APPLICATION OF RECOMBINANT OUTER MEMBRANE PROTEIN IN SERODIAGNOSIS OF LEPTOSPIROSIS

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2008

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Thesis
Entitled

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Nattavut Wongdeethai

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APPLICATION OF RECOMBINANT OUTER MEMBRANE PROTEIN IN SERODIAGNOSIS OF LEPTOSPIROSIS

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ABSTRACT

The rapid diagnosis of leptospirosis is extremely important, and the standard diagnosis of leptospirosis was based on serological testing. Leptospiral recombinant lipoproteins LA0730 and LigA were selected to investigate their serodiagnostic application. Mice were immunized with the recombinant proteins and the specific antibodies obtained were reacted with leptospiral whole-cell antigen. By Western blot technique, mice anti LigA and anti LA0730 reacted with leptospiral cell lysate. By the same technique, rLigA protein reacted with leptospirosis patient sera, while rLA0730 protein did not. Therefore, immunogenic rLigA was used as an ELISA antigen for detecting IgM and IgG in leptospirosis patient sera. Sera of other febrile illnesses and healthy individuals were included as controls. The sensitivity of IgM- and IgG-based ELISA during the acute phase of illness was 70 and 73%, respectively. When the positive results from IgM- and IgG-based ELISA were combined, sensitivity increased to 82%. For convalescent sera, 59 and 68% were positive for IgM- and IgG-based ELISA, respectively; thus, sensitivity also increased (to 77%) using combined ELISA. The specificity of rLigA-based ELISA was 94 and 98% for IgM and IgG ELISA, respectively. Interestingly, when the results from rLigA-based-IgM and IgG-ELISA were combined, 62% of acute phase sera, and 72% of convalescence-phase sera from MAT-negative leptospirosis were diagnosed positive. Therefore, rLigA-based serology has a high potential for the early diagnosis of leptospirosis.

KEY WORDS: *LEPTOSPIRA*/ LEPTOSPIROSIS/ RECOMBINANT PROTEIN/ ELISA/ WESTERN BLOT/ LA0730/ LigA/ IgM/ IgG

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การผลิตและใช้รีคอมบิแนนท์โปรตีนเพื่อการตรวจวินิจฉัยโรคเลปโตสไปโรซีส
(APPLICATION OF RECOMBINANT OUTER MEMBRANE PROTEIN IN SERODIAGNOSIS OF LEPTOSPIROSIS)

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บทคัดย่อ

ความไวในการวินิจฉัยโรคเลปโตสไปโรซีสนั้น มีความสำคัญเป็นอย่างยิ่ง การตรวจหาแอนติบอดีในซีรั่มผู้ป่วย เป็นอีกวิธีหนึ่งที่น่าสนใจในการตรวจวินิจฉัยโรค LA0730 และ LigA โปรตีน ถูกเลือกมาใช้ในการศึกษาครั้งนี้ รีคอมบิแนนท์โปรตีนที่ได้จะนำมาทดสอบคุณสมบัติของโปรตีนว่ามีลักษณะเหมือนกับโปรตีนจากเชื้อเลปโตสไปราในธรรมชาติหรือไม่ โปรตีนทั้งสองชนิดถูกนำไปฉีดในหนูสำหรับการสร้างแอนติบอดี แอนติบอดีที่ได้จะถูกทดสอบกับโปรตีนจากเชื้อด้วยวิธี Western blot พบว่าซีรั่มหนูที่จำเพาะกับ LA0730 และ LigA ไม่ทำปฏิกิริยากับโปรตีนสกัดจากเชื้อเลปโตสไปรา รีคอมบิแนนท์โปรตีนได้ถูกนำไปทดสอบกับซีรั่มคนไข้ ผลปรากฏว่า สามารถตรวจพบแอนติบอดีต่อ LigA โปรตีนได้ในซีรั่มผู้ป่วยเลปโตสไปโรซีส แต่แอนติบอดีไม่ทำปฏิกิริยากับ LA0730 เลย ดังนั้น รีคอมบิแนนท์ LigA โปรตีน จึงถูกนำไปใช้ในการตรวจหาแอนติบอดีในซีรั่มผู้ป่วยเลปโตสไปโรซีส โดยใช้ซีรั่มจากผู้ป่วยโดยโรคที่ทำให้เกิดไข้สูง และคนปกติเป็นกลุ่มเปรียบเทียบโดยวิธี ELISA ผลการศึกษาพบว่า ค่าความไวในการตรวจหาแอนติบอดีในระยะ acute phase ชนิด IgM และ IgG เท่ากับ 70% และ 73% ตามลำดับ และค่าความไวจะสูงขึ้นเป็น 82% เมื่อผลบวกของ IgM และ IgGถูกนำมารวมกัน สำหรับระยะ convalescence phase ค่าความไวให้ที่ 59% และ 68% สำหรับ IgM และ IgG ตามลำดับ และสูงขึ้นเป็น 77% เมื่อผลถูกนำมารวมกัน นอกจากนั้นค่าความจำเพาะของวิธีนี้ยังสูงถึง 94% สำหรับ IgM และ 98% สำหรับ IgG เป็นที่น่าสนใจว่า วิธี ELISA นี้สามารถตรวจพบผลบวกในกลุ่มที่มีผล MAT เป็นลบ โดยพบผู้ป่วยระยะ acute phase ให้ผลบวก 62% และผู้ป่วยระยะ convalescence phase ให้ผลบวก 72% ดังนั้นวิธีการตรวจหาแอนติบอดีต่อ LigA โปรตีนโดยวิธี ELISA นั้นสามารถนำมาพัฒนาเพื่อการวินิจฉัยโรคเลปโตสไปโรซีสในระยะเริ่มแรกของการติดเชื้อได้.

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LIST OF ABBREVIATIONS

Abbreviation or symbol	Term
/	Per
%	Percent
α	Alpha
β	Beta
°C	Degree celsius
Ala	Alanine
Asp	Aspartic acid
Big	Bacterial immunoglobulin-like
bp	base pair
CF	Complement fixation test
CFU/ml	Colony forming unit per milliliter
CNS	Central nervous system
CSF	Cerebrospinal fluid
Cys	Cysteine
DHF	Dengue hemorrhagic fever
DNA	Deoxyribonucleic acid
DW	Distilled water
EDTA	Diaminoethanetetra-acetic acid
e.g.	exampli gratia
ELISA	Enzyme-linked immunosorbent assay
EMJH	Ellinghausen, McCullough, Johnson, Harris
ESR	Erythrocyte sedimentation rate
et al.	et alli
etc.	Et cetera
FN	False negative

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LIST OF ABBREVIATIONS (Continued)

Abbreviation or symbol	Term
FP	False positive
g	Gram(s)
Gly	Glycine
g/mol	Gram(s) per mole
HRP	Horseradish peroxidase
Hsp(s)	Heat shock protein(s)
IFA	Indirect fluorescent antibody test
Ig(s)	Immunoglobulin(s)
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IHA	Indirect hemagglutination assay
Ile	Isoleucine
IP	Intraperitoneal route
IPTG	Isopropyl-β-D-thiogalactopyranoside
kb	kilobase
kDa	Kilo Dalton
LA	Latex agglutination test
LB	Luria Bertani
Leu	Leucine
Lgt	Prolipoprotein diacylglyceryl transferase
Lig	Immunoglobulin-like protein
Lnt	Apolipoprotein transacylase
LPS	Lipopolysaccharide
Lsp	Lipoprotein signal peptidase
M	Molar(s)
mA	Milli Ampere(s)
MAb	Monoclonal antibody
MAT	Microscopic agglutination test

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LIST OF ABBREVIATIONS (Continued)

Abbreviation or symbol	Term
MCAT	Microcapsule test
µg	Microgram(s)
µl	Microliter(s)
mg	Milligram(s)
ml	Milliliter(s)
µg/µl	Microgram(s) per microliter
µg/ml	Microgram(s) per milliliter
mg/ml	Milligram(s) per milliliter
µm	Micrometer(s)
mM	Millimolar(s)
mm	Millimeter(s)
mm^3	Cubicmillimeter(s)
mmol/L	Millimolar(s) per liter
µmol/L	Micromolar(s) per liter
mol%	Mole percent
MW	Molecular weight
NC	Nitrocellulose membrane
ng/ml	Nanogram(s) per milliliter
nm	Nanometer(s)
NPV	Negative predictive value
NSS	Normal saline
OD	Optical density
OMP	Outer membrane protein(s)
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffer saline
PBS-T	PBS containing 0.05% Tween-20
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
pH	Negative logarithm of hydrogen ion activity

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LIST OF ABBREVIATIONS (Continued)

Abbreviation or symbol	Term
Phe	Phenylalanine
pI	Isoelectric point
PMN	Polymorphonuclear neutrophil
PPV	Positive predictive value
PVDF	Polyvinylidene Fluoride Transfer Membrane
r	Recombinant
REA	Restriction endonuclease analysis
RFLP	Restriction fragment length polymorphism
RIA	Radioimmuno assay
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
rpm	Round per minute
RT-PCR	Reverse transcription polymerase chain reaction
SAT	Slide agglutination test
SD	Standard deviation
SDS	Sodium dodecylsulfate
Ser	Serine
SPaseI	Signal peptidase I
SPaseII	Signal peptidase II
spp.	Species
TBS	Tris buffer saline
TBS-T	TBS containing Tween-20
TEMED	N, N,N',N'-tetramethylethylenediamine
Thr	Threonine
TN	True negative
TP	True positive
Tris	Tris (hydroxymethyl aminomethane)
UDW	Ultrapure distilled water

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LIST OF ABBREVIATIONS (Continued)

Abbreviation or symbol	Term
V	Volt(s)
Val	Valine
v/v	Volume by volume
w/v	Weight by volume
WHO	World Health Organization

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CHAPTER I
INTRODUCTION

Leptospirosis is a globally important zoonotic disease caused by pathogenic spirochaetes of the genus *Leptospira*. The disease occurs throughout the world, but its incidence is highest in the tropical regions (WHO, 1999). Chronically infected domestic and wild animal species harboring *Leptospira* in their renal tubules, shed the leptospires into the environment upon urination. Excreted Leptospires continue to survive in suitable moist environments until contact and penetrate to the new hosts via skin abrasions or mucosal surfaces such as conjunctival tissue of the eye (Bharti *et al.*, 2003; Cullen *et al.*, 2002). Dissemination of *leptospira* throughout the infected host can result in a wide range of clinical manifestations of the disease, ranging from a self limiting fever to acute lethal forms and asymptomatic chronic carriage. The severe disease form, known as Weil's syndrome, is an acute febrile illness associated with multiorgan system complications including jaundice, renal failure, meningitis and severe pulmonary hemorrhage form of leptospirosis results in high mortality rates (Niwattayakul *et al.*, 2002; Sehgal *et al.*, 1995; Seijo *et al.*, 2002).

Diagnosis of leptospirosis is complicated by the high degree of cross-reactivity among different serovars. The currently available, microscopic agglutination test (MAT) is laborious. ELISA methods have been developed with a number of modifications (da Silva *et al.*, 1997; Gussenhoven *et al.*, 1997; Levett, 2001; Petchclai *et al.*, 1991; Ribeiro *et al.*, 1995), but most of them depend on proteins derived from whole-cell lysates of *Leptospira*. Currently available veterinarian vaccines are based on inactivated whole cell or membrane preparations of pathogenic leptospires. These types of vaccine confer protective responses through induction of antibodies against leptospiral lipopolysaccharide (de la Pena-Moctezuma *et al.*, 1999). However, these vaccines fail to induce long-term protection against infection and do not provide cross-protection against other different serovars. Recombinant antigens such as LipL32,

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flagellin and heat-shock protein of *leptospira* have also been recently developed for use as diagnostic reagents (Flannery *et al.*, 2001; Park *et al.*, 1999), but the specificity and sensitivity of these antigens in vaccinated animals have not been determined. Identification of leptospiral antigens expressed only during infection could be useful for the development of new diagnostic reagents for serodiagnosis. In order to identify the potential antigens that are expressed during leptospiral infection, one of the clones encodes a gene for leptospiral immunoglobulin-like proteins (LigA) that is only expressed *in vivo* will be further analyzed (Palaniappan *et al.*, 2002).

In this study, the antigenicity of leptospiral immunoglobulin-like protein (LigA) and recombinant 32-kDa unknown lipoprotein, LA0730 were characterized by the preparation of the specific antibodies to recombinant proteins in mice. The reactivity of hyperimmune sera to leptospiral whole cell antigen will be evaluated by ELISA and Western blot techniques, using patient sera and other control serum panels. There after, the application of these recombinant proteins as diagnostic antigens for serodiagnosis of leptospirosis.

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CHAPTER II
OBJECTIVES

1. To identify and characterize the antigenicities of 2 recombinant lipoproteins (LigA, LA0730) whether their epitopes are shared with the native leptospiral membrane proteins.

2. To apply the leptospiral recombinant lipoproteins as specific antigens in the ELISA and Western blot assay.

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CHAPTER III
REVIEW OF THE LITERATURES

I. *LEPTOSPIRA*

1. Historical background

Leptospirosis is a common zoonosis of ubiquitous distribution, caused by infection with pathogenic *Leptospira* species (Levett, 2001). A variety of other infectious diseases that present as undifferentiated febrile syndromes, such as malaria, dengue and influenza, as well as viral hemorrhagic fevers can mimic Leptospirosis (Vinetz, 2001). The leptospirosis was first described by Adolf Weil in 1886. He published a description of four cases of an acute febrile illness with jaundice, severe nervous symptoms, enlargement of liver and spleen, and renal involvement, in term of "Weil's disease". In 1907, Stimson reported a microorganism resembling a spirochaete that demonstrated by silver staining of clumps of spirochetes in the kidney tubules of a patient diagnosed as having yellow fever. He named this organism "*Spirochaeta interrogans*." Later it was realized that this organism was in fact the first *Leptospira* recorded (Faine *et al.*, 1999).

The etiology of leptospirosis was demonstrated independently in 1915 in Japan and Germany. In Japan the spirochete was first isolated by Inada and coworkers. They detected both spirochetes and specific antibodies in the blood of Japanese miners with infectious jaundice, and two groups of German physicians studied German soldiers afflicted by "French disease" in the trenches of northeast France. In 1918, Noguchi described the fine structure of Leptospires and comparing their microscopic and serological features with other spirochaetes. He grew a spirochaete from the liver of yellow fever patient and named it "Spirochaeta icteroides" (Noguchi, 1918). He

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proposed a reclassification of spirochaetes, in which the genus *Leptospira* included the Weil's disease and the yellow fever organism, but hemorrhagic and jaundiced patients with severe leptospirosis were easily confused with those who had yellow fever, because the differences in clinical manifestation were not obvious and the etiological agents and specific laboratory testes for both diseases were still under diagnosed (Faine *et al.*, 1999).

Untilled 1950, the characteristic of *Leptospira* species were classified by Yanagawa and Faine, based on the improved techniques for shadowing, fixing, negative staining, high resolution microscopy and immunolabelling (Faine *et al.*, 1999; Yanagawa and Faine, 1966). Recently, taxonomic changes affected the classification of genus *Leptospira* and advances in the diagnosis of leptospirosis by both serological and molecular methods.

2. Morphology

Leptospires are highly motile, obligate aerobic spirochetes that share features of both gram-positive and gram-negative bacteria (Bharti *et al.*, 2003). All pathogenic *Leptospira* species share the distinctive double membrane architecture of spirochetes (Haake, 2000). As in Gram-positive bacteria, the inner (cytoplasmic) membrane of spirochetes is closely associated with the peptidoglycan cell wall. Spirochetes also have an outer membrane that provides a barrier shielding underlying antigens, such as the endoflagella. However, the spirochetal outer membrane appears to be fluid and labile, which contrasts with the outer membrane of Gram-negative bacteria (Haake and Matsunaga, 2005).

Leptospires are tightly coiled spirochetes, usually 0.25 × 6-25 μm in size that can pass through 0.45 μm filters. The cells have pointed ends, either or both of which are usually bent into a distinctive hook (Levett, 2001). Electron micrograph shows a cylindrical cell body (protoplasmic cylinder) wound helically around an axistyle (0.01-0.02 μm in diameter), which comprises two axial filaments (periplasmic flagella) with polar insertions and located in the periplasmic space. Three types of movement are

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possible: rotation around central axis, progressive movement in the direction of the straight end, and circular motion (Bharti *et al.*, 2003).

3. Taxonomy and Classification

The microorganisms are very delicate and spiral-shaped, hence the name is created. They have a typical terminal hook (Gr. Leptos; delicate, slender, speira; spiral, interrogans; question mark). Leptospires are spirochetes that belong to Phylum *Spirochaetes*, Class *Spirochaetes*, and Order *Spirochateales*. There are three families: *Brachyspiraceae*, *Spirospiraceae* and *Leptospiraceae*. Within *Leptospiraceae*, there are only three genera, *Leptonema*, *Turneria* and *Leptospira*. The genus *Leptospira* was used to divided into only two species, *Leptospira interrogans* comprising all pathogenic strains and the free living saprophytic *Leptospira* belong to the species *Leptospira biflexa* (Bharadwaj, 2004; Levett, 2001).

The taxonomy of *Leptospira* continues to evolve, with implications for diagnosis and epidemiology. They are two major ways to classify *Leptospira*. The first is phenotypic that is clinically useful. *Leptospira* species were divided into numerous serovars, defined by agglutination after cross-absorption with homologous antigen; the agglutinating antigen is mainly lipopolysaccharide. There are more than 250 leptospiral serovars to date. Serovars that are antigenically related have been traditionally grouped into 24 serogroups as shown in **Table 1** (Levett, 2004; Vinetz, 2001). While serogroups have no taxonomic standing, they also proved to be useful for epidemiological understanding. The second mean of classification, most recently updated in 1999 (Brenner *et al.*, 1999), is genotypic in which a number of genomospecies included all serovars of both *L. interrogans* and *L. biflexa*. It divided into 17 genomospecies based on DNA:DNA hybridization under varying stringencies by Southern blot analysis (Vinetz, 2001). DNA hybridization studies have also confirmed the taxonomic status of the monospecific genus *Leptonema* (Brenner *et al.*, 1999; Ramadass *et al.*, 1990). The genotypic classification of Leptospires are supported by multilocus enzyme electrophoresis data (Letocart *et al.*, 1999) and 16s rRNA gene sequence analysis (Hookey, 1993). However, the molecular classification

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is incompatible with the system of serogroups which has long been useful for clinicians and epidemiologists. Moreover, there is a pressing need for simple molecular tools for identification of species and serovars (Levett, 2004).

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Table 1. **Classification of *Leptospira* species**

Species	Serogroups	Serovars	Strains
Pathogens			
L. interrogans	*Australis*	australis	Ballico
	Australis	bratislava	Jez Bratislava
	Bataviae	bataviae	Van Tienen
	Canicola	canicola	Hond Utrecht IV
	Hebdomadis	hebdomadis	Hebdomadis
	Icterohaemorrhagiae	icterohaemorrhagiae	RGA
	Icterohaemorrhagiae	copenhageni	M 20
	Icterohaemorrhagiae	lai	Lai
	Pomona	pomona	Pomona
	Pyrogenes	pyrogenes	Salinem
	Sejroe	hardjo	Hardjoprajitno
L. alexanderi	*Manhao*	manhao3	L 60
L. fainei	*Hurstbridge*	hurstbridge	BUT 6
L. inadai	*Lyme*	lyme	10
L. kirschneri	*Autumnalis*	bim	1051
	Cynopteri	cynopteri	3522 C
	Grippotyphosa	grippotyphosa	Moskva V
	Pomona	mozdok	5621
	Panama	panama	CZ 214K
L. meyeri	*Semaranga*	semaranga	Veldrat Semaranga 173
L. borgpetersenii	*Ballum*	ballum	Mus 127
	Ballum	castellonis	Castellon 3
	Javanica	javanica	Veldrat Bataviae 46
	Sejroe	sejroe	M 84
	Tarassovi	tarassovi	Perepilitsin
L. weillii	*Celledoni*	celledoni	Celledoni
L. noguchii	*Autumnalis*	fortbragg	Fort Bragg
L. santarosai	*Bataviae*	brasiliensis	An 776
	Mini	georgia	LT 117
Genomospecies 1	*Ranarum*	pingchang	80-412
Genomospecies 4	*Icterohaemorrhagiae*	hualin	LT 11-33
Genomospecies 5	*Semaranga*	saopaulo	Sao Paulo
Saprophytes			
Genomospecies 3	*Holland*	holland	Waz Holland (P438)
L. biflexa	*Semaranga*	patoc	Patoc I
L. wolbachii		codice	CDC

Adapted from Levett PN. Leptospira and leptonema. In: Murray PR, Baron EJ, Pfaller MA, *et al*., eds. Manual of clinical microbiology, 8th ed. Washington DC: ASM Press, 2003: 929-36.

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4. Epidemiology and Transmission

Leptospirosis has a worldwide distribution. The incidence of human infection is higher in the tropics than in temperate regions but transmission occurs in both industrialized and developing countries (Bharti *et al.*, 2003). The disease is occurred in seasonal, with peak incidence occurring in summer or fall in temperate regions, where temperature is the limiting factor in survival of leptospires, and during rainy seasons in warm-climate regions, where rapid dessication would otherwise prevent survival (Levett, 2001). The disease is caused by pathogenic spirochaete of the genus *Leptospira* (Faine, 1998; Levett, 2001). The organisms have been found in the urine from cattle, pigs, horses, dogs, rodents and wild animals as they are reservoir hosts. Carrying of leptospires is maintained by the persistent colonization of the proximal renal tubules of carrier animals. An infected animal can remain symptom-free and shed infectious organisms in the urine for its entire lifetime (Faine *et al.*, 1999; Leonard *et al.*, 1992; Thiermann, 1981).

Human and other animals become infected after direct or indirect contact with the urine of an infected animal. The usual portal of entry is through abrasions or cuts in the skin or via the conjunctiva; infection may take place via intact skin after prolonged immersion in water, but this usually follows by skin abrasions and is thus difficult to substantiate (Levett, 2001).

There are extremely rarely recorded examples of leptospires transmitted directly between humans by urine, because humans are accidential carriers and their hygienic habits generally prevent transmission. Human infections may be acquired through occupational, recreational, or avocational exposures. Occupation is a significant risk factor for human (Waitkins, 1986).

Epidemiologic studies indicated that leptospirosis was formerly considered to be primarily an occupational disease, associated with activities such as mining, sewer maintenance, livestock farming and butchering, veterinary medicine, and military manoeuvers. To date, the relative importance of such occupational risks has decreased

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since protective measures have been implemented. In tropical environments, occupational exposure such as rice farming and other agricultural activities is still significant, but so too is the exposure of the general population during activities of daily living, and especially is associated with high seasonal rainfall (Bunnell *et al.*, 2000; Russell *et al.*, 2003; Sanders *et al.*, 1999; Trevejo *et al.*, 1998).

Southeast Asia is an endemic area for leptospirosis, and infection in human has been reported throughout the region, with the serovars included in the major pathogenic species, 70% have been isolated in Asia (Van *et al.*, 1998). In 1999, more than 500,000 leptospirosis cases were reported in China. The reported case fatality rates ranged from 1 to 7.9%. In India approximately 2,000 cases were reported with case fatality rates ranging from 0.7 to 13.9% (Vinetz, 2001).

