22, is up-regulated during host infection (Nally *et al*., 2007) and encodes a lipoprotein with an OmpA domain (Koizumi and Watanabe, 2003) that is strongly recognized by sera from human leptospirosis patients (Gamberini *et al*., 2005). Furthermore, Loa22 is conserved among pathogenic *Leptospira* (Gamberini *et al*., 2005), suggesting that it may play a specific role in disease pathogenesis.

Another highly conserved leptospiral lipoprotein, LipL41 (Shang *et al*., 1996), can provide 71% protection in the hamster model of leptospirosis when administered in combination with recombinant OmpL1 (Haake *et al*., 1999). The LipL41 is one of the important and surface exposed leptospiral outer membrane protein expressed by pathogenic *Leptospira* species only (Shang *et al*., 1996). In the present study, an ELISA using the LipL41 recombinant protein as an antigen was developed for carrying out the serodiagnosis of bovine leptospirosis. The conserved nature of LipL41 among the pathogenic *Leptospira* spp. Suggests that rLipL41 ELISA may exhibit similar performance regardless of the locally predominant serovars. Thus, use of the recombinant LipL41 antigen in ELISA has the potential to become a useful tool for serodiagnosis of bovine leptospiral infection (Mariya *et al*., 2006). The leptospiral antigens that are conserved among the diverse pathogenic leptospires have potential importance in the development of new serodiagnostic and immunoprotective strategies. The present study was therefore carried out to find out the phenotypic conservation of the leptospiral proteins OmpL1 and LipL41, and the genetic conservation of OmpL1 and LipL41 genes among the leptospiral isolates of Andaman Islands and among the reference strains (Natarajaseenivasan *et al*., 2005).

In these studies, we selected all three proteins, *i.e.* LipL32, Loa22 and LipL41 to be expressed in plasmid vector. The PCR-amplified protein gene was ligated in to plasmid vector which had hexahistidine tag molecules and used *E. coli* strain BL21 (DE3)pLysS as host during recombinant protein expression.

The objectives of this study are to produce some selected-recombinant outer membrane protein and evaluate their immunological reactivity with normal and patient sera by Western blot analysis to demonstrate whether each protein is immunogenic and expressed during infection. Another objective is to determine the immunogenicity of recombinant protein by immunization mouse with recombinant protein and determination whether the antibody to recombinant protein can react to Leptospiral native protein or not. This experiment is designed to demonstrate whether the recombinant protein possess the epitopes in common to native Leptospiral protein or not.

CHAPTER II
OBJECTIVES

1. Cloning and expression the leptospiral outer membrane protein *i.e.* LipL32, LipL41 and Loa22.

2. Characterization of four selected-recombinant leptospiral outer membrane protein in their immunological reactivity with patient sera to demonstrate whether each protein is immunogenic and expressed during infection.

3. The selected-recombinant leptospiral protein that could react to patient sera was subjected to immunize mice. The antibody to purified recombinant protein was tested for the reactivity with leptospiral whole cell lysate to determine whether the recombinant protein shared the commom epitope to native Leptospiral protein.

CHAPTER III
LITERATURE REVIEW

3.1 *LEPTOSPIRA* Historical background

Leptospira (from the Greek leptos, meaning fine or thin, and the Latin spira, meaning coil) is a genus of spirochaete bacteria, including a small number of pathogenic and saprophytic species (Ryan, 2004). *Leptospira* was first observed in 1907 in kidney tissue slices of a leptospirosis victim who was described as having died of "yellow fever (Stimson, 1907). The history of leptospirosis or Weil's disease has of course been around for millions of years, but the cause of illness was only identified recently as bacteria. The exact way they cause illness and immunity was still in progress. Although, it was attempt to fully understand the etiologie bacteria for many more years, Adolf Weil's publication in 1886 was credited with the first detailed account of the infection, and it was right that the icteric form of illness he described has been given his name. However the infection was known and reported long before that, with accounts associating fever and jaundice with farming, flooding and livestock floating about in literature and language all over the world, from ancient China to India and Europe. Moving towards recent times, Weil's disease (albeit without a name) was noted in medical reports from the Napoleonic campaigns and travellers to the Americas.

The fact that Weil's paper received so much attention was more an accident of geography and politics than of any unique insight. In the late 19th Century, a lot of medical material was in German, printing made such material readily available, and Weil was in the right place at the right time to be noticed. The illness Weil was described the severe icteric form with jaundice, and it is not as simple to find descriptions of the milder forms, simply because they were so easily misdiagnosed and weren't accepted as a disease until the advent of bacteriology allowed the causes of

illness to be determined. The association of risk was known for decades, even centuries, before the leptospiral bacterium was found and associated as a cause. Spirochetes were found to cause a wide range of illness in the latter half of the 19th Century, but the first credited account of a leptospire isolated from a patient was by Stimpson, in 1907. The paper had a rough ride and was republished a few times before being noticed, but looking at it today we see several important discoveries, such as the concentration of bacteria in the renal tubules, that others took far longer to 'discover' at the time. Stimpson called the bacteria 'Spirocheta interrogans' due to their stained shapes looking like question marks, and the name has remained.

The First World War increased research on all sides, as the trench conditions made case numbers increase beyond all previous records. One of the outcomes of all this wartime research, apart from the knowledge of the bacteria and their virulence, was that each side disclaimed each other for discoveries and even now it is hard to determine precisely who discovered what first. Rats as a carrier (reservoir host) were identified in Japan in the years between discovery of the bacteria and the War. Most of the basic pathology and epidemiology we use today was defined before 1940, and in recent years the research has concentrated on the bacterial DNA sequence and internal cellular processes that confer virulence, immunity and may lead to development of better vaccines.

3.2 Classification

3.2.1 Serological Classification

Classification based on serotyping remains the gold standard, molecular-based taxonomy, which is still in used, classifies *Leptospira* into 12 genomically distinct species: *L. alexanderi, L. biflexa, L. borgpetersenii, L. fainei, L. inadai, Leptospira interrogans, L. kirschneri, L.a noguchii, L. santarosai, L. weilii, L. meyeri* and *L. wolbachii* (Brenner *et al*., 1999; Yasuda *et al*., 1987).

3.2.2 Genotyping Classification

The genus *Leptospira*, including pathogenic and saprophytic species, can be further classified into serological types, *i.e.*, serogroups and serovars, which defined

by a cross agglutination absorption test. The alternative genotypic classification is based on DNA hybridization, thus the leptospires can be assigned to the species level (Faine *et al*., 1999b; Ramadass *et al*., 1992; Yasuda *et al*., 1987). *Leptospira*, together with the genera *Leptonema* and *Turneria*, is a member of the family Leptospiraceae. The genus *Leptospira* is divided into 17 genomospecies based on DNA hybridization studies (Bharti *et al*., 2003; Brenner *et al*., 1999; Levett *et al*., 2006). Leptospires have been classified into more than 230 serovars based on their agglutinating antigens (Brenner *et al*., 1999; Levett *et al*., 2006). These species can be further divided into pathogenic, non-pathogenic and opportunistic/possibly pathogenic *Leptospira* with pathogenic species. The pathogenic *Leptospira* include; *L. interrogans, L. kirschneri, L. santarosai, L. weilii*, *L. alexanderi*, *L. borgpetersenii, L. genomospecies 1* and *L. noguchii*. The non pathogenic *Leptospira* include: *L. biflexa, L. meyeri, L. wolbachii, L. genomospecies 3, L. genomospecies 4, L. genomospecies 5* and opportunistic/intermediate pathogens *Leptospira* include *L. broomi, L. fainei* and *L. inadai* (Perolat, 1998).

Table 3.1 Genotyping classification of *Leptospira* species

Leptospira	**No**	**Serogroup**	**Serovar**	**Strain**	**Species**
Pathogenic	1	Australis	Australis	Ballico	*L. interrogans*
	2	Australis	Bangkok	Bangkok D 92	*L. interrogans*
	3	Australis	Bratislava	Jez Bratislava	*L. interrogans*
	4	Autumnalis	Sutumnalis	Akiyami A	*L. interrogans*
	5	Autumnalis	New	Heusden P2062	*L. interrogans*
	6	Autumnalis	Rachamati	Rachmat	*L. interrogans*
	7	Ballum	Ballum	MUS 127	*L. borgpetersenii*
	8	Bataviae	Bataviae	Van Tienam	*L. interrogans*
	9	Canicola	Canicola	Hound Utrech IV	*L. interrogans*
	10	Cellidoni	Cellidoni	Celledoni	*L. weilii*
	11	Djasiman	Djasiman	Djasiman	*L. interrogans*
	12	Grippotyphosa	Grippotyphosa	Moskva V	*L. kirschener*
	13	Hebdomadis	Hebdomadis	Hebdomadis	*L. interrogans*
	14	Icterohaemorrhagiae	Copenhegeni	M 20	*L. interrogans*
	15	Icterohaemorrhagiae	Icterohaemorrhagiae	RGA	*L. interrogans*
	16	Javanica	Javanica	Veldrat Bataviae 46	*L. borgpetersenii*
	17	Louisiana	Saigon	L 79	*L. interrogans*
	18	Panama	Panama	CZ 214 K	*L. noguchi*
	19	Pomona	Pomona	Pomona	*L. interrogans*
	20	Pyrogenes	Pyrogenes	Salinem	*L. interrogans*
	21	Pyrogenes	Zanoni	Zanoni	*L. interrogans*
	22	Ranarum	Ranarum	ICF	*L. meyeri*
	23	Sarmin	Sarmin	Sarmin	*L. weilii*
	24	Sejroe	Hardjo	Hardjoprajitno	*L. interrogans*
	25	Sejroe	Sejroe	M84	*L. borgpetersenii*
	26	Sejroe	Wolffi	3750	*L. interrogans*
Intermediate	27	Tarassovi	Tarassovi	Perepelicin	*L. borgpetersenii*
Saprophytic	28	Hurstbridge	Hurstbridge	But 6	*L. fainei*
	29	Semaranga	Patoc	Patoc I	*L. biflexa*
	30	Semaranga	Semananga	Veldrat Semarang 173	*L. meyeri*
	31	Andamana	Andaman	CH11	*L. biflexa*
	32	Codice	Codice	CDC	*L. wolbachi*

This species classification has been controversial (Yasuda *et al*., 1987).

3.3 Morphology

Leptospirosis is the zoonotic disease caused by members of the genus, *Leptospira*. They are motile helical spirochaetes that metabolize long chain fatty acids as their carbon source (Slack *et al*., 2006). Although over 200 serovars of *Leptospira* have been described, all members of the genus have similar morphology. *Leptospira* are spiral-shaped bacteria that are 6-20 µm in length and 0.1 µm in diameter with a wavelength of about 0.5 µm (Levett, 2001). One or both ends of the spirochete are usually hooked. Because they are so thin, live *Leptospira* are best observed by darkfield microscopy. The bacteria have a number of freedom degrees; when ready to proliferate via binary fission, the bacterium noticeably bends in the place of the future split. *Leptospira* have a Gram-negative-like cell envelope consisting of a cytoplasmic and outer membrane. However, the peptidoglycan layer is associated with the cytoplasmic rather than the outer membrane, an arrangement that is unique to spirochetes. The two flagella of *Leptospira* extend from the cytoplasmic membrane at the ends of the bacteria into the periplasmic space and are necessary for the motility of *Leptospira* (Picardeau *et al*., 2001).

3.4 Epidemiology and transmission

This has been exemplified by outbreaks in Nicaragua, Brazil, India, Southeast Asia, the United States, and more recently afflicting participants from several countries in the EcoChallenge Sabah 2000 competition in Malaysia (Centers for Disease Control and Prevention (CDC), 2001). It was identified as an emerging infectious disease after recent epidemic outbreaks around the world (Campagnolo *et al*., 2000; Kariv *et al*., 2001). The widely distribution of leptospires results from their ability to colonize in the renal tubules of a diverse group of domestic and wild animals, where they persist, multiply, and are voided in urine. Its unique ability to survive as a free-living organism, either in unprotected water reservoirs or in the soil, allows relentless spread of these microbes (Adler *et al*., 2002). For several centuries, human infection has been known for its association with agriculture and with other occupations in which animal contact is unavoidable (Levett, 1999).

Thailand is the endemic area of the disease's especially in the northeast of the country's (Tangkanakul *et al*., 2000) where most of the people are farmers. Since 1996, the reported cases increased markedly. In 2000, 14,285 leptospirosis cases were reported and 10,217 cases in 2001 with 362 deaths and 171 deaths respectively (Leelarasamee *et al*., 2004). Numerous serovars of leptospires were claimed as the cause of infection even in the same geographical area.

Leptospirosis is transmitted by the urine of an infected animal, and is contagious as long as it is still moist. There is a common misconception that the disease is associated only with rats. Rats, mice and voles are important primary hosts, but a wide range of other mammals including dogs, deer, rabbits, hedgehogs, cows, sheep, raccoons, possums, skunks, and even certain marine mammals are also able to carry and transmit the disease. Dogs may lick the urine of an infected animal off the grass or soil, or drink from an infected puddle. There have been reports of "house dogs" contracting leptospirosis apparently from licking the urine of infected mice that entered the house. Although this behavior is generally associated with some degree of infection risk, the infective bacteria do not live naturally in freshwater and can only survive outside an animal host for a matter of days (Bharti *et al.*, 2003).

The disease therefore requires a population of mammalian carriers, normally together with wetted soil or polluted puddles, to keep the cycle going. The type of habitats most likely to carry infective bacteria are muddy riverbanks, ditches, gulleys and muddy livestock rearing areas where there is regular passage of either wild or farm mammals. There is a direct correlation between the amount of rainfall and the incidence of leptospirosis, making it seasonal in temperate climates and year-round in tropical climates (Ko *et al*., 1999). These spirochetes colonize proximal renal tubules of chronically infected carrier animals such as cattle, horses, dogs, pigs, rats, raccoons, and skunks. Carrier animals then shed leptospires in their urine, and humans acquire leptospirosis by accidental exposure to urine from infected animals or to urine-contaminated water. *L. interrogans* invades humans and other hosts through intact or injured mucous membranes and then disseminates from the site of initial infection via the bloodstream. The postentry period of approximately 10 to 14 days is characterized

by a leptospiremic phase, during which leptospires persist in the blood. The primary lesion during this phase is damaged to the endothelia of small blood vessels, resulting in localized ischemia in kidneys, liver, meninges, and muscles (Bharti *et al*., 2003; Frane *et al*., 1999). Maintenance hosts are carrier animals that harbor leptospires in their proximal renal tubules, in some cases, for the life of the animal. Transmission to new hosts involves either direct or indirect exposure to organisms shed in the urine of infected animals. The paradigm for direct transmission is *Leptospira borgpetersenii* serovar Hardjo infection of cattle. Accidental infection of humans by serovar Hardjo almost always involves a history of contact with cattle. In contrast, transmission of *Leptospira interrogans* serovar Copenhageni or *Leptospira interrogans* serovar Lai from rats to humans is usually indirect (Lo *et al*., 2006).

Leptospirosis is also transmitted by the semen of infected animals (Kiktenko *et al*., 1976). Abattoir workers can contract the disease through contact with infected blood or body fluids. Humans become infected through contact with water, food, or soil containing urine from these infected animals. This may happen by swallowing contaminated food or water or through skin contact. The disease is not known to be spread from person to person and cases of bacterial dissemination in convalescence are extremely rare in humans. Leptospirosis is common among watersport enthusiasts in specific areas as prolonged immersion in water is known to promote the entry of the bacteria. Occupational risk factors include veterinarians, slaughter house workers, farmers, and sewer workers. An outbreak in an inner city environment has been linked to contact with rat urine (Langston and Heuter, 2003). Urine excreted by infected animals contains viable *Leptospira* , which can survive for days to weeks in soil or water (Chang, 1946; Hellstrom and Marshall, 1978). Flooding following heavy rainfall is frequently associated with large outbreaks of leptospirosis (Ko *et al*., 1999; Trevejo *et al*., 1998). It should also be noted that owners of pet rats are at risk due to contamination from wild rats. In June 2006, Sheffield England a 25 year old Male contracted Weils Disease after being infected from pet rats purchased from a petshop.

3.5 Pathogenesis

The underlying pathogenic mechanisms associated with the severe manifestations of leptospirosis are poorly understood but it is assumed that a generalized endothelial dysfunction, as observed in experimental and human leptospirosis, is the main pathogenic mechanism of tissue damage (de Brito *et al*., 1979; Nicodemo *et al*., 1997). Leptospirosis is the most widespread zoonosis in the world and has emerged as an important public health problem in large urban centres of developing countries (Ko *et al*., 1999). The primary lesion caused by leptospiral infection is damaging to the endothelium of small blood vessels, leading to hemorrhage and localized ischemia in multiple organs (Faine *et al*., 1999b).

Leptospirosis is caused by pathogenic spirochaetes of the genus *Leptospira*. The organism affects many mammalian species, including humans. Animals may become inapparent carriers and shedding of leptospires, primarily in the urine, serves as a source of infection for other animals and humans (Palaniappan *et al*., 2004). Its severe disease form, known as Weil's syndrome, is an acute febrile illness associated with multiorgan system complications including jaundice, renal failure, meningitis and pulmonary haemorrhage, with a mortality rate that may exceed 15% (Faine *et al*., 1999b; Marotto *et al*., 1999). Infection with pathogenic serovars of *Leptospira* may result in a very wide spectrum of clinical conditions ranging from subclinical infection to severe multi-systemic disease characterized by jaundice and renal failure.

Pathogenic *Leptospira* are highly motile and invasive organisms (Barocchi *et al*., 2001; Merien and Baranton, 1997), which rapidly disseminate to target organs after entering the host, usually through abrasions in the skin or mucous membranes (Faine *et al*., 1999b). These organisms may be cleared by the humoral immune response, but in carriers they have the ability to colonize and persist in the kidney tubules. The rat (*Rattus norvegicus*) is the principal reservoir associated with urban epidemics of leptospirosis (Ko *et al*., 1999). The broad range of symptoms may lead to significant diagnostic confusion with other common causes of febrile illness, particularly in tropical regions (Levett *et al*., 2000).

The broad host range of pathogenic leptospires indicates an ability to overcome the antimicrobial defense systems of a wide variety of animals. Following entry through the skin or mucosa, *L. interrogans* is exposed to the innate humoral and cellular defense systems, which normally disarm and remove less virulent microbial intruders. Initial leptospiral infection is followed by bacteremia that persists through the incubation period and for 1 to 2 weeks after onset of acute disease. Multiplication in susceptible hosts is rapid, with doubling times of 8 h or less for virulent strains that cause acute fulminating disease (Faine *et al*., 1999b). Mortality from severe forms of the disease is 5-40% (Bharti *et al*., 2003; Ko *et al*., 1999).

Mortality remains significant, generally stemming from delays in diagnosis due to lack of infrastructure and inadequate clinical suspicion, or lack of treatment facilities in developing countries, and may also result from inherent pathogenicity of some leptospiral strains or genetically determined host immunopathological responses (Bharti *et al*., 2003). Furthermore, leptospirosis is fairly rare in non-adults, or is not diagnosed, or is confused with other infectious diseases such as influenza, viral hepatitis, infectious mononucleosis, rickettsiosis, brucellosis, and in the tropics, dengue, as well as with other causes of mild renal failures (Levett, 2001).

3.6 Virulence factor

3.6.1 Lipopolysacharide (LPS) or Endotoxin

Leptospiral lipopolysaccharide (LPS) is a major outer membrane component recognized by Toll-like receptor 2 (TLR2) on macrophages (Werts *et al*., 2001). The acquired immune response to the carbohydrate component of leptospiral LPS confers serovar-specific immunity and underlies the serological classification of the genus *Leptospira* into > 200 serovars (de la Pena-Moctezuma *et al*., 2001). The outer membrane contains a variety of lipoproteins and transmembrane outer membrane proteins (Cullen *et al*., 2002). As expected, the protein composition of the outer membrane differs when comparing *Leptospira* growing in artificial medium with *Leptospira* present in an infected animal (Haake *et al*., 1998; Nally *et al*., 2007; Palaniappan *et al*., 2002). Several leptospiral outer membrane proteins have been shown to attach to the host extracellular matrix and to factor H. These proteins may be

important for adhesion of *Leptospira* to host tissues and in resisting complement, respectively (Barbosa *et al*., 2006; Choy *et al*., 2007; Verma *et al*., 2006).

The outer membrane of *Leptospira*, like those of most other Gram-negative bacteria, contains lipopolysaccharide (LPS). Differences in the highly immunogenic LPS structure account for the numerous serovars of *Leptospira* (Levett, 2001). Consequently, immunity is serovar specific; current leptospiral vaccines, which consist of one or several serovars of *Leptospira* endemic in the population to be immunized, protect only against the serovars contained in the vaccine preparation. Leptospiral LPS has low endotoxin activity (Levett, 2001).

An unusual feature of leptospiral LPS is that it activates host cells via TLR2 rather than TLR4 (Werts *et al*., 2001). The unique structure of the lipid A portion of the LPS molecule may acccount for Que-Gewirth *et al* observation (Que-Gewirth *et al*., 2004). Finally, the LPS O antigen content of *L. interrogans* differs in an acutely infected versus a chronically infected animal (Nally *et al*., 2005). The role of O antigen changes in the establishment or maintenance of acute or chronic infection, if any, is unknown.

3.6.2 Other virulence factor

The virulence mechanisms, and more generally the fundamental understanding of the biology of the causative agents of leptospirosis, remain largely unknown. To date, only a few proteins have been identified as putative virulence factors. Pathogenic leptospires have been shown to express adhesins (Barbosa *et al*., 2006; Merien *et al*., 2000), hemolysins (Lee *et al*., 2002), and many lipoproteins prominent in leptospires and other spirochetes that could play a role in host–cell interactions (Cullen *et al*., 2004). Few leptospiral factors that contribute to the pathogenesis of disease have been identified. The periplasmic endoflagella involved in propeller movement of the bacterium is an important factor in tissue penetration and motility through highly viscous fluids (Perolat *et al*., 1998; Trueba *et al*., 1992). Moreover, chemotaxis for haemoglobin has been demonstrated, which indicates that *Leptospira* may be attracted to abraded skin surfaces (Yuri *et al*., 1993). Many pathogenic *Leptospira* secrete

sphingomyelinase C (SphA) and poreforming haemolysins (SphH), possibly associated with the haemolytic anaemia observed in leptospirosis patients (Lee *et al*., 2002; Segers *et al*., 1992). The ability of pathogenic leptospires to penetrate, disseminate and persist in mammalian host tissues appears to rely on the ability of these organisms to attach to eukaryotic cells and extracellular matrix proteins (Ballard, 1993). The variety of host cells recognized by *Leptospira* suggests the presence of several adhesins. However, the only putative leptospiral adhesin identified to date is a virulence-associated leptospiral surface protein that binds purified fibronectin (Merien *et al*., 2000). In a recent report, pathogenic leptospires were distinguished from saprophytic organisms by their ability to rapidly translocate through a polarized MDCK monolayer without disrupting tight junctions (Barocchi *et al*., 2002). Rapid translocation across tissue barriers may be a mechanism used by this pathogen to invade host organs.

LigA and LigB, encoded by separated genes, are members of the family of bacterial proteins containing immunoglobulin like repeats that have been identified as adhesins relevant to microbial pathogenesis, such as intimin in *Escherichia coli* and invasin in *Yersinia pseudotuberculosis* (Matsunaga *et al*., 2003; Palaniappan *et al*., 2002). The Lig proteins are expressed only on the surfaces of leptospiral pathogens isolated from infected animals and not by saprophytic *Leptospira* species (Matsunaga *et al*., 2003). Moreover, we have recently shown that Lig expression in pathogenic *Leptospira* is induced by a change in the osmolarity of the culture medium that mimics the transition that the bacteria might encounter upon entry into a mammalian host (Matsunaga *et al*., 2005). Their structural resemblance to known adhesins and close association with virulence suggest that the Lig proteins may be MSCRAMMs (microbial surface components recognizing adhesive matrix molecules), an important category of bacterial proteins involved in the colonization of host tissue (Patti *et al*., 1994). Several adhesins that bind host extracellular matrix proteins have been identified in pathogenic spirochetes. This leptospiral adhesin was previously identified as LfhA, a factor H-binding protein in pathogenic *L. interrogans* (Verma *et al*., 2006).

3.6.3 Lipoprotein

Lipoprotein is a biochemical assembly that contains both proteins and lipids. The lipids or their derivatives may be covalently or non-covalently bound to the proteins. Many enzymes, transporters, structural proteins, antigens, adhesins and toxins are lipoproteins. Examples include the high density and low density lipoproteins of the blood, the transmembrane proteins of the mitochondrion and the chloroplast, and bacterial lipoproteins. *Leptospira* have a Gram-negative-like cell envelope consisting of a cytoplasmic and outer membrane. However, the peptidoglycan layer is associated with the cytoplasmic rather than the outer membrane, an arrangement that is unique to spirochetes. The two flagella of *Leptospira* extend from the cytoplasmic membrane at the ends of the bacteria into the periplasmic space and are necessary for the motility of *Leptospira* (Picardeau *et al*., 2001).

The outer membrane contains a variety of lipoproteins and transmembrane outer membrane proteins. As expected, the protein composition of the outer membrane differs when comparing *Leptospira* growing in artificial medium with *Leptospira* presented in an infected animal (Cullen *et al*., 2002). Several leptospiral outer membrane proteins have been shown to attach to the host extracellular matrix and to factor H (Haake *et al*., 1998; Nally *et al*., 2007; Palaniappan *et al*., 2002). These proteins may be important for adhesion of *Leptospira* to host tissues and in resisting complement, respectively (Barbosa *et al*., 2006; Choy *et al*., 2007; Verma *et al*., 2006). The outer membrane of *Leptospira*, like those of most other Gram-negative bacteria, contains lipopolysaccharide (LPS). Differences in the highly immunogenic LPS structure account for the numerous serovars of *Leptospira* (Levett, 2001). Consequently, immunity is serovar specific; current leptospiral vaccines, which consist of one or several serovars of *Leptospira* endemic in the population to be immunized, protect only against the serovars contained in the vaccine preparation. Leptospiral LPS has low endotoxin activity (Levett, 2001). An unusual feature of leptospiral LPS is that it activates host cells via TLR2 rather than TLR4 (Werts *et al.*, 2001). The unique structure of the lipid A portion of the LPS molecule may acccount for Que-Gewirth observation (Que-Gewirth *et al*., 2004). Finally, the LPS O antigen content of *L. interrogans* differs in an acutely infected versus a chronically infected

animal (Nally *et al*., 2005). The role of O antigen changes in the establishment or maintenance of acute or chronic infection, if any, is unknown. A large number of exported lipoproteins and transmembrane outer membrane proteins which may be involved in leptospiral pathogenesis and protective immunity were identified. Currently available vaccines have low efficacy, are serovar-specific, and do not induce long-term protection against infection (Faine, 1994; Levett, 2001). The large number of pathogenic serovars (>200) and the cost of producing a multiserovar vaccine have been the major limitations. Outer membrane proteins that are conserved among pathogenic serovars might be used in a recombinant vaccine without the limitations of currently available whole-cell preparations. It is anticipated that examination of these candidate protective immunogens will provide new approaches for vaccine development. Experimental evidence for fatty acid modification of leptospiral lipoproteins has been described for the outer membrane lipoproteins LipL32 (LIC11352) (Haake *et al*., 2000), LipL36 (LIC13060) (Haake, 1998), and LipL41 (LIC12966) (Shang *et al*., 1996). The cytoplasmic membrane also contains lipoproteins, as demonstrated for LipL31 (LIC11456) and LipL71 (LIC11003) (Haake and Matsunaga, 2002). A total of 184 predicted coding sequences in the *L. interrogans* genome were found to have a lipoprotein signal peptidase cleavage site (Nascimento *et al*., 2004). All proposed lipoproteincoding sequences conform to the rule of having an L, I, V, or F in the -3 and/or -4 position relative to cysteine and most of them have A, G, S, or N in the -1 position relative to cysteine (Haake, 2000).

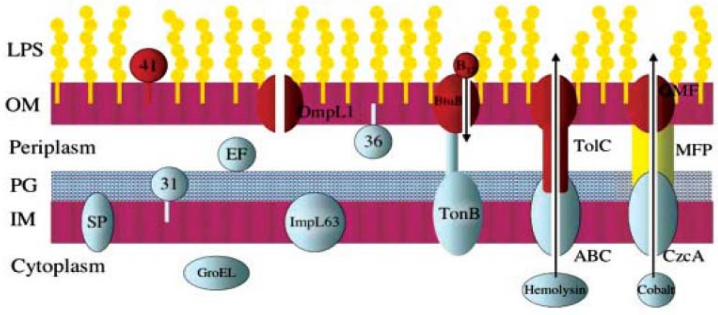


Figure 1. Model of leptospiral membrane architecture. Leptospires have two membranes, an outer membrane (OM) and a cytoplasmic or inner membrane (IM). As in Gram-positive bacteria, the peptidoglycan (PG) cell wall is closely associated with the IM. The leptospiral surface is dominated by lipopolysaccharide (LPS) carbohydrate side chains.

Subsurface proteins include the cytoplasmic protein, GroEL, and the periplasmic endoflagella (EF). The IM contains lipoproteins such as LipL31 and transmembrane proteins such as signal peptidase (SP) and ImpL63. The OMP contains lipoproteins including LipL41 and LipL36, and transmembrane proteins including the porin, OmpL1. Genomic sequence analysis reveals several novel types of outer membrane proteins (OMPs), including TonB dependent OMPs involved in nutrient acquisition. BtuB is an example of a TonB-dependent OMP involved in uptake of vitamin B12. The type I efflux system is represented by TolC, which forms a complex with ATP-binding cassette (ABC) transporters to export hemolysins and other cytoplasmic components. The leptospiral genome also contains genes involved in a three-component efflux system consisting of an outer membrane factor (OMF), membrane fusion protein (MFP), and an inner membrane transporter, in this case CzcA, which is involved in heavy metal detoxification.

3.6.3.1 Biosynthesis of lipoprotein

The structure of the lipid modification in the case of major outer membrane protein of *Escherichia coli* was elucidated chemically in 1973. It was shown that Sulphydryl group of N-terminal Cysteine was modified with a diacylglyceryl group attached through a thioether linkage and the amino group was acylated with a fatty acid (Hantke and Braun, 1973a;, 1973b; Koerner et al., 1973; Preuveneers *et al*., 1973) (shown in figure). The acyl group composition was found to be same as that of membrane phospholipids and therefore membrane phospholipids were suspected to be the lipid donors for the modification (Hantke and Braun, 1973a; 1973b; Koerner *et al.*, 1973; Preuveneers *et al*., 1973).

The Biosynthetic Pathway

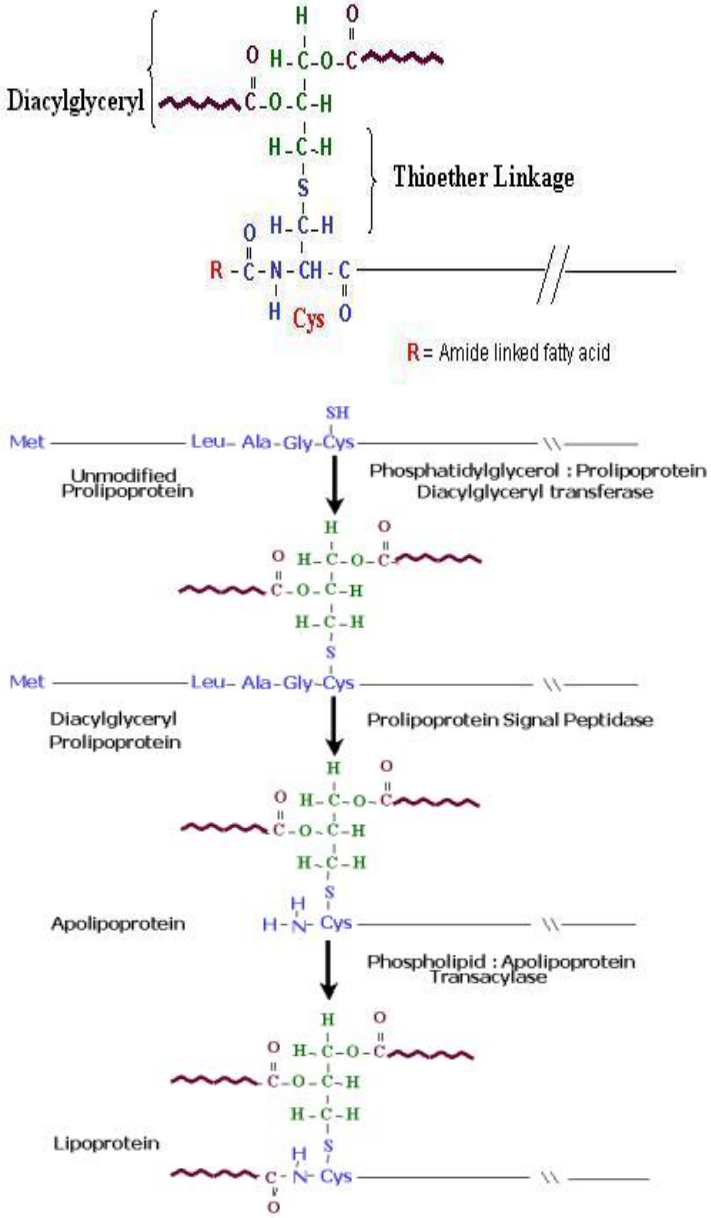


Figure 2. Biosynthesis of lipoprotein. In another important finding it was shown that the precursor for this outer membrane lipoprotein was actually a pre-protein with an N-terminal extension of 20 amino acids possessing the characteristics of a typical signal peptide (Inouye *et al.*, 1977). Naturally, the interest in the biosynthesis of this lipoprotein started immediately. Firstly, the origin of the lipid in the molecule was traced to phospholipids as suspected earlier (Chattopadhyay and Wu, 1977).