In Thailand, Leptospirosis was first reported in 1942 by Yunibandhu and coworkers (Yunibandhu, 1943). In 1972, Leptospirosis was included as one of the 58 reportable infectious diseases under the National Passive Surveillance System. The incidence has dramatically increased since 1996, with a peak in 2000 (Tangkanakul *et al.*, 2005). Recently, more than 2,000-3,000 leptospirosis cases were reported in 2005 and represented an annual incidence rate of approximately 4.61/100,000 population, most cases were reported from the Northeast region, in Thailand, leptospirosis must be markedly under-reported due to lack of awareness of the disease and relatively inaccessible and insuffiently rapid diagnostics (Bharti *et al.*, 2003).

5. Clinical manifestation of Leptospirosis

Leptospirosis at present is grossly underreported because of its protean clinical manifestations. The spectrum of disease ranges from a mild inconsequential febrile illness to a severe fatal form presenting with multiorgan failure conventionally was called “Weil’s disease” (Bharadwaj, 2004). The specific serovars involved depend largely on the geographic location and the ecology of local maintenance hosts. Thus in Europe, serovars *Copenhageni* and *Icterohaemorrhagiae* are usually responsible for infection, while in Southeast Asia, serovar *Lai* is common (Levett, 2001).

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The clinical presentation of leptospirosis is biphasic with the acute or septicemic phase lasting about a week, followed by the immune phase, characterized by antibody production and excretion of leptospires in the urine (Levett, 2001). The incubation period following immersion or accidental laboratory exposure has shown extremes of 2 to 26 days, the usual range being 7 to 13 days and the average of 10 days.

In general typical descriptions of leptospirosis, it can be divided into two distinct clinical syndromes: 90% of patients, presented with a mild anicteric febrile illness (anicteric form), are described as the septicemic stage and the immune stage, and 10% are severely fulminant disease with jaundice and other organ dysfunctions, or weil's syndrome (icterohaemorrhagic form) is a more serious, potentially fatal syndrome (Bharti *et al.*, 2003).

5.1 Anicteric leptospirosis

In the first or septicemic phase, patients usually present with a febrile illness of sudden onset include fever, chills with rigors, headache, myalgias, skin rashes, anorexia, nausea, vomiting, abdominal pain, conjunctival suffusion, prostration and sometimes circulartory collapse, other signs include pharyngeal injection, lymphadenopathy, splenomegaly, hepatomegaly and muscle tenderness (Farr, 1995). The fever may be high and remittent, reaching a peak of 40°C before defervescence. The headache is often severe, resembling that occurring in dengue, with retro-orbital pain and photophobia (Kelley, 1998). Conjunctival suffusion is the prominant characteristic and usually appears on the third or forth day. Myalgias usually involve the muscles in the lower back, thighs, calves, abdomen and paraspinal region and can be severe. Myalgias may cause nuchal rigidity reminiscent of meningitis and can mimic acute abdomen, leading to confusion with surgical intraabdominal emergencies (Berman *et al.*, 1973; Guarner *et al.*, 2001; Morgan *et al.*, 2002). The cutaneous manifestations in mild leptospirosis include transient urticarial, macular or maculopapular, erythematous, or purpuric rash (Paster *et al.*, 1991). The first phase

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lasts 3 to 9 days, followed by 2 or 3 days of defervescence, after which the second or immune phase develops.

The immune stage of anicteric leptospirosis (second phase) is characterized by leptospiruria and correlates with the appearance of IgM antibodies in the serum, preceded by a 1 to 3 days asymptomatic period. The symptomatology in this stage is varied. Fever and earlier constitutional symptoms recur in some patients are less severe at the onset of the immune stage than during the septicemic stage. The duration of the immune stage ranges from 4 to 30 days, and the leptospires are cleared from the blood and CSF after the first 1-2 days of this stage. Leptospiruria develops and persists for 1-3 weeks (Farr, 1995), and signs of meningitis, such as headaches, photophobia, and nuchal rigidity, may develop. Central nervous system (CNS) involvement in leptospirosis most commonly occurs as aseptic meningitis (Edwards and Domm, 1960; Heath and Johnson, 1994; Sperber and Schleupner, 1989) is the hallmark of the immune stage. Complications such as opticneuritis, uveitis (Rathinam *et al.*, 1997; Watt, 1990), irridocyclitis (Beeson *et al.*, 1951), chorioretinitis (Hines, 1984), iritis (Farr, 1995), and other nervous system manifestations, including encephalitis, myelitis, and peripheral neuropathy occur more frequently in the immune phase (Edwards *et al.*, 1990). Prolonged or recurrent uveitis was demonstrated in 2% of patients with onset several months after symptoms of clinical leptospirosis (Alexander *et al.*, 1952).

5.2 Icteric leptospirosis

During the leptospiremic phase of icteric leptospirosis, the symptoms do not suggest leptospirosis until the third to seventh days of illness, when jaundice and azotemia develop. The biphasic course of the disease is obscured by severe and persistent fever, jaundice, and azotemia (Farr, 1995). The severe form of leptospirosis is usually characterized by symptoms of hepatic dysfunction, renal insufficiency, haemorrhage, myocarditis and vascular dysfunction (de Brito *et al.*, 1987; Farr, 1995; Lee *et al.*, 1986), and a high mortality. Haemorrhage can occur as petechiae, purpura, conjunctival haemorrhage, gastrointestinal haemorrhage, and pulmonary haemorrhage.

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Severe pulmonary haemorrhage has been described in China (Dai, 1992), Korea (Park *et al.*, 1989) and Nicaragua (Trevejo *et al.*, 1998; Zaki and Shieh, 1996), where patients died of pulmonary haemorrhage with no significant renal dysfunction or jaundice. Other, less common manifestations of leptospirosis are generalized lymphadenopathy (van Crevel *et al.*, 1994; Zaki and Shieh, 1996), pharyngitis, acalculous cholecystitis (fever, right upper quadrant pain, murphy's sign) (Guarner *et al.*, 2001; Monno and Mizushima, 1993), and adult respiratory distress syndrome (de Koning *et al.*, 1995).

6. Pathogenesis and Virulence factors

Host infection by pathogenic *Leptospira* produced a diverse array of clinical manifestations ranging from subclinical infection to undifferentiated febrile illness to jaundice, renal failure, and potentially lethal pulmonary haemorrhage. The highly variable clinical manifestations of leptospiral infection suggest that a diverse range of events may contribute to acute and chronic infection processes of people and reservoir hosts. Pathogenic leptospires spread immediately and circulate in the blood stream after penetrating skin or mucous membranes. One mechanism of virulence is motility; it is probably important in initial infection and in dissemination of organisms from the site of entry to sites of end-organ damage such as lung, liver, kidney, eye, and brain. In addition, toxin and enzymes produced by leptospires may also contribute to their pathogenicity (Bharti *et al.*, 2003; Farr, 1995). The clinical and pathological features of infection have suggested the presence of an endotoxin. Although several laboratories have recently isolated a lipopolysaccharide-like substance, but this has not been shown to contribute to the pathogenesis of leptospirosis (Cinco *et al.*, 1988; Midwinter *et al.*, 1994; Ono *et al.*, 1984; Vinh *et al.*, 1986).

Leptospira virulence factors such as hemolysins (Kasarov, 1970; Thompson and Manktelow, 1986; Trowbridge *et al.*, 1981), lypopolysaccharide (Isogai *et al.*, 1986), glycolipoprotein (Alves *et al.*, 1992), peptidoglycan (Dobrina, 1995), heat shock protein (Stamm *et al.*, 1991), flagellin (Goldstein and Charon, 1990), and others may contribute to the pathogenesis. Leptospira hemolysins have been suggested to be

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phospholipases, which act on erythrocytes and other cell membranes containing the substrate phospholipids, leading to cytolysis (Kasarov, 1970; Thompson and Manktelow, 1986; Trowbridge *et al.*, 1981). Phospholipases that are clearly involved in the alteration of erythrocyte membranes may also function to degrade the phospholipids of other kinds of cells and thus contribute to pathological changes seen in infections (Kasarov, 1970). Isogai and coworker reported that administration of lipopolysaccharide from *L. interrogans* induced apoptosis of lymphocytes in mice (Isogai *et al.*, 1998; Isogai *et al.*, 1986). Vinh and coworker have shown that the glycolipoprotein fraction from *L. interrogans* was cytotoxic to mouse fibroblast cultures (Vinh *et al.*, 1986).

Heat shock proteins (Hsps) are highly conserve and ubiquitous molecules. Their synthesis is increased to protect either prokaryotic or eukaryotic cells from various insults during the period of stress caused by infection, inflammation, or similar events (Kaufmann, 1990; Young, 1992). The Hsps perform important functions, but also as major antigens of various microbial pathogens. They induce very strong humoral and cellular immune responses in several infections (Park *et al.*, 1999). Homologs of two major Hsps, GroEL and DanK have previously been identified in *L. interrogans* and saprophytic *L. biflexa* under heat shock conditions (Stamm *et al.*, 1991). The Hsp60 of *Leptospira* has been shown to be the ubiquitous "common antigen" of the serovar *hardjo* (Ballard *et al.*, 1990). Each leptospire possesses two flagella (axial filaments) inserted subterminally into the protoplasmic cylinder. The filament, sheath, hook and basal body of leptospiral flagella are similar to the corresponding structures of other bacterial flagella (Smibert, 1973). In 1970, Chang and Faine showed that purified leptospiral flagella were immunogenic and formed a characteristic precipitation line on immunodiffusion. The pattern of cross-reactivity between serovars by immunodiffusion was different from that seen in the agglutination-absorption system used for classification (Chang *et al.*, 1974).

Faine reported that both virulent and avirulent leptospires were phagocytosed by reticuloendothelial-fixed phagocytes in immunized and unimmunized mice. Subsequently, Faine and coworker demonstrated phagocytosis of leptospires by

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macrophages and PMN (polymorphonuclear neutrophils) from different animal species such as mice, guinea pigs and rabbits. Specific opsonization with whole leptospiral antiserum increased the rate of phagocytosis (Faine, 1964). Leptospiral LPS stimulated adherence of neutrophils to endothelial cells and platelets, causing aggregation and suggesting a role in the development of thrombocytopenia (Dobrina, 1995; Isogai *et al.*, 1997). Effects on specific organs and tissue can be described briefly as follows:

Abdomen; the liver shows slight or focal hepatocellular necrosis with focal lymphocytic infiltration, and proliferation of kupffer cells, with cholestasis and round cell infiltration in the periportal area, can usually be observed. Jaundice is the most noticeable clinical finding in cases of hepatic dysfunction, while other laboratory manifestations include decreased serum albumin levels, increased globulin levels, and impaired production of vitamin K-dependent clotting factors were also found (Farr, 1995). The kidneys may be swollen, and yellow-green in jaundiced patients, with subsurface haemorrhages. The constant histological appearances are those of interstitial nephritis, progressing to tubular necrosis in severe cases as well as in renal failure with tubular necrosis. Moreover, splenomegaly was a feature of Weil's description of leptospirosis (Faine *et al.*, 1999).

Cardiopulmonary system; pulmonary involvement in leptospirosis is generally the result of haemorrhage rather than of inflammation. In cases with severe pulmonary haemorrhage, larger interstitial extravasations occur, sometimes into the bronchioles and bronchi (Faine *et al.*, 1999). Cardiac dysfunction may also result in hypoperfusion. Myocarditis, myopericarditis, and cardiac dysrhythmias are well-documented manifestations of leptospirosis (Farr, 1995).

Muscles; the myalgias typical of early disease appear to be due to active invasion of skeletal muscle by leptospires. One of the characteristic lesions of leptospirosis is the degeneration of striated muscle, causing extreme tenderness and spasm (Faine *et al.*, 1999).

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Central nervous system; involvement of the central nervous system (CNS) is common in patients with leptospirosis, although the pathogenesis of meningeal irritation is obscured. Leptospires easily enter the cerebrospinal fluid in the early septicemic phase of the illness, but these are little evidence of inflammatory response in the CSF (Farr, 1995). There may be subarachnoid haemorrhages, and small areas of capillary thrombosis and ischaemic necrosis in the brain and spinal cord. In China, leptospirosis may be one of the causes of cerebral arteritis (Moyamoya disease) (Faine *et al.*, 1999; Matsushima *et al.*, 1997).

Eye; conjunctival suffusion, especially pericorneal, caused by vascular dilatation but not inflammation. The persistence of the leptospires in the aqueous humor may cause a chronic, recurrent latent uveitis, iritis, and iridocyclitis are common (Faine *et al.*, 1999; Farr, 1995).

7. Prevention and Control

Strategies to prevent and control leptospirosis focus mainly on reducing direct contact with infected animals and contaminated water or soil (Faine, 1982). The risk of contact with infected animals may be reduced by the institution of appropriate sanitation measures among veterinarians, farmers, and others who handle animals. Since rodents are important natural reservoirs for leptospires, reduction of rodent populations can reduce the incidence of human leptospirosis in endemic areas. However, in places with poverty and poor sanitation, reduction of exposure to rodents may be difficult. Prevention of indirect exposure, or exposure to contaminated water or soil, is more difficult, but a common recommendation is to wear rubber boots and protective clothing and, where possible, to avoid exposure to contaminated rivers, streams, still water, and mud. However, education through industry or community self-help groups can raise awareness and prevent infections in humans and the animals they keep. The most compelling advocates for the control of leptospirosis are people who have been as patients themselves (Faine, 1998).

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8. Vaccine development

An effective leptospirosis vaccine that is widely available for use in humans has still not been developed. To date, all leptospirosis vaccine candidates have been bacterins (killed bacteria), and are associated with unacceptable side-effects and short-term, highly limited, non-cross reacting efficacy (Vinetz, 2001). As described by Haake in 1999 that two leptospiral outer membrane-associated proteins, OmpL1 and LipL41, of *L. Kirshneri* sorovar *Grippotyphosa* were synergistic in their protective efficacy in a hamster model of haemorrhagic leptospirosis (Haake *et al.*, 1999). Whether the same is true in people has not been shown. Preparations of leptospiral lipopolysaccharide (LPS) can elicit protective immunity, but this is generally serovar specific and a vaccine would require using a combination of commonly prevalent serovars in an area. Given the inherent difficulty in preparing multivalent LPS vaccines the identification of conserved protein antigens for use in vaccination is of critical importance (Bharadwaj, 2004). Moreover, the leptospiral OMPs which stimulate an IgM response and other recently described surface expressed proteins the Lig proteins could also be evaluated as candidates for preparation of subunit vaccines which would have broad-based actions in preventing leptospirosis in humans and animals (Koizumi and Watanabe, 2004).

9. Treatment

Treatment of leptospirosis is most effective when initiated early, during the acute illness and differs depending on the severity and duration of symptoms at the time of presentation. Patients with mild, flu-like symptoms require only symptomatic treatment but should caution to seek further medical help if they develop jaundice (Levett, 2001; Levett, 2004). In patients with severe disease, which any or several of kidney, liver, heart or lung failure, bleeding or meningitis may be presented, late administration of antibiotics has shown clinical efficacy and reduction in mortality rates (Faine *et al.*, 1999; Watt *et al.*, 1988). However, suggestive evidence supported the use of penicillin and doxycycline (Guidugli *et al.*, 2000b). Treatment of leptospirosis patients continues to be supportive management and use of appropriate

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antibiotics. Currently, recommended regimens and dosages are based on the severity of the disease. Doxycycline is recommended for both prophylaxis and mild disease (Guidugli *et al.*, 2000a; Pappas *et al.*, 1985). Ampicillin and amoxicillin are also recommended in mild disease, whereas penicillin G and ampicillin are indicated for severe disease (Watt *et al.*, 1988).

10. Diagnosis of leptospirosis

Laboratory participation in the diagnosis is mandatory because the clinical feature is not specific in either humans or animals. Even where leptospirosis is endemic and well recognized, but the existence of other similar infections can confuse diagnosis (Faine, 1998). The differential diagnosis of leptospirosis depends on the epidemiology of acute febrile illnesses in the particular area. A high index of suspicion is needed in endemic areas, and leptospirosis must be considered when a patient presents with acute onset of fever, headache, and myalgia. However, in locations where dengue fever or malaria is also present, the differentiation may be very difficult because of the similar clinical manifestations (Shieh *et al.*, 2006). The definitive diagnosis of leptospirosis depends on laboratory findings. The presence of leptospires may be confirmed by direct detection of the organisms include microscopy, antigen detection, DNA detection, and culture or indirectly by serological methods.

10.1 Likelihood of diagnosis

The probability of making a diagnosis of leptospirosis by World Health Organization (WHO) criteria depends on the clinical features, the laboratory findings and the history situation. These factors have been scored, so that they may be used to help assess the likelihood of leptospirosis in an acute phase patient. The patients were presumptively diagnosed leptospirosis according to the WHO criteria (**Appendix A**). A presumptive diagnosis of leptospirosis may be indicated if part A or part A and B score of 26 or more or part A, B and C score of 25 or more. A score between 20 and 25 suggested leptospirosis as a possible but unconfirmed diagnosis (Faine, 1982).

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10.2 Clinical features

Several non-specific findings may show increased ESR (Erythrocyte sedimentation rate), mild increases in transaminases, aminotransferases, alkaline phosphatase, and bilirubin (Bharti *et al.*, 2003; Edwards and Domm, 1960; Levett, 2001). Urinalysis showing proteinuria, pyuria, and haematuria. Hyaline and granular casts may also be presented in microscopic analysis (Edwards and Domm, 1960). CSF protein may be normal or slightly raised, while in CSF, glucose is usually normal. In patients with severe jaundice, xanthochromia may occur (Cargill and Beeson, 1947; Edwards and Domm, 1960; Wolff, 1954). Initial CSF examination may show predominance of polymorphs or lymphocytes, but later examination almost invariably shows that lymphocytes predominate (Beeson and Hankey, 1952; Cargill and Beeson, 1947).

In severe leptospirosis, there is peripheral leucocytosis with a shift to the left. Thrombocytopenia is common (Edwards *et al.*, 1982) and renal function is impaired, as indicated by raised plasma creatinine concentrations. The degree of azotemia varies with severity of illness (Alston and Broom, 1958). In icteric patients, the increase in bilirubin is generally out of proportion to other test valves of liver function, serum creatinine phosphokinase and serum amylase also may be raised (Edwards *et al.*, 1982).

10.3 Direct diagnostic methods

10.3.1 Microscopy

Direct microscopic observation is used to visualize organisms directly in body fluids such as blood, urine, CSF, and dialysate fluid or environmental specimens, to check whether cultures have grown, to identify organisms in culture, and to read microscopic agglutination tests (Faine, 1998). Leptospires may be visualized in clinical material by dark-field microscopy or by immunofluorescence or light microscopy after appropriate staining. Dark-field microscopic examination of

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body fluids has been used but is both insensitive and lacking specificity, but most authorities in the field believe that these results are fraught with false positives and false negatives. For this reason, direct dark-field microscopy of clinical specimens usually should not be performed (Levett, 2001). Approximately 10^4 leptospires/ml are necessary for one cell per field to be visible by dark-field microscopy (Turner, 1970).

However, staining methods have been applied to increase the sensitivity of direct microscopic examination. Leptospires were first visualized by silver staining. Immunohistochemical staining techniques using immunoalkaline-phosphatase (Guarner *et al.*, 2001; Meites *et al.*, 2004; Zaki and Shieh, 1996) or immunoperoxidase (Alves *et al.*, 1986) staining methods can readily demonstrate leptospiral antigens and intact *leptospira* in various tissues. Staining with immunofluorescence is most easily visible but it required equipment for determination (Faine, 1998).

10.3.2 Culture

Culture identification of leptospires is the "gold standard" but is difficult to achieve in practice because most clinical laboratories do not use the specialized media required (Vinetz, 2001). Blood cultures should be taken as soon as possible after the patient's presentation. Leptospires survive in commercially available conventional blood culture media for periods of time ranging from 48 hr to 4 weeks (Palmer and Zochowski, 2000). Other samples that may be cultured during the first week of illness include CSF and peritoneal dialysate. Urine can be cultured from the beginning of the second week of symptomatic illness. The duration of urinary excretion varies, but may be several weeks (Bal *et al.*, 1994). Survival of leptospires in voided human urine is limited, so urine should be processed immediately (Turner, 1970), by neutralization of pH and centrifugation. After centrifugation, the sediment is resuspended in buffer and inoculated into semi-solid medium containing 5-fluorouracil; the analog 5-fluorouracil should be added to the media to inhibit contaminants from overgrowing the leptospires, which grow more slowly (Farr, 1995).

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Cultures are incubated at 28-30°C and examined weekly by dark-field microscopy, for up to 13 weeks before being discarded (Levett, 2001; Levett, 2004).

Culture is difficult, requires several weeks of incubation, and has low sensitivity. Blood and CSF specimens can be collected in heparin or sodium oxalate for transport at room temperature, citrate anticoagulation should be avoided since it is inhibitory substance for the growth of *Leptospira* spp. (Wolff, 1954). Fluid media are used for primary culture. Greater yields and faster growth are usually obtained in media containing bovine albumin-Tween 80 media such as EMJH (Ellinghausen, McCullough, Johnson, Harris) than in media containing rabbit serum, 8-10% v/v (Faine, 1994), but leptospires culture media are not routinely available in clinical laboratories. Media with rifampicin, neomycin or other inhibitors have been used for primary isolation from contaminated sources, but leptospires growing in these media must be subcultured quickly into media without antibiotics to preserve their viability (Faine, 1998). Maximum density occurs in 3-10 days, but may be delayed much longer. Subcultures are made into fluid medium when growth is detected, both to maintain viability and as a precaution against contamination by slow-growing microorganisms. Isolated leptospires are identified either by serological methods or more recently, by molecular techniques that have become more widely used for identification of leptospirosis.

10.4 Molecular diagnosis

One mean for direct demonstration of leptospires in human specimens is the molecular detection. DNA-DNA hybridization and polymerase chain reaction (PCR) have been used for *Leptospira* detection in a variety of samples from both human and animals (Bal *et al.*, 1994; Kee *et al.*, 1994; Masri *et al.*, 1997; Merien *et al.*, 1992; Van *et al.*, 1989). Both techniques are sensitive and specific. Moreover, they can differentiate between pathogenic and non-pathogenic species as well as having high potential for early diagnosis of leptospirosis. DNA amplification using PCR assays can detect as few as 1-10 leptospires per ml of sample (Bharadwaj, 2004). However, this method is laborious, expensive, and time consuming. Currently, these techniques

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are still being standardized. Therefore, they are inappropriate for routine work especially in the developing parts of the world where high endemicity of leptospirosis has been established.

Several polymerase chain reaction (PCR) primer pairs have been described for amplification of leptospires, some based on specific gene targets (Renesto *et al.*, 2000), most frequently 16s or 23s rRNA genes (Hookey, 1992; Woo *et al.*, 1997) and repetitive elements (Savio *et al.*, 1994; Woodward *et al.*, 1991; Zuerner *et al.*, 1995; Zuerner and Bolin, 1997), while others have been constructed from genomic libraries (Gravekamp *et al.*, 1993; Kee *et al.*, 1994; Van *et al.*, 1989). Leptospiral DNA has been amplified from serum (Brown *et al.*, 1995; Merien *et al.*, 1995), urine (Bal *et al.*, 1994; Brown *et al.*, 1995; Merien *et al.*, 1995), aqueous humor (Merien *et al.*, 1993), and a number of tissues obtained at autopsy (Brown *et al.*, 2003).

A real-time quantitative PCR assay using TaqMan chemistry, using an 87 bp section of the 16s rRNA gene as its target, to detect leptospires in clinical and environmental samples has been reported (Smythe *et al.*, 2002). This approach, which obviates the need for agarose gel electrophoresis, thus reducing the risk of PCR amplicon contamination, has the potential for highly specific, high-throughput diagnosis of leptospirosis (Vinetz, 2001). Another targeting the LipL32 virulence factor gene using a real-time SYBR green PCR assay is currently being validated (Levett *et al.*, 2005).

PCR is a rapid detection of small numbers of leptospires in clinical samples, and more sensitive than culture for the detection of leptospires in both sera and urine samples from patients with severe leptospirosis, especially during the first few days of infection (Brown *et al.*, 1995). But the limitation of PCR-based diagnosis of leptospirosis is the inability of PCR assays to identify the infecting serovar. While this is not significant for individual patient management, the identity of the serovar has both epidemiological and public health value. Serovar identification requires isolation of the infecting strain from patients or carrier animals (Levett, 2004).

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Several molecular methods, which identify strain differences at the subspecies level can be used as additional typing systems for identification of leptospiral isolates in epidemiological studies. These include restriction endonuclease analysis (REA) (Marshall *et al.*, 1984) of chromosomal DNA by fixed-field gel electrophoresis or pulsed-field gel electrophoresis (PFGE) (Herrmann *et al.*, 1992), DNA hybridization using repetitive sequences (Pacciarini *et al.*, 1992), ribotyping (Perolat *et al.*, 1990; Perolat *et al.*, 1994) and a number of PCR-based approaches. Restriction fragment length polymorphism analysis (RFLP) has been extremely useful (Ralph *et al.*, 1993). In addition, for most bacteria, the number of fragments obtained from typical restriction enzyme is too large, making reliable comparison difficult. However, large amounts of high molecular weight DNA are needed.

10.5 Serological diagnosis

Serology is the most frequently used diagnostic approach for leptospirosis. Because of the difficulties associated with culturing leptospires and the generally low index of clinical suspicion at the time of presentation. IgM antibodies are detectable in the blood 5-7 days after the onset of symptoms (Levett, 2004) and reach to the maximum titre in 3rd or 4th week (Bharadwaj, 2004). Serological methods can be divided into two groups; genus-specific and serogroup-specific. The use of agglutination tests was described soon after the first isolation of the organism and the microscopic agglutination test remains the definitive serologic investigation in leptospirosis. Many serological methods for diagnosis of leptospirosis have been applied.

10.5.1 Microscopic agglutination test

The microscopic agglutination test (MAT) is the reference standard test for serological diagnosis of leptospires because of its high sensitivity and specificity (Cole *et al.*, 1973; Cumberland *et al.*, 1999). The MAT is a serogroup-specific assay and cannot be used to interpret reliably the identity of the infecting serovar. However, the ability of convalescent MAT titers to predict even the infecting serogroup may be

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as low as 40% (Levett, 2003). The MAT used a panel of lived pathogenic leptospiral strains as antigens for detect the diluted patient sera. The results are examined under dark-field microscopy and expressed as the percentage of organisms cleared from the field by agglutination. This assay required significant expertise to perform, interpret, and its use is restricted to a few reference laboratories. Despite these limitations, the MAT has epidemiological value, and it is often used to give an indication of the presumptive serovar or serogroup of leptospires involved in an infection (Levett, 2004).

The definitive diagnosis of leptospirosis was defined as a four-fold or greater increase in the MAT titer, or an isolation of leptospires from clinical specimens. The end-point is the highest dilution of serum in which 50% agglutination occurs. Because of the difficulty in detecting when 50% of the leptospires are agglutinated, the end-point is determined by the presence of approximately 50% free, unagglutinated leptospires, by comparison with the control suspension. The highest dilution of serum which gives the end-point is reported as "MAT titer" of that serum sample. Generally, titers of 1:100 or 1:200 should be considered suggestive of leptospirosis, and a second serum sample is then requested. If a second sample is received, the two serum samples should be tested together. Titers may go as high as 1:25,600, but the range for reactive sera is usually 1:400 to 1:6,400. Some nonspecific reactivity may occur at a 1:50 serum dilution. In endemic areas, a single titer of ≥800 in symptomatic patients is generally indicative of leptospirosis (Faine, 1988), but titers as high as ≥ 1,600 have been recommended (Alexander, 1986). Titres following acute infection may be extremely high or seroconversion may be delayed up to several weeks after recovery (Faine, 1982). The test is generally done with lived spirochetes, but Formalin-killed ones may also be used. If the latter are used, titers will be lower and the reactivity will be somewhat less specific and more cross-reactions are detected (Faine, 1982; Palmer *et al.*, 1987).

Interpretation of the MAT is complicated by the high degree of cross-reaction that occurs between different serogroups, especially in acute phase samples. Patients often have similar titers to all serovars of an individual serogroup. Of note,

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"paradoxical" reactions, in which the highest titers are detected to a serogroup unrelated to the infecting one, are also common (Alston and Broom, 1958; Levett, 2001; Tjalma and Galton, 1965). The broad cross-reactivity in the acute phase, followed by relative serogroup-specificity in convalescent samples, results from the detection in the MAT of both IgM and IgG antibodies and the presence of several common antigens among leptospires (Adler and Faine, 1978; Chapman *et al.*, 1987; Lin *et al.*, 1997).

Diagnosis of leptospirosis by MAT often requires paired serum samples which delays the diagnosis. Moreover, false negative results were frequently reported when the causative leptospire serovar was not included in the panel of typing organisms (Thiermann, 1984). Other alternatives of antibody detection assays have subsequently been developed for early diagnosis of leptospirosis. These include the hemolytic test (Cox *et al.*, 1957), complement fixation test (Robertson and Boulanger, 1963), latex agglutination test (Ramadass *et al.*, 1999; Smits *et al.*, 2000), the slide agglutination test or macroscopic agglutination test (Farr, 1995), the indirect hemagglutination assay (IHA) (Levett and Whittington, 1998; Sulzer *et al.*, 1975), the indirect immunofluorescence test (IFA) (Torten *et al.*, 1966), the microcapsule agglutination test (MCAT) (Arimitsu *et al.*, 1994), the indirect enzyme-linked immunosorbent assay (ELISA) (Adler *et al.*, 1980; Milner *et al.*, 1985), the dot-ELISA (da Silva *et al.*, 1997; Pappas *et al.*, 1985; Watt *et al.*, 1988), the LEPTO-dipstick (Gussenhoven *et al.*, 1997), and the lateral flow assay (Smits *et al.*, 2001).

10.5.2 Complement fixation test

Complement fixation test (CF) was widely used, but the methods were not standardized. CF test was applied to veterinary diagnosis, but species-specific difference was noted (Robertson and Boulanger, 1963). The antigen consists of an acidic heat-treated suspension of pooled leptospiral serogroups. Positive controls should always be included and any sera found to give positive or doubtful results should be referred to a leptospiral reference laboratory for confirmation, while false positive reactions can occur in patients suffering from mycoplasma pneumonia,

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hepatitis A and cytomegalovirus infection, but now CF tests have been replaced by ELISA methods (Adler *et al.*, 1980; Palmer, 1988).

10.5.3 LEPTO Dipstick

The LEPTO Dipstick assay is newly developed test for the diagnosis of leptospirosis and uses a broadly reactive antigen for detecting IgM antibodies. This assay is a commercial test kit from the Royal Tropical Institute (KIT), the Netherlands (Gussenhoven *et al.*, 1997). The test using the principle of immunochromatography which composed of a broadly reactive leptospiral antigens and antihuman IgM dye conjugate coated on cellulose strip to detect IgM antibodies in human sera. In 2001, Smits and coworkers have recently developed another diagnostic test, named lepto lateral-flow for rapid diagnosis of leptospirosis (Smits *et al.*, 2001). They described a lateral-flow assay for the detection of leptospira-specific IgM antibodies in human sera and used a broadly reactive leptospiral antigen to bind to leptospira-specific antibodies presented in the serum and a colloidal gold-labeled anti-human IgM antibody as the detection reagent. The assay is performed by the addition of serum and sample fluid and can be read after 10 minutes. They evaluated this test under actual field conditions in an area highly endemic to leptospirosis. The parameters of evaluation were the validity of the test results, its utility and simplicity and its requirements in terms of skill and equipment (Sehgal *et al.*, 2003; Smits *et al.*, 2001).

In Thailand, sensitivity and specificity of Dipstick method showed 77.5% and 91.3%, respectively when compared with MAT, IHA, MCAT and ELISA methods (Naigowit *et al.*, 2000), similar to Sehgal *et al*, which gave 78.7% sensitivity and 88.3% specificity similar to IgM ELISA (sensitivity 78.5%, specificity 87.6%) (Sehgal *et al.*, 1999). The sensitivities of both LEPTO dipstick and IgM ELISA are at acceptable levels even during the first week of illness when the IgM antibodies start to appear. The dipstick assay may give false-positive results at a weak (1+) staining intensity due to cross-reactivity with samples from patients with a number of other diseases including hepatitis, meningitis, malaria and with rheumatoide factor positive samples. False positive results also occur when low levels of persisting antibodies

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against leptospires are presented due to a previous exposure (Hatta *et al.*, 2000). However, the dipstick assay is cheap and its application dose not require special equipment or training. The dipstick assay is also fast and easy to perform, and the ingredients do not need to be refrigerated (Smits *et al.*, 2000).

10.5.4 Indirect fluorescent antibody test (IFA)

Current diagnostic methods for acute leptospirosis are usually based on the demonstration of serum antibodies using serological tests. Serological approaches have been developed, including the indirect immunofluorescent technique for detection of immunoglobulin IgG, IgM and IgA (Appassakij *et al.*, 1995). A genus-specific immunofluorescent antibody (IFA) test using a single broadly reactive strain of *Leptospira*, especially those regarding the sensitivity of the IFA assay with acute sera. In addition, the specificity of the IFA test and the appropriate cut-off titers have not been evaluated in tropical countries where background endemic leptospiral antibody may be present in patients with other diseases known to mimic leptospirosis. The test is performed on slides containing approximately 10^7 leptospires/ml of *L. interrogans*. The known dilutions of patient's serum and fluorescein isothiocyanate (FITC)-conjugated anti-human immunoglobulin (IgG / IgA / IgM kappa and lambda) antiserum were added, incubated, washed and observed for the bright green fluorescene of leptospires under fluorescent microscope. The diagnosis of leptospirosis examined by IFA method with a diagnostic cut-off value ≥1:100 gave 92.5% sensitivity, 95.0% specificity and 93.8% accuracy (Naigowit *et al.*, 2000) nearly similar to that reported by Appassakij *et al*. with sensitivity and specificity of 91.4% and 97.0% (Appassakij *et al.*, 1995), respectively. However, the IFA method is significantly more sensitive and specific than the other methods, but this technique has some limitations that it cannot be used if there is no fluorescence microscope and needs skilled personnel. Therefore, IFA is suitable for a reference laboratory.

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10.5.5 Indirect hemagglutination assay (IHA)

The indirect hemagglutination assay (IHA) developed at Centers for Disease Control and Prevention (CDC), USA which used sensitized red blood cells have been developed as immunological tool for serodiagnosis of leptospirosis. The extraction of an erythrocyte-sensitizing substance led to the development of both a hemolytic assay requiring complement (Cox *et al.*, 1957) and a hemagglutination assay (Sulzer *et al.*, 1975). These assays detect both IgM and IgG antibodies (Levett and Whittington, 1998). The IHA was shown to have a sensitivity of 92% and specificity of 95% compared with the MAT (Sulzer *et al.*, 1975). This test is available commercially and for many years as the only U.S. Food and Drug Administration-approved product for serological diagnosis of leptospirosis (Levett, 2001). The sensitivity and specificity of the leptospirosis IHA in Thailand shown 73.8% and 97.5%, respectively (Naigowit *et al.*, 2000), but in Hawii showed only 41% sensitivity (Effler *et al.*, 2000). The variation of sensitivity and specificity among population was recommended the serovar that can generally be found in the area, should be selected for preparing the antigen because it gives better sensitivity and specificity (Naigowit *et al.*, 2000).

10.5.6 Latex agglutination test (LA)

A rapid semi-quantitative latex agglutination test (LA) has been developed for the detection of leptospiral antibodies in serum samples of human and animals. The LA was performed on white agglutination card by mixing the patient's serum with reagent (broad reactive leptospiral antigen coated latex particles, and the agglutination will be observed within 2 minutes), the sensitivity of LA test was 84.8% of human serum and 63.1% of animal serum when compared with ELISA test, LA test was slightly lower than plate-ELISA both in human and animal sera were 85.9% and 69.2%, respectively (Ramadass *et al.*, 1999). The LA test was evaluated with group of serum samples from patients with leptospirosis and control patients from Hawii, the Seychelles, Thailand, and the Netherlands. The mean overall sensitivity and specificity were 82.3% and 94.6% (Smits *et al.*, 2000), respectively. The comparison

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of the experiments performed on groups of consecutively collected samples from several regions, which were endemic area of the disease that the latex agglutination assay may have a wide application.

In Thailand, LA showed 94.7% sensitivity, 93.3% specificity and 94.0% accuracy when compared with IFA. When paired sera were examined by the MAT, Dipstick, IFA and LA, LA detected antibodies in 39.9% of the cases in acute phase compared with IFA, Dipstick, and MAT, which gave positive reactions in 18.2, 16.1 and 12.6% of the cases, respectively. In the convalescent serum, LA gave the highest positive rate of 73.4%, followed by Dipstick, IFA and MAT with rates of 68.5, 64.3 and 53.8%, respectively (Naigowit *et al.*, 2001). Therefore, LA test is recommended as a simple, easy to perform and dose not require special skills or equipment. The reagents have a long shelf life, even at tropical temperatures. Together, these factors make the assay suitable for use even at the peripheral level of a health care system as a rapid screening test for leptospirosis (Smits *et al.*, 2000).

10.5.7 Macroscopic agglutination test

Rapid slide macroagglutination test for the screening of large numbers of human sera, available as a commercial kit called Simples Test and further referred to as the slide agglutination test (SAT). A macroscopic slide agglutination test was described in which 12 serovars were combined into four pools for the rapid screening of sera from humans and animals (Galton *et al.*, 1958). Despite the use of an expanded antigen range, false-negative results were reported for sera from populations in areas of endemic leptospirosis (Wolff and Bohlander, 1966). The macroscopic SAT was performed on glass slide by mixing the diluted serum with antigen suspension, and the agglutination will be observed within 4-6 minutes under direct light against a dark background and was recorded as positive or negative. SAT is an excellent screening test for the acute phase of leptospirosis. Its sensitivity is high at the beginning of the infection and low in the convalescent phase. SAT were similar to those of the IgM ELISA for the detection of infection early in the course of the illness,

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but SAT is inexpensive, can be performed more quickly and more easily than ELISA, and could be used by the less well equipped laboratories (Brandao *et al.*, 1998).

10.5.8 Microcapsule agglutination test (MCAT)

A microcapsule agglutination test (MCAT) has also been developed for the serodiagnosis of leptospirosis. This test is based on the passive agglutination of synthetic polymer carriers, in place of red blood cells has been evaluated extensively in Japan and China (Arimitsu *et al.*, 1982; Cui *et al.*, 1991). In an international multicenter evaluation, the MCAT was more sensitive than either the MAT or an IgM-ELISA in early acute phase samples, but failed to detect infections caused by some serovars (Arimitsu *et al.*, 1994). In Thailand, the MCAT was evaluated and showed overall sensitivity, specificity and accuracy were 90.2%, 96.3% and 93.7%, respectively. MCAT is simple, can be performed by unskilled personnel with minimum laboratory facilities. MCAT would be a reliable serodiagnostic test for rapidly screening individuals for leptospirosis (Suputtamongkol *et al.*, 1998).

10.5.9 Enzyme-linked immunosorbent assay

The most important recent advance in the diagnosis of leptospirosis has been the large-scale clinical testing of new rapid serologic assays that use enzyme-linked immunosorbant assay (ELISA) formats. ELISA is widely used because of the ability to measure specific IgM, IgG or other immunoglobulin classes. 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 (Cumberland *et al.*, 1999). Antigens used for diagnosis and epidemiological study were broadly species-reactive derived from boiled leptospires, formalin-killed leptospiral culture, or sonicated leptospiral suspension (Faine, 1998). Recently, recombinant proteins and monoclonal antibodies have been developed for leptospiral serodiagnosis (Flannery *et al.*, 2001; Saengjaruk *et al.*, 2002).

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Comparision between MAT and IgM-ELISA for diagnosis of leptospirosis were described by means of sensitivity and specificity. The sensitivity of IgM detection by ELISA was 52% in the first acute-phase specimen, increasing to 89% and 93% in the second acute-phase and convalescent specimens, respectively. The specificity of the IgM-ELISA was high (≥ 94%) in all specimens, but the sensitivity of the MAT was lower than IgM-ELISA in the first acute-phase specimen (30%), increasing to 63% in the second acute-phase specimen and 76% in the convalescent specimen. The specificity of the MAT was ≥ 97% in all specimen (Cumberland *et al.*, 1999), meaned that detection of IgM antibodies by ELISA showed high sensitivity and specificity if the blood sample is taken several days after the onset of symptoms are first noted, IgM was first detected as early as 3 days after the clinical onset of infection. The MAT detected both IgG and IgM antibodies, but titers in the MAT increase later than ELISA assays. The upper limit of sensitivity of the MAT may be as high by the time of the second acute-phase specimen. Detection of IgM antibodies by ELISA was more sensitive than MAT, and of equivalent specificity. The highest sensitivity was attained when both the IgM-ELISA and MAT were performed on each specimen.

To improve the diagnostic techniques, a dot-ELISA was evaluated for the detection of immunologic markers both in the acute (IgM) and in the convalescent (IgG and IgA) phase of leptospirosis. The overall sensitivities were 98%, 71% and 77% for IgM, IgG and IgA, respectively (da Silva *et al.*, 1997). The dot-ELISA offers good sensitivity, reproducibility, and excellent specificity, yielding accurate results. It permits the use of a single serum sample and requires small amounts of antigen consisting of sonicated strains, thus avoiding the risk of infection to those who perform the test. The technique also permits a reduction of the reaction time needed for the MAT, and provides more sensitive and specific results, mainly at the onset of the disease (Pappas *et al.*, 1985; Watt *et al.*, 1988). In addition, the dot-ELISA for the diagnosis of human leptospirosis has other advantages, such as the possibility of visual readings, reliable results that do not require special equipment, and the possibility of carrying out the test at room temperature, all of which facilitate its use in the field and in less equipped laboratories (da Silva *et al.*, 1997).

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Recently, production of monoclonal antibodies (MAbs) specific for *Leptospira interrogans*, recombinant leptospiral antigen-based ELISA including leptospiral outer membrane proteins (LipL32, OmpL1, LipL41, and LipL36), heat shock protein (Hsp 58) (Flannery *et al.*, 2001), and novel potential antigens that are expressed during leptospiral infection immunoglobulin-like protein (LigA and LigB) (Matsunaga *et al.*, 2003) have been developed as new serodiagnostic test for leptospirosis. Hybridomas were secreting MAbs specific to antigens of pathogenic leptospira. MAbs was used in a dot blot-ELISA for detecting leptospira antigen in urine samples showed high potential for rapid, sensitive, and specific diagnosis of leptospirosis (Saengjaruk *et al.*, 2002). Recombinant protein-based serologic test may achieve high sensitivity and specificity because of the high concentration of immunoreactive antigens which can be used in assays and the lack of nonspecific moieties present in whole-cell preparations. Five recombinant leptospiral proteins were evaluated as antigens in ELISA. The recombinant LipL32 ELISA demonstrated the highest sensitivity in acute (56%) and convalescent (94%) phase of illness, compared with recombinant OmpL1, LipL41, and Hsp58 had sensitivities of 16, 24, and 18% during the acute phase and 72, 44, and 32% during convalescent phase, respectively. Moreover, recombinant LipL32 IgG-ELISA also demonstrated specificity of 95% in healthy and 90-97% in other febrile illness, indicated that recombinant LipL32 can be incorporated in rapid formats, such as dipstick, to facilitate its use in serodiagnosis (Flannery *et al.*, 2001). The diagnostic potential of recombinant *lig* antigens in the kinetic ELISA assay was evaluated by using serum sample of the dog with MAT ≥1,600, which showed reactivity of 76, 41, and 35% to recombinant conserved *ligA*, recombinant Variable *ligA* and recombinant variable *ligB*, respectively. These finding suggested that recombinant antigen to the conserved region of *ligA* and *ligB* can differentiate between vaccinated and naturally infected animals (Palaniappan *et al.*, 2004). Although ELISA techniques showed high sensitivity, specificity and useful in the examination of a large number of samples in the setting of an epidemic but the limitation of this method is that it needs expensive laboratory equipment and skilled personnel. Therefore, it is recommended for use in a reference laboratory for confirmation (Naigowit *et al.*, 2000).

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II. Spirochaetal lipoproteins

Lipoproteins are universal components of eubacterial membranes. The ability of lipoproteins to decorate bacterial membranes provides for a wide variety of essential structural and functional roles. Other lipoproteins function as adhesins, enzymes, transporters, binding proteins, toxins and in a variety of other capacities essential for virulence (Madan and Sankaran, 2002). Bacterial lipoproteins consist of a large group of proteins with many different functions. The characteristic feature of all lipoproteins is a signal sequence in the N-terminal end, followed by a cysteine (Hayashi and Wu, 1990). Periplasmic and outer membrane proteins are synthesized as precursors with a signal peptide at their N-termini in the cytoplasm, and are then translocated across the inner membrane by Sec translocase (Duong *et al.*, 1997; Mori and Ito, 2001). The signal peptide is essential for translocation, but does not determine the final destinations of translocated proteins. Lipidation is directed by the presence of a cysteine-containing 'Lipobox' within the lipoprotein signal peptide sequence and this feature has greatly facilitated the identification of putative lipoproteins by gene sequence analysis.

1. Biosynthesis of lipoproteins

Processing of a lipoprotein precursor, which has a consensus sequence called a lipobox or lipoprotein box around the signal peptide cleavage site (Hayashi and Wu, 1990), to its mature form sequentially takes place on the periplasmic side of the inner membrane. The signal sequence is cleaved by signal peptidase II (SPaseII), also called lipoprotein signal peptidase (Lsp). These lipoprotein signal peptides are quite similar to the signal peptides of secreted proteins, which are cleaved by signal peptidase I (SPaseI). The signal sequence can be divided into three regions: an n-region, an h-region, and a c-region. The n-region is characterized by presence of the positive amino acids lysine and/or arginine, the h-region consists of hydrophobic amino acids, and the c-region has a characteristic region of four amino acids around the cleavage site that is vary well conserved, a so-called lipobox, as show in **Figure 1**. The most conserved amino acids in the lipobox are a leucine in position -3 from the

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cleavage site, an alanine in position -2, and a glycine or an alanine in position -1. The cysteine at position +1 is required: LA(G,A)↓C (von Heijne, 1989), it used for lipoprotein predictions.