Phosphatidylglycerol was shown to be specifically required for the initial modification of Cys of the lipoproteins with diacylglycerol, catalyzed by the enzyme phosphatidylglycerol-prolipoprotein diacylglyceryl transferase (Lai *et al*., 1980; Sankaran and Wu, 1994). N-acylation was shown to be achieved with any of the phospholipids (Lai *et al*., 1980). Discovery of Globomycin, a cyclic pentapeptide antibiotic that specifically inhibited the maturation of lipoproteins led to the identification of a signal peptidase specific for lipoproteins (Inukai *et al*., 1978). This signal peptidase called signal peptidase II required diacylglyceryl modification prior to cleavage (Dev and Ray, 1984; Hussain *et al*., 1980). This meant that diacylglyceryl modification preceded cleavage of the signal peptide. The final modification *i.e.* after the cleavage of the signal peptide, fatty acylation of the amino group of the N-terminal diacylglyceryl modified Cys to form N-acyl diacylglyceryl Cysteine was subsequently identified (Gupta and Wu, 1991; Sankaran *et al*., 1995). The pathway (Gupta and Wu, 1991; Sankaran and Wu, 1994) is shown in the figure above. The genes for these enzymes have been identified by Wu *et al*. in a variety of bacteria and they are found to be highly conserved (Qi *et al*., 1995).

3.7 Clinical manifestation

The degree of illness in leptospirosis varies from asymptomatic to a severe or fatal illness. The most common forms are (1) anicteric and (2) icteric or Weil's syndrome.

3.7.1 Anicteric Leptosrirosis

Anicteric: Initial symptoms last 4-7 days and may mimic flu, including fever, myalgia, conjunctivitis, neck stiffness, nausea and sometimes vomiting. In the second or immune stage of anicteric leptospirosis, fever is usually not present or is low-grade. Headaches, meningitis, myalgia, nausea, vomiting and abdominal pain are also common in this stage. Patients usually recover in about a month, although leptospiruria may continue for several months (Ohio Department, 2007). The primary lesion during this phase is damaging to the endothelia of small blood vessels, resulting in localized ischemia in kidneys, liver, meninges, and muscles (Faine *et al*., 1999b;

Bharti *et al*., 2003). The first phase is characterized most frequently by sudden onset high fever with chills, headache, conjunctival suffusion (without discharge), cough and pulmonary chest pain, abdominal pain, nausea vomiting, and myalgia (especially calves and thighs). The abdominal pain may be similar to "surgical abdomen." Other signs include lymphadenopathy, pharyngeal injection, rash of variable presentation, hepatomegaly, and/or splenomegaly.

In some cases, the illness resolves after about one week with no further manifestations; and in other cases, after one to three days, the illness recurs. If the second phase occurs, symptoms tend to both milder and more varied than in the first phase - except that aseptic meningitis may occur (Olszyna *et al*., 1998). *L. interrogans* invades human and other hosts through intact or injured mucous membranes and then disseminates from the site of initial infection via the bloodstream. The postentry period of approximately 10 to 14 days is characterized by a leptospiremic phase, during which leptospires persist in the blood (Faine *et al*., 1999a).

3.7.2 Icteric Leptospirosis

Icteric: Only 5-10% of cases have this severe form of leptospirosis (Weil's disease), in which the infection progresses to hepatitis, nephritis, meningitis, respiratory distress and hemorrhagic tendencies, with associated jaundice and azotemia. Convalescence may take several months (Ohio Department, 2007). The clinical spectrum ranges from a mild anicteric disease to the more severe forms such as Weil’s syndrome (jaundice, haemorrhagic diathesis and acute renal failure), associated with a 10% mortality and severe pulmonary haemorrhage syndrome (SPHS), for which the case fatality rate can be >50% (McBride *et al*., 2005). This second (immune) phase usually lasts for several days, but may persist for weeks. Icteric leptospirosis or Weil's syndrome is the more severe form and is characterized by symptoms as described above (except not usually biphasic); and after about one week, the development of decreased renal function, pulmonary complications, jaundice, and hemorrhagic manifestations. Renal failure (acute tubular necrosis) with oliguria or anuria may develop after about one week of illness, but does not usually require dialysis, and often returns to normal within months. Early recognition of

decreased renal function is critical to patient survival. Pulmonary complications may include the presence of infiltrates, cough, dyspnea, hemoptysis, and/or chest pain. Decreased hepatic function and jaundice are common, but hepatic failure is seldom a cause of death - though the degree of jaundice correlates with risk of death. Hemorrhagic manifestations most often are epistaxis, bleeding gums, purpura, petechiae; and less frequently, gastrointestinal or subarachnoid bleeding.

Leptospirosis is a worldwide zoonotic disease with varied clinical manifestations ranging from fever, myalgia, and conjunctival suffusion to severe life threatening illness due to involvement of multiple organ systems, e.g. hepatic, renal, central nervous system, etc. Recently, pulmonary complication occurring early in the disease with high case fatality rate have been reported (Singh *et al*., 1999). Two distinct clinical syndromes are encountered in leptospirosis; 90% of patients experience a relatively mild, self-limited, anicteric febrile illness. A much smaller proportion, 5% to 10%, develops icteric leptospirosis, or Weil's disease, which is far more severe and potentially lethal and is characterized by respiratory failure, hepatic, renal, and cardiovascular dysfunction with meningitis (Coursin *et al*., 2000). The incidence of acute renal failure varies widely, from fewer than 10% to over 60% of patients, depending upon the severity of the disease (Visith and Kearkiat, 2005), and is usually non-oliguric (Abdulkader, 1997). Oliguria is a significant predictor of death [odds ratio (OR) 9.0] (Bharti *et al*., 2003). Renal failure is often hypercatabolic, with rapid elevation of blood urea nitrogen and serum creatinine levels and is associated with cholestatic jaundice (Visith and Kearkiat, 2005). Hepato-cellular jaundice can also be observed in patients with severe leptospirosis and profound hepatic ischaemia. Hyperbilirubinaemia (total serum bilirubin over 25 mg/dl) is also not uncommon (Coursin *et al*., 2000; Visith and Kearkiat, 2005). However, a few reports in adults have described patients with anicteric leptospirosis and severe acute renal failure requiring dialytic treatment (Erdinc *et al*., 2006; Peces, 2003).

3.8 Laboratory diagnosis

Prompt diagnosis is critical in preventing severe outcomes, since antibiotics are believed to provide the greatest benefit when initiated early in the course of illness (Faine *et al*., 1999b). Early phase leptospirosis is often not identified or is diagnosed as other causes of acute febrile disease due to its nonspecific clinical presentation (Kaur *et al*., 2003). Misdiagnosis of leptospirosis has become a significant problem as diseases with similar early symptoms, such as dengue, have reemerged in the same places (LaRocque *et al*., 2005). Identification of leptospirosis will therefore need to rely on a high index of clinical suspicion and the use of a rapid and specific laboratory test (Levett *et al*., 2000).

3.8.1 Detection of organism or part of organism

3.8.1.1 Direct microscopic examination

Leptospires may be visualized in clinical material by dark-field microscopy or by immunofluorescence or light microscopy after appropriate staining. Approximately, 10 leptospires/ml are necessary for one cell per field to be visible by dark-ground microscopy (DGM) (Shah *et al*., 1999). A quantitative buffy coat method has been shown to have a sensitivity of approximately 10 leptospires/ml (Clerke *et al*., 2002). Microscopy of blood is of value only during the first few days of the acute illness, while leptospiremia occurs. Dark-field microscopic examination of body fluids such as blood, urine, CSF, and dialysate fluid has been used but is both insensitive and lacks specificity. The drawbacks of DGM on clinical specimens as a diagnostic tool have been that both false positive and false negative diagnosis can be easily made even in experienced hands (Effler *et al*., 2002; O'Keefe, 2002).

Confirmatory diagnosis of clinical leptospirosis may be achieved by each hospital laboratory performing all-purpose bacteriological methods. Direct microscopic examination of blood may frequently demonstrate circulating leptospiras in the first few days of pyrexia. *Leptospira* may be tentatively cultured from blood in the same initial period of illness, in fluid semisynthetic commercial media according to the original Ellinghausen medium. Sero-conversion for leptospiral antibodies is

constantly observed on two serum samples taken in the first few days of illness and ten days thereafter respectively, in each case of current leptospirosis (Cacciapuoti, 1984). Darkfield microscopy is not recommended because it requires more than 104 cells/mL (Turner, 1970) and technical skill to exclude confusing fibrin proteins.

3.8.1.2 Cultivation

Leptospiremia occurs during the first stage of the disease, beginning before the onset of symptoms, and ends by the first week of the illness. Therefore blood cultures should be taken as soon as possible after the patient's presentation. One or two drops of blood are inoculated into 10 ml of semisolid medium containing 5-fluorouracil at the patient's bedside. For the greatest recovery rate, multiple cultures should be performed, but this is rarely possible. Inoculation of media with dilutions of blood samples may increase recovery. Rapid detection of leptospires by radiometric methods has been described. Leptospires survive in conventional blood culture media for a number of days. Rarely, leptospires have been isolated from blood weeks after the onset of symptoms. Isolation of leptospires from clinical samples gives a definitive diagnosis and also aids in identifying the prevalent serovar (Effler *et al*., 2002).

Apart from blood, CSF and dialysate fluid can also be cultured during the first week of illness. Urine can be cultured from the beginning of the second week of symptomatic illness. The duration of urinary excretion varies but may last for several weeks. Survival of leptospires in voided human urine is limited, so urine should be processed immediately by centrifugation, followed by resuspending the sediment in phosphate-buffered saline (to neutralize the pH) and inoculating into semisolid medium containing 5-fluorouracil. Cultures are incubated at 28 to 30°C and examined weekly by dark-field microscopy for up to 13 weeks before being discarded. Contaminated cultures may be passed through a 0.2 µm or 0.45 µm filter before subculture into fresh medium. Though the use of culture confirms diagnosis, it is rarely used, as it is very tedious, complicated, expensive, technically demanding, time consuming, requiring prolonged incubation (minimum 1 month before declaring a sample negative) and may not be successful (low sensitivity). The organism also has a relatively long doubling time (6 to 8 h or more).Additionally they are highly infectious

organisms requiring 'Biosafety level II' facilities (Effler *et al*., 2002). Pathogenic *Leptospira* and saprophytic *Leptospira* spp. are morphologically similar but can be differentiated on the basis of pathogenicity to laboratory animals or growth characteristics at 13°C (Johnson and Harris, 1967). Prolonged incubation of growth and expensive pathogenicity testing in laboratory animals, therefore, rapid and sensitive methods, are needed for differentiating the 2 species. Leptospiral isolation is difficult, time consuming, and potentially biohazardous.

3.8.1.3Antigen detection

Detection of leptospiral antigens in clinical material offer greater specificity than DGM while having the potential for greater sensitivity. Radioimmunoassay (RIA) can detect 10^4sub to 10^5sub leptospires/ml and an enzyme-linked immunosorbent assay (ELISA) method can detect 10^5sub leptospires/ml. A chemiluminescent immunoassay (CLIA) has been applied to human blood and urine but has been less sensitive than earlier ELISA. Recently, immunomagnetic antigen capture has been combined with fluoroimmunoassay to detect as few as 10^2sub leptospires/ml in urine of cattle infected with serovar hardjo (Ahmad *et al*., 2005). Saglam *et al* (2003) designed to investigate the presence of leptospiral antigens in kidney and liver of naturally infected cattle using an immunoperoxidase (IP) staining and Levaditi's staining methods. A total of 39 cattle suspected from leptospirosis were examined histologically and immunohistochemically for the presence of leptospiral antigens. The leptospiral antigens were detected in 16 out of 39 cases (seven kidneys, three livers, and six kidneys and livers) when IP staining method was used, whereas leptospiral antigens were detected in 6 out of 39 cases (four kidneys and two livers) when Levaditi's staining method was used.

3.8.1.4 Molecular diagnosis

A variety of molecular methods have been developed for the specific detection of pathogenic *Leptospira* spp. serovars in clinical samples. These include DNA–DNA hybridization (Terpstra, 1986), *in situ* hybridization (Terpstra, 1987) and DNA probes (LeFebvre, 1987), which have been used mainly for detection of leptospires in urine samples from animals infected experimentally with serovar Hardjobovis. The

polymerase chain reaction (PCR) also has been used to detect *Leptospira* spp. in urine samples from cattle experimentally infected with serovar Hardjobovis (Alt *et al*., 2001; Gerritsen *et al*., 1991; Taylor *et al*., 1997; Van Eys *et al*., 1989; Wagenaar *et al*., 2000; Wagenaar *et al*., 1994). A PCR to detect *Leptospira* spp. in the urine of naturally infected cattle using genus-specific primers has been reported by Talpada *et al* (Talpada *et al*., 2003). Recently, a nested PCR with primers derived from the LipL32 sequence has been reported by Nassi *et al*. using DNA of reference *Leptospira* spp., and by Jouglard *et al* (Jouglard *et al*., 2006) using DNA from clinical samples, including urine and serum. Polymerase chain reaction (PCR) has been developed for the diagnosis of leptospirosis (Gavin *et al*., 2005; Gravekamp *et al*., 1993; Kee *et al*., 1994; Merien *et al*., 1992; Smythe *et al*., 2002; Van Eys *et al*., 1989; Woo *et al*., 1997a; Woo *et al*., 1997c).

A multiplex polymerase chain reaction (PCR) is developed for diagnosing leptospirosis and differentiating pathogenic and saprophytic leptospires. Specific primers are designed to amplify 23S rDNA from pathogenic *Leptospira* and saprophytic *Leptospira* spp. detection and differentiation between pathogenic and saprophytic *Leptospira* spp. by multiplex polymerase chain reaction. The studies are based on PCR using 16S rDNA or 23S rDNA primers (Murgia *et al*., 1997; Woo *et al*., 1997a) and real-time PCR (Woo *et al*., 1997b) are specific for identifying saprophytic *Leptospira* spp. However, PCR with the higher sensitivity and ability to distinguish pathogenic from saprophytic leptospires is still needed.

A conventional and real-time PCR amplification and sequencing method has been developed for an alternate gene target: DNA gyrase subunit B (gyrB). Phylogenetic comparisons are undertaken between pathogenic *Leptospira* 16srRNA and gyrB genes using clustering and minimum evolution analysis. In addition 50 unidentified *Leptospira* isolates were characterised by gyrB sequencing and compared with conventional 16s rRNA sequencing (Slack *et al*., 2006). PCR-based detection methods have been developed but their use has been restricted to the reference laboratory setting, and they are unlikely to be implemented in developing countries, where the major public health burden of leptospirosis exists. Therefore, new strategies

for diagnosis which can aid early case identification and timely administration of antimicrobial therapy need to be identified.

3.8.2 Serological diagnosis

3.8.2.1 Microscopic agglutination test (MAT)

Most cases of leptospirosis are diagnosed by serology (Brandao *et al*., 1998; Effler *et al*., 2000; Flannery *et al*., 2001). Antibodies can become detectable by the 6th to 10th day of disease and generally reach peak levels within 3 to 4 weeks. Antibody levels then gradually recede but may remain detectable for years. Serological methods can be divided into two groups: those, which are genus specific and those which are serogroup specific. The definitive serological investigation in leptospirosis remains the microscopic agglutination test (Arimitsu *et al*., 1994; Brandao *et al*., 1998; Cumberland *et al*., 1999). Laboratory diagnosis of leptospirosis has conventionally been performed by demonstration of antibodies to leptospires in the sera. The traditional serologic test, microscopic agglutination test, has the disadvantage that it is tedious to test against a large battery of serovars (Levett, 2001). Traditionally, the reference method for diagnosis of leptospirosis is the microscopic agglutination test (MAT). However, this test has several drawbacks, including the requirement for a permanent stock of reference strains representing the appropriate serogroups, subjectivity involved in reading the results under dark-field microscopy, inability to differentiate titres of natural infection from vaccinal titres and the failure to identify most chronic shedders (Thiermann, 1984). Moreover, the assay is labour intensive and represents a biohazard to laboratory staff (Mendoza and Prescott, 1992; Surujballi *et al*., 1997). Isolation of leptospires is time consuming, subject to contamination and may require 4–6 months (Ellis *et al*., 1983). However, the standard diagnostic method, the microscopic agglutination test (MAT), requires paired serum samples for proper interpretation and is not adequate for clinical management (Cumberland *et al*., 1999).

Microscopic agglutination testing (MAT), the reference serodiagnostic test, may not be helpful in identifying the infecting serovar in individual cases, but is useful in predicting serogroups present within a population (Levett, 2003). Galton *et al* showed the results obtained by comparing test data from human and animal sera

indicate that the agreement between the MAT and improved microtechnique exceeded 96%. Agreement between the original microtechnique and MAT test was about 87% (Galton *et al*., 1965).

3.8.2.2 Enzyme-linked immunosorbent assay (ELISA)

Because of the complexity of the MAT, rapid screening tests for leptospiral antibodies in acute infection have been developed. Complement fixation (CF) was widely used but methods were not standardized. CF tests have generally been replaced by ELISA methods. IgM antibodies become detectable during the first week of illness allowing the diagnosis to be confirmed and treatment initiated while it is likely to be most effective. Antibody levels are generally low or absent during very early infection. IgM detection has repeatedly been shown to be more sensitive than MAT when the first specimen is taken early in the acute phase of the illness. However most of the commercially available ELISA kits use non-pathogenic *L.biflexa* patoc 1 strain as an antigen. The drawback of this test is that the infective serovar cannot be assessed. Though the test is more sensitive than MAT it is less specific.

An IgM-specific dot-ELISA has been developed in which polyvalent leptospiral antigen was dotted onto nitrocellulose filter disks in microtiter tray wells, allowing the use of smaller volumes of reagents. Further modifications of this approach have been used to detect IgG and IgA, in addition to IgM and have employed an immunodominant antigen and a polyester fabric-resin support in place of nitrocellulose. A commercial slide agglutination assay has been recently found to be as sensitive and specific as an IgM-ELISA, while remaining reactive for a shorter time after recovery than either the IgM-ELISA, or the MAT (Cumberland *et al*., 1999). Whole-*Leptospira*-based serologic assays are commercially available in enzyme-linked immunosorbent assay (ELISA) and other rapid formats, yet clinical evaluations found that these assays have sensitivities of 28 to 72% during acute-phase illness (Effler *et al*., 2002; Levett *et al*., 2001; Smits *et al*., 2000; Smits *et al*., 2001a; Smits *et al*., 2001b; Smits, 1999). Moreover, the sensitivity for these assays may be less than 25% for patients in the critical first week of illness (Effler *et al*., 2002), when treatment with antibiotic therapy may be most effective.

3.8.2.3 A commercial LEPTO dipstick

The dipstick uses a broadly reactive, non-serovar specific antigen for detecting IgM antibodies. Myint *et al* diagnosed of most patients with clinically suspected leptospirosis were tested with *Leptospira* Dip- S-Ticks (PanBio InDx Inc., Baltimore, MD) at Kamphaeng Phet Province, Provincial Hospital to detect IgM antibodies for Leptospiral antigens according to the manufacturer's instructions (Myint *et al*., 2007). The concordance of 45% (51% at S1 and 38% at S2) between the ELISA and dipstick was in contrast to the two assays were considered to have comparable sensitivities (Levett *et al*., 2001). The dipstick added no additional confirmed positive cases to the ELISA results. One additional limitation is that testing of the ELISA and dipstick was performed in different laboratories. Gussenhoven *et al* developed a simple dipstick method for the detection of *Leptospira*-specific IgM antibodies. Evaluation of the assay revealed that the results of the assay are in agreement with the results of an ELISA for the detection of *Leptospira*-specific IgM antibodies. Nearly the same number of serum samples from case patients and controls were found to be positive by the dipstick assay as by the IgM ELISA, and no differences between the detection rates by each of the assays was seen for sera collected at different stages of the disease. This shows that the method is well suited for the early diagnosis of leptospirosis. The dipstick method has the advantage, however, that it is easy to perform, is highly reproducible, and depends on neither special equipment nor refrigeration. The method is well suited for the early diagnosis of leptospirosis (Gussenhoven *et al*., 1997).

3.8.2.4 Indirect fluorescent antibody test (IFA)

On serial testing, the IFA antibody first appeared during the first week of illness, peaked by the fourth week, and generally decreased below 1:400 after the fourth month. The Indirect fluorescent antibody test appears to be moderately sensitive and specific for the initial diagnosis of leptospirosis. It could replace the more complicated and less sensitive MAT assay (Appassakij *et al*., 1995).

3.8.2.5 Indirect hemagglutination assay (IHA)

The indirect hemagglutination assay developed at CDC was shown to have a sensitivity of 92% and specificity of 95% compared with the MAT. Currently there is a paucity of rapid, sensitive and specific tests available for leptospirosis. The indirect hemagglutination assay has been available for leptospirosis screening purposes in the US. Although published reports have shown the indirect hemagglutination assay have high sensitivity and specificity, experience in Hawaii has demonstrated suboptimal sensitivity, limiting its usefulness as a diagnostic test (Effler *et al*., 2000). The indirect hemagglutination assay gave a sensitivity of 73.8% and a specificity of 97.5%, different from the test, which gave a higher sensitivity of 98.0% and a lower specificity of 92.2%. The indirect hemagglutination assay was developed by selecting antigens from *L.interrogans* serovar Bataviae which was the one most often found in Thailand. The indirect hemagglutination test gave more sensitivity and specificity than commercial test kits which used *L. biflexa*. It is recommended that the serovar can generally be found in the area should be selected for preparing the antigen because it gives better sensitivity and specificity.

In comparison between IHA and Slide agglutination test the overall sensitivity of the hemagglutination test is 92% in contrast to 69% for the presumptive slide agglutination test. The specificity is 95% for the hemagglutination test in comparison with 83% for the slide test (Imamura *et al*., 1972). Furthermore, four antigenic pools, each containing three serotypes, are used because of the antigenic disparity of the various leptosipral serotypes. A genus-specific leptospiral hemolytic test has been described in which passive hemolysis of sheep erythrocytes (RBC) was used (Baker and Cox, 1973; Cox *et al*., 1958). This technique was modified to one of passive hemagglutination (HA) for quantitating leptospiral antibodies (Cole *et al*., 1973; Imamura *et al*., 1972).

Effler *et al* confirmed the utility of indirect hemagglutination assay as an initial screening test for the investigation of hospitalized patients clinically suspected of having acute leptospirosis. They did not titrate sera which reacted in the indirect hemagglutination assay, although this is recommended by the manufacturer. Used in

this way, the indirect hemagglutination assay has a low cost and requires no specialized equipment or incubation conditions. These factors make it ideal for laboratories in which leptospirosis is not a frequent diagnosis and also for use in clinical diagnostic laboratories in which resources are limited (Effler *et al*., 2000).

3.8.2.6 Microcapsule agglutination test

A microcapsule agglutination test using a synthetic polymer in place of red blood cells has been evaluated extensively. The microcapsule agglutination test is reportedly more sensitive than either the MAT or an IgM-ELISA in early acute phase samples, but failed to detect infections caused by some serovars. Advantages of this direct agglutination method is that it can be applied without modification to sera from other animal species. A passive microcapsule agglutination test for the diagnosis of leptospirosis was developed by utilizing chemically stable microcapsules instead of sheep erythrocytes. In the test, sonically disrupted antigens of *Leptospira* were sensitized to microcapsules treated with glutaraldehyde. Compared with the microscopic agglutination test, the passive microcapsule agglutination test showed a relatively genus-specific tendency and a 4 to 32-fold-higher sensitivity. The sensitized microcapsule antigens were stable for at least 1 year. The microcapsules coupled with mixed antigens can be used as a serodiagnostic screening test for diseases caused by various types of *Leptospira*. The test, which is very simple and reproducible and requiring no specific training, can be employed easily as a routine test in diagnostic laboratories (Arimitsu *et al*., 1982).

3.9 Production of recombinant leptospiral lipoprotein for serodiagnosis

It has long been appreciated that *Leptospira* species adapt to and survive in vastly different environments, but little is known about the molecular nature of these adaptations. The leptospiral outer membrane lipoprotein LipL36 has provided one example of environmentally regulated protein expression. Immunohistochemical staining demonstrated expression of LipL36 during *in vitro* growth at 30°C but not in infected tissue or at culture temperatures of 37°C, indicating an adaptive response by

the organism to infection which included the diminution of expression of LipL36 (Haake, 1998; Nally *et al*., 2001).

Virulence factors expressed during host infection are expected to elicit specific antibody responses and, thus, may serve as candidate markers for a recombinant protein-based serodiagnostic test. A novel family of surface-associated proteins, *Leptospira* immunoglobulin (Ig)-like proteins (LigA, LigB, and LigC) (Koizumi and Watanabe, 2004; Matsunaga *et al*., 2003; Palaniappan *et al*., 2002), which have bacterial Ig-like (Big) tandemrepeat domains found in virulence factors such as intimin of enteropathogenic *Escherichia coli* (Luo *et al.*, 2000) and invasin of *Yersinia pseudotuberculosis* (Hamburger *et al*., 1999), have been identified. Lig genes are present exclusively in pathogenic and not saprophytic *Leptospira* species strains but not in strains that have been attenuated by culture passaging (Matsunaga *et al*., 2003). Lig proteins are expressed during host infection (Matsunaga *et al*., 2003) and appear to induce strong antibody responses in patients (Koizumi and Watanabe, 2004; Matsunaga *et al*., 2003) and infected animals (Koizumi and Watanabe, 2004; Palaniappan *et al*., 2002; Palaniappan *et al*., 2004). However, previous studies were performed with limited numbers of leptospirosis patients (Koizumi and Watanabe, 2004; Matsunaga *et al*., 2003).

Identification of an effective serodiagnostic marker for infection has been the major barrier to identify recombinant protein-based approaches to improve diagnosis of leptospirosis. Previous studies have focused on detecting antibody responses against a series of proteins which include LipL32 (Bomfim *et al*., 2005; Flannery *et al*., 2001; Guerreiro *et al*., 2001; Haake *et al*., 2000), LipL41 (Flannery *et al*., 2001; Guerreiro *et al*., 2001), and GroEL (Flannery *et al*., 2001; Guerreiro *et al*., 2001; Park *et al*., 1999). However, to date, the performance of these recombinant based serologic assays has not provided adequate sensitivity for identifying acute-phase leptospirosis. More recently, a novel family of surface-associated proteins, Lig proteins, were identified (Koizumi and Watanabe, 2004; Matsunaga *et al*., 2003; Palaniappan *et al*., 2002) which have Big tandem-repeat domains. These proteins were identified through screening of genomic DNA expression libraries with patient (Koizumi and Watanabe,

2004; Matsunaga *et al*., 2003) and infected animal sera (Palaniappan *et al*., 2004) and appear to be preferentially expressed during host infection (Matsunaga *et al*., 2003; Palaniappan *et al*., 2004).

A serodiagnostic assay will ideally use a recombinant LigB fragment derived from a single serovar which detects cross-reactive antibodies elicited during infection with other serovars. Predicted amino acid sequence identities are 92 to 96% among corresponding LigB. A single recombinant LigB fragment may therefore be capable of detecting cross-reactive antibody responses elicited during infections with *L. interrogans* and *L. kirschneri*, which account for the majority of serovars of public health importance (Levett, 2001).

3.10 Treatment and Control

The pathogenic spirochetes are shed in the urine of host animals. Human infection can occur after contact with contaminated water or with urine or tissues of infected animals. Occupations at greatest risk for infection include farmers, abattoir workers, sewer workers, and miners. Recreational activities are increasingly recognized as risk factors for leptospirosis (Centers for Disease Control and Prevention, 2001)**.** Penicillin in large doses, streptomycin, tetracycline, doxycycline, erythromycin and chloramphenicol are leptospirocidal *in vitro*. Evidence concerning the influence of drugs on leptospirosis in humans is conflicting and to be effective should be given before the fourth day of illness. Penicillin G and amoxicillin may be effective as late as seven days into the illness. Leptospirosis is treated with antibiotics, such as doxycycline or penicillin, which should be given early in the course of the disease (Ohio Department, 2007). Intravenous antibiotics may be required for persons with more severe symptoms. Persons with symptoms suggestive of leptospirosis should contact a health care provider.

Water may be contaminated with animal urine. Generally, no environmental specimens are indicated. Public education about the mode of transmission and control of reservoir animals, especially raccoons, opossums, skunks and rodents, should be

considered. Drainage of potentially contaminated waters and soil can be recommended, if feasible. The risk of acquiring leptospirosis can be greatly reduced by not swimming or wading in water that might be contaminated with animal urine. Protective clothing or footwear should be worn by those exposed to contaminated water or soil because of their job or recreational activities (Ohio Department, 2007). Leptospirosis responds well to treatment, especially if started early in the illness. Anicteric or mild leptospirosis is treated with doxycycline 100 mg po bid for 7 days OR ampicillin 500-750 mg po qid OR amoxicillin 500 mg po qid. Icticteric or anicteric (moderate to severe) leptospirosis is treated with IV penicillin G 1.5 million units qid OR IV ampicillin one gram qid OR IV amoxicillin one gram qid OR IV erythromycin 500 mg qid. Jarisch-Herxheimer reactions to therapy may occur, *i.e.*, acute febrile reaction, accompanied by headache and myalgia due to release of toxic products of killed spirochetes. The reaction usually occurs within 24 hours of initiation of therapy and subsides about 24 hours later. Therapy is continued unless symptoms are severe. Renal failure is treated as in other circumstances. Prophylaxis with doxycycline 200 mg/week is noted as effective in some sources. Doxycycline (200 mg/week) seems to have no effect on disease rates, but that it did reduce morbidity and mortality (Olszyna *et al*., 1998; Sehgal *et al*., 2000).

3.11 Immunity

Zhang *et al* reported that OmpL1 and LipL32 were highly conserved among the main endemic strains of *L. interrogans* in China (Zhang *et al.*, 2005). Further identified additional conserved leptospiral protein antigen candidates, such as LipL41 and immunoglobulin- like proteins, and they have been shown to elicit protective immunity in animal models (Branger *et al*., 2001; Haake *et al*., 1999; Koizumi and Watanabe, 2004). Vaccines for domestic livestock and canines give protection for specific serovars, but not for emerging serovars or existing serovars which cross to other species (Ohio Department., 2007). Nonspecific host defenses appear ineffective against the virulent leptospires, which are rapidly killed *in vitro* by the antibody-complement system; virulent strains are more resistant to this leptospiricidal activity than are avirulent strains. Immunity to leptospirosis is primarily humoral; cell-

mediated immunity does not appear to be important, but may be responsible for some of the late manifestations of the disease. Immunity to leptospirosis is serotype specific and may persist for years. Immune serum has been used to treat human leptospirosis and passively protects experimental animals from the disease. The survival of leptospires within the convoluted tubules of the kidneys may be related to the ineffectiveness of the antibody-complement system at this site. Previously infected animals can become seronegative and continue to shed leptospires in their urine, possibly because of the lack of antigenic stimulation by leptospires in the kidneys (Faine *et al*., 1999b; Nazarchuk and Rmanenko, 1998; Yamashiro-Kanashiro *et al*., 1991).

3.12 Vaccine development

Leptospiral outer membrane proteins are potential targets for inducing protective immune responses in the host, and those that are also well conserved among pathogenic serovars make attractive vaccine candidates (Cullen *et al*., 2004; Cullen *et al*., 2005; Haake *et al*., 1999; Haake, 2000). Human vaccines composed of inactivated whole bacterial cell or outer membrane envelope are available in some countries to prevent leptospirosis (Koizumi and Watanabe, 2005; Yan *et al*., 2003). However various kinds of serovar specificity limited the efficacy of protection against different pathogenic leptospires (Guerreiro *et al*., 2001; Sonrier *et al*., 2000). A major focus of research for the prevention of leptospirosis is to identify proteins conserved among pathogenic leptospires, which may generate cross-protection against strains of various serovars (Cullen *et al*., 2003; Haake *et al*., 1993; Haake *et al*., 2000; Matsunaga *et al*., 2002; Matsunaga *et al*., 2003). In addition to the complete genomic sequence information for pathogenic bacteria, CGH analysis was useful as one of the approaches based on reverse vaccinology (Yang *et al*., 2006) for screening vaccine candidates against leptospirosis. Genes that are highly conserved over a broad range of strains could be useful for the development of a proteinbased vaccine capable of protecting hosts against most of the pathogenic serogroups of *L. interrogans* in China (He *et al*., 2007).

Southern blot and preliminary sequence analyses suggest that LfhA may fit those criteria. We are presently assessing the potential use of LfhA in a vaccine for prevention of leptospirosis in humans and domestic animals. The usefulness of LfhA as a vaccinogen is strengthened by the possibility that antibodies which bind LfhA may also physically prevent factor H binding, thereby increasing the susceptibility of the bacterium to complement mediated killing (Verma *et al*., 2006).

Current vaccines against leptospirosis target the lipopolysaccharide coat of leptospires, which is highly variable for the > 200 serovars identified, thus limiting cross-protection (Levett, 2001). Thus, the Lig proteins appear to be closely associated with infection of the mammalian host, suggesting that they may be protective immunogens. Indeed, recent studies have demonstrated that recombinant forms of Lig serve as effective vaccines in animal models (Koizumi and Watanabe, 2004; Matsunaga *et al*., 2006), the multifunctional Lig proteins not only confer advantages on *Leptospira* in initiating and establishing an infection but also make the spirochetes potentially more vulnerable to vaccine-mediated protection due to the possible involvement of Lig in multiple steps of pathogenesis that include the critical process of adhesion. If it can be confirmed that Lig expression is common among pathogenic leptospires, this would provide an additional rationale for studies on the efficacy of Lig-based vaccines and diagnostic tests. Antigens based on conserved proteins, such as OmpL1 and LipL41, protect hamsters from leptospirosis and could elicit broader protection against heterologous strains (Haake *et al*., 1999). Effective protection by Lig vaccines has been demonstrated in two other animal models (Levett *et al*., 2000; Palaniappan *et al*., 2002). Further identification of the Lig sequences required for interaction with the host will aid in vaccine development. There are no effective vaccines for human.