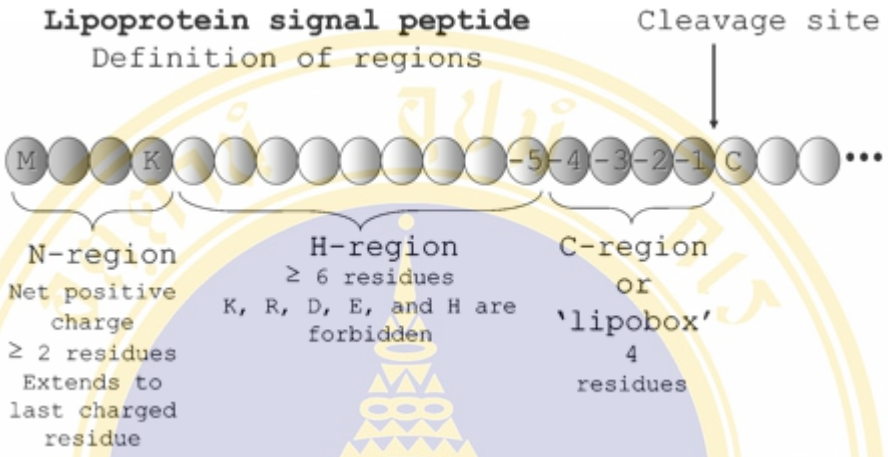

Figure 1. Definition of spirochaetal lipoprotein signal peptide regions.

Biosynthesis of lipoproteins in Gram-negative and Gram-positive bacteria consists of three steps, as shown in **Figure 2**: transfer of a diacylglyceride to the cysteine sulphydryl group of the unmodified prolipoprotein; cleavage of the signal peptide by signal peptidase II, forming an apolipoprotein: and, finally, acylation of the ∝-amino group of the N-terminal cysteine of the apolipoprotein (Sankaran and Wu, 1994). Before the processing of the prolipoprotein, which takes place on the periplasmic side of the inner membrane, the prolipoprotein is exported through the inner membrane by the general secretory pathway that is also used by secretory proteins processed by SPaseI (Hayashi and Wu, 1990). In Gram-negative bacteria, the lipoproteins are anchored to either the inner or the outer membrane, and a single amino acid in position +2 is proposed to determine the final destination of the lipoproteins (Seydel *et al.*, 1999; Yamaguchi *et al.*, 1988). Homologues for all three enzymes required for lipoprotein biosynthesis have been found in Gram-negative bacteria and also in spirochaetes. The first of the three enzymes in the lipoprotein-precessing pathway is phosphadidylglycerol: prolipoprotein diacylglyceryl transferase (Lgt). This enzyme transfers a diacylglyceryl group (containing two fatty acids) from phosphadidylglycerol to the sulfur atom of cysteine. The second enzyme in the

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pathway is prolipoprotein signal peptidase (LspA or signal peptidase II), which proteolytically removes the signal peptide, making cysteine the new N-terminal amino acid. The third enzyme is phospholipid: apolipoprotein transacylase (Lnt), which transfers a third fatty acid from a membrane phospholipid to the nitrogen atom of cysteine. After processing, lipoproteins will have three fatty acids attached to the cysteine residue (Haake, 2000).

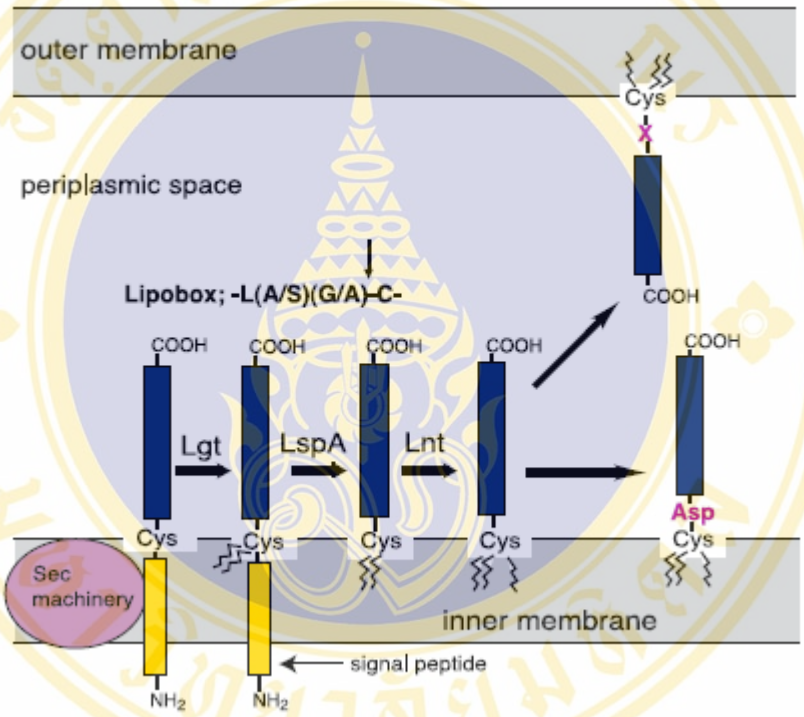

Figure 2. **Biogenesis of lipoproteins.** Consensus lipobox sequences are indicated with a signal cleavage site indicated by an arrow. X represents a residue other than Asp. Lgt, phosphatidylglycerol/prolipoprotein diacylglyceryl transferase; LspA, prolipoprotein signal peptidase (also called SPaseII); Lnt, phospholipid/apolipoprotein transacylase.

Subsequent studies of spirochetal lipoproteins revealed that proteins with disparate Lsp cleavage sites could still be lipid modified. The sequences of 26 spirochetal lipoproteins with experimental evidence of lipid modification were used to difine a spirochete Lsp consensus sequence $[L,A,S]_{-4}[L,V,F,I]_{-3}[I,V,G]_{-2}[A,S,G]_{-1}C_{+1}$ (Haake, 2000). More generally, a small neutral amino acid in the -1 position and a

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hydrophobic residue at -2, -3, or -4 seems to suffice for processing. The spirochetal Lsp consensus sequence is thus less stringent, with the absence of hydrophobic residues in the -3 position compensated for by the substitution of hydrophobic residues in the -4 and also variably in the -2 position. It has been postulated that a lower rate of protein synthesis in spirochetes may have permitted genetic drift of the spirochetal Lsp cleavage site (Haake, 2000).

Three classes of leptospiral outer membrane proteins (OMPs) have been described: transmembrane, lipoprotein, and peripheral membrane proteins. OmpL1 was the first leptospiral OMP to be described. 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). A second class of leptospiral OMPs are lipoproteins that are anchored to the outer membrane by fatty acids attached to an amino-terminal cysteine (Haake, 2000). Analysis of the detergent phase of Triton X-114 extracts of *Leptospira kirschneri* intrinsically labeled with tritiated palmitate indicates that the leptospiral OM contains at least five lipoproteins (Shang *et al.*, 1996). The genes encoding three leptospiral OM lipoproteins, LipL32, LipL36, LipL41, and LipL48, have been described (Haake *et al.*, 2000; Haake *et al.*, 1998; Haake and Matsunaga, 2002; Shang *et al.*, 1996).

LipL32 (the major outer membrane lipoprotein) is known to be the most prominent protein in the leptospiral protein profile and is an immunodominant antigen during human leptospirosis (Flannery *et al.*, 2001; Guerreiro *et al.*, 2001; Haake *et al.*, 2000). LipL41 is a surface-exposed outer membrane lipoprotein that provides synergistic immunoprotection with OmpL1 (Haake *et al.*, 1999; Shang *et al.*, 1996). Haake and coworkers characterized the LipL36 protein and demonstrated that its expression is down-regulated during mammalian infection and can be taken as a marker for studying the mechanisms by which pathogenic leptospires adapt to the host environment (Haake *et al.*, 1998). The third class, comprising the peripheral membrane proteins, contains one member, $P31_{LipL45}$, which exploits the lipoprotein

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secretion pathway to target itself to both the inner and the outer membranes (Matsunaga *et al.*, 2002).

Previously, a global analysis of leptospiral OMPs (Cullen *et al.*, 2002) identified pL21, a novel 21-kDa protein that is the second most abundant constituent of the *Leptospira interrogans* serovar Lai outer membrane proteome was identified and designated LipL21. The LipL21 sequence is well conserved among pathogenic leptospires but could not be detected in saprophytic strains. LipL21 is surface-exposed and immunogenic, being recognized by immune sera from humans and hamsters infected with leptospira (Cullen *et al.*, 2003). Recently, a family of surface-exposed leptospiral proteins has been described with a structural organization similar to that of bacterial adhesins (Matsunaga *et al.*, 2003; Palaniappan *et al.*, 2002). The identification and characterization of a new family of bacterial immunoglobulin-like (Big) domain proteins, referred to as Lig (Leptospiral immunoglobulin-like) proteins. A search of the NCBI database found that homology of the *lig* repeat region was limited to an immunoglobulin-like domain of the bacterial intimin binding protein of *Escherichia coli* (Luo *et al.*, 2000), the cell adhesion domain of *Clostridium acetobutylicum* (Nolling *et al.*, 2001), and the invasin of *Yersinia pseudotuberculosis* (Hamburger *et al.*, 1999). Two *lig* genes, designated *ligA* and *ligB*, and one pseudogene, *ligC*, were identified. The *ligA* and *ligB* appear to be inserted into the outer membrane as lipoproteins and have 10-11 immunoglobulin-like domains. *LigB* has a unique carboxy-terminal non-repeat domain, as shown in **Figure 3**. The organization of *ligC* is similar to that of *ligB* but contains mutations that disrupt the reading frame. The *lig* sequences are present in pathogenic but not saprophytic *Leptospira* species.

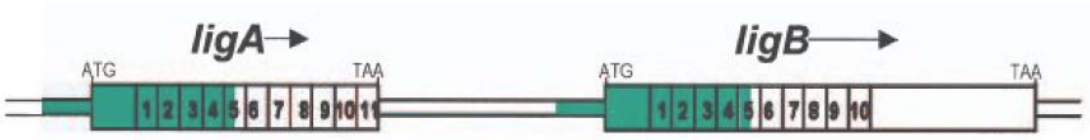

Figure 3. Schematic structure of the *ligAB* locus. The 11 and 10 Big2 domains encoded by *ligA* and *ligB*, respectively, are numbered. The >2 kb segment of DNA sequence identity between the 5' portions of *ligA* and *ligB* is indicated in green.

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LigA and ligB are expressed by a variety of virulent leptospiral strains. Loss of Lig protein and *lig* RNA transcript expression is correlated with the loss of virulence during culture attenuation of pathogenic strains. Thin section immunoelectron microscopy was performed, demonstrating surface-exposure of the Lig proteins. Immunoblot studies with patient sera found that the Lig proteins are a major antigen recognized during the acute host infection. These observations demonstrate that the Lig proteins are a newly identified surface protein of pathogenic *Leptospira*, which by analogy to other bacterial immunoglobulin superfamily virulence factors, may play a role in host cell attachment and invasion during leptospiral pathogenesis. Thus, Lig proteins may have value in serodiagnosis or as a protective immunogen in novel vaccine candidates for the prevention of leptospirosis (Matsunaga *et al.*, 2003).

Lipoproteins are of great interest in understanding the molecular pathogenesis of spirocheates. Because spirochaete lipobox sequences exhibit more plasticity than those of other bacteria, application of existing prediction algorithms to emerging sequence data has been problematic. A novel lipoprotein prediction algorithm is designated by LipoP program and Cello program. These lipoproteins were selected based on their presence only in pathogenic strain, *i.e. Leptospira interrogans* but not in *Leptospira biflexa*. Lipoprotein LA0730 was selected from the Lai genome database and predicted molecular weight about 32 kDa, predicted pI was 8.03, SPII score about 2.74, Cello program prediction score was 1.525. The characteristic feature of lipoproteins are the possession of the signal sequence in the N-teminal end, of which composed of the hydrophobic amino acid and followed by a cysteine (Hayashi and Wu, 1990). The signal sequence is cleaved by signal peptidase II (SPaseII), also called lipoprotein signal peptidase. For the prediction of cleavage sites, LipoP program were used to find SPII score (Juncker *et al.*, 2003). The lipoproteins should be located in the surface of organism and used Cello program to predict the localization of lipoprotein (Yu *et al.*, 2004).

LipoP is a method to predict lipoprotein signal peptides in gram-negative bacteria. This hidden Markov model (HMM) based LipoP program was able to

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distinguish between lipoproteins (SPaseII-cleaved signal peptides), SPaseI-cleaved signal peptides, cytoplasmic proteins, and transmembrane proteins. This approach was able to reach high precise prediction with very low error rates (Juncker *et al.*, 2003). As Juncker reported that the results obtained were predicted 96.8% of the lipoproteins correctly with only 0.3% false positives in a set of SPaseI-cleaved, cytoplasmic and transmembrane proteins. This method was also able to predict the cleavage site in both SPaseI and SPaseII-cleaved signal peptides (Juncker *et al.*, 2003).

Cello program is an approach to predict subcellular localization for gram-negative bacteria. This method uses the support vector machines (SVM) trained by multiple feature vectors based on n-peptide compositions. It is a simple, straightforward implementation of single module, which does not need specialized algorithms or particular input vectors for each subcellular localization site. Furthermore, this method gives a better predictive performances compared to those of recently developed developed multimodular PSORT-B (Yu *et al.*, 2004). Because of its simplicity, this approach can be easily extended to other organisms and should be a useful tool for the high-throughput and large-scale analysis of proteomic and genomic data.

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CHAPTER IV
MATERIALS AND METHODS

1. Human sera specimens

The serum samples used in this study was collected from various sources, *i.e.* from leptospirosis patients and from other febrile disease patients as comparative group. For leptospirosis, patients' sera were divided into three groups based on diagnostic results. The confirmed leptospirosis cases composed of the patients that leptospires could be isolated assigned as culture positive group. The second sera group was positive for IgM-lepto strip (Organon, Brussels, Belgium) but their MAT results were negative. The third sera group was positive in MAT assay, with the paired sera specimens or the single sera from individual. The control group consisted of sera from other febrile disease patients including scrub typhus, dengue hemorrhagic fever, melioidosis, from healthy persons both in and out of endemic areas.

2. Molecular cloning of *LigA*

Recombinant LigA clone was constructed and provided to this study by Asst. Prof. Dr. Potjanee Srimanote from Faculty of Allied Health Sciences, Thammasat University. Leptospiral *ligA* genes were selected to clone the genomic DNA template used in this cloning strategy was derived from *Leptospira interrogans* serovar Icterohaemorrhagiae. In brief, PCR product based on *ligA* gene incorporating with restricted enzyme site at both ends was constructed. PCR product and plasmid vector pQE 30 were then digested with 2 restriction enzymes (*i.e. Bam*HI and *Sph*I) and subjected to ligation. Oligoprimers are designed at downstream region of signal peptide and incorporation of *Bam*HI at sense primer (5'-AAGAAGGGATCCACAGAGCAAGTCACCTGGA-3') and *Sph*I at antisense primer

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(5'-AATGTTTGCATGCTATGGCTCCGTTTTAATAGAGGC-3'). The recombinant plasmid was transformed to *E. coli* M15 [pREP4] and expression was induced by 2 mM IPTG (isopropyl-β-D-thiogalactopyranoside). The transformants are selected based on Ampicillin/Kanamycin antibiotic selection gene on plasmid. Therefore the growing colonies that are resistant to both antibiotics contain the plasmid in their cells. Confirmation will be perform by using PCR amplification on each plasmid, and finally by DNA sequencing.

3. Molecular cloning of lipoprotein LA0730

Recombinant lipoprotein clone, LA0730, was previously constructed and contributed to this study using the pCATCH vector and BL21 (DE3) pLysS plus as *E. coli* host (Kalambaheti, personel communication). LA0730 clone passesses inserted protein of approximately 32 kDa, and SPII score of 2.7 the application of this recombinant lipoprotein in leptospirosis serodiagnosis are proposed to be investigated in this study.

4. Preparation of leptospiral whole cell lysate

Pathogenic and non pathogenic *Leptospira* spp. strains were used in this study (**Table 2**). They were provided by the Department of Microbiology and Immunology, Faculty of Tropical Medicine, Mahidol University. Nine *L. interrogans* strains isolated from the blood of patients with clinically diagnosed leptospirosis were used to study for the presence of LigA phenotype.

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Table 2. *Leptospira* spp. were used in this study

Species	Serovar
L. interogans	Autumnalis
	Icterohaemorhage
L. weilii	Celledoni
L.biflexa	Patoc
L. borgpetersenii	Sejroe
L. interogans (human isolated)	Strain No. 431, 448, 518, 551, 810
	UD9, UT104, UT1064
	L-88, L-383, L-562,
	L-894, L-984, L-1254

All strains of *Leptospira* spp. were grown in liquid Ellinghausen-McCullough-Johnson-Harris (EMJH) medium (Difco, Detroit, Michigan, USA) supplemented with 1% heat inactivated normal rabbit serum and 100 µg/ml 5-fluorouracil at 30°C under aerobic conditions until containing approximately 4×10^4 cells/ml. For the detection of LigA in the whole cell homogenates of the *leptospira* spp., the log phase leptospiral culturing in EMJH media was then subjected to high osmotic pressure by additional of NaCl to concentrated of 120 mM for overnight (Matsunaga *et al.*, 2003), and cells were collected for whole cell lysate preparation. The bacterial suspension was centrifuged at 10,000 ×*g* for 30 minutes at 4°C and the supernatant were discarded. The cells pellet was washed twice with PBS containing 5 mM $MgCl_2$ by centrifugation at 10,000 ×*g* for 10 minutes at 4°C. Then the pellets were resuspended in 500 µl of lysis buffer and 50 µl of lysozyme solution (concentration 20 mg/ml), followed by 3 times sonication. Bacterial cell debris was removed by centrifugation at 5,000 ×*g* for 2 minutes and the supernatant was aliquot and quickly frozen at -70°C. The dry weight was determined by placing 100 µl of the homogenized bacterial lysate into a known weight aluminium foil small container. The container which contained 100 µl of the homogenized bacterial lysate was heated under an infra-red light for 1 hour in

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order to dry the bacterial lysate. The container was weighed again. The weight of bacterial preparation in 100 µl was calculated by subtracting the later container weight with the former container weight without bacterial lysate. The weight difference is the weight of 100 µl of the bacterial lysate. This whole cell lysate was used as antigens in ELISA and Western blot analysis for determination of specific antibody from immunized mice.

5. Miniexpression of recombinant proteins by IPTG induction

This experiment was performed to select the optimal incubation period of the IPTG (isopropyl-β-D-thiogalactopyranoside) induction with an appropriate quantity of recombinant proteins. The selected single colony of *E. coli* clones were subcultured in 20 ml of LB broth, which contained 100 µg/ml of ampicillin and 25 µg/ml of kanamycin for recombinant *ligA* clone and 40 µg/ml of chloramphenicol and 25 µg/ml of kanamycin for recombinant LA0730 clone, and incubated with shaking incubator at 37°C. The culture was incubated until its optical density (OD) was determined by spectrophotometer at 600 nm and reach 0.4-0.6 (approximately 2 hours). Then, 3 ml of culture were collected for electrophoresis. The IPTG was added to final concentration of 2 mM, and keep incubating in shaking incubator for 3 hours, 3 ml fraction of culture were collected every 1 hour intevals after induction by IPTG. Those bacterial cell culture collected at each time point, be consistency 1, 2, 3 hours, then adjusted with LB or NSS to obtain the same A_{600} as the initiation point. These 3 samples, included the control cells at before induction was lysed by adding lysis buffer, then subjected to SDS-PAGE. The cell lysate sample that revealed the highest quatity and purity of protein band was selected for using as the optimal incubation period for IPTG induction.

6. Histidine-tag protein purification in native condition of LigA

The selected single colony of cloning *E. coli* was subcultured in 50 ml of LB broth containing 100 µg/ml of ampicillin and 25 µg/ml of kanamycin and incubated overnight with shaking at 37°C. Then 50 ml were taken to subculture into 450 ml of

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LB broth containing 100 μg/ml of ampicillin and 25 μg/ml of kanamycin (2,000 ml flask was used for the proper aeration) and incubated with shaking at 37°C until its OD was determined by spectrophotometer at 600 nm to reach 0.4-0.6 (approximately 2 hours). Subsequently, IPTG were added to final concentration of 2 mM and keep incubating in shaking incubator using the selected time that maximize the protein yield after IPTG induction. The cell pellet was harvested by centrifugation at 10,000 ×*g* for 10 minutes at 4°C. Supernatant were discarded and keep cell pellet (cell pellet was frozen at -20°C). The cell pellet was resuspended in 12 ml of sonication buffer (lysis buffer), follow by ultrasonic cell disruption sonicator. The cell lysate was drawn in and out through needle No. 18 for ten times in order to shorten the contaminated DNA in cell lysate. This step was necessary if cell lysate is very viscous. Supernatant was collected by centrifugation at 10,000 ×*g* for 10 minutes and kept 5 μl of supernatant and cell debris for SDS-PAGE.

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. Working Ni-NTA agarose resin (Ni-NTA ProBond; Invitrogen, USA) were 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 centrifuged at 1,000 ×*g* for 1 minute the supernatant was removed and avoid the resin get dry. The cell lysate, which contain His_6-fusion protein were added into Ni-NTA resin and incubated by rotating for 30-60 minutes at room temperature. Centrifuged at 1,000 ×*g* for 1 minute and then removed the supernatant and kept 5 μl of supernatant for electrophoresis, and washed resin for 4 times by add 8 ml of washing buffer, mixed together and centrifuged at 1,000 ×*g* for 1 minute and then removed the supernatant and kept 5 μl of washing buffer each time for electrophoresis. The mixture of His_6 fusion protein and Ni-NTA agarose resin were 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 (0.5 mM) was added and flow slowly in column containing washing buffer (0 mM). These two buffers were fused gradually, resulting in increasing concentration of this elution solution, until its concentration reach for the appropriate concentration that

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His_6-fusion protein can be eluted. Ten tubes of every 3 ml of eluted fraction were collected. Each tube was determined for expected protein by SDS-PAGE. The LigA protein-containing tubes were pooled together followed by dialysis with PBS buffer pH 7.2 in a beaker using magnetic stirrer and left at low temperature, approximately 4°C (to prevent prolong protein denaturation) for 48 hours. Protein was analyzed by SDS-PAGE gel electrophoresis and Western blot. The protein quantity was examined using Bradford assay and all prepared protein were kept at -20°C until use.

7. LA0730 Protein extraction procedure of an insoluble fusion protein under denaturing condition

E. coli cells harboring recombinant LA0730 plasmid were inoculated into 50 ml LB broth containing both 40 μg/ml of chloramphenicol and 25 μg/ml of kanamycin and incubated overnight at 37°C. Then 50 ml were taken to subculture into 450 ml of LB broth containing 40 μg/ml of chloramphenicol and 25 μg/ml of kanamycin and incubated with shaking at 37°C until its OD was determined by spectrophotometer at 600 nm to reach 0.4-0.6 (approximately 2 hours). Subsequently, IPTG was added to a final concentration of 2 mM during log phase and further incubation for another 3 hours at 37°C in shaking incubator to induce protein expression. The cell pellets were harvested by centrifugation at 8,000 ×*g* for 20 minutes at 4°C and resuspended in 2.5 ml of PBS buffer and it was mainly detected in both the crude extraction and insoluble fraction. The insoluble protein was then required to solubilization in denaturing agents such as urea prior to purification. The cell lysates were stored at -70°C until used.