CHAPTER IV
MATERIALS AND METHODS

4.1 Leptospiral culture

Leptospiral strain was grown at 30°C in 25 ml of EMJH medium (Ellinghausen and McCullough, 1965) supplemented with 3% heat–inactivated rabbit serum (Appendix A). In this study, *Leptospira interrogans* serovar Lai and serovar Copenhageni were cultured in large volume for genomic preparation.

4.2 Standard molecular biology techniques

4.2.1 Polymerase chain reaction (PCR)

The PCR reaction was performed by using GeneAmp PCR system 9700 (PE-Applied Biosystems) with 35 cycles of 95°C for 5 minute (pre denaturation), 95°C for 1 minute (denaturation), 55°C for 1 minute (annealing), 72°C for 1 minute (extention) and the last cycle at 72°C for 4 minute as final extending. After the last, the PCR product was holded at 4°C.

Table 4.1 Composition of each PCR reaction mixture was shown as follow;

PCR reaction composition	**Volume(µl)**
DNA template (50-100 ng)	1.0
10x PCR buffer (include 2mM $MgCl_2$, Biotool)	2.5
2Mm dNTPs	2.5
Tag DNA polymerase (biotool 1-5 unit)	1.0
Forward primer (10 picomol/µl)	1.0
Reverse primer (10 picomol/µl)	1.0
DW	16.0
Total volume	25.0

4.2.2 Leptospiral genomic DNA extraction

The genomic DNA was extracted with Nucleospin® tissue (MACHEREY-NAGEL, Germany), according to manufactory instruction.

4.2.3 Agarose gel electrophoresis

Agarose gel was prepared at a concentration of 0.8% (w/v) in 1x tris-acetate electrophoresis (TAE) buffer (Appendix B). The solution mixture was melted in microwave oven until completely dissolved. The solution was then cool down to about 60°C at room temperature; then 2 µl of ethidium bromide were added. The solution was mixed to disperse the ethidium bromide, and then was subjected to get-casting tray. The 10 µl of PCR product was throughly mixed with stop mix loading dye, and then it was slowly apply onto the gel slots. Five microliters of lamda *Hind* III molecular weight marker was applied into first well as a DNA marker. The electrophoresis was performed at 75 volts for 30 minutes, and then the DNA was visualized with ultraviolet light from transluminator.

4.2.4 Preparetion of *Escherichai coli* electrocompetent cells

Escherichai coli competent cells were prepared using a method modified from Hanaham (1970). SOB medium of 200 ml volume was prepared in each 1 litre flask for 4 flasks. One ml of a fresh overnight culture of *E. coli* strain DH5α was used to inoculate the medium and was incubated at 37°C with shaking. After reaching the desired optical density (OD_{550nm} = 0.4-0.8), cells were pelleted by centrifugation (4303×g,15 minute, 4°C) and the supernatant was discarded after which the cells were resuspended in ice-cold 10% glycerol. The cells were washed twice with ice-cold 10% glycerol and finally resuspended in a minimal volume of ice-cold 10% glycerol. Each 50 µl aliquot of the resuspended cells was contained approximately 5×10^8 cells. These aliquots were snaped frozen in ethanol chilled with dry ice and subsequently stored at -70°C

4.2.5 Gene transformation by electroporation

This technique was used for transformation of recombinant plasmid into the competent *E. coli* cell. Plasmid vector was diluted with sterile deionized water. The

electrophoretic cuvette was soaked in ice tank before used. The 50 µl of competent cell was mixed with 5 µl of diluted recombinant vector then put into the cuvette. The transformation was performed in EC 100 electroporator (E-C Apparatus Corporation) and using 1800 volts. After electroporation, all bacterial cells was transferred into 2 ml of SOC medium (Appendix A) and incubated at 37°C with agitation for 1 hour. The 100 µl of transformation mixture was grown on LB plate that contained appropriate antibiotics for selection of the positive clone, and bacterial plate was incubated overnight at 37°C.

4.3 Primer design for gene cloning

This study was aimed to clone and express the leptospiral membrane proteins, *i.e*., LipL32, LipL41 and Loa22. These three proteins were displayed by pathogenic *Leptospira* during infection and recognized by host immune system. The major protein of the leptospiral outer membrane is a 32-kDa lipoprotein designated LipL32 (Haake *et al.*, 2000). Another highly conserved leptospiral lipoprotein, LipL41 (Shang *et al.*, 1996), can provide 71% protection in the hamster model of leptospirosis when administered in combination with recombinant OmpL1 (Haake *et al.*, 1999). Koizumi N and Watanabe H suggested that Loa22 was detected among pathogenic leptospires but not among non-pathogenic leptospires, possible involvement of this protein in virulence (Koizumi and Watanabe, 2003).

The genomes of the *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 were viewed by Artemis version 7 program. The PCR primers were designed to amplify the LipL32 (LIC11352), Loa22 (LIC10191) and LipL41 (LIC12966) genes. The forward primers were designed to incorporate the *Xho*I restriction endonuclease site, while the reverse primers were designed to incorporate an *Eco*RI site. The oligo primers of each protein were shown in Table 4.2.

Table 4.2 The primers used in this study

Target protein	Primer
LipL32	For. : tgg tct ctc gag cct aaa aag ctc tt (*XhoI*) Rev.: ttt caa gaa ttc ttc agc agc gat agc t (*EcoRI*)
LpiL41	For. : ggt att ctc gag aga taa aga agg ccg t (*XhoI*) Rev.: tgc gtt gaa ttc atc aac ggc gaa agt a (*EcoRI*)
Loa 22	For. : aga gga ctc gag agc tcc tga gcc ttc a (*XhoI*) Rev.: tgg tgc gaa ttc cgc aaa acg gaa agt g (*EcoRI*)

4.4 Plasmid vector and *E. coli* host strain

4.4.1 pRSET B

The pRSET vector was pUC-derived expression vector. Expression of the gene from pRSET is controlled by T7 promoter. DNA inserts were positioned downstream and in frame with a sequence that encodes an N-terminal fusion peptide. This sequence includes an ATG translation initiation codon, a polyhistidine tag that functions as a metal binding domain in the translated protein, a transcript stabilizing sequence from gene 10 of phage T7, the Xpress^{TM} epitope, and the enterokinase cleavage recognition sequence. It had ampicillin resistance gene that is a selection marker for the positive clone (Figure 4.1)

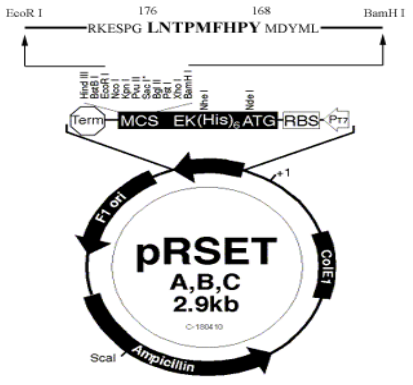


Figure 4.1 The map showed the features of pRSET A, B and C.

4.4.2 BL-21(DE3)plysS-*E. coli* host

The BL-21(DE3)plysS strain was the specific host for expression of T7 regulated genes. The genotypes were *F,ompT,hsdS$_B$(r_B^-,m $_B^-$),gal,dcm,rne*131 (DE3) plysS (Cam^R). This strain carried the DE3 bacteriophage lambda lysogen. These contain the *lac*I gene, the T7 RNA polymerase gene under control of the lacUV5 promotor which is induced by IPTG, and a small portion of the *lac*Z gene. The plysS plasmid promotes lysis and chloramphenicol resistance is a selection marker of this plasmid.

4.4.3 DH5α competent cell

DH5α^{TM} is a well-known, versatile strain that can be used in many cloning applications. The genotype included the following genes; *F$^-$ϕ80lacZΔM15 Δ(lacZYA-argF) U196 endA1 recA1 hsdR17(r_k^-,m_k^+) supE44 thi-1 gyrA96 relA1 phoA*. DH5α^{TM} Cells offer the following benefits: a wide range of efficiencies from >1x10^6 to > 1x10^9 transformants/μg DNA, greatly increased plasmid yield and quality. The DH5α^{TM} competent cell had no antibiotic resistance marker.

4.5 DNA cloning procedure

4.5.1 Plasmid preparation

The bacteria strain containing plasmid vector was grown at 37°C with agitation overnight in LB broth that contained appropriated antibiotic drug. HiYield^{TM} Plasmid Mini Kit was used for plasmid extraction and performed according to manufactory instruction.

4.5.2 PCR product preparation

The PCR reaction was performed according to the protocol that described above (4.2). The appropriated primer was selected to amplify the interested leptospiral gene.

4.5.3 Purification of PCR product or plasmid

HiYield^{TM} Gel/PCR DNA Extraction Kit was used for plasmids and PCR

products, clean up before or after restriction enzyme digestion. The assay was performed according to the manufacture's recommendation.

4.5.4 Restriction enzyme digestion of DNA

Plasmid vectors and PCR products were digested with the same pair of specific restriction enzymes. The reaction mixture was composed of a pair of enzymes, plasmid vectors or PCR products and buffers that recommended for use with each pair of enzymes. After mixing thoroughly, the reaction mixture was incubated in waterbath at 37°C for overnight. Digestion mixture was heat inactivated at 65°C in waterbath for 10 minutes and then purified with HiYield^{TM} Gel/PCR DNA Extraction Kit before the ligation process; each purified PCR product preparation was viewed on agarose gel electrophorasis to estimate the amount of DNA.

4.5.5 Ligation

The ligation mixture composed of 5 µl of digested vector, 5 µl of digested PCR product, 5 µl of deionized water, 4 µl of 5× ligation buffer containing 1 mM ATP and 1 µl of ligase enzyme. After mixing thoroughly, the reaction mixture was incubated in approximately at 13-16°C for overnight. The ligated product was cleaned up by firstly drying under speed dry vacuum (Centrivac concentration, Labcono), then 400 µl of 70% ethanol was added for washing, mixed gently and span down with 12,000 rpm/g for 15 minute. After the washed ethanol was removed, the ligated DNA was dried under vacuum again for 5 minutes and finally was solved in deionized water 20 µl.

4.5.6 Cloning of leptospiral lipoprotein genes

Genes encoding LipL32, LipL41 and Loa22 were cloned into the expression vector pRSET-B via PCR product that incorporated *Xho*I restriction enzyme site at 5' end and *Eco*RI restriction enzyme site at 3' end. The digested PCR products were ligated in-frame into the plasmid of pRSET-B vector, double digested with same enzymes. The resulting ligated plasmid was transformed into *E. coli* host stain DH5α. The LB plates supplemented with 100 µg/ml of ampicillin were used to select *E. coli* clones containing recombinant plasmid pRSET-B. After overnight culturing, the bacterial cell pellet was collected and subjected to boiling to liberate DNA. The

correct clones were screened by PCR based on the primer-pair that used for cloning. Plasmid DNA prepared from the positive clone was also subjected to DNA sequencing, using the primer pairs, T7 promoter and T7 terminater derived from plasmid vector sequence.

The correct plasmid construct was then used to transform *E. coli* strain BL21 (DE3) pLysS, possessed chloramphenicol resistance phenotype. This clone was then required to culture in LB medium containing both ampicillin and chloramphenicol, and one clone was selected for propagation.

4.5.7 PCR screening of positive clone

The colony of DH5α competent cell that contained recombinant plasmid were selected to inoculate in LB broth. Each colony was inoculated in 5 ml of LB broth that contained ampicillin. The culture was grown overnight at 37°C with agitation. Bacterial culture of 1 ml was pelleted by spinning down at 12,000 rpm/g for 5 minutes then deionized water 100 µl was added to the pallet. After mixing thoroughly, the mixture was boiled for 10 minutes and spin down 12,000 rpm/g for 5 minutes. The supernatant was used for PCR according to the same protocol (3.1). The agarose gel electrophoresis was used again for selection of the positive clone that showed the expected size of PCR product. The positive selected clone was grown for plasmid extraction using HiYield ^{TM} Plasmid Mini Kit. The extracted plasmid was used for DNA sequencing analysis to confirm the corrected gene that has been cloned. Genes encoding LipL32, LipL41 and Loa22 were cloned into the expression vector pRSET-B via PCR product that cooperated *Xho*I restriction enzyme site at 5' end and *Eco*RI restricted enzyme site at 3' end. The digested PCR products were ligated in-frame into the appropriate site of expression vector. The resulting fragment was transformed into *XhoI*/*EcoRI* sites in the DH5α expression plasmid. This construct was used to transform *E. coli* BL21(DE3) pLysS competent cell which was then cultured in LB medium containing ampicillin–chloramphenicol. Therefore the growing colonies that are resistant to both antibiotics contain the plasmid in their cells. Confirmation was performed by using PCR amplification on each plasmid, and finally by DNA sequencing.

4.5.8 DNA sequencing

The purified PCR fragment and the primers derived from plasmid vector was sent for DNA sequencing service, at 1st BASE Company. The dye terminator sequencing strategy was employed, which based on the chain-terminator method of Sanger *et al*., 1997 (Gregory *et al*., 1997). In dye terminator sequencing each of the four ddNTPs was labeled with a specific fluorescent dye and so a series of fluorescent dye labeled DNA molecules that differ in size by one base was formed. This mixture of DNA molecules was separated electrophoretically; the wavelength of the fluorescence indicates the dye labeled ddNTP incorporated in each molecule. Nucleotide sequence was performed using the PRISM sequencing kit and an Applied Biosystem 373A automated sequences.

4.6 Expression of recombinant protein

4.6.1 Transformation of constructed recombinant plasmid to BL21 (DE) plysS - *E. coli* host

The positive bacterial clone was cultured at 37°C with agitation overnight in 5 ml of LB broth that contained ampilillin drug (100 µg/ml). HiYield™ Plasmid Mini Kit was used for plasmid extraction. Recombinant plasmid was diluted 1:100 in deionized water. Electroporation technique was used to transform diluted recombinant plasmid to BL2 (DE3)pLysS competent cell according to the protocol that recommended before (4.7.5).

4.6.2 IPTG induction

The overnight culture of recombinant clone was prepared by inoculating one colony of *E. coli* harboring recombinant plasmid was inoculated in 5 ml of LB broth containing the antibiotic drug, and overnight culture was used to inoculate the fresh media in large volume (*i.e.,* 200 ml). The culture broth was shaking for 3-4 hours until OD_{550} reached 0.4- 0.6. The volume 1.5 ml of culture was collected as a pre-induced sample then centrifuged at 12,000 rpm/g for 5 minutes and discarded the supernatant. The volume 100 µl of deionized water was added to resuspend the cell pellet. The remaining culture was induced by addition of IPTG to concentration of 1 mM and

grew at 37°C with shaking incubator for additional 3-4 hours. All cell culture was centrifuged at 8,000 rpm/g at 4°C for 15 minutes to collect the cell pellet and stored in deep freezer until required. In small scale experiment, bacterial cell of 1.5 ml was pelleted and suspended with 500 µl of 1× PBS. Bacterial cells were lysed by sonicator for 5 minutes. The cell lysate was centrifuged at 12,000 rpm/g for 10 minutes, in order to separate the pellet (insoluble protein sample) and supernatant (soluble protein sample). Three samples were then obtained; the pre-induced sample suspension, insoluble protein sample and soluble protein sample. The 50 µl of these 3 samples were mixed with 20 µl of 2× sample buffer (Appendix E) and boiled for 10 minutes. SDS-PAGE was used for determining the predominated protein band.

4.6.3 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of protein preparation

The recombinant protein samples were prepared in sample buffer (two times-concentrations), boiled for 10 minutes and span down 5 minutes at 12,000 rpm/g, and supernate was collected. The protein was loaded onto 12% (w/v) separating gel and 4% (w/v) stacking gel. A standard molecular weight marker was applied into the first well of each gel (Appendix B). Electrophoresis was carried out in a Mini PROTEIN® 3 cell (BIO-RAD). Separation was carried out in electrode buffer (Appendix C) with constant voltage of 200 volts. Electrophoresis was stopped when the blue dye reached the end of the gel. Gel was stained with 0.25% Coomassie brilliant blue solution (Appendix) for 1 hour and destained with destaining solution (Appendix C) at room temperature until the protein band was visible.

4.6.4 Western blot analysis

After protein samples were separated according to molecular weight using SDS-PAGE, the proteins were transferred from the gel onto a nitrocellulose membrane that was soaked in absolute methanol before used. Electrophoretic transfer was performed at 35 volts for 16 hours. The blotted nitrocellulose membrane was blocked with blocking buffer (5% skim milk in 1x TBS with 0.05% Tween® 20) (Appendix D) for 2 hours at room temperature, then incubated with primary antibody in blocking buffer at 37°C for exactly 1-2 hours with agitation. The membrane was further

incubating at 4°C overnight. After incubation overnight, the nitrocellulose membrane was washed for 4 times with washing buffer (TBS-0.05% Tween® 20) (Appendix D) for 5 minutes. The membrane was reacted with secondary antibody conjugated with horse redish peroxidase (HRP) at appropriated dilution in blocking buffer for 2 hours at 37°C with agitation and then washed for 4 times as above. After washing, the membrane was reacted with freshly prepared substrate solution (4-chloro-1-napthol) (Appendix D) at room temperature until the bands were appeared. The reaction was stopped by soaking membrane in distilled water. The molecular weight of the visible bands was determined by comparing with the standard molecular weight marker.

4.6.5 Immunostaining method

The blotted nitrocellulose membrane was blocked with blocking buffer (5% skim milk in 1x TBS with 0.05% Tween® 20) (Appendix D) for 2 hours at room temperature, then incubated with primary antibody in blocking buffer at 37°C for exactly 1-2 hours with agitation. The membrane was further incubated at 4°C overnight. After incubation overnight, the nitrocellulose membrane was washed for 4 times with washing buffer (TBS-0.05% Tween® 20) (Appendix D) for 5 minutes. The membrane was reacted with secondary antibody conjugated with horseradish peroxidase (Sigma) at appropriated dilution in blocking buffer for 2 hours at 37°C with agitation and then washed for 4 times as above. After washing, the membrane was reacted with freshly prepared substrate solution (4-chloro-1-napthol) (Appendix D) at room temperature until the band was appeared. The reaction was stopped by soaking membrane in distilled water. The molecular weight of the visible bands was determined by comparing with the standard molecular weight markers.

4.6.6 Immunoblot analysis

The recombinant proteins were fractionated on a 12% SDS–PAGE and electrotransferred to nitrocellulose membranes. The membranes were incubated with 5% (w/v) non-fat dried milk in PBS-T and, after washing four times for 5 minute with PBS-T, they were further incubated with each mouse anti-recombinant proteins, rLipL32, rLipL41 and rLoa22 serum in 5% non-fat dried milk–PBS-T for 2 hours. Following a repeat of the PBS-T wash as described above, the membranes were

incubated with goat anti-mouse IgG peroxidase conjugate (Sigma) in 5% non-fat dried milk–PBS-T, washed, and revealed with freshly prepared substrate solution (4-chloro-1-napthol). Alternatively, the blotting nitrocellulose membranes were incubated with sera from patients diagnosed with leptospirosis. The blot was developed using goat anti-human IgG peroxidase conjugate (Sigma) as described above.

4.6.7 Preparation of leptospiral whole cell lysate

Leptospires were cultured and harvested by centrifugation at 10,000 rpm/g for 30 minutes at 4°C. The cell pellet was washed twice with 5 mM $MgCl_2$-PBS by centrifugation at 10,000 rpm/g for 10 minutes at 4°C and resuspended in 500 µl of lysis buffer and 50 µl of lysozyme solution (10 mg/ml of stock), followed by 3 times sonication. The total bacteria membrane was collected by centrifugation at 14,000 rpm/g for 20 minutes at 4°C and discarded the supernatant. The whole cell lysate was used as antigen for determination whether the antibody to recombinant protein can react to leptospiral native protein.

4.6.8 Determination of His-tag recombinant protein

Detection of histidine fusion marker in each recombinant protein, the immunostaining was performed to the transferred membrane protein, using the mouse anti-His tag as primary antibody and anti-mouse immunoglobulin conjugated with HRP or alkaline phosphatase was used as secondary antibody. After reacting with specific substrate, as described earlier, the protein bands possessed Histidine tag fusion protein were viewed and their molecular weights was assessed by comparing to standard markers.

4.6.9 Group of serums in this study

Serum samples in this study were divided into 8 groups, i.e., leptospirosis patient sera with MAT positive titer more than or equal to 1:100, MAT negative with culture positive result, Dipstick positive sera with MAT negative, normal people in endemic area sera, normal people out of endemic area, scrub typhus patient sera, melloidosis patient sera and dengue haemorrhagic fever patient sera.

Table 4.3 Groups of human serum for test

Serum	Number
Confirmed leptospirosis cases	
Paired serum with MAT titer ≥ 100	30
Leptospiral culture positive with MAT negative	39
Leptospiral dipstick positive with MAT negative	16
Control group	
Normal people in endemic area sera	30
Normal people out of endemic area sera	31
Scrub typhus patient sera	30
Melloidosis patient sera	30
Dengue haemorrhagic fever patient sera	31

4.7 Purification of recombinant protein

4.7.1 Preparation of inclusion bodies for purification under denaturing conditions

One colony of recombinant clone was inoculated in 50 ml of LB broth containing the antibiotic drug and growing overnight at 37°C in shaking incubator. The 5 ml volume of the overnight culture were inoculated into 300 ml of fresh LB broth containing antibiotic drug and grew at 37°C with shaking until OD_{550} reached 0.6– 1.0. The culture was induced with 300 µl of 1M IPTG to obtain the final concentration of 1M IPTG and grew at 37°C with shaking incubator for 4 hours. All bacterial cell culture was centrifuged at 8,000 rpm/g for 15 minutes at 4°C to collect the cell pellet. The cell pellet was stored overnight at -70°C

4.7.2 Purification of His-tagged proteins from E. coli under native conditions

The cell pellet from an *E. coli* expression culture was thewed on ice. One gram of wet cell pellet was resuspended 2-5 ml LEW buffer (Appendix E). Pipette up and down, or use stirring until complete resuspension without visible cell aggregates.

Perform this step on ice. And lysozyme was added to a final concentration of 1 mg/ml, and then was stirred on ice for 30 minutes. the cell suspension was sonicated on ice according to the instructions provided by the manufacturer. The crude lysate was centrifuged at 10,000 rpm/g for 30 minute at 4°C to remove cellular debris. The protein solution was stored at -70°C.

Purification of the His_6 fusion protein was performed using Ni-NTA chromatography method. This method was based on the affinity of the His_6 affinity tag to Ni^{2+} ions immobilized on a chromatography support resin. The recombinant protein was solubilized with 8 M urea and purified with Protino® Ni-TED Resin gravity flow column chromatography (MACHEREY-NAGEL) under denaturing condition. The target 6x His-tagged recombinant proteins were eluted into 15 fractions with gradient concentration of immidazole ranging from 0 to 0.5 M. Working Ni-NTA agarose resin was prepared by adding sterile Milli Q deionized water in 1:4 (resin:deionized water) ratio and adjusted with 12 ml of lysis buffer. The agarose-resin tubes were mixed by inverting the tubes 10 times and then left for 5 minutes at room temperature. The column was clamped in a vertical position to allow the resin to settle completely, and then removed lysis buffer out. The protein solution, which contained His_6 fusion protein was added into Ni-NTA resin and incubate by rotating for 1 hours at room temperature and washed resin for 4 times by addition of 8 ml of LEW buffer, mixed together and remove this buffer. The mixture His_6 fusion protein and Ni-NTA agarose resin was applied to the gravity-flow column. The column was clamped in a vertical position to allow the resin to settle completely. After the resin was settled. The elution buffer with gradient concentration on immidazole from 0-0.5 M was added and flew slowly on column.

Fifteen tubes of every 3 ml of eluted fraction was collected. Each tube was determined for the protein band by SDS-PAGE gel electrophoresis and Western blot. The protein quantity was examined using Bradford assay and all prepared protein was kept at -20 °C until use.

4.7.3 Dialysis of purified protein

The eluted lysate fractions that contained the band of His-fusion protein were filled into dialysis tube and immerged in PBS pH 7.2 (Appendix E) for 48 hours at 4°C, with two changes of buffer. The dialysed proteins were concentrated and dried by lyophilizer.

4.8 Purified protein concentration

The quantitative assay of protein sample was performed with Bio-Rad protein assay dye reagent which based on the method described by Bradford (Bradford, 1976). The employed assay procedures was followed the microassay procedure described in the Bio-Rad protein assay manual. To assay protein concentration in the sample, 16 µl of the purified recombinant protein was added to the reaction containing 144 µl distilled water to obtain 1:10 dilution followed by adding of 40 µl of Bio-Rad protein assay stocked reagent. The protein sample was diluted with deionized distilled water to 1:100 and 1:500 dilution, prepared in total volume of 160 µl followed by adding of 40 µl of Bio-Rad protein assay dye reagent. The reaction was incubated for at least 5 minutes at room temperature and measured for absorbance at 595 nm. Protein concentration in the sample was estimated from the protein standard curve (ranging from 5 to 50 µg/ml) that performed as recommended in the Bio-Rad protein assay manual.

4.9 Immunogenicity of recombinant protein

4.9.1 Mouse immunization

The purified recombinant LipL32, LipL41 and Loa22 proteins were selected to immunize male ICR mice. The 3 male ICR mice were used for immunization with each recombinant protein. Mice were purchased from National Laboratory Animal Center, Mahidol University, Thailand. Individual mice in each group was injected with 10 µg/dose of each recombinant protein mixed with Imject® Alum (Pierce) in 1:1 ratio via intraperitoneal route for 3 doses. The Alum composed of an aqueous solution of aluminum hydroxide (40 mg/ml) and magnesium hydroxide (40 mg/ml) plus inactive

stabilizers. Mice were reinjected twice in two weeks interval with 10 µg/dose of recombinant protein via the same route. Five days after the last injection, mice were test-bled individually via eye-bleed. The collected sera were tested for antibodies against each recombinant protein antigen and also with the whole cell lysate of *Leptospira* by Western blot analysis.

4.9.2 ELISA based recombinant protein for human Leptospirosis

Individual well of 96 well-microtiter plates was coated with 100 µl per well of purified recombinant protein dissolving in coating buffer (Carbonate-bicarbonate buffer, pH 9.6) at an optimized concentration. The plates were incubated overnight at 4°C in moist-chamber. The next day, unbound antigens was washed off with PBS (pH 7.4) containing 0.05 % Tween 20 (PBS-T) for four times, and 200 µl of blocking solution(1% BSA) was added to each well to block the remaining adsorption sites for 1 hour at 37°C. One hundred microlitres of single dilution that been optimized was employed among different sera group (*i.e.,* Leptospirosis patient with MAT positive, Dengue patient, normal human serum in endemic area, normal human serum in out of endemic area, Scrub typhus patient sera and Melliodosis sera). The diluted sera were added into the well after removal of blocking solution. Blank wells containing diluents instead of serum were included in ELISA plate. The plates were incubated at 37°C for 2 hours. The unbound antibodies were removed by four times washing with PBS-T. Then goat anti-human Ig conjugated to horseradish peroxidase (Southern Biotech) conjugated (1:4,000 in PBS-T) was added into each well for evaluation of total Ig based ELISA, while Goat anti-human IgM conjugated to horseradish peroxidase (Southern Biotech) was used to evaluate IgM based ELISA. After incubation at 37°C for 1 hour, the plates were washed 5 times with PBS-T and 100 µl of freshly prepared ABTS® peroxidase substrate solution was used as a chromogenic enzyme substrate. The plate was incubated in the dark for 30 minutes at room temperature. Then the reaction was stopped with 100 µl of 1% SDS. The optical density of the reaction wells was spectrometrically measured by microtiter plate reader at 405 nm against the blank.

4.10 Statistic analysis

The sensitivity, specificity and accuracy of the recombinant LipL32 LipL41 and Loa 22 based-total Ig ELISA, were analyzed by Galen's method (Galen, 1979). Mean and standard deviation of optical densities were used to analyze in ELISA.

Table 4.4 The 2 x 2 table of Galen's method was used to determine sensitivity and specificity based on following formular;

		Reference method (MAT)		Total
		No. of Positive	No. of Negative	
Test Assay (ELISA)	No. of Positive	TP	FP	TP+FP
	No. of Positive	FN	TN	FN+TN
	Total	TP+FN	FP+TN	TP+TN+FP+FN

TP (True positive) = Number of serum sample that positive results with both, the reference method and the test assay

FP (False positive) = Number of serum sample that negative results with the reference method but the test gives positive result

FN (False negative) = Number of serum sample that positive results with the reference method but test gives negative result

TN (True negative) = Number of serum sample that negative results with both, the reference method and the test assay

Sensitivity

The sensitivity of a test is a probability that the test procedure or result will be positive when a disease is not present. It can be expressed in percentage as shown in the formula below:

[True positive cases ÷ (True positive cases + false negative cases)] × 100

Specificity

The specificity is the probability that the test procedure of result will be

negative when the disease is not present. It can be expressed in percentage as shown in the formula below:

[True negative cases ÷ (True negative cases + false positive cases)] × 100

Accuracy

The efficacy (accuracy) of the test refers to the level of agreement between the test result and the true clinical state. It can be expressed in the following formula as below:

[(True positive cases + true negative cases) ÷ Total number of cases] × 100

One-way ANOVA

The One-way ANOVA analysis is from SPSS program version 11.5 is use to determine whether means O.D. values of MAT positive serum group are significantly different ($p<0.05$) from those of normal serum group and other febrile illness serum group.

CHAPTER V
RESULTS

5.1 Putative OMP for cloning the LipL32, LipL41, and Loa22 gene

The immunoreactive protein antigens residing in the outer membrane of leptospiral cells reported in recent years are LipL21, LipL32, LipL41, Loa22 and OmpL1. They are associated with infection in animals and man as ample evidences are available to show that these proteins are expressed *in vivo* during the infection (Barnett *et al.*, 1999; Matsunaga *et al.*, 2003). This study was thus aimed to clone some outer membrane proteins i.e., LipL32, LipL41 and Loa22 for application as serodiagnosis antigens. Bioinformatic tool now used to predict their proteomic properties shown in Table 5.1. The PSORTB program (Gardy *et al.*, 2005) indicated that the LipL32, LipL41 and Loa22 were located at the outer membrane of leptospires. LipoP (Juncker *et al.*, 2003) and Splip (Setubal *et al.*, 2006) score revealed LipL32 and LipL41 as lipoproteins while Loa22 is not. The full length PCR product was amplified with primers, containing desired site of restriction enzyme. The PCR product contained restricted enzyme sites at both ends were digested and ligated to pRSET-B plasmid vectors digested with same set of restriction enzymes. Positive recombinant clones containing LipL32, Loa22 and LipL41 possessed the inserted DNA of about 720 bp, 430 bp and 973 bp, respectively, shown in Figure 5.1.

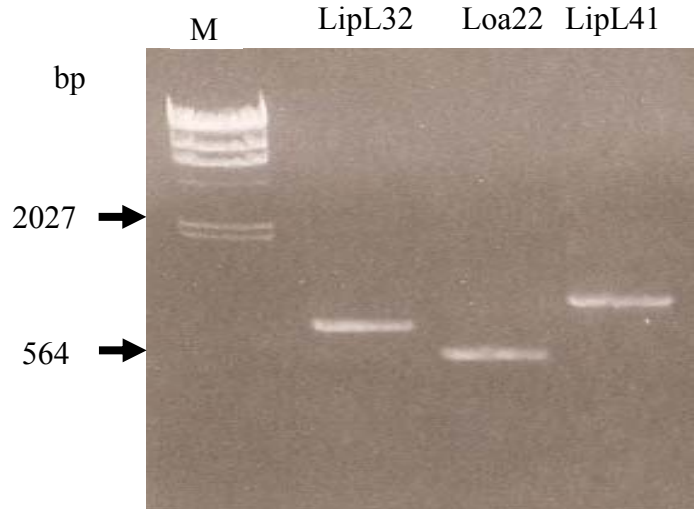


Figure 5.1 The PCR products derived from insert DNA of transforming colonies of the following recombinant clone; LipL32, Loa22 and LipL41 of which their insert size were about 720 bp, 430 bp and 973 bp, respectively.

DNA sequencing was performed on the plasmid of recombinant clone by using the sequencing primers derived from T7 promotor and T7 terminator regions. The sequence of LipL32, LipL41 and Loa22 were viewed by sequencer 4.5 demo program (Tippmann, 2004). The sequences were aligned with each reference sequence obtained from genome database. It was confirmed that all three genes were successfully cloned into the expression vector in the correct orientation, and inframed amino acid sequence.

Table 5.1 the properties of three recombinant proteins

Gene name	**LipL32 (Lic11352)**	**LipL41 (Lic12966)**	**Loa 22 (Lic10191)**
Size of PCR insert (bp)	720	973	430
Prediction MW (kDa)	29.61	38.93	20.91
Protein MW after purification (kDa)	35	27	27
LipoP	SpII score = 15.73	SpII score = 10.63	SpII score = 18.70
Splip	Lipoprotein	Lipoprotein	Not-lipoprotein
Location	Outer membrane	Outer membrane	Non-cytoplasmic

5.2 Expression of the recombinant proteins

The plasmid pRSET-B was employed as expression vector, composing of the coding of 6 His residues, that allowed the expression of recombinant proteins with 6 His-tag located at the N-terminus**.** Each recombinant gene was transformed to *E. coli* BL21(DE3)pLysS host, which facilitated the past bacterial cell lysis.

All recombinant clones were only expressed after induction with 1mM IPTG, as shown in Figure 5.2 lane B, no product was observed before induction. Histaq maker was determined in each bacterial cell lysate to reveal the molecular weight size of expressed protein. The obtained molecular weight sizes of recombinant proteins LipL32, LipL41 and Loa22 were 35 kDa, 27 kDa and 27 kDa, respectively.

For protein expression of each bacterial clone, to determine whether each recombinant protein was presented as solubilized from or packed in the inclusion body. Bacterial cell pellet was lyzed by sonication and then separated into pellet and supernate. Proteins from these two fractions were viewed by SDS-PAGE, and Western Blot analysis was used to confirm the presence of His-tag fusion protein (Figure 5.2). The solubilized supernatant fraction of LipL32 and Loa22 recombinant protein gave the partial His-tag band marker. While LipL41 recombinant protein was mostly presented in the insoluble protein fraction in pellet. The molecular weight of LipL32 recombinant protein was expected about 32 kDa, while the obtained molecular weight of His-tag protein was about 35 kDa. LipL41 recombinant protein was expected as 41 kDa while the Western Blot result of the His-tag protein revealed the smaller size of 27 kDa. The molecular weight of recombinant Loa22 protein was expected as 22 kDa while the molecular weight of obtained His-tag protein revealed larger of 27 kDa.

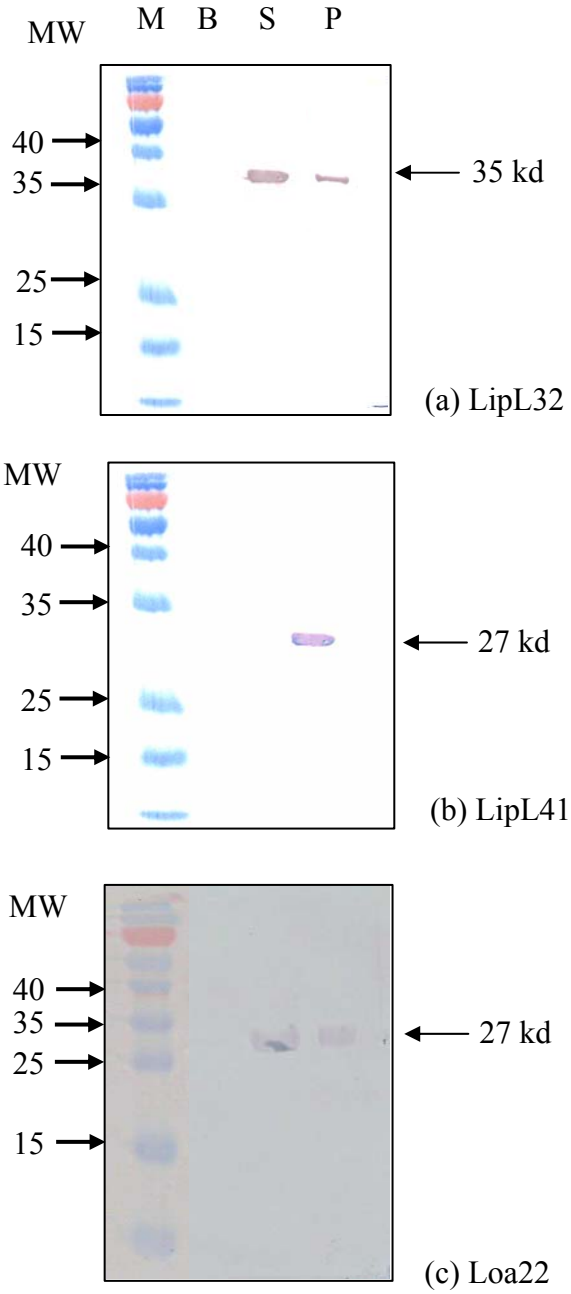


Figure 5.2 Western Blot analysis of recombinant protein LipL32 (a), LipL41 (b) and Loa22 (c) expressed from pRSET-B vector. Bacterial cells were disrupted with sonicator and seperated by centrifugation into supernatant (S) and pellet (P) fractions. Samples from each fraction were separated by 12% SDS-PAGE followed by the Western Blot Assay to confirm the His-tag protein. M, B, S and P denoted as Standard protein molecular weight, cell lysate collected before induction, supernatant and pellet fractions, respectively.

5.3 Purification of the recombinant proteins

The Ni-TED resin affinity flow column (MACHEREY-NAGEL) under denaturing condition was performed to purify of the recombinant proteins. The solubilized forms of recombinant proteins, LipL32 and Loa22, were thus purified under native condition, while the insoluble recombinant LipL41 protein required solubilization with buffer containing 8 M urea (denaturing condition). Fifteen tubes of every 3 ml of eluted fraction were collected. Each fractionated tube was determined for expected protein band by SDS-PAGE.

The target His-tagged recombinant proteins were eluted into 15 fractions with gradient concentration of immidazole ranging from 0-0.5M. For recombinant protein LipL32, the large amount of purified proteins was presented in fractions E1 to E10 (Figure 5.3). All eluted protein fractions that were visible on SDS PAGE were collected and subjected to dialysis in 0.01 M PBS, pH 7.2. The fractions E1 to E11 of recombinant protein LipL41 contained the large amount of protein (Figure 5.4), while the factions E1-E13 of recombinant protein Loa22 were also collected (Figure 5.5). The pool-fraction was subjected to Western blot analysis for confirmation of His-tag marker. The purified recombinant LipL32 protein gave the band at 35 kDa and the purified recombinant LipL41 and Loa22 gave 27 kDa as same as the result from preliminary experiment in Figure 5.3. The amount of each purified protein was quantitated by Bio-Rad protein assay dye reagent as described in materials and methods. The protein concentration of rLipL32 was 250 μg/ml, rLipL41 was 260 μg/ml and rLoa22 was measured as 360 μg/ml.

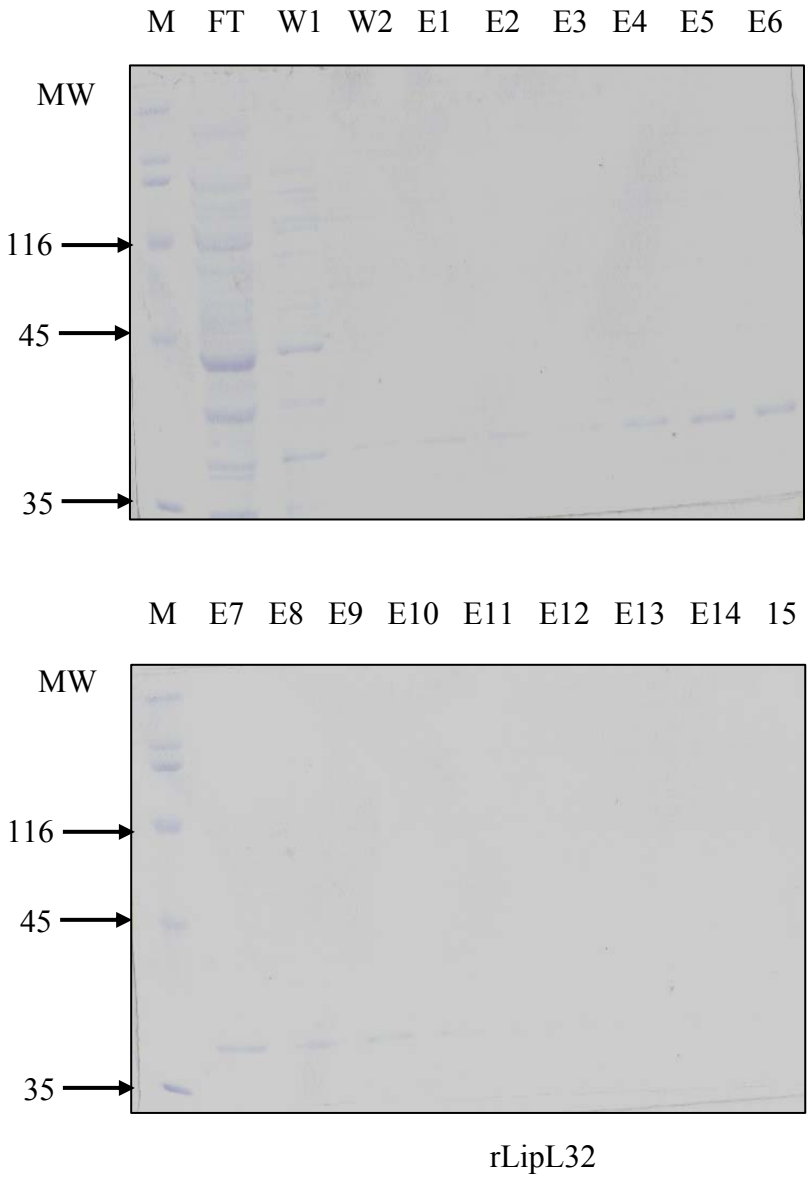


Figure 5.3 The SDS-PAGE analyses of recombinant LipL32 protein was purified by nickel affinity column chromatography. The purified protein band showed clear in E1-E10 fraction.

Lane M:	Standard protein molecular weight
Lane FT:	Flow through
Lane W1-W2:	Fractions collected from washing step
Lane E:	Fractions collected from elution step

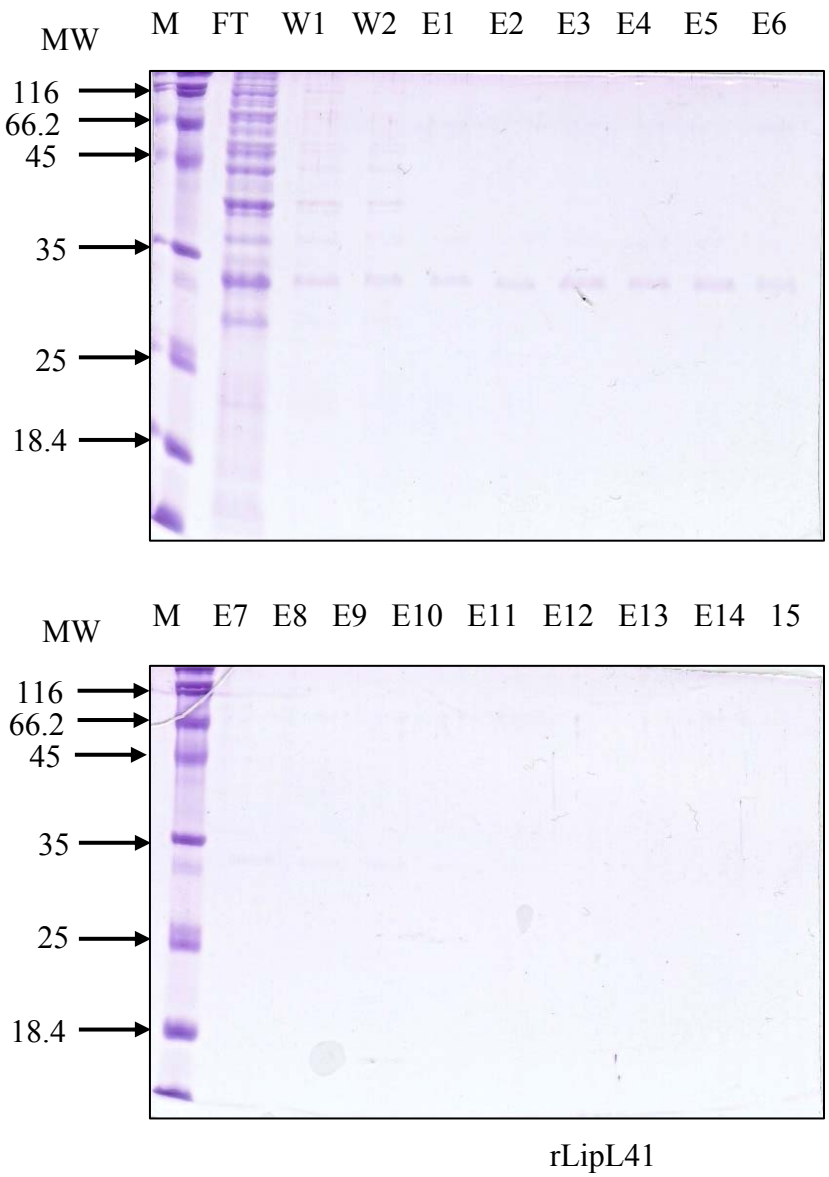


Figure 5.4 The SDS-PAGE analysis of recombinant LipL41 protein was purified by nickel affinity column chromatography. The purified protein band showed clear in E1-E11 fraction.

Lane M:	Standard protein molecular weight
Lane FT:	Flow through
Lane W1-W2:	Fractions collected from washing step
Lane E:	Fractions collected from elution step

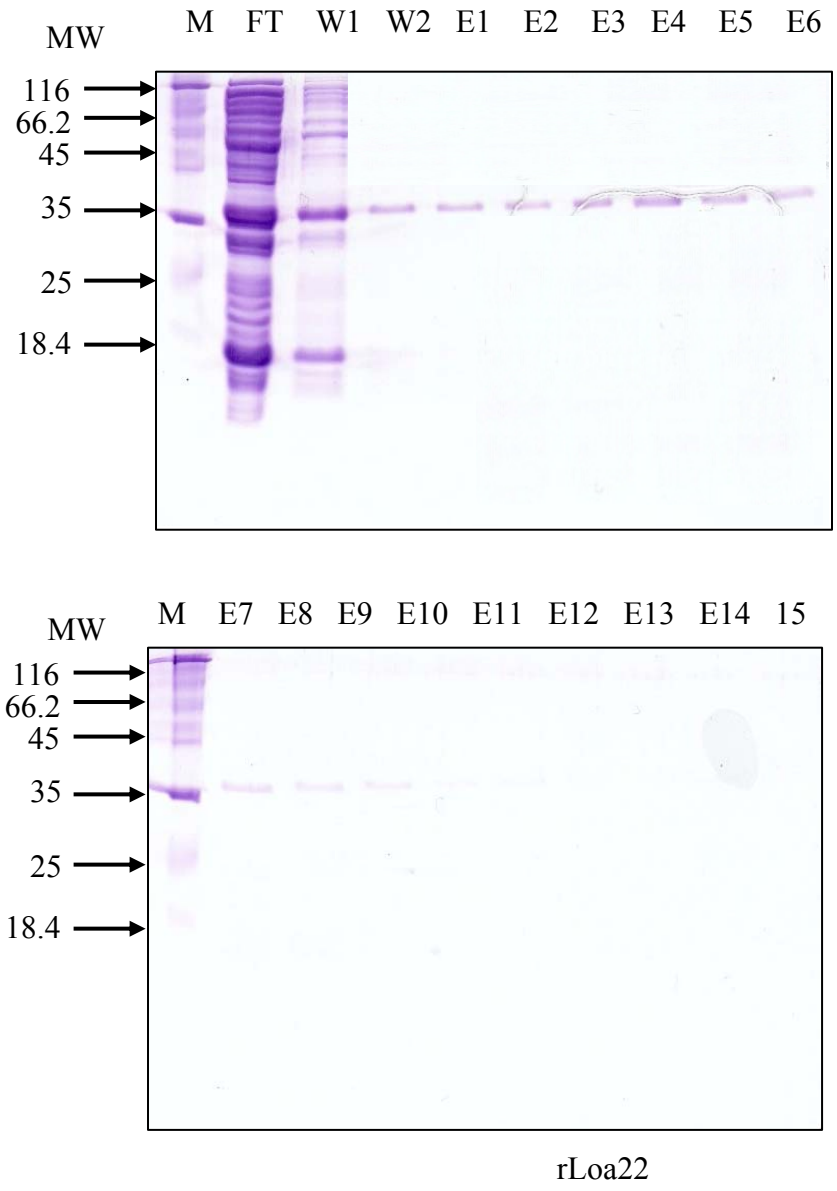


Figure 5.5 The SDS-PAGE analysis of recombinant Loa22 protein was purified by nickel affinity column chromatography. The purified protein band showed clear in E1-E13 fraction.

Lane M:	Standard protein molecular weight
Lane FT:	Flow through
Lane W1-W2:	Fractions collected from washing step
Lane E:	Fractions collected from elution step

5.4 Mice immunization with recombinant proteins

Antibody was raised against each recombinant protein in mice, The purified recombinant LipL32, LipL41 and Loa22 proteins were selected to immunize male ICR mice. The 3 male ICR mice were used for immunization with each recombinant protein. Individual mice in each group were injected with 10 μg/dose of each recombinant protein mixed with Imject® Alum (Pierce) in 1:1 ratio via intraperitoneal route for 3 doses by two weeks-interval.

The antibody titer based on ELISA assay against each recombinant protein was performed. Mice antibody to LipL32 protein was higher than 1:128,000, while antibody titer to rLipL41 protein was 1:128,000 and lastly antibody titer to rLoa22 protein was 1:256,000.

The Western blot analysis also used to determine antibody titer to each recombinant protein. Each recombinant protein at concentration of about 100 μg protein applied in large turf of SDS-PAGE and transformed to nitrocellulose membrane. The antibody dilution was reacted to each antigen strips. Anti-rLipL32 was revealed higher than 50,000 as shown Figure 5.6 lane 10. The antibody titers of rLipL41 and rLoa22 were 100,000 (Figure 5.7 lane 11) and 80,000 (Figure 5.8 lane 9), respectively.

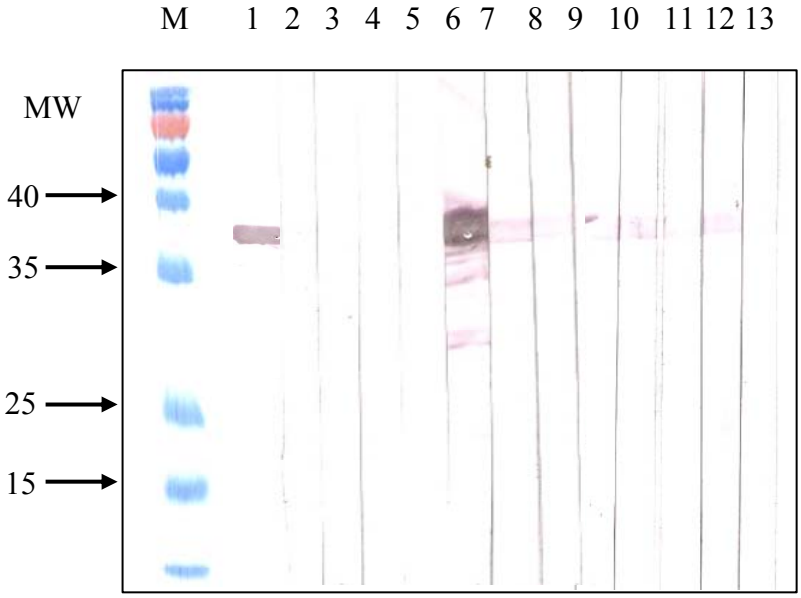


Figure 5.6 Serum titers of immunized mice sera against purified recombinant LipL32 protein. The recombinant LipL32 protein was blotted onto a nitrocellulose membrane, then cut into strips and used for testing with mice sera (pre- immunization and post-immunization) and histidine fusion taq marker was shown as positive control. The result indicated that anti-rlipL32 titer 1:50,000 in lane 10 and was selected as optimal titer to react with leptospiral whole cell lysate.

Lane MW = Protein molecular weight marker
Lane 1 = Histidine fusion taq marker
Lane 2-5 = Reactivity with pre-immunized mice sera using the serial dilutions from 1:1,000, 1:10,000, 1:100,000 and 1:1,000,000
Lane 6-13 = Reactivity with mice sera (immunized mice) using the dilutions from 1:1,000, 1:10,000, 1:20,000, 1:40,000, 1:50,000, 1:80,000, 1:100,000 and 1:1,000,000

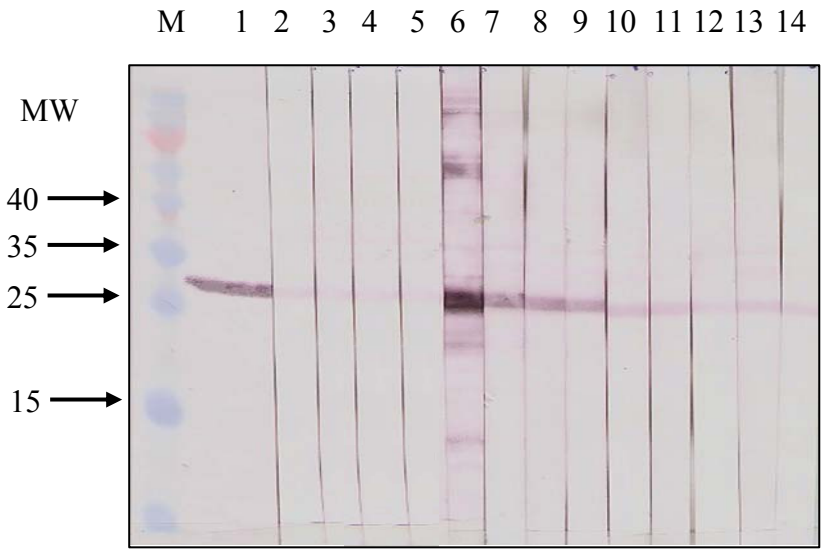


Figure 5.7 Serum titers of immunized mice sera against purified recombinant LipL41 protein. The recombinant LipL41 protein was blotted onto a nitrocellulose membrane, then cut into strips and used for testing with mice sera (pre- immunization and post-immunization). The result indicated that anti-rlipL41 titer 1:100,000 in lane 11 and was selected as optimal titer to react with leptospiral whole cell lysate.

Lane MW = Protein molecular weight marker

Lane 1 = Histidine fusion taq marker

Lane 2-5 = Reactivity with pre-immunized mice sera using the serial dilutions from 1:1,000, 1:10,000, 1:100,000 and 1:1,000,000

Lane 6-14 = Reactivity with mice sera (immunized mice) using the dilution from 1:1,000, 1:10,000, 1:20,000, 1:40,000, 1:50,000, 1:100,000, 1:200,000, 1:400,000, and 1:1,000,000

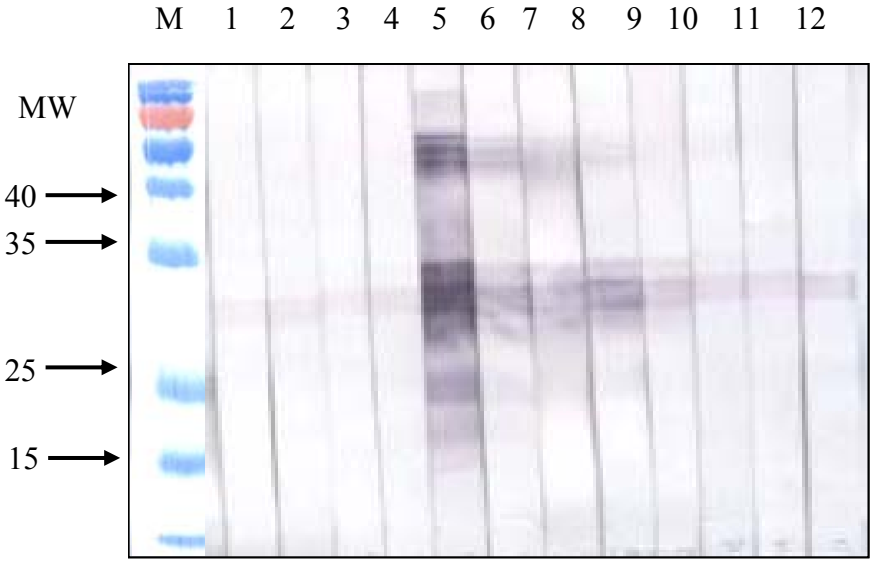


Figure 5.8 Serum titers of immunized mice sera against purified recombinant Loa22 protein. The recombinant Loa22 protein was blotted onto a nitrocellulose membrane, then cut into strips and used for testing with mice sera (pre- immunization and post-immunization). The result indicated that anti-rloa22 titer 1:80,000 in lane 9 and was selected as optimal titer to react with leptospiral whole cell lysate.

Lane MW = Protein molecular weight marker

Lane 1-4 =Reactivity with pre-immunized mice antisera using the serial dilutions from 1:1,000, 1:10,000, 1:100,000 and 1:1,000,000

Lane 5-11 = Reactivity with mice antisera (immunized mice) using the dilutions from 1:1,000, 1:10,000, 1:20,000, 1:40,000, 1:80,000, 1:100,000, and 1:1,000,000

Lane 12 = Histidine fusion taq marker

5.5 Immunoreactivity of recombinant protein to leptospirosis pateint's sera

Western blot assay was performed to test reactivity of recombinant proteins against patient sera which were positive in MAT assay, in comparative to the normal human sera from endemic area. Each purified recombinant protein was individually blotted on a nitrocellulose membrane and cut into strips for testing with individual human serum at dilution of 1:100.

All three recombinant proteins were tested with 11 cases of MAT positive human sera and 4 cases of human sera from endemic area. The MAT positive human sera and human sera in endemic area, were able to react to rLipL32 (Figure 5.9), while there was no reactivity of both rLipL41 and rLoa22 to any tested sera (data not show).

Figure 5.9 Detection of anti-rLipL32 antibodies response in human sera from MAT positive patients and human sera in endemic area by Western blot assay. The thick arrow indicated the position of rLipL32 protein and corresponded to the following bands in individual samples.

Lane M = Standard protein molecular weight
Lane 1-4 = rLipL32 react with normal human sera
Lane 5-15 = rLipL32 react with MAT positive human sera
Lane 16 = rLipL32 react with anti histidine tag as control

5.6 Reactivity of mice anti-recombinant protein against leptospiral serovars panel

The collected mice immune sera were tested for antibodies against each recombinant protein antigen and also with reactivity to the whole cell lysate of *Leptospira* by Western blot analysis. The mouse antiserum was firstly absorbed 8 times with *E. coli* BL-21(DE3) pLysS fixed cell, to eliminate the antibody to *E. coli* cell. The whole cell lysate of several *Leptospira* species, 17 stains were employed as antigen panel in Western blot.

The optimal dilution 1:50,000 of anti-rLipL32 mice sera were selected to react with blotted nitrocellulose membrane, based on the less non-specific background determined in Figure 5.6. In lane 2 of Figure 5.10 (a) and (b) revealed the reactivity of anti-rLipL32 mice sera with the purified rLipL32 protein with the predominated band at the expected size of 37 kDa, relevant to the size of His-taq maker in lane 1. This result showed that immunized mouse can produce the antibody to purified rLipL32 protein and after absorbed with *E. coli* cell, the mouse anti-rLipL32 was specific to rLpiL32 protein as no reactivity was detected in *E. coli* lysate (lane 3). The anti-rLipL32 reacted with leptospiral whole cell lysis have reactive band at 32 kDa.

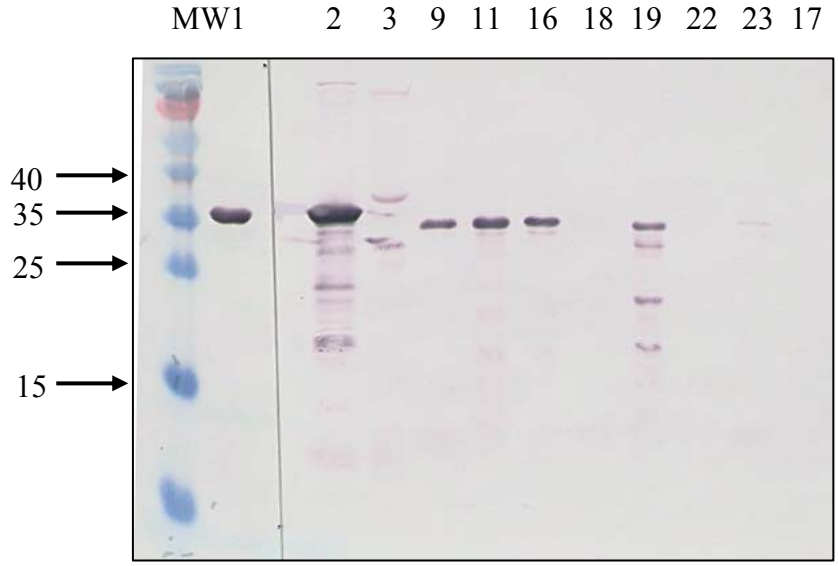
The anti-rLipL41 mice serum was used in dilution 1:100,000 for determining reactivity with rLipL41 protein, *E. coli* BL-21(DE3)pLysS and whole cell lysate. The result showed that anti-rLipL41 could react with rLipL41 protein at the expected size as 27 kDa as according to the positive control of His-taq maker in lane 1, and this antisera could react with some of leptospiral whole cell lysate at the expected size of 41 kDa, Figure 5.11 (a) and (b).

The immunized mice anti-rLoa22, at dilution of 1:80,000 was used to react with rLoa22, *E. coli* BL-21(DE3) pLysS and the native leptospiral cell lysate. The result showed that the reactive band of anti-rLoa22 with rLoa22 at 27 kDa while the reactive band of anti-rLoa22 among leptospiral cell lysate has band in expect size at 22 kDa and 25 kDa in Figure 5.12 (a) and (b). These results showed that rLoa22 protein can induce immune system in mice to produce anti-rLoa22, of which possessed the

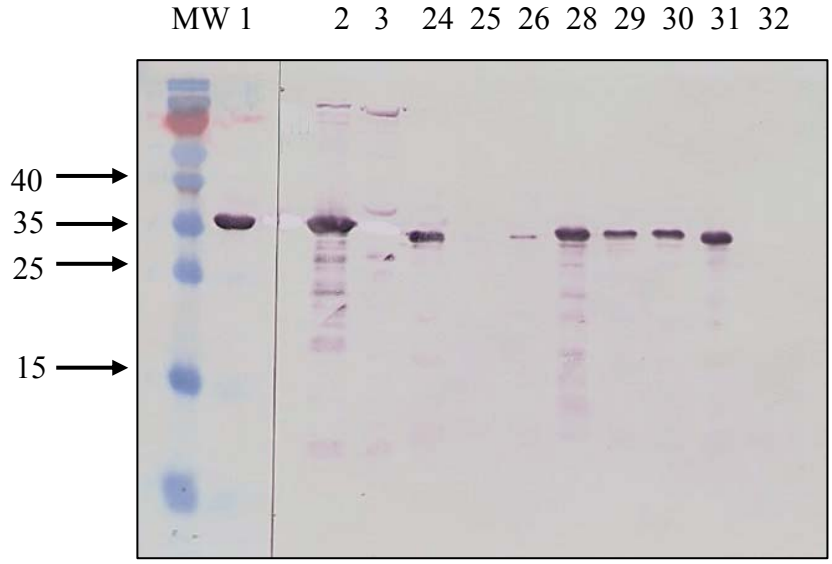
immunoreactivity to some *Leptospira* whole cell lysate. It was thus indicated that rLoa22 has the same epitope with native protein of *Leptpspira* because it has band in expect size at 22 kDa and 25 kDa. The addition reactive band approximately 25 kDa, of anti Loa22, implying that 25 kDa protein shared antigenicity to both Loa22 and rLoa22.

Seventeen strains of *Leptospira* species were used in this study to react with anti-rLipL32, anti-rLipL41 and anti-rLoa22 mice sera. All of three anti-recombinant proteins were have no reactivity with the *L. biflexa* serovar Patoc, which is the non-pathogenic Leptospire in stain no.17 and have no reaction with *L. interrogans* serovar Pomona, *L. borgpeterseni* serovar Sejroe, *L. meyeri* serovar Ranarum and *L. santarosai* serovar Shermani. While the reactive bands were shown against other pathogenic strains, showed reactivity band with *L. interrogans* serovar Copenhageni, *L. interrogans* serovar Djasiman, *L. borgpetersenii* serovar Javanica, *L. interrogans* serovar Pyrogenes *L. interrogans* serovar new, *L. borgpetersenii* serovar Mini, *L. kirshneri* serovar Cynopteri, *L. noguchii* serovar Louisiana, *L. noguchii* serovar Panama and *L. wolffii* serovar Korat.

In summary, mice anti-recombinant proteins in this study showed the specifically reacted with some leptospiral serovar. The anti-rLipL41 could not react to *L. weilli* serovar Sarmin, while anti-rLipL32 and anti-rLoa22 could. The anti-rLoa22 could react to *L. interrogans* serovar Wolffi, while anti-rLip32 and anti-rLip41 could not detect.



(a)



(b)

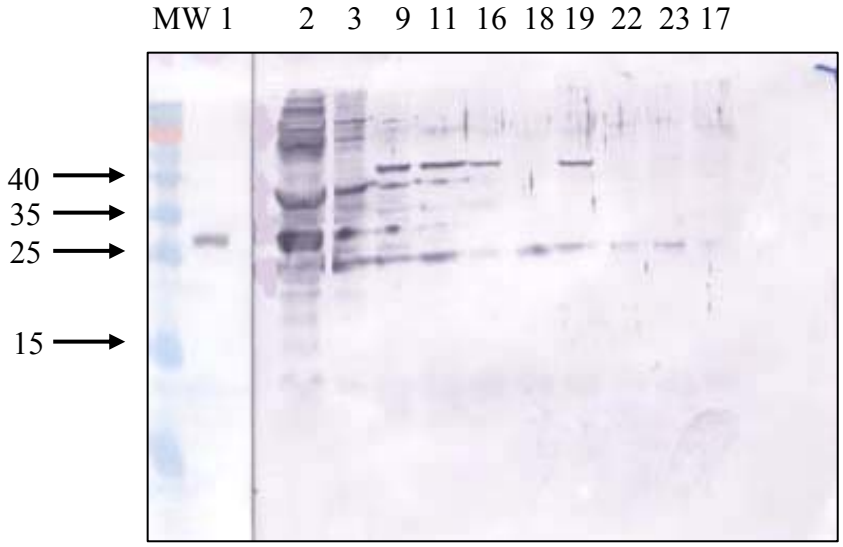
Figure 5.10 The anti-rLipL32 mice sera dilution 1:50,000 reacted with *Leptospira* species cell lysate. The positive control band was rLipL32 protein reacted with mouse anti-Histidine (1:2000) as shown in lane 1(a) and (b). Lane 2, the reactivity of rLipL32 protein with anti-rLipL32 mice sera. The negative control band was *E. coli* BL-21(DE3) pLysS cell lysate reacted with anti-rLipL32 mouse serum as shown in lane 3(a) and (b).