After induction, the induced *E. coli* pellet was resuspened in 2.5 ml of lysis solution (BugBuster® Master Mix, Novagen) and then mixed gently until homogenous. The cell lysates were harvested by centrifugation at 8,000 ×*g* for 15 minutes at 4°C. Supernatant was decanted and kept on ice. Subsequently, the pellet was resuspended in 4 ml of the denaturant buffer (8 M Urea at pH 8.0) (**Appendix D**) and lysed them by sonicated in short pulses of 30 seconds for two times followed by centrifugation at 8,000 ×*g* for 15 minutes at 4°C. Supernatant was decanted and kept

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on ice. The supernate and the pellet suspension obtained during the extraction process were determined by SDS-PAGE using 10 μl of each sample and boiled for 5 minutes with 5 μl of SDS-PAGE sample buffer. After re-centrifugation, the remaining pellet can be futher solubilized in 4 ml of denaturant buffer (8 M Urea at pH 8.0) and the protein profiles of each fraction were observed by SDS-PAGE and the histidine fusion tag of recombinant LA0730 was detected by Western blot analysis.

8. Purification procedure of an insoluble fusion protein under denaturing condition

To purify the histidine tag fusion protein expressed in the insoluble form, the solubilized protein of induced recombinant *E. coli* cell lysate (prepared as described in method 7) was loaded into polyacrylamide gel under non-denaturing condition with no mercaptoethanol and 10% SDS, then subjected to purification process by Electro-Eluter (Model 422, Bio-Rad). The composition of the gel was as described in **Appendix E**. The electrophoresis was carried out in a descending direction in Tris-glycine buffer at constant voltage of 200 volts for about 1 hour. After electrophoresis was complete, the gel was stained in 0.25 M KCl at 4°C for 2-3 hours, then the white band that contained recombinant fusion protein was excesed for elution.

Purification of protein band from acrylamide gel was performed by using Electro-Eluter (Model 422, Bio-Rad) based on the method described in the manufacturer's instruction. To purify protein from polyacrylamide gel after electrophoresis, a gel slice containing the expected protein band was excised from a polyacrylamide gel and transferred into glass tube of the machine. Electrophoresis was performed in a descending direction in Protein Elution buffer (25 mM Tris base, 192 mM Glycine and 0.1% SDS) with constant current of 8-10 mA per glass tube for 3-5 hours. After the elution was completed, then removed the electro-eluter module from the buffer tank. The buffer in glass tube was discarded and carefully collected the liquid in the membrane cap into microfuge tube. This elution would be contained the eluted protein. The elution fractions of the purified protein were pooled and subjected to dialysis in PBS pH 7.2 for 24 hours at 4°C, with two changed of PBS

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buffer. The dialysed protein was concentrated by centrifugation at 4°C 3,000 ×*g* in Amicon Ultra 15 tube (Millipore) to reduce the fraction volume and keep at -70°C for long term storage.

9. Protein concentration assay

The quantitative assays of protein samples were performed with Bio-Rad protein assay dye reagent (Bio-Rad laboratories, USA), 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 examined protein concentration in the sample, 8 μl of the purified recombinant protein was added to the reaction containing 792 μl distilled water to obtain 1:100 dilution followed by adding 10 μl of the diluted protein into 200 μl of Bio-Rad protein assay dye reagent concentration. The reaction was incubated for at least 5 minutes at room temperature and measured the absorbance at wavelength 595 nm. Protein concentration in the sample was estimated from the protein standard curve that performed as recommended in the Bio-Rad protein assay manual.

10. Immunization and absorption of anti-sera

Animal experiments were carried out following the Guidelines and Policies of the National Research Council of Thailand (NRCT), and were approved by the Animal Ethics Committee of the Faculty of Tropical Medicine, Mahidol University.

BALB/c mice, aged 4-6 weeks with 25-30 g in weight purchased from National Laboratory Animal Center, Mahidol University, Thailand, were immunized with one dose of 10 μg of purified protein was mixed with Imject® Alum (Pierce) that contained an aqueous solution of aluminum hydroxide (40 mg/ml) and magnesium hydroxide (40 mg/ml) plus inactive stabilizers via intraperitoneal (IP) route and followed by two doses of 10 μg of purified protein Imject® Alum at 10 day intervals.

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Blood was collected by cardiac puncture two weeks after the third immunization and serum was stored at 4°C.

In order to eliminate non-specific background antibodies, mice anti-sera were cross-adsorbed with heat inactivated appropriate *E. coli* host strain harbouring the vector used for the expression of protein. An overnight culture of the appropriate *E. coli* strain was washed twice in PBS (or NSS) and then resuspended in PBS (NSS) at a density of approximately 2×10^{10} CFU/ml. The suspension was incubated at 56°C for 2 hours after cooling to room temperatured, the bacterial suspension was mixed with an equal volumn of mice anti-sera supplemented with 0.02% NaN_3. Absorption involved incubating the mixture at 37°C for 4 hours or alternatively overnight at 4°C. After each absorption, the bacteria were removed by centrifugation at 4,000 ×*g* for 10 minutes. The process was repeated 6-8 times. After the final absorption, the bacteria were removed by centrifugation, and the supernatant were filtered through 0.45 μM Millipore filter. The absorbed anti-sera were stored for short periods at 4°C or long term at -20°C.

11. Evaluation of enzyme-linked immunosorbent assay (ELISA) to detect anti-recombinant protein antibodies

Indirect ELISA was employed in evaluation of LigA and LA0730 proteins antigen againsted sera from leptospirosis patients. ELISA was performed in microtiter plate. Checkerboard titration of an antigen against leptospirosis sera was performed in order to establish the optimal conditions of antigen, antibody, conjugated and the duration of enzyme reaction.

12. Checkerboard titration

A recombinant protein was diluted with carbonate-bicarbonate buffer (coating buffer) pH 9.6 to the concentration of 2-10 μg/ml. One hundred microliters of antigen dilution were added to each well of flat-bottom microtiter plate. The plate was

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incubated at room temperature for 1 hour and followed by an overnight at 4°C in moisting chamber. After an overnight incubation, the unabsorbed antigen was removed from the plate by 3 times washing with 0.1 M PBS, pH 7.4 containing 0.05% Tween 20 (PBS-T). The surface's wells with unbound antigen were blocked with 300 µl of 0.2% BSA (Bovine serum albumin) and 0.2% Gelatin in PBS-T, incubated for at least 1 hour at 37°C or room temperature, and washed as the above, 3 times by washing buffer. Tested serum sample was diluted with PBS-T buffer into different titers and 100 µl of each dilution were put in a concentration of antigen by duplicated well. The reaction occured at 37°C for 1 hour and then washed for 3 times by washing buffer. The immune complexes were combinded with 100 µl of a dilution of conjugate (anti-human immunoglobulin in PBS-T containing 1% skim milk). Each of peroxidase conjugated rabbit immunoglobulin to human IgG dilution, 1:1,000, 1:2,000, and 1:4,000 were applied to each well, incubated at 37°C for 2 hours and then washed for 4 times by washing buffer. The reactions were visualized with 100 µl of substrate ABTS [2,2-azino-di-(3-ethyl-benzthiazoline sulfonate)](KPL, Gaithersburg MD, USA) and incubated in the dark at room temperature. Absorbance values were measured at wavelength 405 nm by ELISA reader (TECAN, Austria). The appropriate concentration of antigen, the reference serum dilutions and conjugate dilutions were used for further studies.

13. Sodium dodecyl sulphate-polyacrylamide gradient gel electrophoresis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to visualize purified recombinant proteins and whole cell lysates of *Leptospira* species.

An antigen was analyzed by SDS-PAGE consisting of 5% (w/v) stacking gel and 13% (w/v) separating gel. The separating gel was prepared in a vertical slab minigel electrophoresis system, under reducing condition as described by Laemmli (1970), overlaid with isopropanal and allowed to polymerize for 20 minutes at room temperature. After isopropanol was removed, a stacking gel solution was prepared

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and poured on the top of separating gel and inserted a multi-well comb or a single well comb. Polymerization of gel was allowed at least 45 minutes, leave gel set and washed the well with electrode buffer or DW to get rid of unpolymerised acrylamide mix. A volume of individual sample was treated with a volume of sample buffer (0.25 M Tris-HCl, pH 6.8, 8% SDS, 40% glycerol, 20% mercaptoethanol and 1% bromphenol blue), then heat at 95°C for 10 minutes. Samples were applied with standard molecular weight marker at least one well of each gel for calculation of the relative molecular weight of protein. The separation of all samples were done by constant voltage 200 V for mini-gel (1.5 hours for 12% gel) or 300 V for big gel or 20 mA for each 0.75 mm-thick gel for day time running (double for 2 gels) (also double for 1.5 mm-thick gel [such as big gel spacer]) or 8-12 mA for overnight running depending on the percent gel and the thickness of gel until the tracking dye reached to the bottom of the gradient gel. The known protein standard markers were simultaneously separated in the gel. The protein products were alternatively analyzed following staining by coomassie brilliant blue and western blot analysis.

14. Protein staining

The separating proteins in a gel were stained with coomassie brilliant blue K250 for one hour. Excess stain was removed by placing the gel in destaining solution with several changes of the solution until the background of the gel was clear.

15. Western blot analysis

Immunoreactive components of the antigens were identified by Western blot technique. The fractionated proteins in a gel were electrotransferred onto nitrocellulose membrane or PVDF membrane (pre-activated membrane by methanol) by semi-dry transfer blotter. The electrophoretic transfer was accomplished with a constant electric current at 15 V for 1 hour. After blotting, the gel was stained with coomassie brilliant blue stain to assure complete transfer and check band on nitrocellulose membrane by staining with 0.1% Ponseua S for 5 minutes, washed by DW for many times before subjected to react with serum.

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The non specific proteins binding on nitrocellulose sheet were blocked with PBS-T buffer containing 5% skim milk at room temperature for 30 minutes on the rocking platform and then, the membrane was incubated with the tested sera diluted in PBS-T buffer containing 0.02% skim milk at 37°C for 4 hours with agitation. After that, the membrane was further incubated at 4°C overnight. After overnight incubation, the nitrocellulose membrane was washed 4 times with PBS-T buffer for 5 minutes each and then reacted with diluted goat anti-mice immunoglobulins conjugated with alkalinephosphatase in PBS-T buffer for 1-16 hours at 37°C with agitation and washed for 4 times as above, finally washed with DIG III solution (0.1 M Tris-HCl, pH 9.5, 0.1 M NaCl, 50 mM $MgCl_2$) for 5 minutes. After the final washed, freshly prepared substrate solution (10 ml of DIG III, 45 µl of NBT, 35 µl of BCIP for alkaline phosphatase or DAB for peroxidase) were added and incubated in the dark at room temperature until the bands can be visualized. The reaction was stopped by washing with distilled water until background become clear. The relative molecular weight of the reacted bands was determined by their relative mobility compared to the standard molecular weight marker.

16. Statistic analysis

The diagnostic parameters, *i.e.* sensitivity, specificity, accuracy, positive predictive value (PPV) and negative predictive value (NPV) of the recombinant LigA based-IgM/IgG ELISAs, were evaluated according to the method described by Vecchio (Vecchio, 1966). Mean, standard deviation of optical densities were used to analyzed in ELISA. The ELISA has a set of characteristics that reflects the formation expected in correctly classify the patients with MAT assay, which was recommended as gold standard by WHO. The outcomes obtained as true positive, false positive, true negative and false negative. These characteristics can be easily displayed by a simply binary table often referred of as a “2 by 2” table, as shown below,

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		MAT		
		Positive	Negative	Total
ELISA	Positive	TP	FP	TP+FP
	Negative	FN	TN	FN+TN
	Total	TP+FN	FP+TN	TP+TN+FP+FN

TP (True positive) = Number of patients with MAT positive in whom the test is positive

FP (False positive) = Number of patients without MAT positive in whom the test is positive

FN (False negative) = Number of patients with MAT positive in whom the test is negative

TN (True negative) = Number of patients without MAT positive in whom the test is negative

16.1 Sensitivity

The sensitivity of a test is a probability that the test procedure or result will be positive when a disease is present. It can be expressed in percentage as shown in the formula below:

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

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16.2 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

16.3 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

16.4 Positive predictive value

The positive predictive value is the probability that the subject has the disease, given they have tested positive. It can be expressed in percentage as shown in the formula below:

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

16.5 Negative predictive value

The negative predictive value is the probability that the subject does not have the disease, given that they have tested negative. It can be expressed in percentage as shown in formula below:

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

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CHAPTER V
RESULTS

1. Human sera specimens

The serum samples used in this study included leptospirosis patients and patients suffering from other febrile illness as comparative group. The other febrile disease included scrub typhus, dengue hemorrhagic fever, melioidosis. For leptospirosis cases, patients' sera were further sub-divided into three groups based on diagnostic results. Firstly, the patients that leptospires could be isolated assigned as culture-positive group. Secondly, sera group was positive for IgM-lepto dipstick but their MAT assay were negative. Finally, sera group was positive in MAT assay, of which the paired sera or the single sera samples were collected. The serum from healthy persons both in and out of endemic area was included as the controls.

Serum samples were divided into seven groups of subjects (**Table 3**): Group 1 included 67 patient sera that MAT positive (paired sera from 22 patients and single serum from 45 patients). Group 2 included 37 patients who had MAT negative composed of 26 patients of culture positive (paired sera from 16 patients and singles sera from 10 patients) and 11 patients of IgM-lepto dipstick positive (2 singles sera and 9 pair sera). Group 3 included 61 patients of scrub typhus. Group 4 included 30 patients of melioidosis. Group 5 consisted of 43 patients of Dengue hemorrhagic fever (DHF) and group 6 and 7 consisted of healthy individuals sera composed of 30 healthy endemic areas in group 6 and 80 healthy individuals from non endemic area in group 7. Only single serum samples were collected from all subjects of group 5, 6 and 7.

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Table 3. Human sera specimens

Group	Serum samples	Single serum	Pair serum	Total
	Confirmed Leptospirosis case			
1	- MAT positive	45	22	89
2.1	- MAT negative, Culture positive	10	16	42
2.2	- MAT negative, IgM dipstick positive	2	9	20
	Other disease			
3	- Scrub typhus	61	-	61
4	- Melioidosis	30	-	30
5	- Dengue hemorrhagic fever	43	-	43
	Normal control			
6	- High endemicity	30	-	30
7	- Low endemicity	80	-	80
	Total	301	94	395

2. Production of recombinant protein

Two recombinant leptospiral lipoproteins were investigated in this study; LA0730 and LigA. The two proteins demonstrate characteristic feature of lipoproteins which are the possession of the signal sequence in the N-terminal end containing the hydrophobic amino acid and followed by a cysteine (Hayashi and Wu, 1990). These signal sequence is cleaved by signal peptidase II (SPaseII), also called lipoprotein signal peptidase. For the prediction of cleavage sites, LipoP program was used to find the SPII score (Juncker *et al.*, 2003). The lipoproteins should be localized on the surface of organism as predicted by Cello program (Yu *et al.*, 2003). These lipoproteins were selected based on their presence exclusively in pathogenic strains, *i.e. Leptospira interrogans* but not in *Leptospira biflexa*. Lipoprotein LA0730 was selected from the Lai genome database and predicted molecular weight to be 32 kDa, the predicted pI is 8.03, SPII score is about 2.74, Cello program prediction score is 1.525 suggesting its outermembrane localization. LA0730 gene was aimed to be cloned into pCATCH vector at *Nco*I and *Bam*HI sites. At the 5'-end of sense primer was incorporated with *Nco*I enzyme site; 5'-CTGTACCCATGGA GTTGTCCGAGTCTAT-3', while at the 3' end of LA0730 antisense primer, *Bam*HI

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site was incorporated 5'-TTTCCAGGATCCTTCAGGATAAGCTTTA-3'. Each constructed recombinant plasmid was then transformed into BL21 (DE3) *E. coli* host strain, harbouring pLysS.

LigA is a member of the family of bacterial proteins containing immunoglobulin-like repeats. Several proteins in this member have been identified as adhesins involved in microbial pathogenesis, such as intimin in *Escherichia coli* and invasin in *Yersinia pseudotuberculosis*. The LigA protein was expressed only on the surfaces of leptospiral pathogens isolated from infected animals and not by saprophytic *Leptospira* species. Lig is presented in low-passage number culture of infective organisms but was absent in highly passage number of attenuated leptospiral culture that have lost virulence in a hamster model of leptospirosis (Matsunaga *et al.*, 2003). Sera from patients recovering from leptospirosis contain antibodies to Lig proteins, confirming their expression by infectious spirochetes. Thus, the Lig proteins appeared to be closely associated with infection of the mammalian host, suggesting their roles as protective immunogens and diagnostic candidate. *LigA* sequence was selected from *Leptospira interrogans* serovar Icterohaemorrhagiae genome database (Accession number AY221109). Recombinant *ligA* clone was constructed from PCR product derived from the primer pairs designed from *ligA* gene incorporating with restriction enzyme site at both ends. PCR product and plasmid vector pQE 30 were then digested with 2 restriction enzymes (i.e. *Bam*HI and *Sph*I) and subjected to ligation. Oligoprimers are designed at downstream region of signal peptide and incorporation of *Bam*HI at sense primer (5'-AAGAAGGGATCC ACAGAGCAAGTCACCTGGA-3') and *Sph*I at antisense primer (5'-AATGTTT GCATGCTATGGCTCCGTTTTAATAGAGGC-3'). The recombinant plasmid was transformed to *E. coli* M15 [pREP4].

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3. Characterization of recombinant protein LA0730

3.1 Protein extraction procedure by denaturating condition

The lipoprotein LA0730 recombinant protein was successfully expressed by induction with 1 mM IPTG during log phase, and it was mainly detected in the insoluble fraction. The insoluble protein was then required to solubilization in denaturing agents such as urea prior to purification.

LA0730 recombinant clone was cultured in 300 ml of LB broth to log phase growth and induced with 1 mM IPTG. After induction, the induced *E. coli* pellet was harvested by centrifugation and lysed in BugBuster solution. At this point, enzyme (Benzonase® Nuclease) lyses cell and reduces the viscosity of the extraction. After re-centrifugation, the pellet of the lysate that mostly contained inclusion body was collected and subjected to solubilization in 8 M Urea buffer (pH 8.0). SDS-PAGE profile of the *E. coli* lysate was performed and stained with Coomassie brilliant blue K250, the result was shown in **Figure 4**. The supernatant contained crude solubilized protein of *E. coli* cells after extraction by Bugbuster solution was shown in lane S/N1. The cell pellet fraction was solubilized in 8 M Urea buffer and contained the prominent recombinant protein was shown in lane U1-U5 and P. The histidine fusion tag of recombinant LA0730 was detected by Western blot analysis and revealed the molecular weight of 37 kDa, as shown in **Figure 5**. The histidine fusion protein was mostly confined in the pellet, as only the faint band was revealed in supernate fraction (lane S/N1). As the pellet fraction was insolubilized in water, it was then required the denaturating buffer, 8 M Urea, to dissolve the pellet protein.

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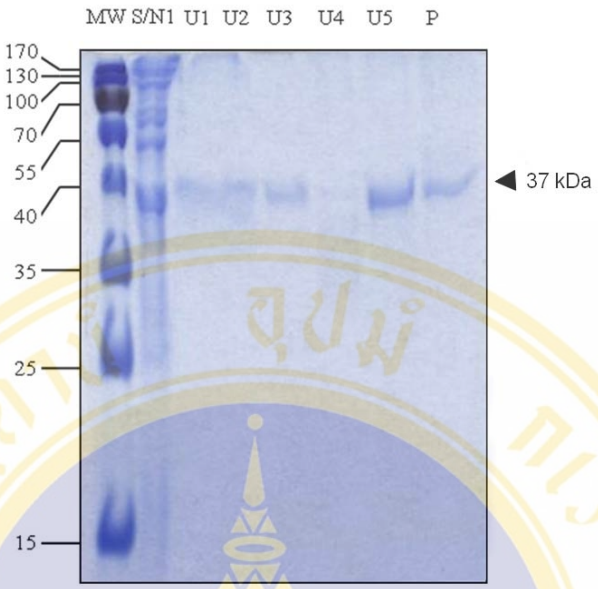

Figure 4. The product of recombinant LA0730 protein after solubilization under denaturing condition. After IPTG induction, the induced *E. coli* pellet was harvested by centrifugation and lysed in BugBuster solution. The SDS-PAGE profile of the *E. coli* lysate was performed and stained with Coomassie brilliant blue. The supernatant contained crude solubilized protein in *E. coli* cells was shown in lane S/N1. The cell pellet fraction was solubilized in 8 M Urea buffer and contained the prominent recombinant protein was shown in lane U1-U5 and P. Arrow indicates 37 kDa protein band of LA0730.

Lane MW; Protein molecular weight marker

Lane S/N1; The first supernate obtained after Bugbuster treatment

Lane U1-U5; The fraction of solubilized *E. coli* lysate in 8 M Urea that was contained the recombinant protein product

Lane P; The cell pellet of the recombinant protein product

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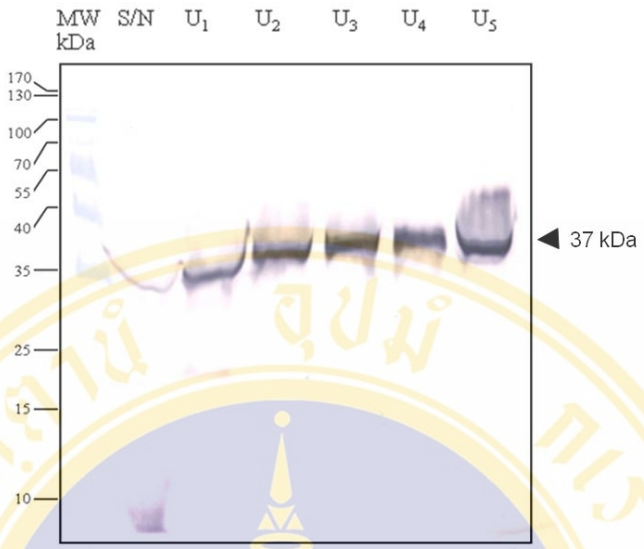

Figure 5. Histidine fusion tag of LA0730 revealed by Western blot. The protein samples were collected during the Bugbuster extraction procedure. The histidine fusion protein was mostly confined in the pellet, as only the faint band was revealed in supernate fraction (lane S/N), while the histidine fusion marker was clearly viewed in pellet fraction, solubilized in denaturating buffer, 8 M Urea. Arrow head indicates 37 kDa protein band of LA0730.

Lane MW; Protein molecular weight marker

Lane S/N; The first supernate obtained after Bugbuster treatment

Lane U1-U5; The fraction of solubilized *E. coli* pellet in 8 M Urea that contained the recombinant protein product

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3.2 Electroeluted fraction of protein band from SDS-PAGE of urea solubilized protein

The LA0730 protein could not be purified by Ni-NTA affinity column due to its inability to bind to the Nickle molecule on the resin. Therefore, it was subjected to purification process by using Electro-Eluter (Model 422, Bio-Rad). The urea solubilized *E. coli* pellet was prepared and subjected to SDS-PAGE under native condition, without mercaptoethanol and 10% SDS in the sample buffer and separating gel. The protein profile was stained with 0.25 M KCl at 4°C for 1-2 hours, then the white band that contained recombinant fusion protein was excised for elution. The Electro-eluted fraction was viewed by Coomassie brilliant blue stain as shown in **Figure 6**. All fractions of the purified protein were pooled and subjected to dialysis. The protein and histidine fusion tag were revealed as 37 kDa protein band using coomassie blue K250 staining and Western blot analysis (**Figure 7**).