The common labelings of each gel were as follows;

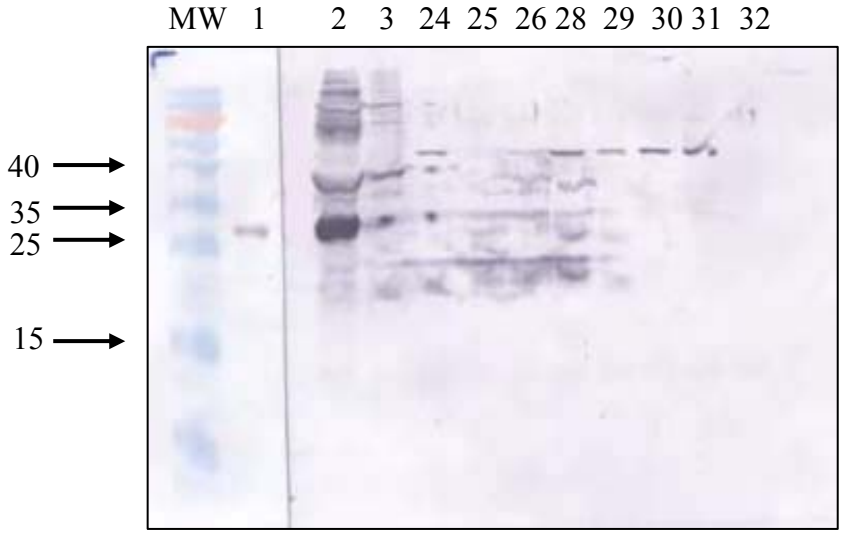
Lane 1 Histidine fusion taq of each recombinant protein

Mice antisera to each recombinant protein were reacted to following proteins and whole cell lysates of the following panel;

Lab strain no.	2	Purified recombinant protein
Lab strain no.	3	*E. coli* BL-21(DE3)pLysS cell lysate
Lab strain no.	9	*L. interogans* serovar Copenhageni
Lab strain no.	11	*L. interogans* serovar Djasiman
Lab strain no.	16	*L. interogans* serovar Javanica
Lab strain no.	18	*L. interogans* serovar Pomona
Lab strain no.	19	*L. interogans* serovar Pyrogenes
Lab strain no.	22	*L. interogans* serovar Sejroe
Lab strain no.	23	*L. weilii* serovar Wolffi
Lab strain no.	17	*L. interogans* serovar Patoc
Lab strain no.	24	*L. biflexa* serovar New
Lab strain no.	25	*L. borgpetersenii* serovar Ranarum
Lab strain no.	26	*L. borgpetersenii* serovar Sarmin
Lab strain no.	28	*L. borgpetersenii* serovar Mini
Lab strain no.	29	*L. borgpetersenii* serovar Cynopteri
Lab strain no.	30	*L. borgpetersenii* serovar Louisiana
Lab strain no.	31	*L. borgpetersenii* serovar Panama
Lab strain no.	32	*L. borgpetersenii* serovar Shermani

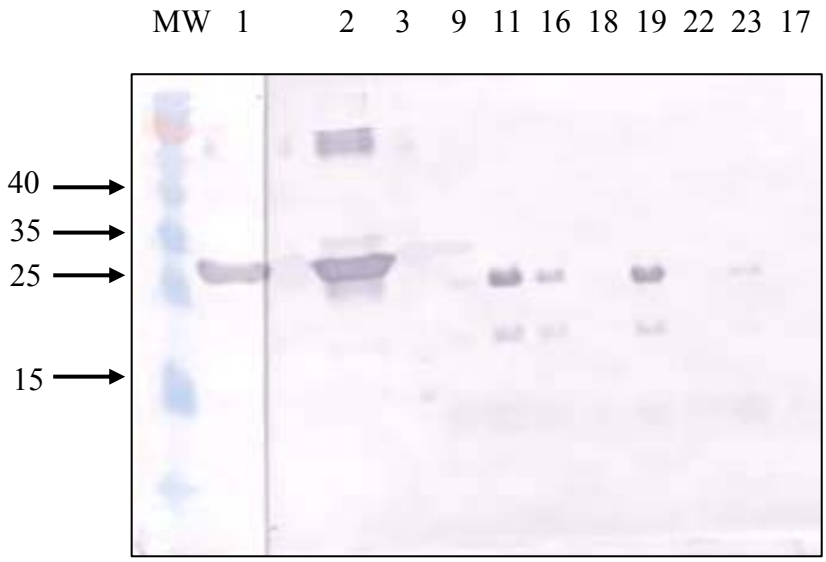


(a)

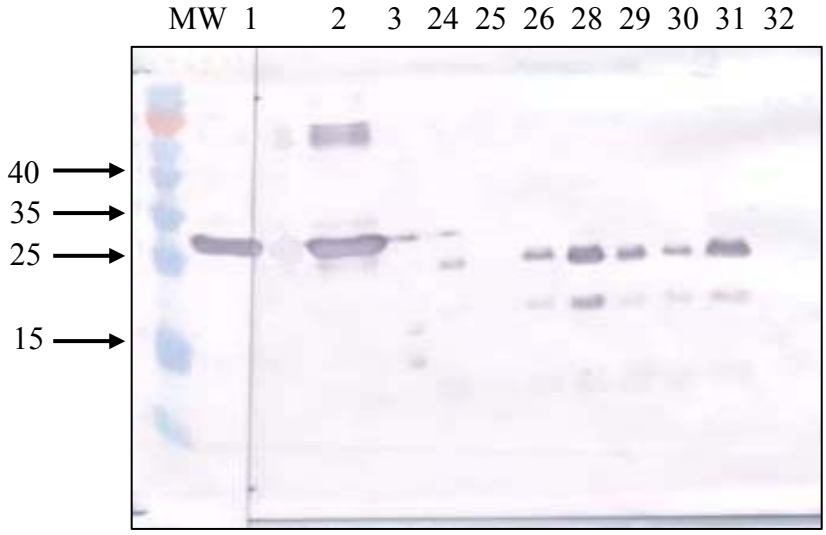


(b)

Figure 5.11 The anti-rLipL41 mice sera dilution 1:100,000 reacted with *Leptospira* species cell lysate. The positive control band was rLipL41 protein reacted with mouse anti-Histidine (1:2000) as shown in lane 1(a) and (b). Lane 2 showed the reactivity of rLipL41 protein with anti-rLipL41 mice sera. The negative control band was *E. coli* BL-21(DE3) pLysS cell lysate reacted with anti-rLipL41 mouse serum as shown in lane 3(a) and (b).



(a)



(b)

Figure 5.12 The anti-rLoa22 mice sera dilution1:80,000 reacted with *Leptospira* species cell lysate. The positive control band was rLipL41 protein reacted with mouse anti-Histidine (1:2000) as shown in lane 1(a) and (b). Lane 2 showed the reactivity of rLipL41 protein with anti-rLipL41 mice sera. The negative control band was *E. coli* BL-21(DE3) pLysS cell lysate reacted with anti-rLoa22 mouse serum as shown in lane 3 (a) and (b).

Table 5.2 Reactivity of anti recombinant proteins mice sera among Leptospiral serovar panel

Lab strain No.	Species	Serovar	Serogroup	Anti LipL32	Anti LipL41	Anti Loa 22
9	*L. interogans*	Copenhageni	Icterohaemoragiae	+	+	+
11	*L. interrogans*	Djasiman	Djasiman	+	+	+
16	*L. borgpetersenii*	Javanica	Javanica	+	+	+
18	*L. interrogans*	Pomona	Pomona	-	-	-
19	*L. interrogans*	Pyrogenes	Pyrogenes	+	+	+
22	*L. borgpetersenii*	Sejroe	Sejroe	-	-	-
23	*L. interrogans*	Wolffi	Sejroe	-	-	+
17	*L. biflexa*	Patoc	Patoc	-	-	-
24	*L. interrogans*	New	Autumnalis	+	+	+
25	*L .meyeri*	Ranarum	Ranarum	-	-	-
26	*L. weilli*	Sarmin	Sarmin	+	-	+
28	*L. borgpetersenii*	Mini	Mini	+	+	+
29	*L. kirshneri*	Cynopteri	Cynopteri	+	+	+
30	*L. noguchii*	Saigon	Louisiana	+	+	+
31	*L. noguchii*	Panama	Panama	+	+	+
32	*L. santarosai*	Shermani	Shermani	-	-	-

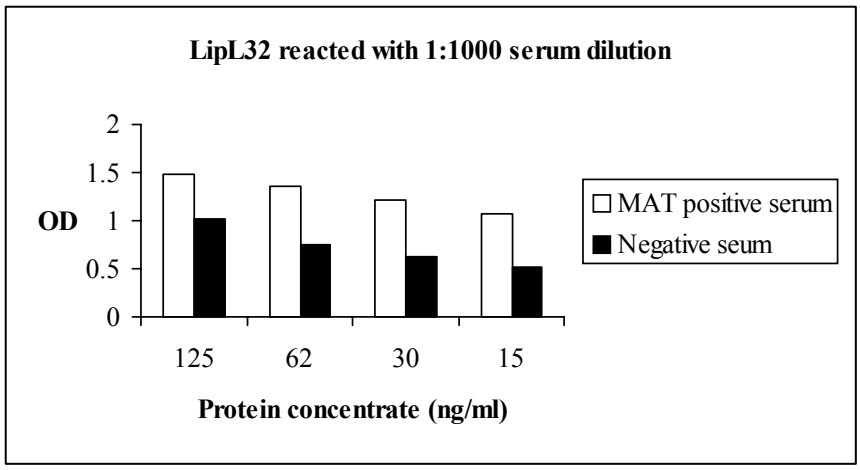
5.7 Immunoreactivity with human serum by ELISA method

5.7.1 Optimization concentration of recombinant proteins and sera dilution

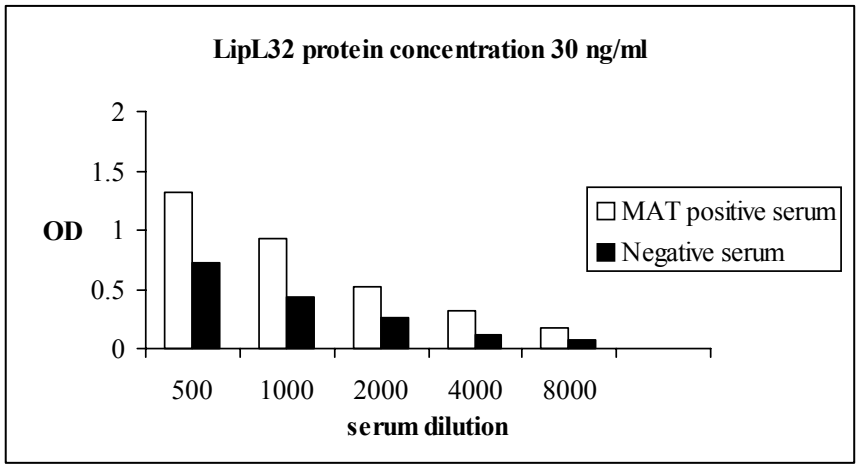
The standard ELISA procedure was employed as described in material and methods. To determine the optimum concentration of recombinant protein, the antigen concentration was varied from 125, 62, 30 and 15 ng/ml, and then reacted to the positive sera with MAT positive and MAT negative serum at fixed dilution of 1:1000. In addition, the fixed concentration of HRP conjugate at dilution of 1:4000 was used. At each point of protein concentration, the difference of OD value between positive and negative serum among each recombinant protein revealed similar trend as shown for rLipL32 in Figure5.13 a, rLipL41 shown in Figure5.14 a, and rLoa22 shown in Figure5.15 a. Among these three recombinant proteins, the concentration of 30 ng/ml were selected, as the liberated OD was optimized in the range of 0.5 – 1.

In order to determine the optimum concentration of sera, the serial dilution of antibody at 1:500, 1:1000, 1:2000, 1:4000 and 1:8000 were performed. These sera dilutions were performed against fixed antigen concentration of 30 ng/ml in all three recombinant proteins. The highest difference of OD value between positive and negative sera at dilution of 1:1000 as shown for rLipL32 in Figure 5.13 b, rLipL41 shown in Figure5.14 b and rLoa22 shown in Figure5.15 b.

Thus the protein concentration of 30 ng/ml was selected as the optimal antigen among rLipL32, rLipL41and rLoa22 protein, for coating the ELISA plate. While the sera dilution 1: 1000 was selected based on the optimum range of positive OD value between 0.5 – 1.

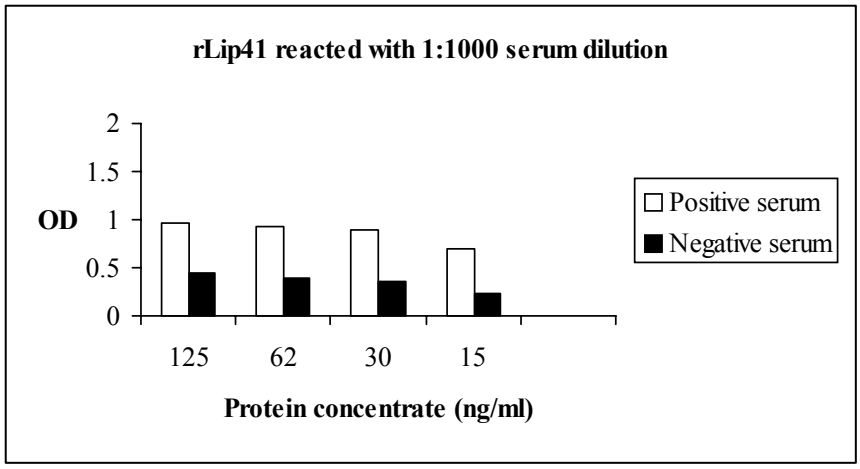


(a)

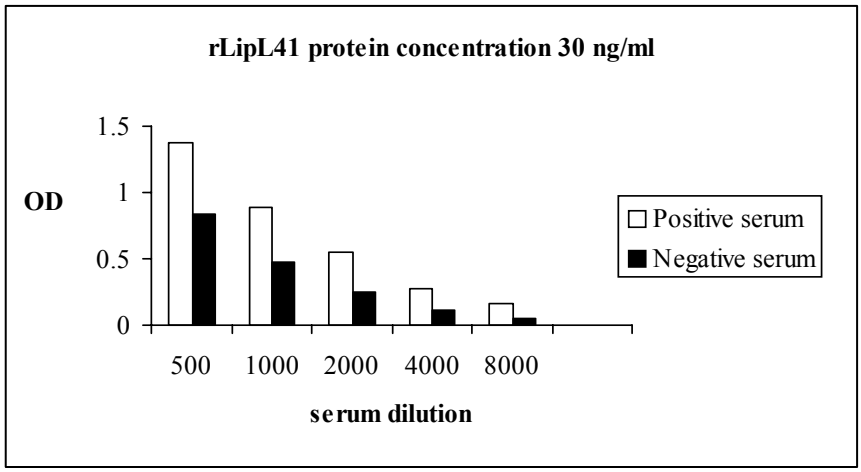


(b)

Figure 5.13 Optimization for rLipL32 total Ig based ELISA assay at different LipL32 protein concentrations (a) and different serum dilutions (b). The absorbance values (OD at 405 nm. on the Y-axis) were plotted for MAT positive patient serum (white bar) and MAT negative serum (black bar).

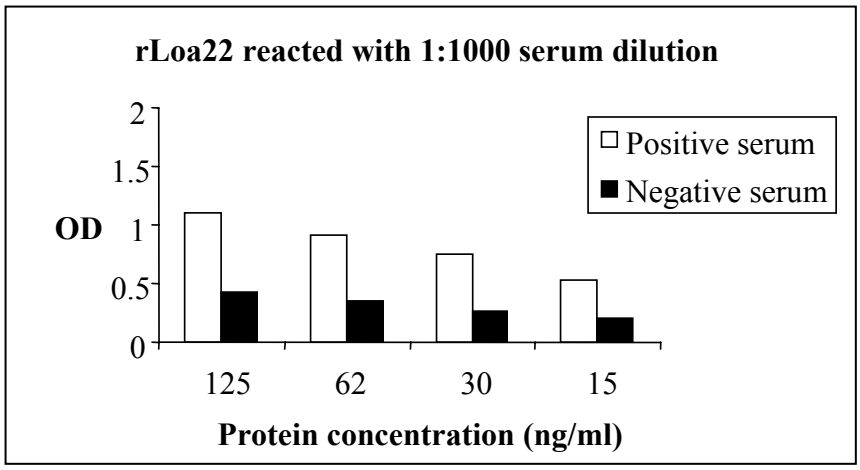


(a)

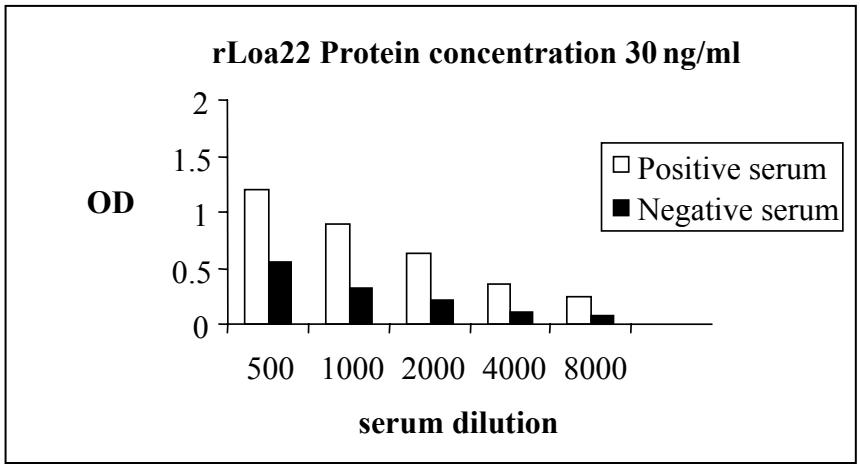


(b)

Figure 5.14 Optimization for rLipL41 total Ig based ELISA assay at different LipL41 protein concentrations (a) and different serum dilutions (b). The absorbance values (OD at 405 nm. on the Y-axis) were plotted for MAT positive patient serum (white bar) and MAT negative serum (black bar).



(a)



(b)

Figure 5.15 Optimization for rLoa22 total Ig based ELISA assay at different rLoa22 protein concentrations (a) and different serum dilutions (b). The absorbance values (OD at 405 nm. on the Y-axis) were plotted for MAT positive patient serum (white bar) and MAT negative serum (black bar).

5.7.2 The ELISA based total Ig reactivity against recombinant protein among different human sera group

Among three recombinant proteins, LipL32, LipL41 and Loa22, the protein at concentration of 30 ng/ml was selected to react with human serum dilution of 1:1000. Eight groups of sera were included as follows;

The suspected leptospirosis cases were included as follow;

1. 30 patient sera with MAT positive
2. 16 patient sera of which MAT negative but positive in Lepto dipstick.
3. 39 patient sera with culture positive, but negative in MAT test

The control groups with negative in MAT were included as follow;

1. 30 sera samples from people lived in endemic area (assigned as HE)
2. 31 sera samples from people lived out of endemic area (assigned as HOE)
3. 30 sera of scrub typhus patients (assigned as ST)
4. 30 sera s of melloidosisosis patients (assigned as MEL)
5. 31 sera s of dengue hemorrhagic patients as DEN)

Each diagnostic test usually requires the cut off value to differentiate positive from negative subject.

In order to select the cut off OD value to differentiate MAT positive serum from other control groups, the SD value was calculated among control group with negative in MAT test, and the cut off value was derived from either mean plus one or 2SD value. The control groups included people lived in endemic area, people lived out of endemic area, scrub typhus patients, dengue hemorrhagic patients and melloidosisosis patients. The differentiation of MAT positive patients from other control group was based on the OD value higher than mean plus 1 or 2SD, and the percentage of positive cases for each group were demonstrated.

For the rLipL32 total Ig ELISA, the OD means plot of each sera group with error bar (95% CI) were revealed in figure 5.16. The one-way ANOVA was used to determine the significant of mean difference ($p<0.05$) among studied groups. The cut off value to differentiate MAT positive from other control group was calculated by mean of five control groups of MAT negative plus 1SD (0.62) or 2SD (0.75) (Table 5.3).

For the rLipL41 total Ig ELISA, the OD means plot of each sera group with error bar (95% CI) were shown in Figure 5.17. The one-way ANOVA was used to determine the significant of mean difference ($p<0.05$) among studied groups. The cut off value to differentiate MAT positive from other control group was calculated by mean of five control groups of MAT negative plus 1SD (0.62) or 2SD (0.67) (Table 5.4).

For the rLoa22 total Ig ELISA, the OD means plot of each sera group with error bar (95% CI) were shown in Figure 5.18. The one-way ANOVA was used to determine the significant of mean difference ($p<0.05$) among studied groups. The cut off value to differentiate MAT positive from other control group was calculated by mean of five control groups of MAT negative plus 1SD (0.58) or 2SD (0.62) (Table 5.5).

The mean OD response to recombinant protein was correlated well with antibody detected by MAT, thus usually higher than sera with negative in MAT. The mean OD of MAT positive was thus significantly higher than normal control group and patients with febrile illness, whose their MAT results were negative.

Table 5.3 One-way ANOVA analyzed among means of OD values derived from anti rLipL32 detected based on total Ig ELISA assay.

group by disease (I)	group by disease (J)	Mean Difference (I-J)	Mean	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Lepto MAT pos			0.83297			
MAT negative	Lepto Dipstick pos	0.18822	0.64475	0.561	-0.08946	0.46589
	Lepto culture pos	0.48481 *	0.34815	0	0.25761	0.71202
	HOE	0.41407 *	0.41890	0	0.19661	0.63152
	HE	0.2631 *	0.56987	0.003	0.05783	0.46836
	ST	0.22373	0.60923	0.058	-0.00362	0.45108
	DHF	0.28971 *	0.54326	0.002	0.06807	0.51135
	melloidosis	0.54703 *	0.28593	0	0.33719	0.75687

* The mean difference is significant at the 0.05 level, in comparative to Lepto MAT positive group.

Mean of Control group: HOE, HE, ST, DHF and Melloidosis = 0.485438
Cut off 1: mean + 1SD = 0.485438+ 0.132313= 0.617751
Cut off 2: mean + 2SD = 0.485438+ 0.264626= 0.750064

As shown in Table 5.3 the mean OD of MAT positive group was significantly higher that other control groups, except in scrup typhus group. In suspected Leptospirosis cases of which Lepto Dipstick was positive, the mean OD was quite high and not significantly different from the patients with MAT positive.

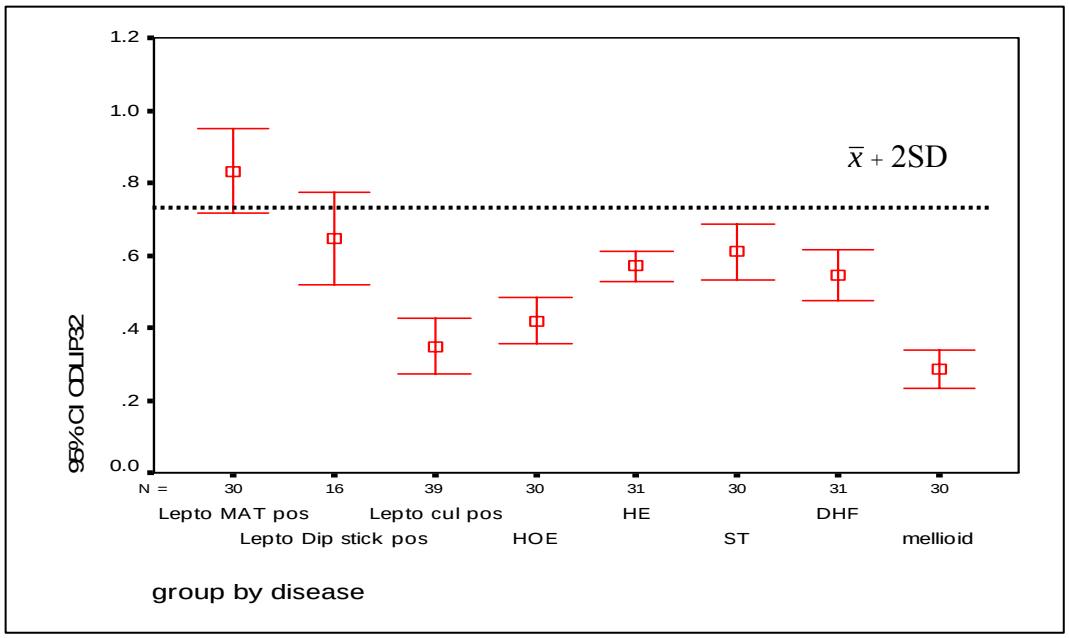


Figure 5.16 The mean-OD of ELISA assay based on the recombinant LipL32 protein among all serum groups. The cut-off value based on $\bar{x}$ + 2SD (0.75) was indicated as dash line.

Table 5.4 One-way ANOVA analyzed among means of OD values derived from anti rLipL41 detected based on total Ig ELISA assay.

group by disease (I)	group by disease (J)	Mean Difference (I-J)	Mean	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Lepto MAT pos			0.90420			
MAT negative	Lepto Dipstick pos	0.28907	0.61512	0.095	-0.02223	0.60038
	Lepto culture pos	0.49233 *	0.41187	0	0.23811	0.74655
	HOE	0.3162 *	0.58800	0.005	0.05883	0.57357
	HE	0.29065 *	0.61355	0.01	0.04191	0.5394
	ST	0.323 *	0.58120	0.004	0.06449	0.58151
	DHF	0.40436 *	0.49984	0	0.13167	0.67705
	melloidosis	0.29617 *	0.60803	0.011	0.04137	0.55096

* The mean difference is significant at the .05 level, in comparative to Lepto MAT positive group.

Mean of Control group: HOE, HE, ST, DHF and Melloidosis = 0.578124
Cut off 1: mean + 1SD = 0.578124+0.045784= 0.623908
Cut off 2: mean + 2SD = 0.578124+0.091568= 0.669692

For anti LipL41, the mean-OD of MAT positive cases were significantly higher than other control group shown in Table 5.4.

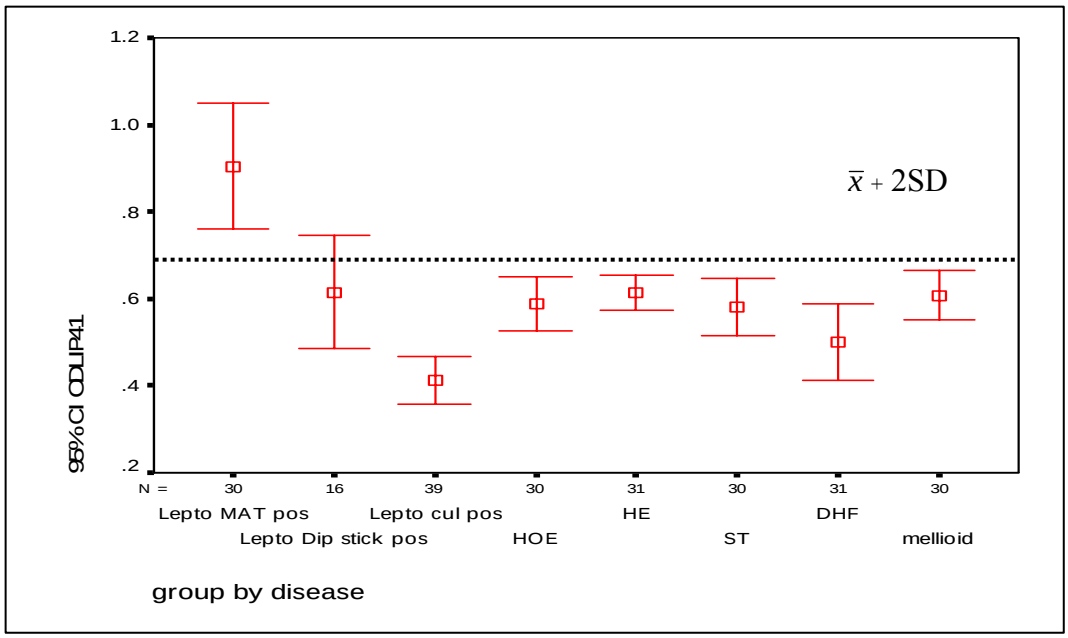


Figure 5.17 The mean-OD of ELISA assay based on the recombinant LipL41 protein among all serum groups. The cut-off value based on $\bar{x}$ + 2SD (0.67) was indicated as dash line.

Table 5.5 One-way ANOVA analyzed among means of OD values derived from anti rLoa22 detected based on total Ig ELISA assay.

group by disease (I)	group by disease (J)	Mean Difference (I-J)	Mean	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Lepto MAT pos			0.82353			
MAT negative	Lepto Dipstick pos	0.2036	0.61994	0.344	-0.06373	0.47092
	Lepto culture pos	0.37512 *	0.44841	0	0.16849	0.58176
	HOE	0.35227 *	0.47127	0	0.14502	0.55951
	HE	0.25821 *	0.56532	0.003	0.05772	0.4587
	ST	0.24793 *	0.57560	0.012	0.03193	0.46394
	DHF	0.27579 *	0.54774	0.031	0.01269	0.53889
	melloidosis	0.3119 *	0.51163	0	0.10335	0.52045

* The mean difference is significant at the .05 level, in comparative to Lepto MAT positive group.

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Mean of Control group: HOE, HE, ST, DHF and Melloidosis = 0.534312
Cut off 1: mean + 1SD = 0.534312+0.042823= 0.577135
Cut off 2: mean + 2SD = 0.534312+0.085646= 0.619958

For anti Loa22, the mean-OD of MAT positive cases were significantly higher than other control group shown in Table 5.5.

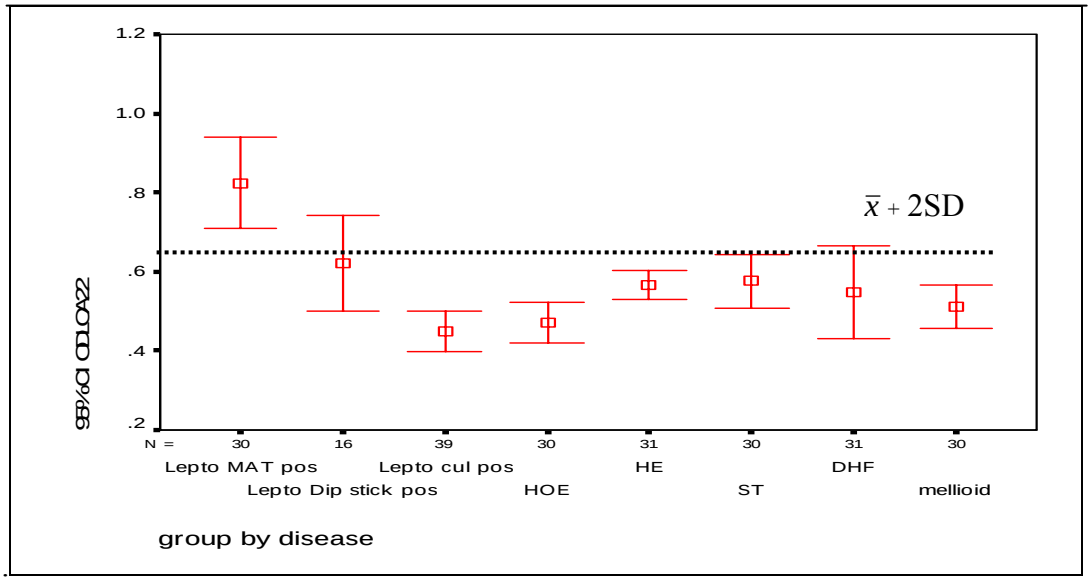

Figure 5.18 The mean-OD of ELISA assay based on the recombinant Loa22 protein among all serum groups. The cut-off value based on $\bar{x}$ + 2SD (0.62) was indicated as dash line.

5.7.3 Number of positive cases based on defined cut off value

The antibody response to recombinant proteins, LipL32, LipL41 and Loa22 response as differential diagnosis were evaluated among MAT positive group (as gold standard) and various control groups. The number of cases possessed OD higher than mean +1SD or mean +2SD were counted and then tabulated as percentage in Table 5.6, 5.7, 5.8 and also graphs in Figure 5.19, 5.20, 5.21.

ELISA based on three recombinant proteins revealed the highest positive percentage among Leptospirosis patient with MAT positive. The mean plus 2SD was cut off value for differentiation of three anti-recombinant proteins, anti-rLipL32 was showed the percentage of OD in MAT positive group at 57% while anti-rLipL41 and anti-rLoa22 were 70% and 77%.

The patient with culture positive was detected based on total Ig ELISA, the result showed that the percentages of OD of this group were quite low for three anti-recombinant proteins as in Figure 5.19, 5.20, 5.21.