4. Immunoreactivity of LA0730 protein to normal and immunized mice sera

The purified protein of LA0730 was used to immunize mice *via* intraperitoneal route. The doses used were 10 μg in the first dose and boosted twice with 20 μg in two-week intervals. The recombinant LA0730 fusion protein was electrophoresed and blotted onto a nitrocellulose membrane, then cut into strips and used for testing with mice anti-sera (pre and post-immunized sera), followed by goat anti-mice IgG conjugated to alkaline phosphatase (Southern Biotech, USA) at dilution 1:2,000 as secondary antibody and detected by NBT/BCIP substrate. The two-fold serial dilution (starting at 1:400 dilution) of immunized mice sera were reacted to each strip of the recombinant fusion proteins. The immunesera titer was revealed to be higher than 1:102,400 (lane 1-7). Although the overall intensities were weaker, the background reactivity between normal serum against purified protein LA0730 was shown titer higher than 1:102,400 similar to those of immunized mice serum as shown in **Figure 8** (lane 8-14).

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Figure 6. SDS-PAGE profile of Electroeluted fraction of protein band derived from urea solubilized protein preparation of LA0730 clone. The expected 37 kDa protein band of LA0730 is indicated by arrow head.

Lane MW; Protein molecular weight marker

Lane 1-9; The electro-eluted fraction of protein band of urea solubilized protein, obtained from each eluted chamber

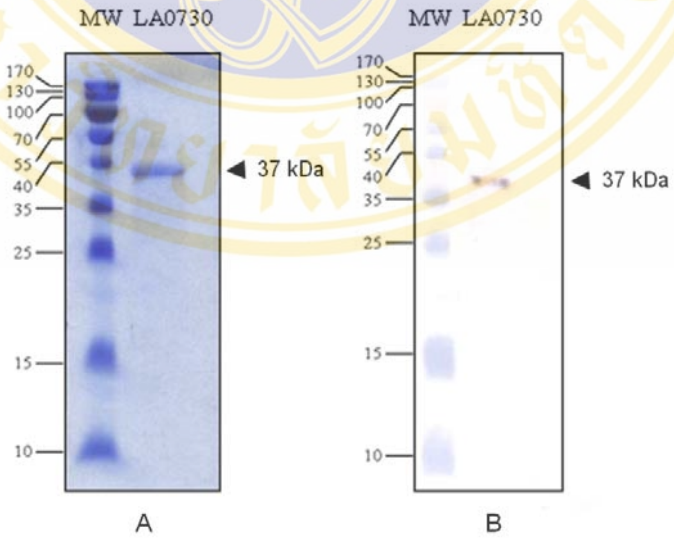

Figure 7. SDS-PAGE and histidine fusion tag of LA0730 protein obtained from electroelution of urea solubilized protein. Gel stained with Coomassie brilliant blue shown in 7A, while histidine fusion tag shown in 7B. The 37 kDa LA0730 proteins are indicated by arrow heads.

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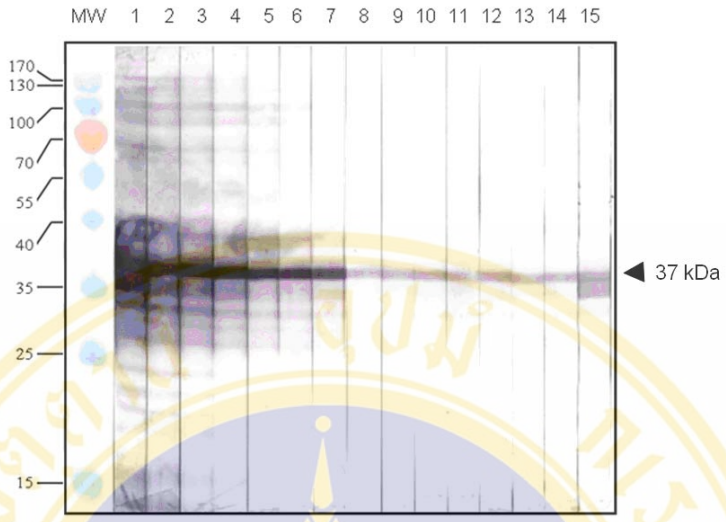

Figure 8. Immunoreactivity of LA0730 protein to normal and immunized mice sera. The LA0730 protein was immunoblotted onto a nitrocellulose membrane, then cut into strips and used for testing with mice sera (pre- and post-immunized) followed by goat anti-mice IgG conjugated to alkaline phosphatase (Southern Biotech, USA) at dilution 1:2,000 as secondary antibody.

Lane MW; Protein molecular weight marker

Lane 1-7; LA0730 protein reacted with immunized mice anti-sera of serial dilution from 1:400-1:25,600

Lane 8-14; LA0730 protein reacted with mice anti-sera (before immunized) of serial dilution from 1:400-1:25,600

Lane 15; LA0730 protein reacted with anti-histidine (1:2,000 dilution)

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5. Western blot analysis of LA0730 with MAT-positive leptospirosis patient sera

To examine the antigenicity of recombinant protein LA0730 whether they could react to leptospirosis patients' sera, Western blot analysis was performed and the positive reaction was focused at the expected molecular weight of recombinant protein (37 kDa). Urea solubilized protein was run on SDS-PAGE and the proteins were blotted onto nitrocellulose membranes. The membranes were then blocked, cut into strips and then used for testing with each leptospirosis patient's sera. The blotted nitrocellulose membranes were reacted with 1:100 dilution of leptospirosis patients' sera overnight, washed and then further incubated with 1:2,000 dilution of goat anti-mice IgG conjugated with HRP (Southern Biotech, USA), and detected by enzymatic reaction. The 16 samples with positive in MAT results were used in this experiment. All sera were likely to react with other protein components in crude urea solubilized of LA0730 but only the faint positive band of LA0730 at the expected molecular weight of 37 kDa were seen as shown in **Figure 9**.

Apart from Western blot assay using crude urea extract, the purified LA0730 protein were also used to react against leptospirosis sera which were clinically diagnosed as leptospirosis and shown high titers of MAT. **Figure 10** depict the results of the Western blot assay of sera from two leptospirosis patients. The purified LA0730 protein was blotted onto PVDF membrane. Then, react to the serial dilutions of each serum *i.e.* 1:200, 1:400 and 1:800 respectively. The immunoblot was further reacted to goat anti-mice IgG conjugated with HRP at 1:2,000 dilution (Southern Biotech, USA), and detected by DAB substrate. For comparative study, two normal healthy sera in endemic area were included. Although, the previous experiment revealed that many protein composites in the crude urea solubilized protein from clone LA0730 could react with leptospirosis patients' sera, the purified LA0730 protein, on the other hands, could not react either with normal healthy sera in endemic area and leptospirosis patients' sera, as shown in **Figure 10**.

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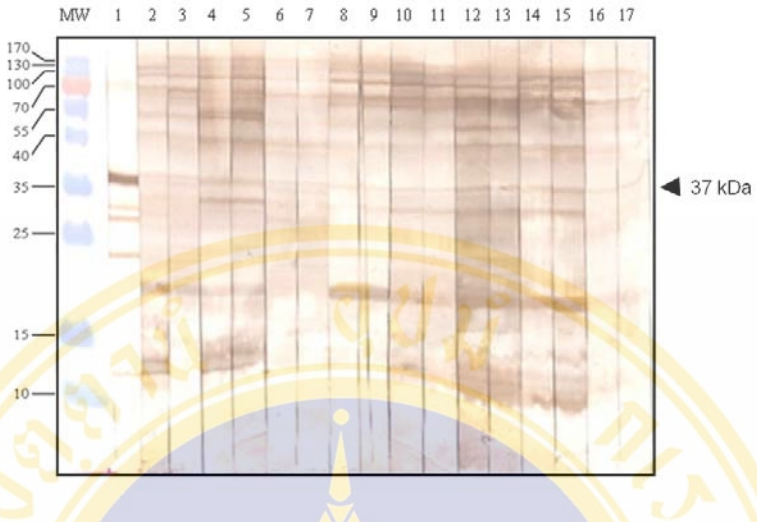

Figure 9. Western blot analysis of LA0730 with human serum positive to MAT test. Urea solubilized fraction protein was employed to react with leptospirosis patients' sera of 1:100 dilution.

Lane MW; Protein molecular weight marker

Lane 1; Urea solubilized protein reacted with anti-histidine antibodies

Lane 2-17; Urea solubilized protein reacted with human serum positive to MAT test

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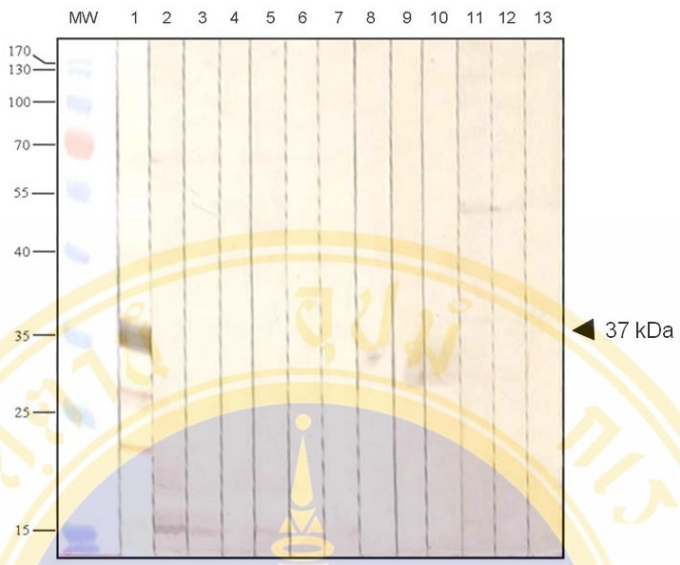

Figure 10. Immunoreactivity of purified LA0730 protein to human serum. The purified LA0730 protein was blotted onto PVDF membrane, then, reacted to serial dilution 1:200, 1:400 and 1:800 of individual serum. The serums included in this experiment were two MAT positive sera and two normal healthy persons from endemic area.

Lane MW; Protein molecular weight marker

Lane 1; Purified LA0730 protein reacted with anti-histidine fusion marker

Lane 2-4; MAT positive sera (No. 1) of dilution 1:200, 400, 800, respectively

Lane 5-7; MAT positive sera (No. 2) of serial dilution as above

Lane 8-10; Healthy person from endemic area (No. 1) of serial dilution as above

Lane 11-13; Healthy person from endemic area (No. 2) of serial dilution as above

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6. Immunoreactivity of mice anti-serum to LA0730 against native leptospiral protein

The purified LA0730 protein was used to immunize mice. It was then examined whether the anti-serum to recombinant protein would react with native leptospiral cell lysate using Western blot analysis. The mice anti-serum specific to recombinant LA0730 protein was firstly absorbed with formalin-fixed *E. coli* host cell, in order to eliminate the non specific antibodies. The total membrane proteins of *Leptospira* species were employed as antigen in Western blot. The blotted nitrocellulose membranes were reacted with dilution of 1:10,000 from mice anti-LA0730 serum and pre-immunized sera. It was found that, mice anti-serum to LA0730 was not react to leptospiral antigen but cross reactivity between anti-LA0730 serum and *E. coli* cell lysate could be identified at the molecular weight marker of approximately 37 kDa. The antigenicity of LA0730 was more related to *E. coli*, while leptospiral native whole cell lysate did not shared antigenic epitope to LA0730. The results were shown in **Figure 11**.

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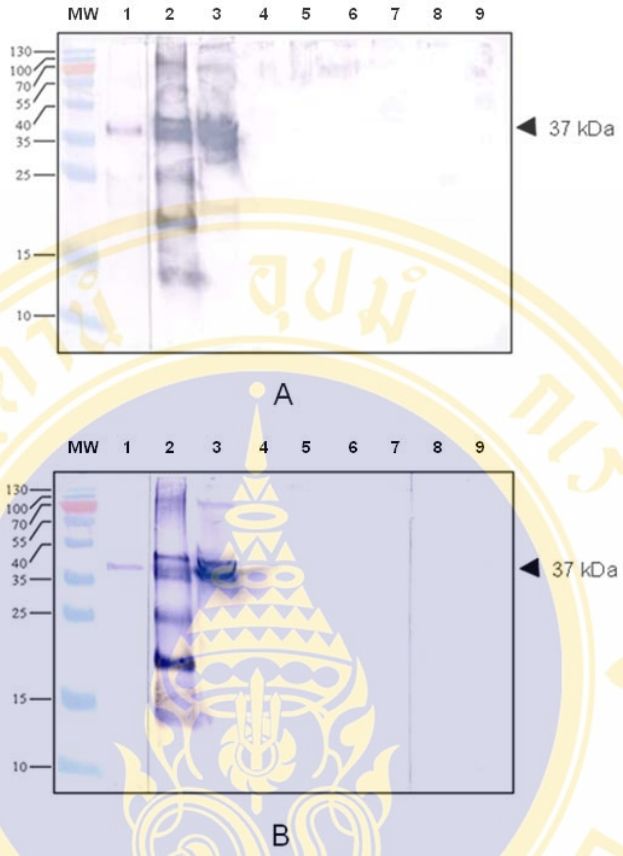

Figure 11. Immunoreactivity of anti-serum to LA0730 against native leptospiral protein. The whole cell lysate proteins of various leptospiral serovars were employed as antigen of which the panels were as follows. The blotted nitrocellulose membranes were reacted with the 1:10,000 dilution of mice anti-LA0730 serum and pre-immunized mice anti-serum. Lane MW; Protein molecular weight marker. Mice anti LA0730 were reacted to following protein panels; lane 1A, 1B as control; Crude LA0730 protein reacted with anti-histidine. Lane 2A, 2B; Crude LA0730 protein as positive control. Lane 3A, 3B; *E. coli* BL21 (DE3), harbouring pLysS as negative control. Whole cell lysate proteins of *Leptospira* species reacted with mice anti-LA0730 serum; (4A-8A) *L. interogans* (human isolate), (9A) *L. interogans* strain Autumnalis, (4B) *L. weilii* serovar Celledoni, (5B) *L. interogans* serovar Icterohaemorhage, (6B) *L.biflexa* serovar Patoc, (7B) *L. borgpetersenii* serovar Sejroe. Pre-immunized mice serum was reacted to leptospiral panel protein; (8B) *L. weilii* serovar Celledoni, (9B) *L.biflexa* serovar Patoc.

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7. Characterization of recombinant LigA protein.

7.1 Protein purification of LigA protein under native condition

For preparation and purification of recombinant LigA proteins, the LigA was inducely expressed in M15 [pREP4] *E. coli* host by Isopropyl-β-D-thiogalactopyranoside (IPTG). The bacterial cells were collected and then the pellet was resuspended in lysis buffer and subject to sonication until complete cell lysis was obtained. The crude extraction was subjected to centrifugation to remove cell debris and the supernatant was loaded onto a 2 ml Ni-nitrilotriacetic acid resin column (Ni-NTA). The column was allowed to be equilibrated with supernatant for at least one hour and the flow-through supernatant was then collected. The column was then washed with 40 ml of LEW buffer. The recombinant LigA was then eluted gradienlly with 30 ml of LEW buffer composing of immidazole of the concentration ranged from 0 to 0.5 M. Three ml fractions were sequentially collected and analyzed by SDS-PAGE gel electrophoresis as shown in **Figure 12**. Fractions containing the recombinant LigA were pooled and subjected to dialysis. The recombinant LigA protein had molecular weight about 60 kDa and its histidine fusion tag could be detected in Western blot analysis.

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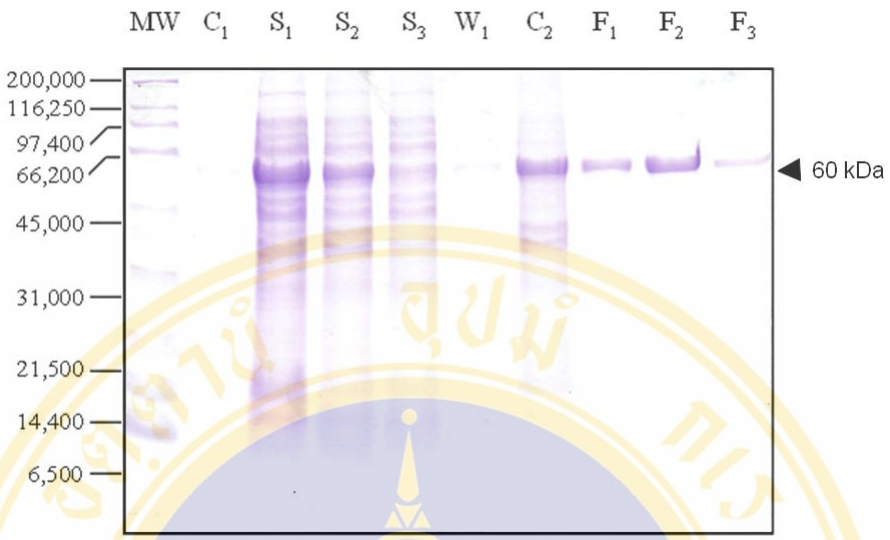

Figure 12. Protein purification of LigA protein under native condition. LigA Protein was subjected to 12% SDS-PAGE and determined by Coomassie brilliant blue stain.

MW; Protein molecular weight marker
C1; Control (Purified LigA protein)
S1-S3; Flow-through supernate
W1; Wash solution
C2; Crude extracted protein
F1-F3; Purified Protein eluted by LEW buffer containing gradient concentration of immidazole phase indicate the molarity of immidazole

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8. Serum titer of mice anti-LigA against purified LigA protein

Each of six female BALB/c mice (6 weeks old) was immunized via intraperitoneal route with purified recombinant LigA protein. The doses used were 10 μg of purified recombinant LigA in 0.2 ml of PBS containing 5 μg of alum adjuvant in the first dose and boosted with 20 μg in two-week interval. The mice were bled by cardiac puncture two week after the second booster. The sera of all mice were pooled and cross-absorbed with heat inactivated whole cells of a M15 [pREP4] *E. coli* strain harbouring pQE30 vector. Then the pooled immunized mice serum was reacted with the recombinant fusion proteins and the serum titer to each recombinant protein was higher than 1:512,000 by Western blot technique as shown in **Figure 13**.

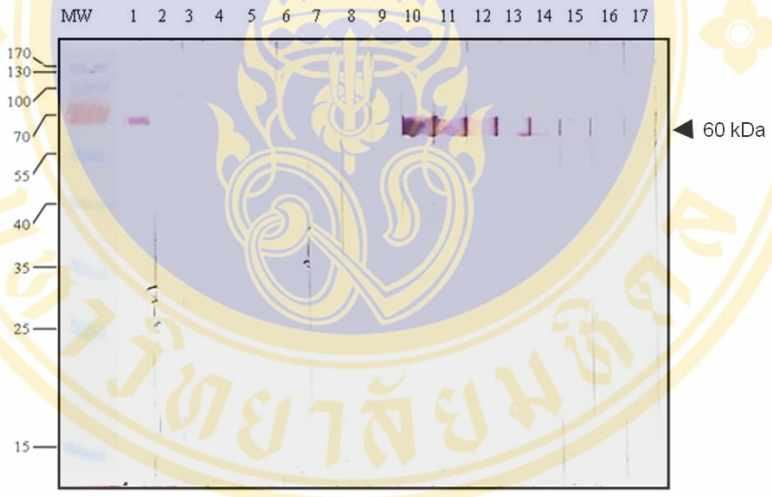

Figure 13. Serum titer of mice anti-LigA against purified LigA protein. The LigA protein was immunoblotted onto a nitrocellulose membrane, then cut into strips and used for testing with mice anti-sera (pre and post-immunized sera) and histidine fusion tag marker was shown as positive control.

Lane MW; Protein molecular weight marker
Lane 1; Histidine fusion tag marker
Lane 2-9; Reactivity with pre-immunized mice anti-sera using the two-fold serial dilution from 1:4,000-1:512,000
Lane 10-17; Reactivity with mice anti-sera (Immunized mice) using the two-fold serial dilution from 1:4,000-1:512,000

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9. Western blot of mice anti-LigA against whole cell lysate of *Leptospira* spp.

It was then examined whether the anti-serum to purified LigA protein would react with native leptospiral cell lysate or not by Western blot analysis. The pooled mice anti-serum specific to recombinant LigA protein was firstly absorbed with *E. coli* M15 [pREP4], harbouring pQE30 lysated, in order to eliminated the non specific reaction against *E. coli* host. The whole cell lysate protein of *Leptospira* species was employed as antigen in Western blot. The blotted nitrocellulose membranes were reacted with 1:64,000 dilution of mice anti-LigA serum and 1:4,000 dilution of pre-immunized mice anti-serum and histidine fusion marker of protein as positive control. It was found that, no reaction band of mice serum was detected to leptospiral antigen and cross reaction between anti-LigA serum and *E. coli* cell lysated could not found in the expected band molecular weight marker. It was suggested that the antigenicity of recombinant LigA protein whether their epitopes were not shared with both the *E. coli* and leptospiral protein. The results were shown in **Figure 14**.

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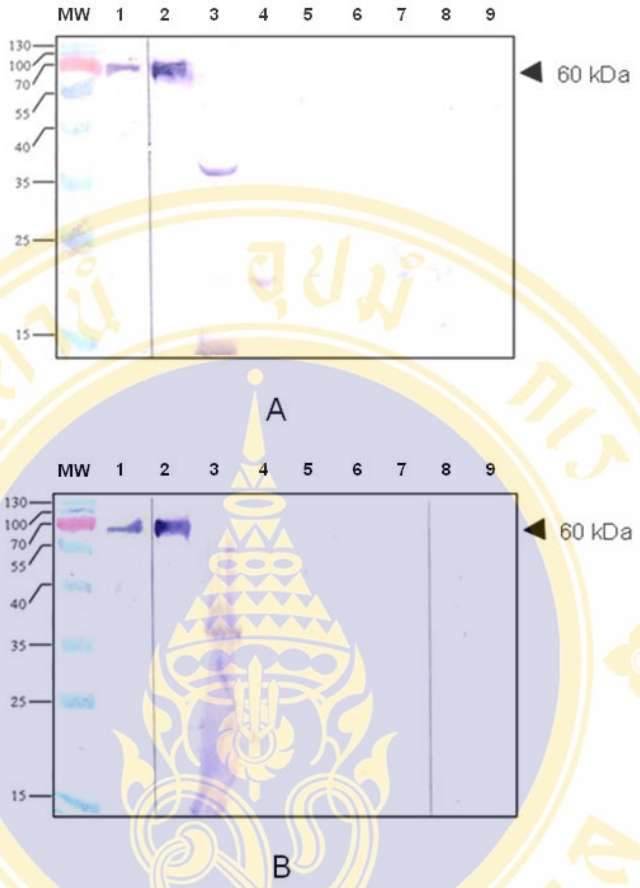

Figure 14. Western blot of mice anti-LigA againsted whole cell lysated of *Leptospira* spp. The total membrane proteins of various leptospiral serovars were employed as antigen in Western blot. The blotted nitrocellulose membranes were reacted with the 1:64,000 dilution of mice anti-LigA serum and pre-immunized mice anti-seum. Lane MW; Protein molecular weight marker. Mice anti-LigA was reacted to following protein panels; lane 1A, 1B as control; LigA protein reacted with anti-histidine. Lane 2A, 2B; LigA protein as positive control. Lane 3A, 3B; *E. coli* M15 [pREP4], harbouring pQE30 as negative control. Whole cell lysate proteins of *Leptospira* species reacted with mice anti-LigA serum; (4A-8A) *L. interogans* (human isolate), (9A) *L. interogans* strain Autumnalis, (4B) *L. weilii* serovar Celledoni, (5B) *L. interogans* serovar Icterohaemorhage, (6B) *L.biflexa* serovar Patoc, (7B) *L. borgpetersenii* serovar Sejroe. Pre-immunized mice serum was reacted to leptospiral panel protein; (8B) *L. weilii* serovar Celledoni, (9B) *L.biflexa* serovar Patoc.

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10. Reactivity of anti-sera to LigA against leptospiral whole cell prepared from cells culturing under high osmolarity condition

The expression of Lig proteins was found to be lost during attenuated culturing of leptospires while several lines of evidence indicated that expression of Lig proteins was increased during infection of the mammalian host. Matsunaga and coworkers (Matsunaga *et al.*, 2003) found that the expressions of Lig proteins were regulated by osmolarity during culturing. The log phase leptospiral culturing in EMJH media was then subjected to high osmotic pressure by additional of NaCl to concentrated of 120 mM for overnight, and cells were collected for whole cell lysate preparation. Nine serovars of *Leptospira* spp. were included in this experiment. SDS-PAGE profile of whole cell lysate and Western blot with mice anti-LigA serum were performed. *E. coli* M15 [pREP4], harbouring pQE30 cell lysated was used as negative control while purified LigA protein was used as positive control. The blotted nitrocellulose membranes were reacted overnight with 1:64,000 dilution of mice anti-LigA sera, washed and then further incubated with 1:4,000 dilution of goat anti-mice IgG conjugated with alkaline phosphatase (Southern Biotech, USA), and detected by enzymatic reaction staining (BCIP and NBT substrate). It was shown that among all leptospires and *E. coli* M15 [pREP4], harbouring pQE30 no immunoreactive band was found while a single band of purified recombinant LigA histidine tag protein was revealed (**Figure 15**).

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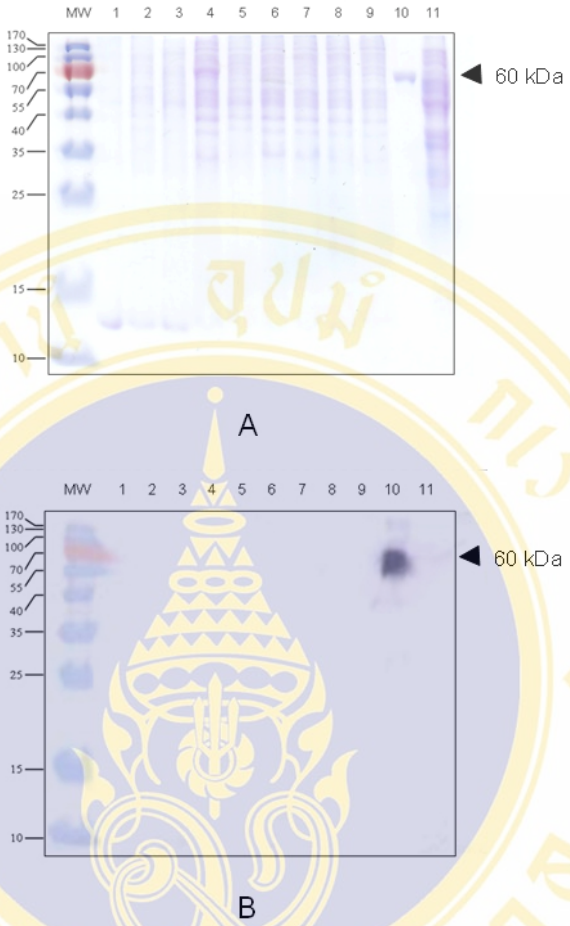

Figure 15. SDS-PAGE of leptospiral whole cell lysate preparation after induction by osmotic pressure. The SDS-PAGE and Western blot analysis was performed against mice anti-purified LigA sera. Gel stained with Coomassie brilliant blue (17A) and Western blot analysis (17B) was treated with mice anti-LigA (dilution 1:64,000). Whole cell homogenate of leptospiral cell as antigen specific to anti-LigA and purified LigA protein used as positive control while *E. coli* M15 [pREP4], harbouring pQE30 cell lysated used as negative control. Lane MW; Protein molecular weight marker**,** Lane 1; whole cell homogenate of UD9**,** Lane 2; whole cell homogenate of UT104**,** Lane 3; whole cell homogenate of UT1064**,** Lane 4; whole cell homogenate of L-88**,** Lane 5; whole cell homogenate of L-383**,** Lane 6; whole cell homogenate of L-562**,** Lane 7; whole cell homogenate of L-894**,** Lane 8; whole cell homogenate of L-984**,** Lane 9; whole cell homogenate of L-1254**,** Lane 10; Purified LigA protein**,** Lane 11; *E. coli* M15 [pREP4], harbouring pQE30 cell lysated

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11. Western blot pattern of human serum with positive MAT against purified LigA Protein

In order to examine antigenicity of the purified recombinant LigA protein, Western blot assay was carried out and tested against leptospirosis sera sample which were clinically diagnosed as having *Leptospira* infection and shown high MAT titer. **Figure 16** revealed the results of the Western blot assay of sixteen sera from patients with leptospirosis. The purified recombinant LigA protein was run on SDS-PAGE and the proteins were blotted into nitrocellulose membranes. The membranes were then blocked, cut into strips and used for testing with leptospirosis patients' sera, the blotted nitrocellulose membranes were reacted with 1:100 dilution of leptospirosis patients' sera overnight. The positive reactivity was focused at the expected molecular weight of recombinant LigA protein at about 60 kDa. The results were shown that some anti-leptospires serum samples could react with recombinant LigA protein. There were four serum samples that Western blot results revealed strongly positive at lane 3, 5, 14 and 15 and seven sera samples shown partially positive at the expected molecular weight of 60 kDa at lane 2, 9, 10, 11, 12, 13 and 16, suggesting that recombinant LigA protein could be applied as antigen for serodiagnosis.

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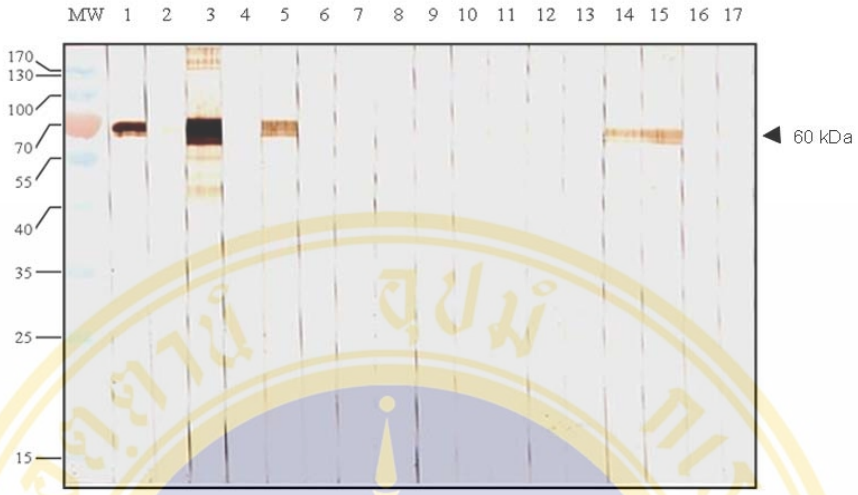

Figure 16. Western blot pattern of human serum with positive MAT against purified LigA protein. Purified recombinant LigA protein was run on SDS-PAGE and the proteins were immunoblotted onto nitrocellulose membranes, cut into strips and reacted with leptospirosis patients' sera of 1:100 dilution and anti-histidine tag for positive control.

Lane MW = Protein molecular weight marker
Lane 1 = Purified recombinant LigA protein reacted with anti-histidine
Lane 2-17 = Purified recombinant LigA protein reacted with human serum positive to MAT test

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12. Optimization of LigA-based ELISA assay

An indirect ELISA was established to detect IgM and IgG antibodies specific to LigA in all serum samples. To determine the optimum concentration of antigen for plate-coating, LigA protein concentration was varied from 2,000 ng/ml with serially two-fold dilution until 12.5 ng/ml as shown in **Figure 17A**. The serum used in this assay composed of the pooled MAT positive sera and pooled sera of normal individual of endemic area. The LigA concentration of 500 ng/ml was selected due to the large different of OD value between the positive and negative control (**Figure 17A**). To determine the optimum concentration of antibody to LigA protein, the LigA concentration of 500 ng/ml was selected to test with serial dilution of pooled MAT positive sera and pooled sera of normal individual of endemic area from 400 to 25,600 fold for IgM (**Figure 17B**) and 500 to 32,000 fold for IgG (**Figure 17C**), in order to determine the appropriated serum dilution for LigA-ELISA based assay. Due to the large different of OD value between the positive and negative control, the optimum concentration of IgM at 1:800 dilution and IgG at 1:4,000 dilution were selected for ELISA assay and anti-human Ig-HRP was used as a conjugated antibody, 1:4000 dilution was selected from the checkerboard titration tested (data not shown).

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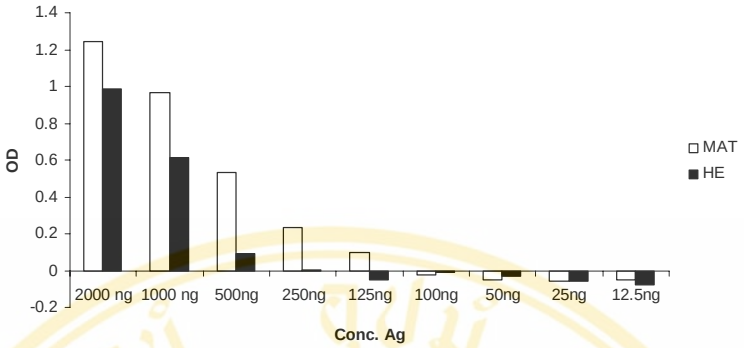
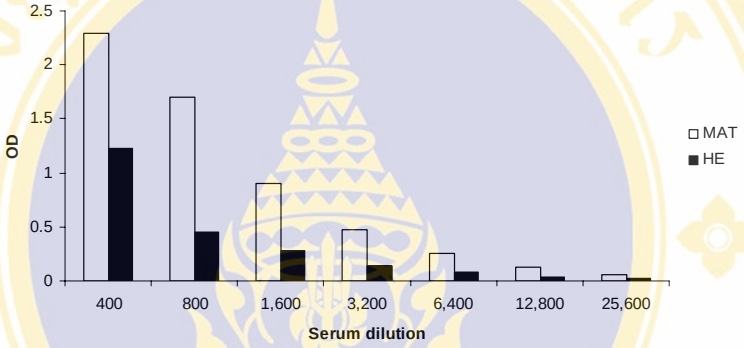
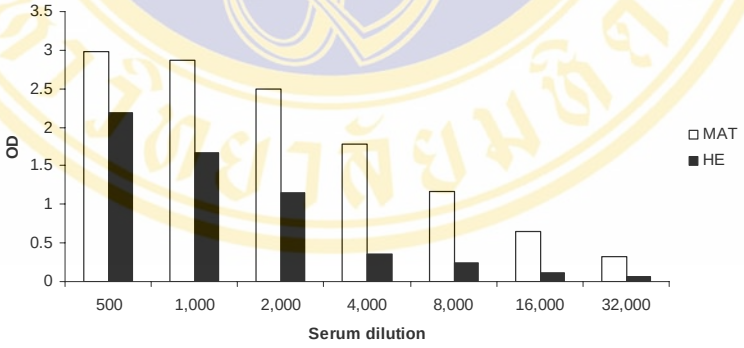


(A)



(B)



(C)

Figure 17. Checkerboard titration of indirect ELISA assay. ELISA assay using different rLigA protein concentrations varied from 2,000 to 12.5 ng/ml, and reacted to pooled MAT positive (white bar) and normal from endemic area (black bar) (A). The selected optimal concentration of 0.5 µg/ml of rLigA protein were incubated with sera that were serial diluted from 400 to 25,600 fold for IgM (B) and 500 to 32,000 fold for IgG (C), in order to determine the appropriated serum dilution for LigA-ELISA based assay.

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13. LigA IgM and IgG based ELISAs

The selected optimal condition of rLigA based ELISA were used to evaluate the ability of recombinant protein for detection the total immunoglobulin to leptospiral LigA protein in patient sera. Eight groups of sera were used; 89 patient sera with MAT positive samples, 42 patient sera of which *Leptospira* could be isolated but their MAT results were negative, 20 patient sera with IgM dipstick positive but their MAT results were negative, 61 scrub typhus patient sera, 30 melioidosis patient sera, 43 dengue hemorrhagic patient sera, 30 sera samples from healthy individual of endemic area and 80 sera samples from healthy in non endemic area.

The mean and standard deviation of OD value in both IgM and IgG based ELISA assay among all sera groups were evaluated by SPSS 11.5 program (**Table 4**). The difference among mean of each sera group with 95% confidence interval was evaluated by one-way ANOVA analysis. There were no significant difference at $p<0.05$ among suspected leptospirosis cases, *i.e.* MAT positive group and group of positive *Leptospira* culture but negative in MAT for IgM-based ELISA (**Table 5**). The MAT positive group was not significant difference to IgM dipstick positive group in IgG-based ELISA (**Table 6**), but they were significant difference ($p<0.05$) to other febrile control group, also healthy control group in both of IgM and IgG based ELISAs. This protein could be applied to differentiate leptospirosis patient to the normal individual and also to other febrile illness patient. Recombinant LigA may be suitable to use as antigen for detection human Ig as differential diagnosis.

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Table 4. Mean and SD of IgM and IgG based ELISAs

Group	N	Mean IgM	SD IgM	Mean IgG	SD IgG
MAT positive	89	1.29	0.64	1.13	0.74
Culture positive	42	1.16	0.59	0.55	0.51
IgM positive	20	0.92	0.64	1.00	0.88
Scrub Typhus	61	0.70	0.49	0.26	0.25
Melioidosis	30	0.36	0.18	0.42	0.12
Dengue hemorrhagic fever	43	0.68	0.35	0.20	0.10
Healthy endemic	30	0.47	0.21	0.27	0.11
Healthy non endemic	80	0.51	0.21	0.27	0.08
Total	395	0.81	0.57	0.53	0.58

Table 5. One-way ANOVA table, Multiple Comparisons of IgM based ELISA

Group (I)	Group (J)	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
MAT	Culture positive	0.02	0.02	0.97	-0.05	0.10
	IgM positive	0.10	0.03	0.03*	0.01	0.19
	Scrub typhus	0.18	0.02	0.00*	0.12	0.25
	melioidosis	0.28	0.03	0.00*	0.20	0.36
	dengue hemorrhagic fever	0.15	0.02	0.00*	0.08	0.22
	healthy endemic	0.22	0.03	0.00*	0.14	0.30
	healthy non endemic	0.20	0.02	0.00*	0.14	0.26

* The mean difference is significant at the .05 level.

Table 6. One-way ANOVA table, Multiple Comparisons of IgG based ELISA

Group (I)	Group (J)	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
MAT	Culture positive	0.10	0.01	0.00*	0.06	0.14
	IgM positive	0.03	0.02	0.54	-0.02	0.09
	Scrub typhus	0.17	0.01	0.00*	0.14	0.21
	Melioidosis	0.09	0.02	0.00*	0.05	0.14
	Dengue hemorrhagic fever	0.18	0.01	0.00*	0.14	0.22
	healthy endemic	0.14	0.02	0.00*	0.10	0.19
	healthy non endemic	0.14	0.01	0.00*	0.11	0.17

* The mean difference is significant at the .05 level.

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13.1 IgM based ELISA of LigA

The selected condition using LigA concentration of 500 ng/ml and sera dilution of 1:800, was applied in IgM-based ELISA. The cut off levels were determined from OD values (**Figure 18**) of the healthy endemic control group. The mean and the standard deviation (SD) of the OD of the serum samples of 30 healthy subjects were 0.47 and 0.21, respectively. When the mean+3SD value equal to 1.1 was used as the cut-off limit between positive and negative LigA based-IgM ELISA, it was found that none of the sera from melioidosis, healthy in endemic and non endemic area were positive and 11 of the 61 sera of scrub typhus (18%) and 4 of the 43 sera of dengue hemorrhagic fever (9%) revealed positive. Forty-seven out of sixty-seven (70%) of acute phase sera and 13 out of 22 (59%) of convalescence sera of leptospirosis MAT-positive were positive while 17 out of 37 (46%) of acute phase sera and 14 out of 25 (56%) of convalescence sera of leptospirosis MAT-negative were positive. In leptospirosis MAT-negative were divided into two groups, group 1 (blood culture positive), 14 out of 26 (54%) of acute phase sera and 10 out of 16 (63%) of convalescence sera were positive and group 2 (IgM-dipstick positive), 3 out of 11 (27%) of acute phase samples and 4 out of 9 (44%) of convalescence phase samples were positive (**Table 7**). As shown in **Table 8** the sensitivity of the LigA based-IgM ELISA in comparison to MAT test for all 67 acute phase serum samples was 70%, while the sensitivity for all of the 22 convalescence phase sera was 59% (**Table 9**). The slightly higher in the percentage of IgM positive serum in acute phase serum than in convalescence phase was relevant to the fact that IgM was mostly produced in acute phase of immunity. The specificity of the IgM ELISA was 94% and the test accuracy of LigA based-IgM ELISA for acute phase sera was 89%, while the test accuracy of LigA based-IgM ELISA for convalescence phase sera was 91%. The positive predictive value and negative predictive value of LigA based-IgM ELISA for acute sera were 76% and 92%, while the convalescence sera were 46% and 96%, respectively. The results were shown in **Table 10**.

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Table 7. Results of the recombinant LigA based-IgM ELISAs of leptospirosis sera.

Group Leptospirosis confirmed cases (n=104)	Percentage of positive result	
	IgM-ELISA	
	Acute	Convalescent
1. *Leptospira* MAT positive	47/67 (70%)	13/22 (59%)
2. *Leptospira* MAT negative	17/37 (46%)	14/25 (56%)
2.1 *leptospira* blood culture positive	14/26 (54%)	10/16 (63%)
2.2 IgM-dipstick positive	3/11 (27%)	4/9 (44%)

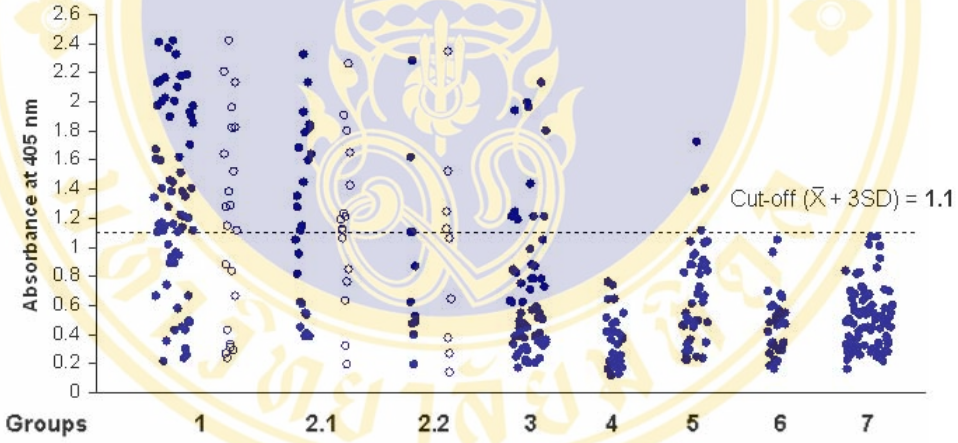

Figure 18. Results of the recombinant LigA based-IgM ELISA of sera of all groups. The defined cut-off value (1.1), which differentiated positive from negative result is indicated as dashed line. Group 1, sera of patients who were positive for MAT. Group 2, sera of patients who were *Leptospira* blood culture positive (2.1) and IgM-dipstick positive (2.2). Group 3-5, sera of patients with Scrub typhus, Melioidosis and Dengue hemorrhagic fever (DHF) and group 6 and 7 were sera of healthy in endemic area and non endemic area, respectively. •; Acute phase serum. o; Convalescence serum.

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Table 8: **Comparison between MAT and IgM based ELISA assay in acute leptospirosis subjects.**

		MAT		
		Positive	Negative	Total
ELISA	Positive	47	15	62
	Negative	20	229	249
	Total	67	244	311

Sensitivity	= (47/67) × 100	= 70%
Specificity	= (229/244) × 100	= 94%
Positive predictive value (PPV)	= (47+229/311) × 100	= 89%
Negative predictive value (NPV)	= (47/62) × 100	= 76%
Accuracy	= (229/249) × 100	= 92%

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Table 9: Comparison between MAT and IgM based ELISA assay in convalescence leptospirosis subjects.

		MAT		
		Positive	Negative	Total
ELISA	Positive	13	15	28
	Negative	9	229	238
	Total	22	244	266

Sensitivity	= (13/22) × 100	= 59%
Specificity	= (229/244) × 100	= 94%
Positive predictive value (PPV)	= (13+229/266) × 100	= 91%
Negative predictive value (NPV)	= (13/28) × 100	= 46%
Accuracy	= (229/238) × 100	= 96%

Table 10. Summary of sensitivity, specificity, accuracy, positive and negative predictive value of LigA based-IgM ELISA assay in comparison to MAT assay. Acute and convalescence phase sera of suspected leptospirosis cases, other febrile illness and healthy controls were included to determine the cut-off values of 1.1.

LigA based-IgM (cut-off =1.1)	Sensitivity	Specificity	Accuracy	Positive predictive value	Negative predictive value
Acute phase sera	70% (47/67)	94% (229/244)	89% (276/311)	76% (47/62)	92% (229/249)
Convalescence phase sera	59% (13/22)	94% (229/244)	91% (242/266)	46% (13/28)	96% (229/238)

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13.2 IgG based ELISA of LigA

For the LigA based-IgG ELISA, the selected condition using LigA concentration of 500 ng/ml and sera dilution of 1:4,000, was applied in IgG-based ELISA. The mean and the standard deviation (SD) of the OD of the serum samples of 30 healthy subjects were 0.27 and 0.11, respectively. When the mean+3SD value of 0.6 was used as the cut-off limit between positive and negative LigA based-IgG ELISA (**Figure 19**), it was found that none of the sera from dengue hemorrhagic fever, healthy in endemic and non endemic area were positive and 3 of the 61 sera of scrub typhus (5%) and 1 of the 30 sera of melioidosis (3%) revealed positive. Fourty-nine out of sixty-seven (73%) of acute phase sera and 15 out of 22 (68%) of convalescence phase sera of leptospirosis MAT-positive were positive, while 15 out of 37 (41%) of acute phase sera and 13 out of 25 (52%) of convalescence phase sera of leptospirosis MAT-negative were positive. In leptospirosis MAT-negative were divided into two groups, group 1 (blood culture positive), 9 out of 26 (35%) of acute phase sera and 8 out of 16 (50%) of convalescence sera were positive and group 2 (IgM-dipstick positive), 6 out of 11 (55%) of acute phase samples and 5 out of 9 (56%) of convalescence phase samples were positive (**Table 11**). As shown in **Table 12** the sensitivity of the LigA based-IgG ELISA in comparison to MAT test for all 67 acute phase serum samples was 73%, while the sensitivity for all of the 22 convalescence phase sera was 68% (**Table 13**). The specificity of the IgG ELISA was 98% and the test accuracy of LigA based-IgG ELISA for acute serum was 93%, while that of convalescence phase sera was 96%. The positive predictive value and negative predictive value of LigA based-IgG ELISA for acute sera were 92% and 93%, while the convalescence sera were 79% and 97%, respectively. The results were summarized in **Table 14**.