The result of anti-rLipL32 based on ELISA of people lived in endemic area (HE) and people who lived out of endemic area (HOE) were showed 6% and 7% while anti-rLipL41 of HE and HOE were 29% and 20%. For anti-rLoa22, the percentage of people lived in endemic area (HE) and people who lived out of endemic area (HOE) were 26% and 7%, respectively. For febrile illness groups as Scrub typhus, Dengue fever and Melloidosisosis were tested. The anti-rLipL32 was showed the percentage of OD in Scrub typhus, Dengue fever and Melloidosisosis were 20%, 19% and 0% while anti-rLipL41 was 33%, 23% and 40%, respectively. For anti-rLoa22 were showed 43%, 26% and 23% in Scrub typhus, Dengue fever and Melloidosisosis, respectively. Among antibody responses to three recombinant proteins, anti rLipL32 was detected in less number among MAT negative sera, in comparative to anti LipL41 and anti Loa22 responses, implying the more specific antigenicity of LipL32 for Leptospirosis.

Table 5.6 Percentage of positive cases for anti rLipL32 based on the OD value higher than cut off 1 ($\bar{x}$ + 1SD) and cut off 2 ($\bar{x}$ + 2SD)

group by disease	N	rLipL32	
		Cut off 1	Cut off 2
		OD ≥ 0.62 (case)	OD ≥ 0.75 (case)
Lepto MAT pos	30	20 (67%)	17 (57%)
Lepto Dip stick pos	16	8 (50%)	5 (31%)
Lepto cul pos	39	4 (10%)	2 (5%)
HOE	30	2 (7%)	2 (7%)
HE	31	9 (29%)	2 (6%)
ST	30	12 (43%)	6 (20%)
DEN	31	9 (29%)	6 (19%)
melloidosis	30	0 (0%)	0 (0%)

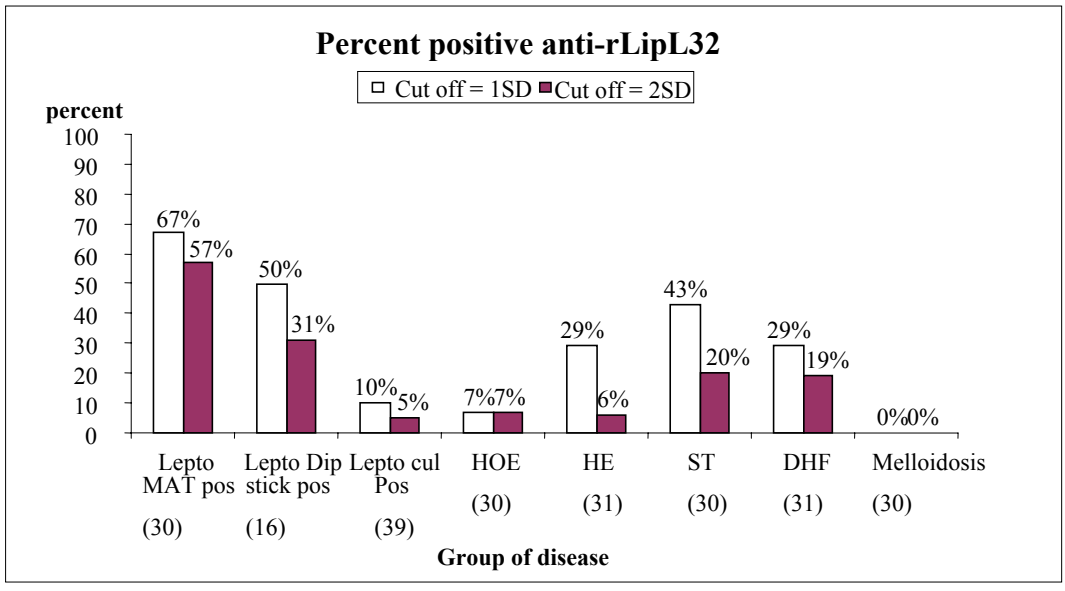

Figure 5.19 Bar graph revealed the percentage of positive cases for anti rLipL32, which were justified based on the OD value higher than cut off 1 ($\bar{x}$ + 1SD) and cut off 2 ($\bar{x}$ + 2SD).

Table 5.7 Percentage of positive cases for anti rLipL41 based on the OD value higher than cut off 1 ($\bar{x}$ + 1SD) and cut off 2 ($\bar{x}$ + 2SD).

group by disease	N	rLipL41	
		Cut off 1	Cut off 2
		OD ≥ 0 .62 (case)	OD ≥ 0.67 (case)
Lepto MAT pos	30	22 (73%)	21 (70%)
Lepto Dip stick pos	16	6 (38%)	6 (38%)
Lepto cul pos	39	5 (13%)	1 (3%)
HOE	30	14 (47%)	6 (20%)
HE	31	14 (45%)	9 (29%)
ST	30	11 (37%)	10 (33%)
DHF	31	10 (32%)	7 (23%)
melloidosis	30	15 (50%)	12 (40%)

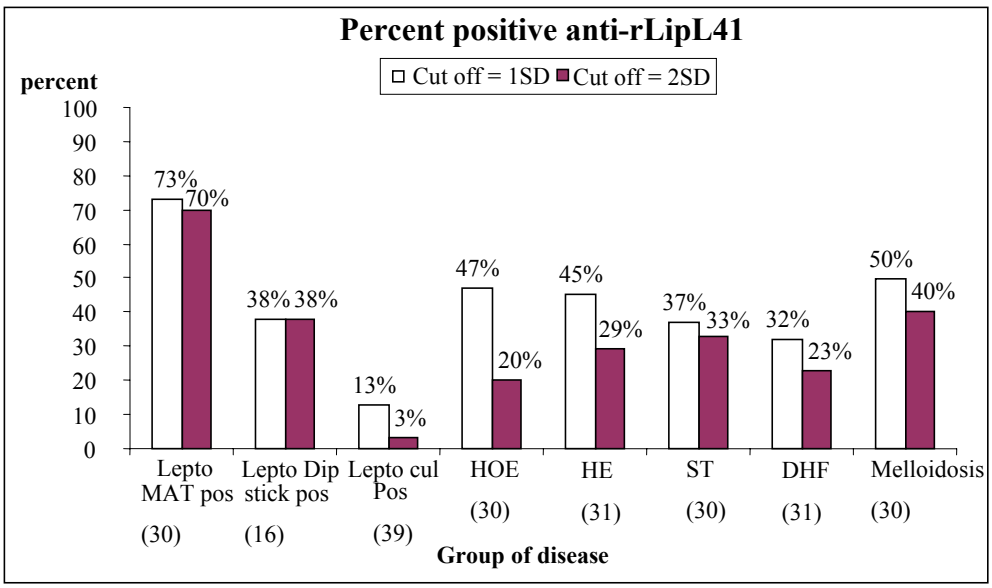

Figure 5.20 Bar graph revealed the percentage of positive cases for anti rLipL41, which were justified based on the OD value higher than cut off ($\bar{x}$ + 1SD) and cut off 2 ($\bar{x}$ + 2SD).

Table 5.8 Percentage of positive cases for anti rLoa22 based on the OD value higher than cut off 1 ($\bar{x}$ + 1SD) and cut off 2 ($\bar{x}$ + 2SD).

Group by disease	N	rLoa22	
		Cut off 1	Cut off 2
		OD ≥ 0.58 (case)	OD ≥ 0.62 (case)
Lepto MAT pos	30	24 (80%)	23 (77%)
Lepto Dip stick pos	16	7 (44%)	7 (44%)
Lepto cul pos	39	8 (21%)	5 (13%)
HOE	30	5 (17%)	2 (7%)
HE	31	12 (39%)	8 (26%)
ST	30	15 (50%)	13 (43%)
DHF	31	10 (32%)	8 (26%)
melloidosis	30	12 (40%)	7 (23%)

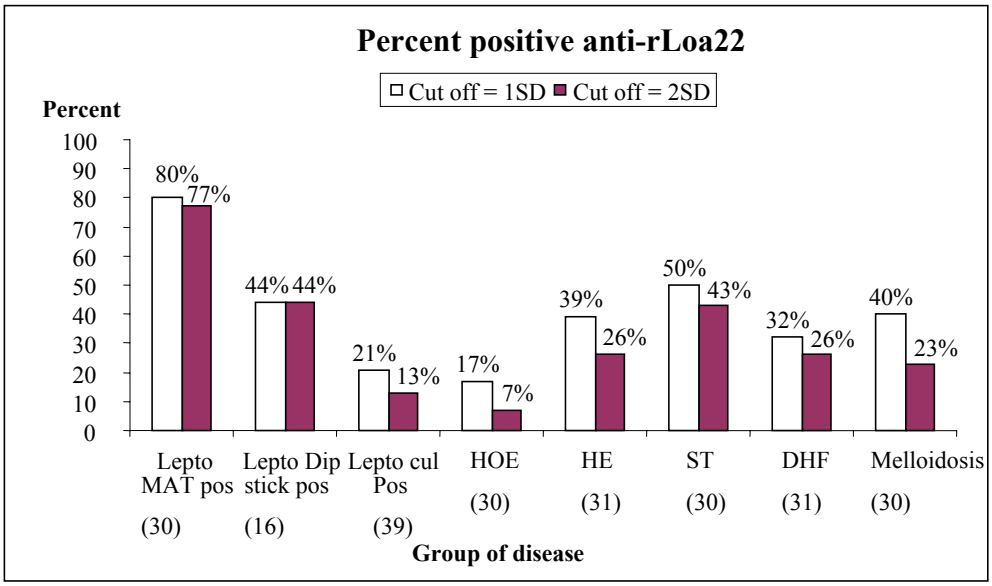

Figure 5.21 Bar graph revealed the percentage of positive cases for anti rLoa22, which were justified based on the OD value higher than cut off 1 ($\bar{x}$ + 1SD) and cut off 2 ($\bar{x}$ + 2SD).

5.7.4 Sensitivity, specificity and accuracy of the ELISA assay to each recombinant protein, in comparative to MAT assay

The positive case of ELISA assay for each recombinant was based on the OD that was higher than the cut off 2 value as mean plus 2SD. In each studied group, the result based on ELISA was compared to MAT assay as gold standard diagnostic test, and the the 2x2 table Galen's method was employed to determine sensitivity, specificity and accuracy as showed in table 5.9.

In cut off 2 was found that the percentage of control group based on ELISA when reactive with rLipL32 in people lived in endemic area (HE) was 6% while the cut off 1 was 29%. In febrile illness groups as Scrub typhus, the percentage of cut off 1 was 43% while the cut off 2 was 20%. From this resulted we suggested to used cut off 2 of percentage for rLipL32. As shown in Fig 5.19 the positive response of anti rLipL32 was revealed based on 2 OD values (cut off 1, cut off 2). Among the normal human sera of endemic area, the positive cases based on cut off 2 was 6%, while based on cut off 1 was quite high at 29%. The OD value at cut off 2 was thus selected to differentiate the infective from normal individual. The positive cases among normal human residence in Bangkok (HOE) were detected in low percentage, based on anti LipL32. The positive cases of anti LipL32 identified among patients with febrile illness were in less number when justification based on cut off 2 than the cut off 1 value. The positive cases based on anti LipL32 response was not detected in melloidosisosis patient, while the higher numbers of positive cases were revealed based on antibody response to LipL41 and Loa22. The efficacy of each recombinant protein as antigen in ELISA was thus calculated based on the positive cases derived from cut off 2 value as revealed in table 5.9.

Table 5.9 The 2X2 table constructed to evaluate the efficient in diagnostic between MAT and ELISA of three recombinant proteins.

(a) rLipL32

		ELISA rLip32		Total
		Elisa Neg	Elisa Pos	
MAT	MAT Neg	184	23	207
	MAT pos	13	17	30
Total		197	40	237

(b) rLipL41

		ELISA rLip41		Total
		Elisa Neg	Elisa Pos	
MAT	MAT Neg	156	51	207
	MAT pos	9	21	30
Total		165	72	237

(c) rLoa22

		ELISA rLoa22		Total
		Elisa Neg	Elisa Pos	
MAT	MAT Neg	157	50	207
	MAT pos	7	23	30
Total		164	73	237

(d) Summary of the sensitivity, specificity and accuracy of ELISA based on three recombinant proteins

	LipL32	LipL41	Loa22
Sensitivity	56.67%	70%	76.67%
Specificity	88.89%	75.36%	75.85%
Efficacy	84.81%	74.68%	75.95%

5.8 Application of IgM based ELISA for Leptospirosis diagnosis

During first week of Leptospirosis infection, the IgG level was not significantly raised, thus the serodiagnosis like MAT assay turned negative, while the total Ig based ELISA assay can detected only 1-2% among acute phase sera. The attempt to detect IgM response to the recombinant proteins was performed to increase sensitivity of ELISA test. The optimal condition used was the same as total Ig based ELISA, but using the IgM conjugate of 1:4000 dilution. The sera dilution of 1:1000 was used to determine the presence of both IgG and IgM in each sample.

The mean of IgM level were determined in various studied groups, and SD value was calculated in individual. The cut off value for differentiation was calculated from mean of control group that included the sera with MAT negative i.e.,, normal people lived in endemic area and out of endemic area, scrub typhus, dengue and melloidosisosis patient sera. The cut off value was thus assigned based of mean OD among those control group plus 1 SD. The percentage positive of IgM based ELISA reactive with rLipL32, rLipL41 and rLoa22 in culture positive were showed 18%, 15% and 15%, respectively. Positive IgM response among three recombinant proteins could be detected among other febrile illness patient, of which the substantial percentage (17-37%) were identified in scrub typhus and dengue, which the low percentage (0-4%) was identified in melloidosisosis patient. The high percentage of IgM response was also revealed among normal human sera, thus the solely relied on IgM response should be considerable.

Table 5.10 The cases of anti LipL32 IgM response that OD was higher than cut off 1

group by disease	N	Mean	Std. Deviation	OD ≥ 0.66
Lepto MAT positive	30	0.52367	0.273459	9 (30%)
Lepto culure positive	39	0.48808	0.296085	7 (18%)
HOE	24	0.51087	0.245864	4 (17%)
HE	30	0.65963	0.333736	15 (50%)
ST	30	0.57600	0.280228	11 (37%)
DHF	30	0.55463	0.264361	7 (23%)
Melloidosis	30	0.26437	0.164975	1 (4%)

Mean of Control group: HOE, HE, ST, DHF and Melloidosis = 0.5131

Cut off 1: mean + 1SD = 0.662284

Cut off 2: mean + 2SD = 0.811469

Table 5.11 The cases of anti LipL41 IgM response that OD was higher than cut off 1

group by disease	N	Mean	Std. Deviation	OD ≥ 0.65
Lepto MAT positive	30	0.28870	0.225791	2 (7%)
Lepto culure positive	39	0.40272	0.322333	6 (15%)
HOE	24	0.54221	0.267474	6 (25%)
HE	30	0.71450	0.363705	16 (53%)
ST	30	0.37350	0.282647	5 (17%)
DHF	30	0.38283	0.296200	5 (17%)
Melloidosis	30	0.10703	0.133968	0 (0%)

Mean of Control group: HOE, HE, ST, DHF and Melloidosis = 0.424014

Cut off 1: mean + 1SD = 0.649335

Cut off 2: mean + 2SD =0.874657

Table 5.12 The cases of anti Loa22 IgM response that OD was higher than cut off 1

group by disease	N	Mean	Std. Deviation	OD ≥ 0.65
Lepto MAT positive	30	0.42880	0.271103	7 (23%)
Lepto culture positive	39	0.40638	0.290303	6 (15%)
HOE	24	0.53733	0.262881	6 (25%)
HE	30	0.68613	0.343947	15 (50%)
ST	30	0.49840	0.271616	8 (27%)
DHF	30	0.44793	0.243178	5 (17%)
Melloidosis	30	0.20233	0.167954	0 (0%)

Mean of Control group: HOE, HE, ST, DHF and Melloidosis = 0.474424

Cut off 1: mean + 1SD = 0.650555

Cut off 2: mean + 2SD = 0.826686

5.9 Evaluation of IgM and IgG response among culture positive cases

Among sera collected during acute phase, only a few number were positive based on IgG response, thus it was attempted to detected the IgM response, which may increase efficacy of diagnosis.

For anti rLipL32 the OD value derived from, IgM and IgG response was scattering plotted for each sera sample as shown in Figure 5.22. The mean plus1SD of 0.66 was defined as cut off as mentioned in section 5.8. Among 39 culture positive cases, 2 cases were positive for IgG, which additional of 7 cases were positive for IgM.

For anti rLipL41 the OD value derived from, IgM and IgG response was scattering plotted for each sera sample as shown in Figure 5.23. The mean plus1SD of 0.65 was defined as cut off as mentioned in 5.8 section. Among 39 culture positive cases, 1 cases were positive for IgG, which additional of 6 cases were positive for IgM.

The rLoa22 the OD value derived from, IgM and IgG response was scattering plotted for each sera sample as shown in Figure 5.24. The mean plus1SD of 0.65 was defined as cut off as mentioned in 5.8 section. Among 39 culture positive cases, 5 cases were positive for IgG, which additional of 6 cases were positive for IgM.

These results revealed that although MAT assay could not reveal any antibody reactivity, IgG and IgM based on ELISA could reveal some specific antibody response to each leptospiral antigen.

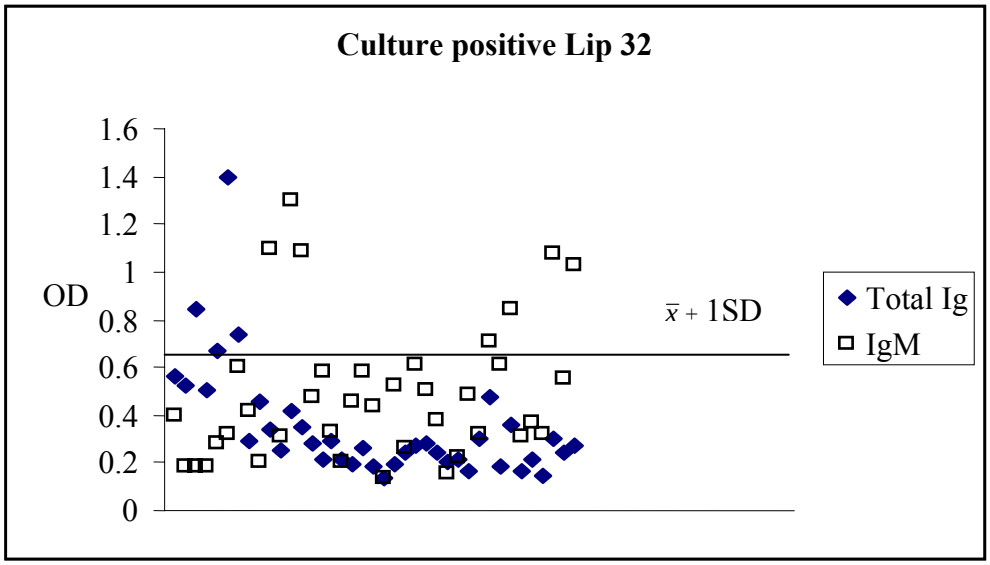

Figure 5.22 The OD scatter plot derived from IgM and IgG of anti LipL32 among patients of culture positive sera. The cut-off OD value defined at $\bar{x}$ + 1SD (0.66) was derived from mean of control group was indicated as back line.

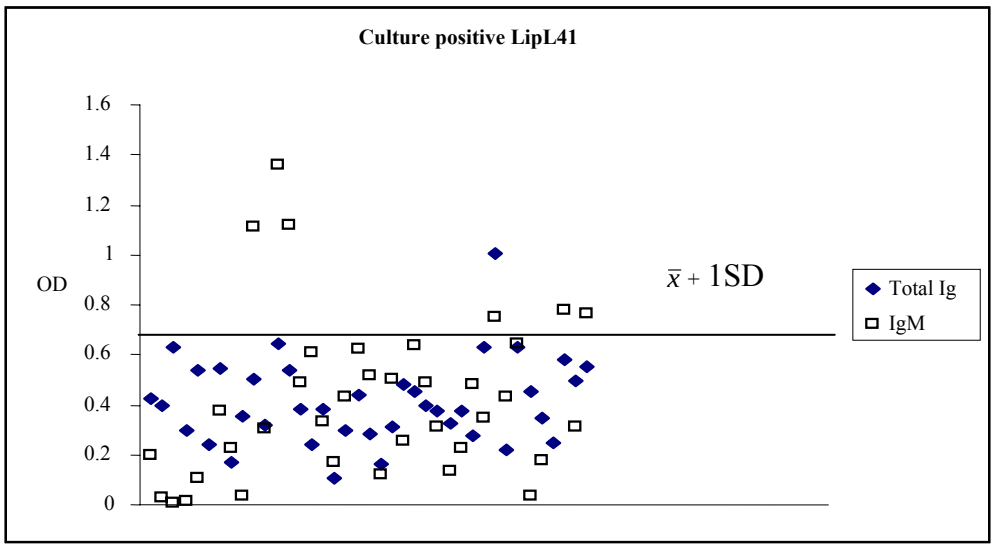

Figure 5.23 The OD scatter plot derived from IgM and IgG of anti LipL41 among patients of culture positive sera. The cut-off OD value defined at $\bar{x}$ + 1SD (0.65) was derived from mean of control group was indicated as back line.

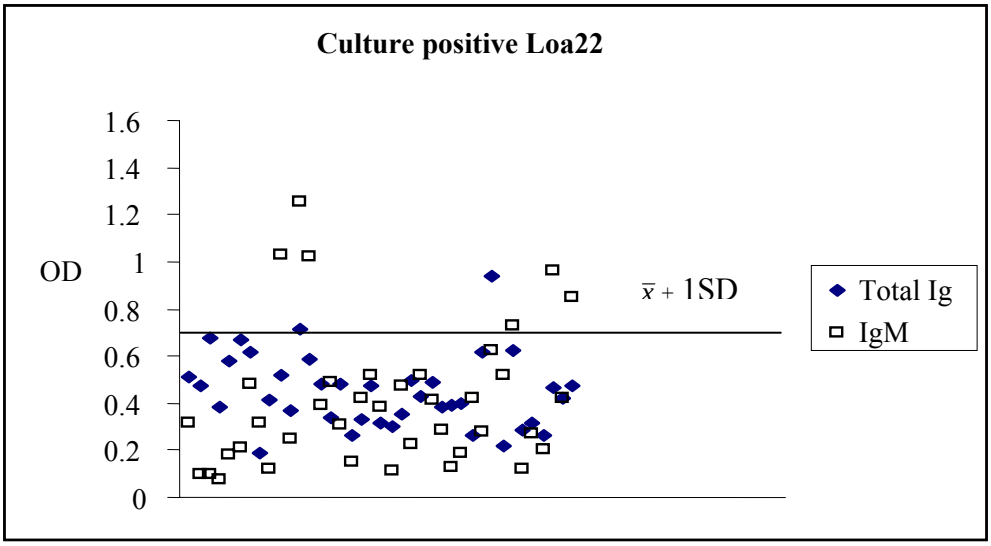

Figure 5.24 The OD scatter plot derived from IgM and IgG of anti Loa22 among patients of culture positive sera. The cut-off OD value defined at $\bar{x}$ + 1SD (0.65) was derived from mean of control group was indicated as back line.

5.10 Evaluation of IgG based ELISA assay in suspected Leptospirosis cases

More suspected Leptospirosis cases with MAT negative were evaluated for IgG response to recombinant protein and the positively was justified based on mean plus 2SD describe in section 5.7.2.

The comparative study groups composed of;

1. Leptospirosis case with MAT negative
2. Leptospirosis cases with Dipstick positive but MAT negative
3. Leptospirosis cases with culture positive

The cut off OD value as mean plus 2SD was used to differentiate the positive percentage of antibody response to recombinant protein. As shown in Table 5.13, although antibody response could not be demonstrated by MAT assay, approximately 40-60% of those sera could react to three constructed recombinant protein at the positive OD level. The samples with positive reactivity to recombinant protein were also correlated with Dipstick assay. Lastly the IgG reactivity to the recombinant protein was not maturely raised at that early collection time point.

Table 5.13 The percentage of positive cases in ELISA based IgG against each recombinant protein

(a) Cut off OD of anti LipL32 ≥ 0.75

group by disease	total	Cases ≥ 0.75	Percentage (%)
MAT negative	154	66	43
Dipstick positive	16	5	31
Leptospirosis culture positive	39	2	5

(b) Cut off OD of anti LipL41 ≥ 0.67

group by disease	total	Cases ≥ 0.67	Percentage (%)
MAT negative	154	68	44
Dip stick positive	16	6	38
Leptospirosis culture positive	39	1	3

(c) Cut off OD of anti Loa22 ≥ 0.62

group by disease	total	Cases ≥ 0.62	Percentage (%)
MAT negative	154	90	58
Dipstick positive	16	7	44
Leptospirosis culture positive	39	5	13

CHAPTER VI
DISCUSSION

6.1 Strategy for leptospirosis diagnosis

People who contract leptospirosis, either directly through contact with infected animals or indirectly from the contaminated environment, often develop kidney disease, liver failure or pulmonary hemorrhage (Sitprija *et al.*, 2003). The clinical presentation of leptospirosis in humans is variable, and can range from a mild flu-like illness to a severe disease with pulmonary hemorrhage, renal failure, and occasionally death (Levett *et al.*, 2001). Consequently, leptospirosis is easily mistaken for other febrile illnesses including influenza, dengue fever, meningitis, or hepatitis. Current vaccines against leptospirosis target the lipopolysaccharide coat of leptospires, which is highly variable for the 200 serovars identified, thus limiting cross-protection (Levett, 2001). Therefore, rapid and appropriate laboratory diagnostic tests are needed to aid clinical case identification and to facilitate the implementation of rapid outbreak investigations for optimal treatment and patient management. Laboratory confirmation of human leptospirosis relies mainly on serological assays aimed at the detection of specific antibodies in serum samples. The microscopic agglutination test (MAT) is considered the standard serologic test that is specific and provides useful epidemiologic data in the form of presumptive serogroup (Cumberland *et al.*, 1999). However, this assay is not suitable for routine laboratories since it is technically demanding, costly, and requires the maintenance of live, hazardous stock serovar cultures and also requires analyses of paired sera to verify the seroconversion which delays the diagnosis (Faine *et al.*, 1999). Ideally, a diagnostic test should be easy to perform, rapid and using only a single specimen (da Silva *et al.*, 1997). Some potentially useful screening tests for use in all routine laboratories have been proposed. Among these serologic approaches, enzyme-linked immunosorbent assay (ELISA) for both IgG- and IgM-leptospiral antibodies have been developed (Adler *et*

al., 1980); and several commercial test kits namely, Leptospirosis IHA, Leptospira MC Test, LEPTO Dipstick, Smart Q™ *Leptospira* test and *Leptospira* IgM ELISA test. are available (Smits *et al.*, 2000).

Conventional laboratory diagnosis usually depends on bacterial isolation and serological techniques like microscopic agglutination test (MAT), ELISA and dipstick assay (Plank and Dean, 2000). Culturing confirmation of leptospire is laborious and does not provide a rapid diagnosis. Isolation of leptospires from human blood is possible in the acute phase of the disease that lasts for up to about 10 days. Culture in EMJH (Ellinghausen McCullough Johnson and Harris) medium may take up to 2 months and does not provide an emergency diagnosis. Serological techniques are mainly used; however, antibodies are undetectable before 8–10 days after disease onset.

The prominent antigen expressed on their cell surface is lipopolysaccharides (LPS) and outer membrane proteins (OMPs). The immense serovar diversity among pathogenic leptospires has been attributed to differences in the structure and composition of LPS (Bulach *et al.*, 2000). Preparations of leptospiral LPS can elicit protective immunity, but this is generally serovar specific. It has been shown that combinations of recombinant leptospiral OMPs (Branger *et al.*, 2001; Brown, 1991) or LPS free outer membrane preparations can also elicit protective immunity in laboratory animals. Utilizing a small number of different serovars, OMPs have been shown to provide heterologous protection. Given the inherent difficulty in preparing multivalent LPS vaccines, the identification of conserved protein antigens for use in vaccination and diagnosis of leptospirosis is of critical importance (Sonrier *et al.*, 2000). In addition, the characterization of leptospiral components contributing to pathogenesis would aid in the development of improved diagnostic strategies.

LigA and LigB, encoded by separate genes, are members of the family of bacterial proteins containing immunoglobulinlike repeats that have been identified in adhesins relevant to microbial pathogenesis, such as in *Escherichia coli* and invasin in *Yersinia pseudotuberculosis*. The Lig proteins are expressed only on the surfaces of

leptospiral pathogens isolated from infected animals and not by saprophytic *Leptospira* species (Matsunaga *et al.*, 2003).

The diagnosis of human leptospirosis continues to be a serious medical and public health problem in Thailand. Thailand is the endemic area of the disease especially in the northeast of the country where most of the people are farmers. Since 1996, the reported cases increased markedly. In 2000, 14,285 leptospirosis cases were reported and 10,217 cases in 2001 with 362 deaths and 171 deaths respectively (Tangkanakul *et al.*, 2001). Numerous serovars of leptospires were claimed as the cause of infection even in the same geographical area. It is frequently under diagnosed, because of several factors including the nonspecific symptoms early in the disease, the inappropriate sample collection, the unavailability of testing facilities, and the difficulty of performing both culture and the reference serological test – MAT (Tansuphasiri *et al.*, 2005). Serodiagnosis of human leptospirosis by an IgM-specific ELISA assay is often used as an alternative to MAT in routine diagnostic laboratories. The MAT detects both IgG and IgM antibodies but the MAT titers are usually low during the acute stage of the disease and, hence, diagnosis based on a single serum sample is difficult (Romero *et al.*, 1998).

Some spirochaetal outer membrane Lipoproteins have been characterized by a structure called lipobox (Haake, 2000). Preparations of leptospiral LPS can elicit protective immunity, but this is generally serovar specific (Faine *et al.*, 1999). Recent work has shown that combinations of recombinant leptospiral outer membrane proteins (OMPs) (Branger *et al.*, 2001; Haake *et al.*, 1999) or LPS free outer membrane preparations can also elicit protective immunity in laboratory animals. Utilizing a small number of different serovars, outer membrane preparations have been shown to provide heterologous protection (Sonrier *et al.*, 2000). Given the inherent difficulty in preparing multivalent LPS vaccines, the identification of conserved protein antigens for use in vaccination and diagnosis of leptospirosis is of critical importance.

Freeze fracture electron microscopy studies have shown that *Leptospira* spp. have approximately one-tenth the content of transmembrane OMPs as comparison

with *Escherichia coli*, despite their similar genetic capacities (Haake, 1991). OmpL1 is the only leptospiral transmembrane OMP which has been identified to date and is thought to be a heat-modifiable porin which is presented in the outer membrane in small amounts (Haake *et al.*, 1993; Shang *et al.*, 1995). In contrast, the leptospiral outer membrane contains relatively abundant amounts of lipoproteins, which associate with the outer membrane via N-terminal fatty acids. The major protein of the leptospiral outer membrane is a 32-kDa lipoprotein designated LipL32 (Haake *et al.*, 2000). In a recent evaluation of recombinant leptospiral protein antigens by enzyme-linked immunosorbent assay, LipL32 was found to be the immunodominant antigen (Flannery *et al.*, 2001). A less-abundant leptospiral OMP designated LipL36 was found to be downregulated in host-adapted leptospires, suggesting that it is not involved in pathogenesis after entry into the mammalian host (Barnett *et al.*, 1999). Another highly conserved leptospiral lipoprotein, LipL41 (Shang *et al.*, 1996), can provide 71% protection in the hamster model of leptospirosis when administered in combination with recombinant OmpL1 (Haake *et al.*, 1999). A model of leptospiral membrane architecture has begun to be assembled (Zuerner *et al.*, 2000), but preliminary investigations utilizing one-dimensional electrophoresis indicate that there are at least 16 protein constituents of the leptospiral outer membrane (Haake, 1991), suggesting that this model is far from complete. The immunodominant role of these outer membrane protein of *Leptospira*, lead to their application for serodiagnostic antigen.

6.2 Selection of three recombinant leptospiral lipoproteins for study

To develop a diagnostic test, the ideal antigen should be immunogenic in nature and conserve among diverse pathogenic leptospiral strains. Major focus of the present study was the production of recombinant leptospiral OMPs and to determine how best they could be exploited in the field of serodiagnosis. The leptospiral antigens that express during infection have potentially important implications in the development of new serodiagnostic and immunoprotective strategies (Natarajaseenivasan *et al.*, 2004). Recombinant protein-based ELISA is a suitable and safe procedure for the examination of a large number of sera as it involves an

immunodominant antigen and lacks non-specific moieties presented in whole-cell preparations.

LipL32 is highly conserved in pathogenic *Leptospira* across the globe, as evidenced by the LipL32 sequences submitted from various parts of the world, further highlighting the effectiveness of the protein for use as an antigen in ELISA, Haake *et al.* and Tahiliani *et al.* (2005) reported to the use of purified rLipL32 for developing the specific IgG ELISA for human serum samples. The major protein of the leptospiral outer membrane is a 32-kDa lipoprotein designated as LipL32 (Haake and Matsunaga, 2005). Flannery *et al.* (2001) also evaluated of recombinant leptospiral protein antigens by enzyme-linked immunosorbent assay based on LipL32. Shang *et al.* (1996) reported that LipL41 is one of the important and surface exposed leptospiral outer membrane protein expressed by pathogenic *Leptospira* species only. The recent completion of the genome sequences of pathogenic *Leptospira* strains has led to the identification of putative determinants that may play a role in virulence (Bulach *et al.*, 2006; Nascimento *et al.*, 2004; Ren *et al.*, 2003). One such determinant, Loa22, is up-regulated during host infection (Nally *et al.*, 2007) and encodes a lipoprotein with an OmpA domain (Koizumi and Watanabe, 2003) that is strongly recognized by sera from human leptospirosis patients (Gamberini *et al.*, 2005). Furthermore, Loa22 is conserved among pathogenic *Leptospira* (Gamberini *et al.*, 2005; Koizumi and Watanabe, 2003; Yang *et al.*, 2006), suggesting that it may play a specific role in pathogenesis. However, its role has not been elucidated until now, because targeted mutagenesis was not previously feasible in pathogenic. Ristow *et al.* characterized a mutant obtained in 2007 by insertion of the transposon Himar1 into a gene encoding a putative lipoprotein, Loa22, which has a predicted OmpA domain based on sequence identity. The resulting mutant did not express Loa22 and was attenuated in virulence in the guinea pig and hamster models of leptospirosis, whereas the genetically complemented strain was restored in Loa22 expression and virulence. Loa22 was expressed during host infection and exposed on the cell surface, it is therefore necessary for virulence of *L. interrogans* in the animal model (Ristow *et al.*, 2007). In this study LipL32, LipL41 and Loa22 were selected for study their immunogenicity and evaluated as an antigen for serodiagnosis based on ELISA assay.

6.3 Expression and purification recombinant proteins processes

The recombinant Leptospiral protein was constructed by using *E. coli* expression system. The purified proteins were used as antigens in development of immuno-diagnostic test. The experimental strategy included cloning the PCR fragments of each leptospiral proteins genes into pRSET-B vector, of which had histidine fusion protein located at at N-terminal. DNA sequencing confirmed that all three genes were successfully cloned into vector in the correct orientation, with the complete open reading frame. Three leptospiral proteins were expressed in *E. coli* in form of histidine fusion proteins. The recombinant proteins can be purified using an affinity column by using 6x His-tagged as carrier peptide. The 6x His-tagged is smaller than other affinity tags, poorly immunogenic in most species and rarely interferes with protein structure or function (Crowe *et al.*, 1996).

Expression of LipL32 and Loa22 recombinant proteins in *E. coli* revealed the partially presented as solubilized form while LipL41 recombinant protein was mainly located in inclusion bodies. As foreign protein located in the inclusion bodies was increase with inactive formation which requires solubilization in denaturant (8 M urea). The refolding process was then required to obtain proteins in the native conformation.

The expressed recombinant proteins of LipL32, Loa22 and LipL41 revealed the single band at molecular weight of about 35 kDa, 27 kDa and 27 kDa, respectively. Two purified recombinant proteins, LipL32 and Loa22 possessed the additional molecular weight from the predicted molecular weight, and also the polyhistidine tagged peptide was included. The rLipL41 in this study possessed MW of 27 kDa, of which less than the expected of 41 kDa. Analysis of inserted DNA revealed one base mismatch from LipL41 database, that caused a stop codon in protein translation, so the protein product of only 24 kDa was predicted to express. The obtained 27 kDa of rLipL41 was thus related to the analysed insert DNA.

During the protein expression process, some condition might induce the overproduction of proteins in cytoplasm of *E. coli* that often accompanied by their misfolding and segregation into inclusion bodies (Baneyx, 1999). More experiments were required to find optimal condition for each expressed protein.

The recombinant fusion proteins from LipL41 clones were purified using protino® Ni-TED Resin gravity-flow column chromatography under denaturing condition. It was attempted to prepare LipL32 and Loa22 in solubilized from and subjected to Ni affinity column under native condition.

6.4 Immunogenic of recombinant proteins

All the purified recombinant proteins, LipL32, LipL41 and Loa22 were prepared to immunize mice. The ICR mice were immunized by each recombinant protein. These recombinant proteins possessed the immunogenic property since the antibody titer was raised higher than 128,000 based on ELISA assay.

Mice anti-rLipL32 reacted to its own recombinant LipL32 at molecular weight of 35 kDa, which was reactive to other leptospiral whole cell lysate at approximately 32 kDa, confirming the presense of 32 kDa outer membrane protein among *Leptospira*. No reactivity to *L. biflexa*, confirming an absence of LipL32 in *L. biflexa* (Parma et al., 1997). No reactivity to a few serogroup i.e. *L. interrogans* serovar Pomona, *L. borgpeterseni* serovar Sejroe, *L. meyeri* serovar Ranarum, *L. santarosai* serovar Shermani, and *L. weilii* serovar Wolffi implying the diversity of the Icterochaemaragiae's rLipL32 to LipL32 in other serogroup.

Due to preparation of rLipL41 in denatured from, the conformation of the rLipL41 was not similar to native protein in nature, but antibody produced against these epitopes could react with other leptospiral whole cell lysate. The immunogenic of antibody to LipL41 revealed the reactive band at molecular weight about 41 kDa among the whole cell lysate. No reactivity to *L. biflexa* and to a few serogroup i.e. *L. interrogans* serovar Pomona, *L. borgpeterseni* serovar Sejroe, *L. meyeri* serovar

Ranarum, *L. santarosai* serovar Shermani, *L. weilli* serovar Sarmin and *L. weilli* serovar Sarmin, indicating an absence of LipL41 or antigenic diversity of in those serogroup, in comparison to antigenicity of rLipL41.

The anti-rLoa22 reacted with recombinant Loa22 at molecular weight 27 kDa while anti-rLoa22 reacted with other leptospiral whole cell lysate, revealed two bands about 22 kDa and 25 kDa. No reactively to *L. biflexa*, confirming an absence of Loa22 in *L. biflexa*. No reactivity to a few serogroup i.e. *L. interrogans* serovar Pomona, *L. borgpeterseni* serovar Sejroe, *L. meyeri* serovar Ranarum and *L. santarosai* serovar Shermani.

These results above indicated that all three of recombinant proteins antigen shared epitope with Leptosiral antigen because antibody to recombinant protein can react with some *Leptospira* whole cell lysate. The recombinant protein might be good antigenic to detect specific antibody to *leptospira* in human serum.

6.5 Reactivity of recombinant proteins with human serum by Western blot assay

Each recombinant protein was used as antigen in Western blot assay and tested with leptospirosis patient sera and normal human sera. The recombinant LipL32 protein (37 kDa) could react with all sera of the MAT positive cases of leptospirosis and normal human sera. The normal volunteers might have been also exposed to leptospires. For the recombinant LipL41 and Loa22 were no immunoreactivity with MAT positive sera and normal sera determined by Western blot assay, suggesting that the denatured antigens, prepared during Western blot procedure might be modified and would not be recognized by antibody. ELISA required more preserved from of antigen, was alternative assay to evaluate an efficiency of each antigen.

6.6 Optimized condition of ELISA method

The more sensitive ELISA method was selected to evaluate these three recombinant proteins for use as diagnostic antigen. ELISA assay was appropriated

when the purified recombinant protein was used, as only small quantity was required, in addition, mass number of subjects could be performed, and results were come out in 1 day process, but allowed the Ag to be coated overnight. Another benefit of ELISA assay was its OD value report that was sensitive to use for differential diagnosis, although the hard step was setting up the accurate cut off value.

The optimized condition of ELISA was determined using the MAT positive serum as true positive and dengue patient serum as true negative. The concentration of coating antigen was quite low as 30 ng/ml for all three recombinant proteins, that was economized to use. The serum dilution of each subject was chosen as 1:1000, as the OD value was optimum in the range of 0.5 – 1.0, while the OD was too low, when sera dilution of 1:2000 was used. Our ELISA detected both IgM and IgG class. The total Ig-HRP conjugate was utilized that could be represented detection of IgG class, as the total Ig prepared from serum that more that 80% was IgG class.

6.7 Community base line of antibody response to recombinant protein

The MAT assay was considered as standard serologic test, of which its main target antigen was LPS coat of leptospires, thus the antigen panel required inclusion of many Leptospiral serogroups, in order to cover the individual immunoreactivity that could be presented. In contrast our ELISA assay detected antibody against outer membrane protein antigen. The evaluation of new ELISA assay was performed in comparative to standard MAT assay. The patient's sera with positive in MAT assay were included in comparative to sera with negative in MAT. As the ideal test was aimed to differentiate normal from infected individuals, and patients infected with other febrile illness, so normal person lived in both endemic and non-endemic area, in addition to patients of dengue fever, scrub typhus and melioidosis were included. It was noted that antibody to membrane protein was revealed in comparison to antibody to LPS among cases in community.

6.8 Application of IgG - ELISA assay

The investigation of anti- rLipL32, anti- rLipL41 and anti- rLoa22 were performed in various studied group, with focusing on the OD derived from the fixed titre at 1:1000 of total Ig class. In order to differentiate Leptospirosis patients from normal or patients of other febrile illness, mean of OD value was then calculated from OD value from all control groups, of which their MAT results were negative. The cut off value was then assigned from mean plus 1 SD (cut off 1) and mean plus 2 SD (cut off 2), and positives cases possessed OD higher than cut off value were counted.

Among 3 recombinant proteins, anti- LipL32 gave the lowest background of IgG response among normal individuals. Based on cut off 2, the positive cases among HE group identified by anti-LipL32 was 6%, while anti LipL41 and anti Loa22 was high as 29 and 26% respectively. Antibody response to leptospiral recombinant antigen in HE group was usually higher than HOE group, probably due to the more exposure to Leptospiral antigen among normal individual living in the field. Among febrile illness patients, i.e. dengue, scrub typhus, the less number of positive cases were identified based on anti- LipL32, while no positive case was detect among melloidosis patients. A few patients among scrub typhus and dengue patients who gave strong positive in ELISA assay might indicate the co-infection, that have been reported sometimes, although no reactivity in MAT could be displayed. In this study anti-LipL32 was then likely to be more specific to identify Leptospirosis.

6.9 Application of IgM - ELISA assay

The IgG response was not able to be detected during acute phase sera, thus their IgM response at this point that was mostly higher than IgG, should be apply in diagnosis. The based line of IgM response of individual recombinant antigen was determined among control group, i.e. normal human and patients of other febrile illness. Mean OD of IgM to anti LipL32, LipL41 and Loa22 were 0.51, 0.42 and 0.47 respectively, and the cut off 1 OD value was derived from mean plus 1SD. As shown in Table 5.10, 5.11, 5.12, a few more cases were identified as positive based on IgM

response. Among 39 cases of acute sera when focused in anti-LipL32, positive IgG class was revealed in 2 cases, with the additional 7 cases were positive in IgM class. The high OD of IgM response should also be regarded as positive for Leptospirosis infection. Among each 30 patients of scrub typhus, dengue and melliodosis, a few more positive cases of IgM were identified, that probably regarded as positive Leptospirosis coinfection. The high numbers of IgM positive cases were observed in normal human of endemic area, indicating recent exposure to leptospiral antigen, and protective antibody among this group, so they maintained healthy. For the accurate diagnosis, the justification of OD value should be accompanied with individual's health condition, whether they expressed any illness or not. Generally, OD value of IgG response among normal healthy were low, thus indicated as negative.

In some suspected patients, although their MAT results were negative, some were positive by IgM Dip stick (Organon, Brussels). Among the 16 cases positive in IgM Dip stick, 5 of them (31%) had positive anti- rLipL32. In addition, among 154 cases with MAT negative, approximately 40% of them had immunoreactivity to recombinant protein, suggesting that MAT was not the only reliable diagnosis as about 40% might be the misdiagnostic cases. MAT assay probably not the good gold standard due to the different cut-off titres ranging from 1 in 100 to 1 in 800 were employed for diagnosis. Although, the cut-off titers as low as 1:100 was employed as positive cases in Thailand, more than 40% of MAT negative possessed IgG response to recombinant antigen. The ELISA screening on the anti-LipL32 based on both IgG and IgM may perform as alternative Leptospirosis diagnosis, to avoid misdiagnostic cases, as this procedure was easy to perform with the available recombinant protein.

Lastly, based on the MAT as the serological standard assay for Leptospirosis, relative sensitivity and specificity of IgG response of all three recombinant protein based on ELISA was determined, anti LipL32 revealed an efficacy of 84%, in comparison to 74% and 75% in LipL41 and Loa22 respectively. Detection of IgG antibodies by ELISA is more sensitive than the MAT and gives a positive result earlier in the acute phase of the disease. It is easier to perform and can easily accommodate a large number of samples and gives a less subjective result than MAT (Winslow,

1997). The fewer efficacies in comparative to MAT might due to immunoreactivity to the different type of target antigen: as MAT was detect antibody reactivity to LPS, while ELISA detect antibody reactivity to conserved outer membrane protein, however the efficacy to confirm as Leptospirosis infection was reasonable high as 84% as comparative to MAT.

CHAPTER VII
CONCLUSION

Leptospirosis, a zoonotic disease of global importance, occurs in urban environments of industrialized and developing countries, as well as in rural regions worldwide. Mortality remains significant, generally stemming from delays in diagnosis due to lack of infrastructure and inadequate clinical suspicion, or lack of treatment facilities in developing countries, and may also result from inherent pathogenicity of some leptospiral strains or genetically determined host immunopathological responses (Bharti *et al.*, 2003). Although whole Leptospira antigen can be used in serodiagnostic tests to detect either IgM or IgG antibodies (Adler *et al.*, 1980), extensive quality control measures are necessary to monitor batch-to-batch variability in antigen composition inherent in growing large cultures of *Leptospira* (Faine *et al.*, 1999). Recombinant-antigen-based assays may circumvent this problem and furthermore may be produced at lower cost, an important consideration for implementation in developing countries. Detection of IgM antibodies by ELISA is more sensitive than the MAT and gives a positive result earlier in the acute phase of the disease. It is easier to perform and can easily accommodate a large number of samples and gives a less subjective result than MAT (Winslow, 1997). ELISA was thus selected as protocol for diagnosis based on their sensitivity and requirement of less amount of proteint antigen, as low as 30 ng/ml in this study. Optimization of ELISA assay was performed, and the following standard ELISA test was employed in a single diluted serum of 1:1000, with the optimized Ag, and conjugated Antibody concentration.

The recombinant protein was constructed based on cloning the PCR fragments encoded for each leptospiral protein into pRSET-B vector, of which had histidine fusion protein located at at N-terminal. DNA sequencing confirmed that all three genes were successfully cloned into vector in the correct orientation, with the

complete open reading frame. The three Leptospiral recombinant proteins were successfully produced, and were demonstrated to share antigenicity with native Leptospiral protein.

The rLipL32 was demonstrated to be the most specific for diagnosis, as sera from melioidosis patients had no reactivity, although few dengue and scrub typhus could react. A few patients among scrub typhus and dengue patients who gave strong positive in ELISA assay might indicate the co-infection, that have been reported sometimes, although no reactivity in MAT could be displayed. In addition, rLipL32 seem to be more sensitive than MAT, and could detect more Leptospirosis cases (31%) among cases that were positive in IgM dipstick In addition, among 154 cases with MAT negative, approximately 40% of them had immunoreactivity to recombinant protein, suggesting that MAT was not the only reliable diagnosis as about 40% might be the misdiagnostic cases. MAT assay probably not the good gold standard due to the different cut-off titres ranging from 1 in 100 to 1 in 800 were employed for diagnosis. Although, the cut-off titers as low as 1:100 was employed as positive cases in Thailand, more than 40% of MAT negative possessed IgG response to recombinant antigen. The ELISA screening on the anti-LipL32 based on both IgG and IgM may perform as alternative Leptospirosis diagnosis, to avoid misdiagnostic cases, as this procedure was easy to perform with the available recombinant protein.

The high numbers of IgM positive cases were observed in normal human of endemic area, indicating recent exposure to leptospiral antigen, and protective antibody presented among this group, so they maintained healthy. For the accurate diagnosis, the justification of OD value should be accompanied with individual's health condition, whether they expressed any illness or not. Generally, OD value of IgG response among normal healthy were low, thus indicated as negative for infection. Our research demonstrated the ELISA assay as alternative serodiagnostic of Leptospirosis, it was not perfect, but possessed a few benefits over conventional MAT assay.

BIBLIOGRAPHY

Ohio Department of Health Disease Fact Sheet.Available from/URL: www.odh.ohio.gov/pdf/IDCM/lepto.pdf [accessed 2007 Dec, 1]

Abdulkader RC. Acute renal failure in leptospirosis. Ren Fail 1997; 19: 191-8.

Adler H, Vonstein S, Deplazes P, *et al*. Prevalence of *Leptospira* spp. in various species of small mammals caught in an inner-city area in Switzerland. Epidemiol Infect 2002; 128: 107-9.

Adler B, Murphy AM, Locarnini SA, Faine S. Detection of specific anti-leptospiral immunoglobulins M and G in human serum by solid-phase enzyme-linked immunosorbent assay. J Clin Microbiol 1980; 11: 452-7.

Ahmad SN, Shah S, Ahmad FM. Laboratory diagnosis of leptospirosis. J Postgrad Med 2005; 51: 195-200.

Alt DP, Zuerner RL, Bolin CA. Evaluation of antibiotics for treatment of cattle infected with *Leptospira borgpetersenii* serovar Hardjo.J Am Vet Med Assoc 2001; 219: 636-9.

Appassakij H, Silpapojakul K, Wansit R, *et al*. Evaluation of the immunofluorescent antibody test for the diagnosis of human leptospirosis. Am J Trop Med Hyg 1995; 52: 340-3.

Arimitsu Y, Kobayashi S, Akama K, Matuhasi T. Development of a simple serological method for diagnosing leptospirosis: a microcapsule agglutination test. J Clin Microbiol 1982; 15: 835-41.

Arimitsu Y, Kmety E. Evaluation of the one-point microcapsule agglutination test for diagnosis of leptospirosis. Bull World Health Organ 1994; 72: 395-9.

Baker LA, Cox CD. Quantitative assay for genus-specific leptospiral antigen and antibody. Appl Microbiol 1973; 25: 697-8.

Ballard S, Segers P, Bleumink-Pluym, *et al*. Molecular analysis of the hsp(groE) operon of *Leptospira interrogans* serova Copenhageni. Mol Microbiol 1993; 8: 739-51.

Barbosa AS, Abreu PAE, Neves FO, *et al*. A newly identified leptospiral adhesin mediates attachment to laminin. Infect Immun 2006; 74: 6356-64.

Baneyx F. Recombinant protein expression in *Escherichia coli*. Curr Opin Biotechnol 1999; 10: 411-21.

Barnett JK, Barnett D, Bolin CA *et al*. Expression and distribution of leptospiral outer membrane components during renal infection of hamsters. Infect Immun 1999; 67: 853-61.

Barocchi MA, Ko M, Ferrer SR, *et al*. Identification of new repetitive element in *Leptospira interrogans* serovar Copenhageni and its application to PCR-based differentiation of *Leptospira* serogroups. J Clin Microbiol 2001; .39: 191-5.

Barocchi MA, Albert IK, Mcdonald KL, Piley LW. Rapid translocation of polarized MDCK cell monolayers by *Leptospira interrogans*, an invasive but nonintracellular pathogen. Infect Immun 2002; 70: 6926-32.

Bharti AR, Nally JE, Ricaldi JN, *et al*. Leptospirosis: a zoonotic disease of global importance. Lancet Infect Dis 2003; 3: 757-71.

Bomfim MRQ, Ko A, Koury MC. Evaluation of the recombinant LipL32 in enzyme-linked immunosorbent assay for the serodiagnosis of bovine leptospirosis. Vet Microbiol 2005; 109: 89-94.

Brandao AP, Camargo ED, da Silva ED, *et al*. Macroscopic agglutination test for rapid diagnosis of human leptospirosis. J Clin Microbiol 1998; 36: 3138-42.

Branger C, Sonrier C, Chatrenet B, *et al*. Identification of the hemolysis-associated protein 1 as a cross-protective immunogen of *Leptospira interrogans* by adenovirus-mediated vaccination. Infect Immun 2001; 69: 6831-8.

Brenner DJ, Kaufmann AF, Sulzer KR. Further determination of DNA relatedness between serogroups and serovars in the family Leptospiraceae with a proposal for *Leptospira alexanderi* sp. and four new *Leptospira* genomospecies. Int J Syst Bacteriol 1999; 49 Pt 2: 839-58.

Brown JA, LeFebvre RB, Pan MJ. Protein and antigen profiles of prevalent serovars of *Leptospira interrogans*. Infect Immun 1991; 59: 1772-7.

Bulach DM, Kalambaheti T, de la Pena-Moctezuma A, Adler B. Functional analysis of genes in the rfb locus of *Leptospira borgpetersenii* serovar Hardjo subtype Hardjobovis. Infect Immun 2000; 68: 3793-8.

Bulach DM, Zuerner RL, Wilson P *et al.* Genome reduction in *Leptospira borgpetersenii* reflects limited transmission potential. PNAS 2006; 103: 14560-5.

Cacciapuoti B. The diagnosis of leptospirosis in the hospital microbiology laboratory. Boll Ist Sieroter Milan 1984; 63: 237-43.

Campagnolo ER, Warwick MC, Marx HL, *et al*. Analysis of the 1998 outbreak of leptospirosis in Missouri in humans exposed to infected swine. J Am Vet Med Assoc 2000; 216: 676-82.

Centers for Disease Control and Prevention (CDC). Update: outbreak of acute febrile illness among athletes participating in Eco-Challenge-Sabah 2000--Borneo, Malaysia, 2000. MMWR Morb Mortal Wkly Rep 2001; 50: 21-4.

Chang SL. Studies on *Leptospira Icterohaemorrhagiae*: a critical study of the effect of penicillin on *Leptospira Icterohaemorrhagiae in vitro* and in Leptospirosis in guinea pigs. J Clin Invest 1946; 25: 752-60.

Chattopadhyay PK, Wu HC. Biosynthesis of the covalently linked diglyceride in murein lipoprotein of *Escherichia coli*. Proc Natl Acad Sci USA 1977; 74: 5318-22.

Champagne MJ, Higgins R, Fairbrother JM, *et al*. Detection and characterization of leptospiral antigens using a biotin/avidin double-antibody sandwich enzyme-linked immunosorbent assay and immunoblot. Can J Vet Res (1991); 55: 239-45.

Choy HA, Kelley MM, Chen TL, Moller AK, *et al*. Physiological osmotic induction of *Leptospira interrogans* adhesion: LigA and LigB bind extracellular matrix proteins and fibrinogen. Infect Immun 2007; 75: 2441-50.

Clerke AM, Leuva AC, Joshi C, Trivedi SV. Clinical profile of leptospirosis in South gujarat. J Postgrad Med 2002; 48: 117-8.

Cole JR, Sulzer CR, Pursell AR. Improved microtechnique for the leptospiral microscopic agglutination test. Appl Environ Microbiol 1973; 25 976-80.

Coursin DB, Updike SJ, Maki DG. Massive rhabdomyolysis and multiple organ dysfunction syndrome caused by leptospirosis. Intensive Care Med 2000; 26: 808-12.

Cox CD, Stover RC, Treick RW. Serological studies on hemolytic antigen from *Leptospira*. Proc Soc Exp Biol Med 1958; 98: 265-9.

Crowe J, Masone BS, Ribbe J. One-step purification of recombinant proteins with the 6xHis tag and Ni-NTA resin. Methods Mol Biol 1996; 58: 491-510.

Cullen PA, Cordwell SJ, Bulach DM, *et al*. Global analysis of outer membrane proteins from *Leptospira interrogans* serovar Lai. Infect Immun 2002; 70: 2311-8.

Cullen PA, Haake DA, Bulach DM, *et al*. LipL21 is a novel surface-exposed lipoprotein of pathogenic *Leptospira* species. Infect Immun 2003; 71: 2414-21.

Cullen PA, Haake DA, Adler B. Outer membrane proteins of pathogenic spirochetes. FEMS Microbiol Rev 2004; 28: 291-318.

Cullen PA, Xu X, Matsunaga J, *et al*. Surfaceome of *Leptospira* spp. Infect Immun 2005; 73: 4853-63.

Cumberland P, Everard CO, Levett PN. Assessment of the efficacy of an IgM-ELISA and microscopic agglutination test (MAT) in the diagnosis of acute leptospirosis. Am J Trop Med Hyg 1999; 61: 731-4.

da Silva MV, Nakamura PM, Camargo ED, *et al*. Immunodiagnosis of human leptospirosis by dot-ELISA for the detection of IgM, IgG, and IgA antibodies. Am J Trop Med Hyg 1997; 56: 650-5.

de Brito T, Bohm GM, Yasuda PH. Vascular damage in acute experimental leptospirosis of the guinea-pig. J Pathol 1979; 128: 177-82.

de la Pena-Moctezuma A, Bulach DM, Adler B. Genetic differences among the LPS biosynthetic loci of serovars of *Leptospira interrogans* and *Leptospira borgpetersenii*. FEMS Immunol Med Mic 2001; 31: 73-81.

Dey S, Mohan CM, Ramadass P, Nachimuthu K. Diagnosis of leptospirosis by recombinant antigen based single serum dilution ELISA. Indian J Med Res 2008; 128: 172-7.

Dev IK, Ray PH. Rapid assay and purification of a unique signal peptidase that processes the prolipoprotein from *Escherichia coli*. J Biol Chem 1984; 259: 11114-20.

Effler PV, Domen HY, Bragg SL, Aye T, Sasaki DM. Evaluation of the indirect hemagglutination assay for diagnosis of acute leptospirosis in Hawaii. J Clin Microbiol 2000; 38: 1081-4.

Effler PV, Bogard AK, Domen HY, *et al*. Evaluation of eight rapid screening tests for acute leptospirosis in Hawaii. J Clin Microbiol 2002; 40: 1464-9.

Ellinghausen H, McCullough WG. Nutrition of *Leptospira pomona* and growth of 13 other serotypes: fragtionation of oleic albumin complex and a medium of bovine albumin and polysorbate 80. Am J Vet Res 1965; 26: 45-51.

Ellis WA, Bryson DG, O'Brien JJ, Neill SD. Leptospiral infection in aborted equine foetuses. Equine Vet J 1983; 15: 321-4.

Erdinc FS, Koruk ST, Hatipoglu CA, Kinikli S, Demiroz AP. Three cases of anicteric leptospirosis from Turkey: mild to severe complications. J Infect 2006; 52: e45-8.

Faine S. *Leptospira* and Leptospirosis. Melbourne: CRC Press; 1994a. p. 55-73.

Faine S. Metabolism, Nutrition and Cultivation (Chapter 5). *Leptospira* and Leptospirosis. Melbourne: CRC Press; 1994b: 77-90.

Faine S. Taxonomy, Classification and Nomenclature (Chapter 8). *Leptospira* and Leptospirosis. Melbourne: CRC Press; 1994c. p. 117-44.

Faine S. Pathogenesis, Virulence and Immunity (Chapter 10). *Leptospira* and Leptospirosis. Melbourne: CRC Press; 1994d.: 154-76.

Faine S, Adler B, Bolin C, perolat P. Clinical leptospirosis in humans (Chapter 12). Melbourne, Australia: Medsci; 1999a.: 17-28

Faine S, Adler B, Bolin C, Perolat P. *Leptospira* and Leptospirosis. Melbourne, Australia: MediSci; 1999b. p. 73-91

Flannery B, Costa D, Carvalho FP, *et al*. Evaluation of recombinant *Leptospira* antigen-based enzyme-linked immunosorbent assays for the serodiagnosis of leptospirosis. J Clin Microbiol 2001; 39: 3303-10.

Galton MM, Sulzer CR, Santarosa CA, Fields MJ. Application of a microtechnique to the agglutination test for leptospiral antibodies. Appl Microbiol 1965; 13: 81-5.

Gamberini M, Gomez RM, Atzingen MV, *et al*. Whole-genome analysis of *Leptospira interrogans* to identify potential vaccine candidates against leptospirosis. FEMS Microbiol Lett 2005; 244: 305-13.

Gardy JL, Laird MR, Chen F *et al*. PSORTb v.2.0: expanded prediction of bacterial protein subcellular localization and insights gained from comparative proteome analysis. Bioinformatics 2005; 21: 617-23.

Gavin PJ, Thomson Jr. RB, Donnersberger DR, *et al*. Detection of leptospiral DNA by real-time polymerase chain reaction in acute Weil syndrome. Infect Immun 2005; 13: 187-9.

Gerritsen MJ, Olyhoek T, Smits MA, Bokhout BA. Sample preparation method for polymerase chain reaction-based semiquantitative detection of *Leptospira interrogans* serovar Hardjo subtype hardjobovis in bovine urine. J Clin Microbiol 1991; 29: 2805-8.

Gravekamp C, Van de Kemp H, Franzen M, *et al*. Detection of seven species of pathogenic leptospires by PCR using two sets of primers. J Gen Microbiol 1993; 139: 1691-700.

Gregory SG, Howell GR, Bentley DR. Genome mapping by fluorescent fingerprinting. Genome Res 1997; 7: 1162-8.

Guerreiro H, Croda J, Flannery B, *et al*. Leptospiral proteins recognized during the humoral immune response to leptospirosis in humans. Infect Immun 2001; 69: 4958-68.

Gupta SD, Wu HC. Identification and subcellular localization of apolipoprotein N-acyltransferase in *Escherichia coli*. FEMS Microbiol Lett 1991; 62: 37-41.

Gussenhoven G, van der Hoorn M, Goris M, *et al*. LEPTO dipstick, a dipstick assay for detection of *Leptospira*-specific immunoglobulin M antibodies in human sera. J Clin Microbiol 1997; 35: 92-7.

Haake DA, Champion CI, Martinich C, *et al*. Molecular cloning and sequence analysis of the gene encoding OmpL1, a transmembrane outer membrane protein of pathogenic *Leptospira* spp. J Bacteriol 1993; 175: 4225-34.

Haake DA, Martinich C, Summers TA, *et al*. Characterisation of leptospiral outermembrane lipoprotein LipL36: Downregulation associated with late-log phase growth and mammalian infection. Infect Immun 1998; 66: 1579-87.

Haake DA, Mazel MK, McCoy AM, *et al*. Leptospiral outer membrane proteins OmpL1 and LipL41 exhibit synergistic immunoprotection. Infect Immun 1999; 67: 6572-82.

Haake DA, Chao G, Zuerner RL, *et al*. The leptospiral major outer membrane protein LipL32 is a lipoprotein expressed during mammalian infection. Infect Immun 2000; 68: 2276-85.

Haake DA, Matsunaga J. Characterization of the leptospiral outer membrane and description of three novel leptospiral membrane proteins. Infect Immun 2002; 70: 4936-45.

Haake DA, Martinich C, Summers TA, *et al*. Characterization of leptospiral outer membrane lipoprotein LipL36: downregulation associated with late-log-phase growth and mammalian infection. Infect Immun 1998; 66: 1579-87.

Haake DA, Matsunaga J. Leptospiral membrane proteins--variations on a theme. Indian J Med Res 2005; 121: 143-5.

Haake DA, Walker EM, Blanco DR, Bolin *et al*. Changes in the surface of *Leptospira interrogans* serovar Grippotyphosa during in vitro cultivation. Infect Immun 1991; 59: 1131-40.

Hamburger ZA, Brown MS, Isberg RR, Bjorkman PJ. Crystal structure of invasin: a bacterial integrin-binding protein. Science 1999; 286: 291-5.

Hantke K, Braun V. The structure of covalent binding of lipid to protein in the murein-lipoprotein of the outer membrane of *Escherichia coli*. Hoppe Seylers Z Physiol Chem 1973a; 354: 813-5.

Hantke K, Braun V. Covalent binding of lipid to protein. Diglyceride and amide-linked fatty acid at the N-terminal end of the murein-lipoprotein of the *Escherichia coli* outer membrane. Eur J Biochem 1973b; 34: 284-96.

Hartman E and Van Houten *et al*. Serodiagnosis of canine leptospirosis by solid-phase enzyme-linked immunosorbent assay. Vet Immunol Immunopathol (1984); 7: 33-42.

He P, Sheng YY, Shi YZ, *et al*. Genetic diversity among major endemic strains of *Leptospira interrogans* in China. BMC Genomics 2007; 8: 204.

Hellstrom JS, Marshall RB. Survival of *Leptospira interrogans* serovar Pomona in an acidic soil under simulated New Zealand field conditions. Res Vet Sci 1978; 25: 29-33.

Hsieh WJ, Chang YF, Chen Cs, Pan MJ. Omp52 is a growth-phase-regulated outer membrane protein of *Leptospira santarosai* serovar Shermani. FEMS Microbiol Lett 2005; 243: 339-45.

Hussain M, Ichihara S, Mizushima S. Accumulation of glyceride-containing precursor of the outer membrane lipoprotein in the cytoplasmic membrane of *Escherichia coli* treated with globomycin. J Biol Chem 1980; 255: 3707-12.

Imamura S, Matsui H, Ashizawa Y. Studies on indirect hemagglutination test for leptospirosis. Jpn J Exp Med 1972; 42: 563-8.

Inouye S, Wang S, Sekizawa J, *et al*. Amino acid sequence for the peptide extension on the prolipoprotein of the *Escherichia coli* outer membrane. Proc Natl Acad Sci USA 1977; 74: 1004-8.

Inukai M, Takeuchi M, Shimizu K, Arai M. Mechanism of action of globomycin. J Antibiot (Tokyo) 1978; 31: 1203-5.

Johnson RC, Harris VG. Differentiation of pathogenic and saprophytic letospires growth at low temperatures. J Bacteriol 1967; 94: 27-31.

Jouglard SD, Simionatto S, Seixas FK, *et al*. Nested polymerase chain reaction for detection of pathogenic leptospires. J Microbiol 2006; 52: 747-52.

Juncker AS, Willenbrock H, Von Heijne G, *et al*. Prediction of lipoprotein signal peptides in Gram-negative bacteria. Protein Sci 2003; 12: 1652-62.

Kariv R, Klempfner R, Barnea A, *et al*. The changing epidemiology of leptospirosis in Israel. Emerg Infect Dis 2001; 7: 990-2.

Kaur IR, Sachdeva R, Arora V, Talwar V. Preliminary survey of leptospirosis amongst febrile patients from urban slums of East Delhi. J Assoc Physicians India 2003; 51: 249-51.

Kee SH, Kim IS, Choi MS, Chang WH. Detection of leptospiral DNA by PCR. J Clin Microbiol 1994; 32: 1035-9.

Kiktenko VS, Balashov NG, Rodina VN. Leptospirosis infection through insemination of animals. J Hyg Epidemiol Microbiol Immunol 1976; 21: 207-13.

Ko AI, Galvao RM, Dourado RC, *et al*. Urban epidemic of severe leptospirosis in Brazil. Salvador Leptospirosis Study Group. Lancet 1999; 354: 820-5.

Koerner D, Surks MI, Oppenheimer JH. *In vitro* formation of apparent covalent complexes between L-triiodothyronine and plasma protein. J Clin Endocrinol Metab 1973; 36: 239-45.

Koizumi N, Watanabe H. Molecular cloning and characterization of a novel leptospiral lipoprotein with *OmpA* domain. FEMS Microbiology Lett 2003; 226: 215-9.

Koizumi N, Watanabe H. Leptospiral immunoglobulin-like proteins elicit protective immunity. Vaccine 2004; 22: 1545-52.

Lai SH, Philbrick WM, Wu HC. Acyl moieties in phospholipids are the precursors for the fatty acids in murein lipoprotein of *Escherichia coli*. J Biol Chem 1980; 255: 5384-7.

Langston CE, Heuter KJ. Leptospirosis. A re-emerging zoonotic disease. Vet Clin North Am Small Anim Pract 2003; 33: 791-807.

LaRocque RC, Breiman RF, Ari MD, *et al*. Leptospirosis during dengue outbreak, Bangladesh. Emerg Infect Dis 2005; 11: 766-9.

Lee SH, Kim S, Park SC, Kim MJ. Cytotoxic activities of *Leptospira interrogans* hemolysin *SphH* as a pore-forming protein on mammalian cells. Infect Immun 2002; 70: 315-22.

Leelarasamee A, Chupaprawan C, Chenchittikul M, Udompanthurat S. Etiologies of acute undifferentiated febrile illness in Thailand. J Med Assoc Thai 2004; 87: 464-72.

LeFebvre RB. DNA probe for detection of the *Leptospira interrogans* serovar Hardjo genotype hardjo-bovis. J Clin Microbiol 1987; 25: 2236-8.

Levett PN. Leptospirosis: re-emerging or re-discovered disease. J Med Microbiol 1999; 48: 417-8.

Levett PN, Branch SL. Detection of dengue infection in patients investigated for leptospirosis in Barbados. Am J Trop Med Hyg 2000; 62: 112-4.

Levett PN. Leptospirosis. Clin Microbiol Rev 2001; 14: 296-326.

Levett PN, Branch SL, Whittington CU. Two methods for rapid serological diagnosis of acute leptospirosis. Clin Diagn Lab Immunol 2001; 8: 349-51.

Levett PN. Usefulness of serologic analysis as a predictor of the infecting serovar in patients with severe leptospirosis. Clin Infect Dis 2003; 36: 447-52.

Levett PN, Morey RE, Galloway RL, *et al*. *Leptospira broomii* sp. nov., isolated from humans with leptospirosis. Int J Syst Evol Microbiol 2006; 56: 671-3.

Lo M, Bulach DM, Powell DR, *et al*. Effects of temperature on gene expression patterns in *Leptospira interrogans* serovar Lai as assessed by whole-genome microarrays. Infect Immun 2006; 74: 5848-59.

Luo Y, Frey EA, Pfuetzner RA, *et al*. Crystal structure of enteropathogenic *Escherichia coli* intimin-receptor complex. Nature 2000; 405: 1073-7.

Mariya R, Chaudhary P, Kumar AA, *et al*. Evaluation of a recombinant LipL41 antigen of *Leptospira interrogans* serovar Canicola in ELISA for serodiagnosis of bovine leptospirosis. Comp Immunol Microbiol Infect Dis 2006; 29: 269-77.

Marotto PC, Nascimento CM, Eluf-Neto J, *et al*. Acute lung injury in leptospirosis: clinical and laboratory features, outcome, and factors associated with mortality. Clin Infect Dis 1999; 29: 1561-3.

Matsunaga J, Young TA, Barnett JK, Barnett D, Bolin CA, Haake DA. Novel 45-kilodalton leptospiral protein that is processed to a 31-kilodalton growth-phase-regulated peripheral membrane protein. Infect Immun 2002; 70: 323-34.

Matsunaga J, Barocchi M, Croda J, *et al*. Pathogenic *Leptospira* species express surface-exposed proteins belonging to the bacterial immunoglobulin superfamily. Mol Microbiol 2003; 49: 929-45.

Matsunaga J, Sanchez Y, Xu X, Haake DA. Osmolarity, a key environmental signal controlling expression of leptospiral proteins LigA and LigB and the extracellular release of LigA. Infect Immun 2005; 73: 70-8.

Matsunaga J, Werneid K, Zuerner RL, *et al*. LipL46 is a novel surface-exposed lipoprotein expressed during leptospiral dissemination in the mammalian host. Microbiol 2006; 152: 3777-86.

McBride AJ, Athanazio DA, Reis MG, Ko AI. Leptospirosis. Curr Opin Infect Dis 2005; 18: 376-86.

Mendoza L, Prescott JF. Serodiagnosis of leptospirosis in pigs using an axial filament enzyme-linked immunosorbent assay. Vet Microbiol 1992; 31: 55-70.

Merien F, Amouriaux P, Perolat P, Baranton G, Saint Girons I. Polymerase chain reaction for detection of *Leptospira* spp. in clinical samples. J Clin Microbiol 1992; 30: 2219-24.

Merien F, Baranton G. Invasion of Vero cells and induction of apoptosis in macrophages by pathogenic *Leptospira interrogans* are correlated with virulence. Infect Immun 1997; 62: 5477-82.

Merien F, Truccolo J, Baranton G, Perolat P. Identification of a 36-kDa fibronectin-binding protein expressed by a virulent variant of *Leptospira interrogans* serovar Icterohaemorrhagiae. FEMS Microbiol Lett 2000; 185: 17-22.

Merien F, Portnoi D, Bourhy P, *et al*. A rapid and quantitative method for the detection of *Leptospira* species in human leptospirosis. FEMS Microbiol Lett 2005; 249: 139-47.

Murgia R, Riquelme N, Baranton G, Cinco M. Oligonucleotides specific for pathogenic and saprophytic *leptospira* occurring in water. FEMS Microbiol Lett 1997; 148: 27-34.

Myint KS, Gibbons RV, Murray CK, *et al*. Leptospirosis in Kamphaeng Phet, Thailand. Am J Trop Med Hyg 2007; 76: 135-8.

Nally JE, Timoney JF, Stevenson B. Temperature-regulated protein synthesis by *Leptospira interrogans*. Infect Immun 2001; 69: 400-4.

Nally JE, Chow E, Fishbein MC *et al*. Changes in Lipopolysaccharide o antigen distinguish acute versus chronic *Leptospira interrogans* infections. Infect Immun 2005; 73: 3251-60.

Nally JE, Whitelegge JP, Bassilian S, *et al*. Characterization of the outer membrane proteome of *Leptospira interrogans* expressed during acute lethal infection. Infect Immun 2007; 75: 766-73.

Nascimento AL, Ko AI, Martins EA, *et al*. Comparative genomics of two *Leptospira interrogans* serovars reveals novel insights into physiology and pathogenesis. J Bacteriol 2004; 186: 2164-72.

Natarajaseenivasan K, Vijayachari P, Sharma S, *et al*. Phenotypic & genotypic conservation of ompL1 & lipL41 among leptospiral isolates of Andaman Islands. Indian J Med Res 2005; 122: 343-7.

Natarajaseenivasan K, Vijayachari P, Sugunan AP, *et al*. Leptospiral proteins expressed during acute & convalescent phases of human leptospirosis. Indian Journal of Medical Research 2004; 120: 151-9.

Nazarchuk LV, Romanenko LI. Natural antileptospirosis immunity in donor-capable subjects. Fiziol Zh 1998; 44: 19-23.

Nicodemo AC, Duarte MI, Alves VA, *et al*. Lung lesions in human leptospirosis: microscopic, immunohistochemical, and ultrastructural features related to thrombocytopenia. Am J Trop Med Hyg 1997; 56: 181-7.

O'Keefe JS. A brief review on the laboratory diagnosis of leptospirosis. N Z Vet J 2002; 50: 9-13.

Olszyna DP, Jaspars R, Speelman P, *et al*. Leptospirosis in the Netherlands, 1991-1995. Ned Tijdschr Geneeskd 1998; 142: 1270-3.

Palaniappan RU, Chang YF, Jusuf SD, *et al*. Cloning and molecular characterization of an immunogenic LigA protein of *Leptospira interrogans*. Infect Immun 2002; 70: 5924-30.

Palaniappan RU, Chang YF, Hassan F, *et al*. Expression of leptospiral immunoglobulin-like protein by *Leptospira interrogans* and evaluation of its diagnostic potential in a kinetic ELISA. J Med Microbiol 2004; 53: 975-84.

Park SH, Ahn BY. Expression and immunologic characterization of recombinant heat shock protein 58 of *Leptospira* species: a major target antigen of the humoral immune response. DNA Cell Biol 1999; 18: 903-10.

Parma AE, Seijo A, Lucchesi PM, *et al*. Differentiation of pathogenic and non-pathogenic leptospires by means of the polymerase chain reaction. Rev Inst Med Trop Sao Paulo 1997; 39: 203-7.

Patti JM, Allen BL, McGavin MJ, Hook M. MSCRAMM-mediated adherence of microorganisms to host tissues. Annu Rev Microbiol 1994; 48: 585-617.

Peces R. Acute renal failure in severe leptospirosis. Nephrol Dial Transplant 2003; 18: 1235-6.

Perolat P, Chappel RJ, Adler B, *et al*. *Leptospira fainei* sp. nov., isolated from pigs in Australia. Inter J Syst Bacteriol 1998; 48: 851-8.

Picardeau M, Brenot A, Saint Girons I. First evidence for gene replacement in *Leptospira* spp. Inactivation of *L. biflexa* flaB results in non-motile mutants deficient in endoflagella. Mol Microbiol 2001; 40: 189-99.

Plank R, Dean D. Overview of the epidemiology, microbiology, and pathogenesis of *Leptospira* spp. in humans. Microbes Infect 2000; 2: 1265-76.

Preuveneers MJ, Peacock D, Crook EM, *et al*. D-3-hydroxybutyrate dehydrogenase from *Rhodopseudomonas spheroides*. Kinetic mechanism from steady-state kinetics of the reaction catalysed by the enzyme in solution and covalently attached to diethylaminoethylcellulose. Biochem J 1973; 133: 133-57.

Qi HY, Sankaran K, Gan K, Wu HC. Structure-function relationship of bacterial prolipoprotein diacylglyceryl transferase: functionally significant conserved regions. J Bacteriol 1995; 177: 6820-4.

Que-Gewirth NL, Ribeiro AA, Kalb SR, *et al*. A methylated phosphate group and four amide-linked acyl chains in *Leptospira interrogans* lipid A. The membrane anchor of an unusual lipopolysaccharide that activates TLR2. J Biol Chem 2004; 279: 25420-9.

Ren SX, Fu G, Jiang XG *et al*. Unique physiological and pathogenic features of *Leptospira interrogans* revealed by whole-genome sequencing. Nature 2003; 422: 888-93.

Ramadass P, Jarvis BDW, Corner RJ, *et al*. Genetic characterization of pathogenic *Leptospira* species by DNA hybridization. Int J Sys Bacteriol 1992; 42: 215-9.

Ramadass P, Samuel B, Nachimuthu K. A rapid latex agglutination test for detection of leptospiral antibodies. Vet Microbiol 1999; 70: 137-40.

Rathinam SR. Ocular leptospirosis. Curr Opin Ophthalmol 2002; 13: 381-6.

Ribotta MJ, Higgins R, Gottschalk M, *et al*. Development of an indirect enzyme-linked immunosorbent assay for the detection of leptospiral antibodies in dogs. Can J Vet Res 2000; 64: 32-7.

Ristow P, Bourhy P, da Cruz McBride FW *et al*. The *OmpA*-like protein Loa22 is essential for leptospiral virulence. PLoS Pathog 2007; 3: 97.

Romero EC, Caly CR, Yasuda PH. The persistence of leptospiral agglutinins titers in human sera diagnosed by the microscopic agglutination test. Rev Inst Med Trop Sao Paulo 1998; 40: 183-4.

Saglam YS, Temur A, Aslan A. Detection of leptospiral antigens in kidney and liver of cattle. Dtsch Tierarztl Wochenschr 2003; 110: 75-7.

Sankaran K, Wu HC. Lipid modification of bacterial prolipoprotein. Transfer of diacylglyceryl moiety from phosphatidylglycerol. J Biol Chem 1994; 269: 19701-6.

Sankaran K, Gupta SD, Wu HC. Modification of bacterial lipoproteins. Methods Enzymol 1995; 250: 683-97.

Segers R, van Gestel J, van Eys G, *et al*. Presence of putative sphingomyelinase genes among members of the family Leptospiraceae. Infect Immun 1992; 60: 1707-10.

Sehgal SC, Sugunan AP, Murhekar MV, *et al*. Randomized controlled trial of doxycycline prophylaxis against leptospirosis in an endemic area. Int J Antimicrob Agents 2000; 13: 249-55.

Setubal JC, Reis M, Matsunaga J, Haake DA. Lipoprotein computational prediction in spirochaetal genomes. Microbio 2006; 152: 113-21.

Shah I, Warke S, Deshmukh CT, Kamat JR. Leptospirosis-an under-diagnosed clinical condition. J Postgrad Med 1999; 45: 93-4.

Shang ES, Summers TA, Haake DA. Molecular cloning and sequence analysis of the gene encoding LipL41, a surface-exposed lipoprotein of pathogenic *Leptospira* species. Infect Immun 1996; 64: 2322-30.

Shang ES, Exner MM, Summers TA *et al*. The rare outer membrane protein, OmpL1, of pathogenic *Leptospira* species is a heat-modifiable porin. Infect Immun 1995; 63: 3174-81.

Singh SS, Vijayachari P, Sinha A, *et al*. Clinico-epidemiological study of hospitalized cases of severe leptospirosis. Indian J Med Res 1999; 109: 94-9.

Sitprija V, Losuwanrak K, Kanjanabuch T. Leptospiral nephropathy. Seminars in Nephrol 2003; 23: 42-8.

Slack A, Symonds M, Dohnt M, Smythe L. Identification of pathogenic *Leptospira* species by conventional or real-time PCR and sequencing of the DNA gyrase subunit B encoding gene. BMC Genomics 2006; 6: 95-8

Smits HL, van der Hoorn MA, Goris MG, *et al*. Simple latex agglutination assay for rapid serodiagnosis of human leptospirosis. J Clin Microbiol 2000; 38: 1272-5.

Smits HL, Chee HD, Eapen CK, *et al*. Latex based, rapid and easy assay for human leptospirosis in a single test format. Trop Med Int Health 2001a; 6: 114-8.

Smits HL, Eapen CK, Sugathan S, *et al*. Lateral-flow assay for rapid serodiagnosis of human leptospirosis. Clin Diagn Lab Immunol 2001b; 8: 166-9.

Smits HL, Ananyina YV, Chereshsky A, *et al*. International multicenter evaluation of the clinical utility of a dipstick assay for detection of *Leptospira*-specific immunoglobulin M antibodies in human serum specimens. J Clin Microbiol 1999; 37: 2904-9.

Smythe LD, Smith IL, Smith GA, *et al*. A quantitative PCR (TaqMan) assay for pathogenic *Leptospira* spp. BMC Infect Dis 2002; 2: 13-9

Sonrier C, Branger C, Michel V, *et al*. Evidence of cross-protection within *Leptospira interrogans* in an experimental model. Vaccine 2000; 19: 86-94.

Surujballi OP, Marenger RM, Eaglesome MD, *et al*. Development and initial evaluation of an indirect enzyme-linked immunosorbent assay for the detection of *Leptospira interrogans* serovar Hardjo antibodies in bovine sera. Res Vet Sci 1997; 61: 260-6.

Tahiliani P, Kumar MM, Chandu D, *et al*. Gel purified LipL32: a prospective antigen for detection of leptospirosis. J Postgrad Med 2005; 51: 164-8.

Talpada MD, Garvey N, Sprowls R, *et al*. Prevalence of leptospiral infection in Texas cattle: implications for transmission to humans. Vector Borne Zoonotic Dis 2003; 3: 141-7.

Tangkanakul W, Siriarayaporn P, Pool T, *et al*. Environmental and travel factors related to leptospirosis in Thailand. J Med Assoc Thai 2001; 84: 1674-80.

Tangkanakul W, Tharmaphornpil P, Plikaytis BD, *et al*. Risk factors associated with leptospirosis in northeastern Thailand, 1998. Am J Trop Med Hyg 2000; 63: 204-8.

Tansuphasiri U, Deepradit S, Phulsuksombati D, Tangkanakul W. A test strip IgM dot-ELISA assay using leptospiral antigen of endemic strains for serodiagnosis of acute leptospirosis. J Med Assoc Thai 2005; 88: 391-8.

Taylor MJ, Ellis WA, Montgomery JM, *et al*. Magnetic immuno capture PCR assay (MIPA): detection of *Leptospira borgpetersenii* serovar Hardjo. Vet Microbiol 1997; 56: 135-45.

Terpstra WJ, Schoone GJ, Schegget J. Detection of leptospiral DNA by nucleic acid hybridization with 32P- and biotin-labelled probes. J Med Microbiol 1986; 22: 23-8.

Terpstra WJ, Schoone GJ, Ligthart GS, ter Schegget J. Detection of *Leptospira interrogans* in clinical specimens by *in situ* hybridization using biotin-labelled DNA probes. J Gen Microbiol 1987; 133: 911-4.

Thiermann AB. Isolation of leptospires in diagnosis of leptospirosis. Mod Vet Pract 1984; 65: 758-9.

Tippmann HF. Analysis for free: comparing programs for sequence analysis. Brief Bioinform 2004; 5: 82-7.

Trevejo RT, Rigau-Perez JG, Ashford DA, *et al*. Epidemic leptospirosis associated with pulmonary hemorrhage-Nicaragua, 1995. J Infect Dis 1998; 178: 1457-63.

Trueba GA, Bolin CA, Zuerner RL. Characterization of the periplasmic flagellum proteins of *Leptospira interrogans*. J Bacteriol 1992; 174: 4761-8.

Turner LH. Leptospirosis Maintenance, isolation and demonstration of leptospires. Trans R Soc Trop Med Hyg 1970; 64: 623-46.

Van Eys GJ, Gravekamp C, Gerritsen MJ, *et al*. Detection of leptospires in urine by polymerase chain reaction. J Clin Microbiol 1989; 27: 2258-62.

Verma A, Hellwage J, Artiushin S, *et al*. *LfhA*, a novel factor H-binding protein of *Leptospira interrogans*. Infect Immun 2006; 74: 2659-66.

Visith S, Kearkiat P. Nephropathy in leptospirosis. J Postgrad Med 2005; 51: 184-8.

Wagenaar J, Zuerner RL, Alt D, Bolin CA. Comparison of polymerase chain reaction assays with bacteriologic culture, immunofluorescence, and nucleic acid hybridization for detection of *Leptospira borgpetersenii* serovar Hardjo in urine of cattle. Am J Vet Res 2000; 61: 316-20.

Wagenaar JA, Segers RP. Rapid and specific detection of pathogenic *Leptospira* species by amplification of ribosomal sequences. Mol Biotechnol 1994; 2: 1-14.

Werts C, Tapping RI, Mathison JC, *et al*. Leptospiral lipopolysaccharide activates cells through a TLR2-dependent mechanism. Nat Immunol 2001; 2: 346-52.

Winslow WE, Merry DJ, Pirc ML, Devine PL. Evaluation of a commercial enzyme-linked immunosorbent assay for detection of immunoglobulin M antibody in diagnosis of human leptospiral infection. J Clin Microbiol 1997; 35: 1938-42.

Woo TH, Smythe LD, Symonds ML, *et al*. Rapid distinction between *Leptospira interrogans* and *Leptospira biflexa* by PCR amplification of 23S ribosomal DNA. FEMS Microbiol Lett 1997a; 150: 9-18.

Woo TH, Patel BK, Smythe LD, *et al*. Identification of pathogenic *Leptospira* genospecies by continuous monitoring of fluorogenic hybridization probes during rapid-cycle PCR. J Clin Microbiol 1997b; 35: 3140-6.

Woo TH, Patel BK, Smythe LD, *et al*. Comparison of two PCR methods for rapid identification of *Leptospira* genospecies interrogans. FEMS Microbiology Letters 1997c; 155: 169-77.

Yamashiro-Kanashiro EH, Benard G, *et al*. Cellular immune response analysis of patients with leptospirosis. Am J Trop Med Hyg 1991; 45: 138-45.

Yan Y, Chen Y, Liou W, *et al*. An evaluation of the serological and epidemiological effects of the outer envelope vaccine to *leptospira*. J Chin Med Assoc 2003; 66: 224-30.

Yang HL, Zhu YZ, Qin JH, *et al*. In silico and microarray-based genomic approaches to identifying potential vaccine candidates against *Leptospira interrogans*. BMC Genomics 2006; 7: 293-6.

Yasuda PH, Streigerwalt AG, Sulzer KA, *et al*. Deoxyribonucleic acid and relatedness between serogroups and serovars in the family Leptospiraceae with proposal for seven *Leptospira* species. Int J Syst Bacteriol 1987; 37: 404-15.

Yuri K, Takamoto Y, Okada M, *et al*. Chemotaxis of leptospires to hemoglobin in relation to virulence. Infect Immun 1993; 61: 2270-2.

Zhang XY, Yu Y, He P, *et al*. Expression and comparative analysis of genes encoding outer membrane proteins LipL21, LipL32 and OmpL1 in epidemic leptospires. Biochim Biophys Res Commun 2005; 37: 649-56.

Zuerner R, Haake D, Adler B, Segers R. Technological advances in the molecular biology of Leptospira. J Mol Microbiol Biotechnol 2000; 2: 455-62.

Zuerner RL, Knudtson W, Bolin CA, Trueba G. Characterization of outer membrane and secreted proteins of *Leptospira interrogans* serovar Pomona. Microb Pathog 1991; 10: 311-22.

APPENDIX

APPENDIX A
LEPTOSPIRAL CULTURE MEDIA

1. EMJH medium

The 100 ml of EMJH medium consist of 90 ml of EMJH base solution and 10 ml of 10× stock. The 10× stock of 200 ml volume are prepared by adding the following ingredients:

Bovine serum albumin (BSA)	20	g
Sodium pyruvate	0.4	g
Sodium acetate anhydrous	0.2	g
Tween 80	2.5	g
$FeSO_4 \cdot 7H_2O$	0.02	g
1.5% $CaCl_2$	1.4	ml
1.5% $MgCl_2 \cdot 2H_2O$	1.4	ml
0.4 % $ZnSO_4 7H_2O$	2	ml
0.3 % $CuSO_4 5H_2O$	0.2	ml
0.1 % Vitamin B12	0.4	ml
20 % glycerol	1	ml
0.1 % $MnSO_4 4H_2O$	2	ml

The EMJH medium is steriled by filtration through 0.2 μm Millipore membrane and stored at 4°C. The 100 ml of ten time stock will be added to 900 ml EMJH based, as the working media. In addition, 3% heated-inactivated rabbit sera will be supplement to EMJH media, before using.

2. *E. coli* culture media

2.1 LB broth

Tryptone	10	g
Yeast extract	5	g
NaCl	5	g

Distilled water	1000	ml

Shake until the solutes have dissolved, sterilize by autoclaving for 15 minute at 121°C, 15Ib/square inches and store at 4°C.

2.2 LB agar

Tryptone	10	g
Yeast extract	5	g
NaCl	5	g
Agar	20	g
Distilled water	1000	ml

Shake until the solutes have dissolved, sterilize by autoclaving for 15 minute at 121°C, 15Ib/square inches and store at 4°C.

2.3 SOC medium (Prepare freshly before use)

SOB	19.4	ml
1 M Glucose	0.4	ml
1 M $MgCl_2$	0.2	ml

2.4 Ampicillin stock solution (100 mg/ml)

The solution was prepared by dissolving 1 g of ampicillin powder in 10 ml of distilled water, filter sterilization and dispense the solution in aliquots. The solution was stored at -20°C. The working concentration of 100 μg/ml is included in bacterial media.

2.5 Kanamycin stock solution (50 mg/ml)

The solution was prepared by dissolving 1 g of Kanamycin powder in 20 ml of distilled water, filter sterilization and dispense the solution in aliquots. The solution was stored at -20°C. The working concentration of 50 μg/ml is included in bacterial media.

2.6 Chloramphenical stock solution (40 mg/ml)

The solution was prepared by dissolving 1.2 g of Chloramphenical powder in 30 ml of 95% ethanol, filter sterilization and dispense the solution in aliquots. The solution was stored at -20°C.

2.7 IPTG (1 M)

The solution was prepared by dissolving 5 g of IPTG (MW 238.3) in 21 ml of distilled water, filter sterilization, store in aliquots at -20°C.

2.8 1× PBS pH 7.2

NaCl	8.0 g
K_2HPO_4	1.21 g
KH_2PO_4	0.34 g

The buffer was prepared by dissolved all reagents, adjust to 1,000 ml with distilled water, pH 7.2 and Sterile by autoclaving for 15 minute at 121°C, 15Ib/square inches and store at 4°C.

APPENDIX B
REAGENT AND BUFFER FOR AGAROSE GEL ELECTROPHORESIS

1. 0.8 % (w/v) agarose gel

Agarose powder	0.8	g
TAE buffer	100	ml

Melted in microwave oven until completely dissolved

2. 50×TAE (Tris-Acetic acid EDTA) buffer

Tris	242	g
Glacial acetic acid	57.1	ml
Na_2EDTA	37.2	g

Adjust pH to 8.3 and adjust volume to 1,000 ml with distilled water. This buffer is diluted 1:5 for used.

3. Gel loading dye

Sucrose	5.00	g
$Na_2EDTA{\cdot}2H_2O$	0.74	g
Bromphenol blue	0.01	g
Distilled water	20	ml

The solution was prepared by dissolved all reagents and adjusted pH to 7.0. The solution was stored at 4^oC or room temperature.

APPENDIX C
REAGENT FOR SDS-PAGE AND STAINING

1. 1.5M Tris-HCl pH 8.8, 0.4% SDS (resolving gel buffer)

Tris base	18.15 g
SDS	0.4 g
Distilled water	100 ml

Dissolves in distilled water, adjust to pH 8.8 with 1N HCl and adjust to 100 ml final volume with distilled water, store at 4°C.

2. 0.5M Tris-HCl pH 6.8, 2% SDS (stacking gel buffer)

Tris base	6.05 g
SDS	2 g
Distilled water	100 ml

Dissolves in distilled water, adjust to pH 6.8 with 1N HCl and adjust to 100 ml final volume with distilled water, store at 4°C.

3. Ammonium persulfate (10% w/v)

Dissolving 0.1 g of ammonium persulfate in 1 ml of distilled water and store at -20°C.

4. Electrode buffer pH 8.3 (5×)

Tris base	15 g
Glycine	72 g
SDS	5 g

Dissolves in UDW, adjust to pH 8.3 and adjust to 1000 ml final volume with UDW, store at 4°C.

5. 2× sample buffer (SDS reducing buffer)

0.5 M Tris-HCl pH 6.8,2 % SDS	4.0	ml
Glycerol	2.0	ml
10% SDS	1.7	ml
2-mercaptoethanol	1.0	ml
1% Bromphenol blue	1.3	ml
Distilled water	10	ml

The mixture was stored at -20°C in small aliquots.

6. Separating gel preparation (12%)

Polyacrylamide separating gel is prepared by mixing the following reagent:

1.5 M Tris-HCl pH 8.8, 0.4% SDS	2.5	ml
40% Acrylamide	3.1	ml
UDW	3.24	ml

The reagents are gently mixed and the polymerization is initiated by adding 50 µl of 10% ammonium persulphate and 10 µl of TEMED. The gel is poured on the vertical gel electrophoresis and overlay with UDW. The gel is polymerizing for 30 minutes.

7. Stacking gel preparation (4%)

Stacking gel is prepared by mixing the following reagents:

0.5 M Tris-HCl pH 8.8, 0.4% SDS	12.5	ml
40% Acrylamide	0.5	ml
UDW	3.2	ml

The reagents are gently mixed and the polymerization is initiated by adding 50 µl of 10% ammonium persulphate and 10 µl of TEMED. The gel is poured on the vertical gel electrophoresis and overlay with UDW. The gel is polymerizing for 30 minutes.

8. Coomassie brilliant blue (protein staining buffer)

Coomassie brilliant blue	1.25	g
Methanol	250	ml

Glacial acetic acid	50	ml

Dissolve and adjust volume to 500 ml with distilled water. Filter the solution with a Whatman No. 1 paper and store at room temperature.

9. Destaining solution

Methanol	200	ml
Glacial acetic acid	50	ml
Glycerol	25	ml

Dissolve and adjust volume to 1000 ml with distilled water and store at room temperature.

APPENDIX D
REAGENT AND BUFFER FOR WESTERN BLOT ANALYSIS

1. Boltting buffer (10×)

Tris base	30.3	g
Glycine	72	g

Dissolve and adjust volume to 1000 ml with distilled water and store at room temperature.

2. Transblot buffer

10× blotting buffer	200	ml
Methanol	400	ml

Adjust volume to 2000 ml with distilled water and store at 4°C.

3. 10× Tris buffer saline (TBS) pH 7.4

Tris-HCl	60.6	g
NaCl	87.7	g

Adjust pH to 7.4 and adjust volume to 1000 ml with distilled water. This buffer is diluted 1:10 for used.

4. Blocking buffer (5% skim milk in TBS-T)

1X TBS	100	ml
Skim milk	5	g
Tween®20	150	µl

The solution is freshly prepared before used and heating on hot plate for dissolve the skim milk powder.

5. Washing buffer (0.05% Tween-20 in TBS pH 7.4)

1X TBS	1000 ml
Tween®20	250 µl

The solution was mixed well and stored at room temperature.

6. Substrate solution for peroxidase enzyme

Part A:	4-Chloro-1-naphthal	0.015 g
	Methanol	5 ml
Part B:	1X TBS	25 ml
	30% (v/v) H_2O_2	20 µl

Part A and B is freshly prepared and combined immediately before use.

7. Substrate solution for alkaline phosphatase enzyme

1x buffer 3	10 ml
NBT (75µg/ml)	45 µl
BCIP (50µg/ml)	35 µl

The solution was freshly prepared and combined immediately before use.

8. Nitro blue tetrezolium chloride (NBT) 75 µg/ml

The solution was prepared by dissolved 1 g of NBT (MW 433.6 g/mol) in 13.3 ml of 70% DMF. The solution is light sensitive and should be stored at -20°C.

9. 5-Bromo-4-chloro-3-indolyl phosphate (BCIP) 50 µg/ml

The solution was prepared by dissolved 1 g of BCIP (MW 433.6 g/mol) in 20 ml of 100% DMF. The solution is light sensitive and should be stored at -20°C.

APPENDIX E
BUFFER FOR PURIFICATION UNDER DENATURING CONDITION

1. Lysis-Equilibration-Wash (LEW) buffer, pH 8.0

50 mM NaH_2PO_4	7.8 g
300 mM NaCl	17.5 g

Adjust pH to 8.0 and adjust volume to 1000 ml with distilled water, sterilize by autoclaving 15 minutes at 121^{o}C, 15Ib/square inches and store at room temperature.

2. Denaturing solubilization buffer, pH 8.0

LEW buffer	30 ml
Urea	14.41 g

LEW buffer containing 8 M urea of desired volume is prepared, and adjust to pH 8.0 and adjusted volume to 30 ml with LEW buffer. This buffer is prepared freshly before use.

3. Denaturing elution buffer (500 mM immidazole), pH 8.0

LEW buffer with 8 M urea	15 ml
Immidazole	0.127 g

This buffer is the denaturing solubilization buffer containing 500 mM immadazole. This buffer is prepared freshly before use.

APPENDIX F
REAGENT FOR PREPARATION OF LEPTOSPIRAL WHOLE CELL LYSATE

1 Lysis buffer

1 M $MgCl_2$ (5mM $MgCl_2$)	1	ml
1 M Tris pH 8.0 (20mM Tris pH 8.0)	4	ml
1 M NaCl (150mM NaCl)	30	ml
1 M EDTA (2mM EDTA)	0.4	ml
2 mg Lysozyme/ml (20mg/ml)	400	mg

Mix all reagents together, adjust the volume to 200 ml with distilled water and solution was stored at 4^oC.

APPENDIX G
REAGENT AND BUFFER FOR ELISA

1. 0.05 M carbonate-bicarbonat buffer pH 9.6 (Coating buffer)

Na_2CO_3	1.59	g
$NaHCO_3$	2.93	g

Adjust to pH 9.6 with HCl and dissolve in final volume of 1000 ml with distilled water. Sterilize by autoclaving and store at 4°C.

2. PBS-T (Washing Buffer)

PBS-Tween® 20 (0.05%)		
1X PBS	500	ml
Tween® 20	100	ml

The solution was mixed well and stored at 4^oC.

3. 1% BSA (Blocking solution)

Bovine serum albumin (BSA)	1	g
Distilled water	100	ml

The solution was mixed well and prepared freshly before use.

4. Diluent

0.2 % gelatin, 0.2 BSA in PBS-T (Prepare freshly before use)

5. 1% SDS (stop reaction)

Sodium dodecyl sulfate	1	g
Distilled water	100	ml

6. Substrate solution

ABTS® Peroxidase substrate solution A	100 ml
ABTS® Peroxidase substrate solution B	100 ml

Mixed solution A and solution B is freshly prepared ratio 1:1 and combined immediately before use.

BIOGRAPHY

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