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Table 11. Results of the recombinant LigA based-IgG ELISAs of leptospirosis sera.

Group Leptospirosis confirmed cases (n=104)	Percentage of positive result	
	IgG-ELISA	
	Acute	Convalescent
1. *Leptospira* MAT positive	49/67 (73%)	15/22 (68%)
2. *Leptospira* MAT negative	15/37 (41%)	13/25 (52%)
2.1 *leptospira* blood culture positive	9/26 (35%)	8/16 (50%)
2.2 IgM-dipstick positive	6/11 (55%)	5/9 (56%)

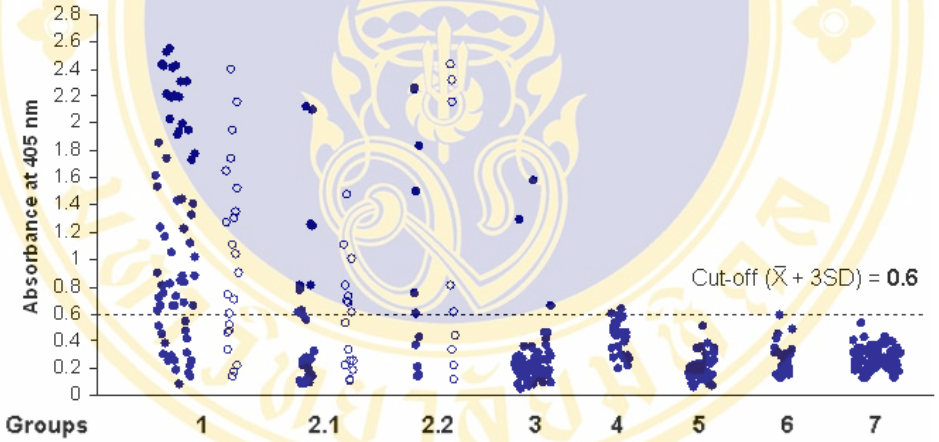

Figure 19. Results of the recombinant LigA based-IgG ELISA of sera of all groups. The defined cut-off value (0.6) which differentiated positive from negative result is indicated as dashed line. Group 1, sera of patients who were positive for MAT. Group 2, sera of patients who were *Leptospira* blood culture positive (2.1) and IgM-dipstick positive (2.2). Group 3-5, sera of patients with Scrub typhus, Melioidosis and Dengue hemorrhagic fever (DHF) and group 6 and 7 were sera of healthy in endemic area and non endemic area, respectively. •; Acute phase serum. o; Convalescence serum.

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Table 12: **Comparison between the positive results by IgG based ELISA for acute leptospirosis**

		MAT		
		Positive	Negative	Total
ELISA	Positive	49	4	53
	Negative	18	240	258
	Total	67	244	311

Sensitivity	= (49/67) × 100	= 73%
Specificity	= (240/244) × 100	= 98%
Positive predictive value (PPV)	= (49+240/311) × 100	= 93%
Negative predictive value (NPV)	= (49/53) × 100	= 92%
Accuracy	= (240/258) × 100	= 93%

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Table 13: **Comparison between the positive results by IgG based ELISA for convalescence leptospirosis**

		MAT		
		Positive	Negative	Total
ELISA	Positive	15	4	19
	Negative	7	240	247
	Total	22	244	266

Sensitivity	= (15/22) × 100	= 68%
Specificity	= (240/244) × 100	= 98%
Positive predictive value (PPV)	= (15+240/266) × 100	= 96%
Negative predictive value (NPV)	= (15/19) × 100	= 79%
Accuracy	= (240/247) × 100	= 97%

Table 14. Summary of sensitivity, specificity, accuracy, positive and negative predictive value of LigA based-IgG ELISA assay in comparison to MAT assay. Acute and convalescence phase sera of suspected leptospirosis cases, other febrile illness and healthy controls were included to determine the cut-off values of 0.6.

LigA based-IgG (cut-off =0.6)	Sensitivity	Specificity	Accuracy	Positive predictive value	Negative predictive value
Acute phase sera	73% (49/67)	98% (240/244)	93% (289/311)	92% (49/53)	93% (240/258)
Convalescence phase sera	68% (15/22)	98% (240/244)	96% (255/266)	79% (15/19)	97% (240/247)

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When the results of the recombinant LigA based-IgM and IgG ELISAs of all acute phase sera were combined, 55 out of 67 (82%) of acute phase sera and 17 out of 22 (77%) of convalescence phase sera of leptospirosis MAT-positive revealed positive, while 23 out of 37 (62%) of acute phase sera and 18 out of 25 (72%) of convalescence phase sera of leptospirosis MAT-negative were positive. For blood culture positive, 17 out of 26 (65%) of acute phase sera and 13 out of 16 (81%) of convalescence sera were positive and 6 out of 11 (55%) of acute phase samples and 5 out of 9 (56%) of convalescence phase samples of IgM-dipstick positive were positive. The sensitivity of the LigA based-IgG ELISA for all 104 acute phase serum samples was 75% (78 out of 104 sera were positive) and the sensitivity for all of the 47 convalescence phase sera was 74% (35 out of 47 sera were positive). The results were shown in **Table 15**.

Table 15. Results of the recombinant LigA based IgM and IgG ELISAs of leptospirosis sera.

Group Leptospirosis confirmed cases (n=104)	Percentage of positive result	
	Combined IgM and IgG ELISA	
	Acute	Convalescent
1. *Leptospira* MAT positive	55/67 (82%)	17/22 (77%)
2. *Leptospira* MAT negative	23/37 (62%)	18/25 (72%)
2.1 *leptospira* blood culture positive	17/26 (65%)	13/16 (81%)
2.2 IgM-dipstick positive	6/11 (55%)	5/9 (56%)

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CHAPTER VI
DISCUSSION

The exposure to pathogenic bacteria would stimulate the immune system to generate antibodies directed against bacterial cell surface or membrane antigens. Serology is performed to diagnose human leptospirosis, which has been used in routine laboratory. Laboratory diagnosis of leptospirosis can also be made by examining the organism (Levett, 2003). Leptospiral culture is the most specific diagnostic methods but it was difficult to perform and required several weeks of incubation. Culturing examination has low sensitivity and the specialized culture media could be prepared in few clinical laboratories. The microscopic agglutination test (MAT) is the reference test for serological diagnosis of leptospires because of its high sensitivity (Cole *et al.*, 1973; Cumberland *et al.*, 1999). Although, MAT is a serogroup-specific assay, it cannot be used to identity the infecting serovar correctly. However, the ability of convalescent MAT titers to predict even the infecting serogroup may be as low as 40% (Levett, 2003). MAT assay also required significant expertise to perform, interpret therefore its use was restricted to a few reference laboratories. Despite these limitations, the MAT has epidemiological value, and it is often used to give an indication of the presumptive serovar or serogroup of leptospires involved in an infection (Levett, 2004). Diagnosis of leptospirosis by MAT often requires paired serum samples which delay the diagnosis. Moreover, false negative results were frequently reported when the causative leptospire serovar was not included in the panel of typing organisms (Thiermann, 1984). Other alternative antibody-detection assays have subsequently been developed for early diagnosis of leptospirosis.

Recombinant antigen from cell surface or membrane protein may serve as ideal candidate for development of novel vaccines and improved diagnostic tests. Identification of an effective serodiagnostic marker for infection has been the major

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barrier to develop new diagnosis assay. Previous studies focused on detecting antibody responses against a series of proteins which included LipL21 (Cullen *et al.*, 2002), LipL32 (Flannery *et al.*, 2001; Guerreiro *et al.*, 2001; Haake, 2000), LipL36 (Haake *et al.*, 1998), LipL41 (Flannery *et al.*, 2001; Guerreiro *et al.*, 2001; Mariya *et al.*, 2006), 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. Recently, two recombinant leptospiral lipoproteins LA0730 and LigA were prepared and characterized. In this study, LigA was selected and evaluated as the antigen in IgM and IgG based-ELISAs for detecting antibodies in the patients' sera.

1. Selection of leptospiral LA0730 lipoproteins

A few bioinformatics criteria were utilized to select lipoprotein from leptospiral genome database. The candidate antigen for serodiagnosis should express during infection and can induce an immune response. Many lipoproteins were shown to play a role in immunity, these lipoproteins were then in focus for detailed study. The first criteria was based on their location at the cell surface by using the Cello program. Moreover, the selected gene should be presented only in pathogenic *Leptospira interrogans* not presented in saprophytic *Leptospira*. The outer membrane protein of molecular weight about 32 kDa was shown to react with human leptospirosis sera (Biswas *et al.*, 2005). LA0730 protein of predicted MW of 32 kDa was thus selected, of which it's predicted properties included; pI was 8.03, SPII score about 2.74, Cello score was 1.525. LA0730 was likely to be lipoprotein and located at outer membrane of *Leptospira* spp.

The hidden Markov model-based LipoP program used in this study showed improved accuracy for lipoprotein recognition in general for Gram-negative bacteria, but includes many non-spirochaetal lipoproteins in its training set (Setubal *et al.*, 2006). Although its sensitivity for detection of spirochaetal lipoproteins (compared to PSORT) has improved, but allows charged amino acids in the lipobox, which is not consistent with a lipoprotein sequence (Setubal *et al.*, 2006).

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Another novel lipoprotein prediction method, SpLip, was developed. The SpLip algorithm was constructed as a hybrid of a lipobox weight matrix approach supplemented by a set of lipoprotein signal peptide rules allowing for conservative amino acid substitutions and on an understanding of the biochemistry of lipoprotein signal peptides. Moreover, this algorithm was designed specifically tailored to identify spirochaetal lipoproteins and appeared to be more accurate than the hidden Markov model-based LipoP program (Setubal *et al.*, 2006).

Cello is an amino-acid-composition SVM (support vector machine)-based method available to predict bacterial protein localization. This method was simple and generated predictions for all proteins submitted, in some cases, with quality scores. However, it might not provide a high-confidence result due to this method forces predictions for every sequence analyzed.

Gardy and Brinkman reported that PSORTb and Proteome Analyst were discussed to be the high-precision predictive methods, but they did not always return a result (Gardy and Brinkman, 2006). Moreover, they presented a series of alternative analytical approaches, any of which can assign a protein to a single location with a high degree of precision, can be used as the starting point for more focused localization. The approaches of interest are homology based inference using databases of proteins with annotated localization sites and methods based on sequence features that report the presence or absence of a signal peptide, For example, the outer membrane proteins are characterized by the presence of β-barrel outer membrane proteins and Proteome Analyst and PSORTb provided the highest predictive performance for identification of outer membrane β-barrel proteins with respect to precision (Gardy and Brinkman, 2006).

2. Expression and purification of recombinant LA0730 protein

Expression of LA0730 in *E. coli* in this study was predominantly in the form of inclusion bodies. BugBuster Protein Extraction Reagent is formulated for the gentle disruption of the cell wall of *E. coli*, resulting in the liberation of soluble protein. It

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provides a simple, rapid, low cost alternative to mechanical methods such as French Press or sonication for releasing expressed target proteins in preparation for functional studies or purification. The proprietary formulation utilizes a mixture of non-ionic detergents that is able to perforate cell wall without denaturing soluble protein.

The recombinant fusion protein LA0730 required solubilization in 8M Urea buffer and was not able to adhere Ni-NTA affinity chromatography system as its histidine fusion tag could not access to Nikle molecule. The denatured protein still encountered drastic change in high concentration of urea, which may lead to the incorrectly folded proteins. Therefore, we came to the conclusion that a quick change in urea concentration might gradually change protein conformation, thus leading to the formation of incorrectly folded aggregates. On the contrary, a gradient change in urea concentration may stabilize the correctly folded intermediates of different refolding stages and direct the refolding process to further develop in a correct way until the final native conformation was reached (Gu *et al.*, 2001). Therefore, Electro-Elution of selected protein band was used to purify recombinant LA0730.

3. Selection of recombinant LigA protein

LigA (Leptospiral immunoglobulin like protein A) is member of the family of bacterial proteins containing immunoglobulin-like repeats (Palaniappan *et al.*, 2002) that have been identified in adhesins relevant to microbial pathogenesis, such as intimin in *Escherichia coli* (Luo *et al.*, 2000) and invasin in *Yersinia pseudotuberculosis* (Hamburger *et al.*, 1999). The Lig protein was expressed only on the surfaces of leptospiral pathogens isolated from infected animals and not by saprophytic *Leptospira* spp (Matsunaga *et al.*, 2003). In addition, sera from patients recovering from leptospirosis contain antibodies to Lig proteins, confirming their expression by infectious spirochetes (Matsunaga *et al.*, 2003; Palaniappan *et al.*, 2002). Thus, the Lig proteins appear to be closely associated with infection of the mammalian host, suggesting that they may be protective immunogens (Koizumi and Watanabe, 2004; Palaniappan *et al.*, 2006). Indeed, recent studies have demonstrated that recombinant Lig protein was served as effective antigen for serodiagnosis.

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Two homologous immunogenic proteins, LigA and LigB are conserved at the amino-terminal region but are variable at the carboxyl-terminal region exist in various pathogenic leptospires. The six repeat domains in the N-terminal of these proteins exhibit strong homology with each other, whereas repeat domains in the C-terminal of these proteins show great diversity. Therefore, the sequence diversity found in their C-termini may contribute to tissue specificity for leptospiral infection (Koizumi and Watanabe, 2004). Previous studies found that the C-terminal Big domain regions of LigA (domains 7 to 12) and LigB (domains 7 to 11) are seroreactive as well (Matsunaga *et al*., 2003; Koizumi and Watanabe, 2004; Palaniappan *et al*., 2004). As the full length of LigA and LigB protein were quite large about 128 and 212 kDa, respectively, only the portion of Lig protein was then selected for cloning. Croda *et al*. (2008) selected the N-terminal of LigB protein (amino acid position 131 to 649) while in this study, C-terminal of LigA was selected to clone (amino acid position 707 to 1217), express and evaluate in serodiagnosis.

4. Expression and purification of recombinant LigA protein

The purification procedure can be classified as "immobilized metal affinity chromatography" and is based on the affinity of consecutive histidine residues of the affinity tag to Ni^{2+} ions immobilized on an organic chelator, usually diamino acetic acid (Porath *et al.*, 1975). This chromatographic method allows the purification of fusion proteins containing poly-histidine affinity tag under native and denaturing condition (Crowe *et al.*, 1995). A series of vectors have been developed for expressing foreign proteins in *E. coli*. These vectors permit: (a) expression of native proteins; (b) expression of fusion proteins bearing six consecutive histidine residues. The following benefit has been demonstrated: (a) substantial over-expression of divergent foreign proteins using these vectors in *E. coli*; (b) simple and efficient affinity purification of over-expressed proteins from *E. coli* cell extracts using metal chelate columns (Ghosh and Lowenstein, 1996).

The purification of LigA protein was performed by using native conditions. The 6xhistidine tag was used as a ligand for the affinity purification of the expressed

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proteins on columns containing Nickel ions chelated to imminodiacetic acid-agarose. This affinity interaction was believed to be a result of coordination of a nitrogen on the imidazole moiety of polyhistidine with a vacant coordination site on the metal. The metal was immobilized to a support through complex formation with a chelate that was covalently attached to the support. 6xHis tags offer several advantages for protein purification. The small size of the 6xHis tag renders it less immunogenic than other larger tags. It was no charged at physiological pH and therefore usually shows the least interference with protein structure and function (Lewis *et al.*, 2001). The high affinity of the 6xHis tag for Ni-NTA allows efficient purification on commercially available, high-capacity matrices (5-10 mg protein per ml Ni-NTA resin) at a moderate cost per preparation. Finally, purification is simple and extremely robust, with the 6xHis tag-Ni-NTA system being the only affinity purification principle that could be performed under both native and denaturing conditions (Hochuli *et al.*, 1987). Therefore, the histidine tag usually did not need to be removed for downstream applications following purification. After purification, LigA protein was subject to characterization by ELISA and Western bolt technique.

5. Osmotic induction of *Leptospira* spp.

The *ligA* contained gene encoded a lipoprotein signal peptide, thus LigA expressed in *E. coli* is lipidated (Koizumi and Watanabe, 2004), indicating that there is *sec*-dependent export of LigA across the cytoplasmic membrane followed by lipidation of the lipobox cysteine by leptospiral lipoprotein signal peptidase. It is possible that once LigA reaches the surface of the spirochete as a lipoprotein, it is subject to proteolytic removal of its lipid anchor, resulting in the formation of LigA, which can be recovered from the culture supernatant (Matsunaga *et al.*, 2005). Differential Lig expression was recapitulated in culture: Lig was presented in low-passage-number growth of infective organisms but was absent in highly passaged, culture-attenuated leptospires that lost virulence in a hamster model of leptospirosis (Matsunaga *et al.*, 2003). Previous publication discovered that the change in osmolarity encountered during this transition caused a dramatic increase in expression of the two surface-exposed Lig proteins, LigA and LigB, in cultures of pathogenic

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strains of *Leptospira*, the Lig expression facilitated the identification of sodium chloride as the primary molecular signal that controlled *lig* expression *in vitro*, indicating that sodium chloride acted through its effect on osmolarity (Matsunaga *et al.*, 2005). An association of virulence with Lig expression was also suggested by diminished levels of Lig in nonpathogenic strains and pathogenic leptospires that have lost virulence after extensive subculturing in an artificial medium (Matsunaga *et al.*, 2003; Palaniappan *et al.*, 2002).

Mice anti-LigA sera produced in this study was not reacted to leptospiral whole cell lysate when using Western blot technique. The leptospiral whole cell lysate obtained from the culture grown under osmotic pressure was then subjected to Western blot assay against mice anti-LigA sera. These treated leptospiral whole cell still could not react to anti-LigA indicating either LigA was not presented in *in vitro* culture or even after osmolarity pressure. Western blot immunoassay was performed to allow different natures of antigen preparation in denaturation form, performed in Western blot. No immunoreactivity of mice anti-LigA to leptospiral cell implied that the epitope of recombinant LigA was not similar to the native form presented in leptospiral cell.

6. Immunogenic of recombinant LA0730 and LigA protein

The ability of a peptide to react specifically with the functional binding site of a complementary antibody is known as its antigenic reactivity or antigenicity. This knowledge is obtained along reductionist lines by turning the biological question of antigen recognition into the purely chemical phenomenon of protein-peptide interactions described in terms of atomic forces and non-covalent bonds. The situation is quite different with immunogenicity which is the ability of the peptide to induce an immune response in a competent host. Whereas antigenicity can be reduced to the level of chemistry, such a reduction is not achievable in the case of immunogenicity which depends on many complex interactions with various elements of the host immune system. These cellular and regulatory mechanisms cannot be controlled by adjusting the structure of the peptide in a predetermined manner (Regenmortel, 2001).

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LA0730 and LigA protein were used to test the antigenicity and immunogenicity by ELISA and Western blot analysis.

LA0730 and LigA protein were selected to immunize mice. The LA0730 recombinant protein possessed the immunogenic property as the antibody titer to recombinant LA0730 protein was higher than 1:102,400 determined by Western blot assay. However, the background reactivity of pre-immunized serum to purified protein LA0730 was also presented, indicating that this protein had the common epitope that natural antibody could react to. The immunized mice serum could not react to total membrane of *Leptospira* spp. but could react to *E. coli* cell lysate (negative control) by Western blot assay. This may be due to the wrong folding structure conformation of recombinant protein after solubilization under denaturing conditions, leptospiral antigen would change the epitopes to be different from. The anti-LA0730 serum that could react predominantly to *E. coli* indicated that recombinant LA0730 possessed the common epitope presented in *E. coli*. The distorted conformation after denaturing of the recombinant proteins is considered to be the major factor for non-reactivity. Improvement of the refolding procedure may induce correct folding structure of protein. Leptospiral LA0730 proteins were evaluated as antigen in Western blot technique. The purified LA0730 protein could not react either with normal healthy sera in endemic area and leptospirosis patients' sera. The major factor may be caused by wrong folding conformation, suggesting that this protein may be inappropriate for serodiagnosis.

The antibody titer of recombinant LigA protein was revealed to be higher than 1:512,000 determined by Western blot assay and the background reactivity could not be detected in pre-immunized serum. The high titer of anti-LigA indicated that LigA protein was good antigenicity and immunogenicity that could stimulate the immune response. The anti-LigA mice serum could not react to total membrane of *Leptospira* spp. by Western blot assay. This may suggest that recombinant LigA's epitope might not be similar to leptospiral native epitope. As the repeated passage of leptospiral culture would lost the expression of LigA protein (Matsunaga *et al.*, 2003; Palaniappan *et al.*, 2002), the membrane protein prepared from *in vitro* culture in this

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study might not be recognized by anti-LigA. The attempt to induce expression of LigA protein by culturing *Leptospira* in high osmotic pressure (Matsunaga *et al.*, 2005) was done, but these whole cell lysate still could not react to anti-LigA. Since the purified LigA protein could react with leptospirosis patients' sera, suggesting that in natural infection the antibody against recombinant LigA protein was produced, thus this recombinant LigA may be appropriate for serodiagnosis. ELISA technique by purified LigA protein was then evaluated for detection of leptospirosis.

7. LigA based-IgM and IgG ELISAs

In this study, the recombinant LigA was used as the antigen in the IgM and IgG based-ELISAs for detection of antibodies in the patients' sera. The ELISAs' sensitivities were 70% and 73% for IgM and IgG based ELISAs, respectively, when performed on the sera obtained during acute phase of illness. While the results of IgM and IgG based-ELISAs were combined, the sensitivity increased to 82% when conducted to the samples during the same period. For convalescence, 59% and 68% were positive for IgM and IgG based-ELISAs respectively, thus its sensitivity increased to 77% using combined ELISAs as well.

MAT is the standard method to diagnose leptospirosis in routine laboratory when LigA-based ELISA was applied to detect antibody among the sera of blood culture-positive patients at the acute phase of infection the sensitivity was revealed as 65% and it increased to 81% when tested on the convalescence sera. This finding indicated that the LigA based-ELISAs are more sensitive than MAT and/or blood culture and then should be considered as a rapid serological alternative before the culture result becomes available (Srimanote *et al.*, 2008).

As reported in Croda *et al*., 2007 study, recombinant Lig protein IgM based assays provided higher sensitivity than that of whole cell *Leptospira* based assay since anti-rLig IgM antibodies were detected but IgM ELISA results based on whole leptospiral cell lysate was negative. Croda *et al.* performed Western blot analysis to detect IgM and IgG antibodies using the recombinant N-terminus Lig as the antigen

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and found that 92% and 57% were positive for MAT/ELISA positive sera, and MAT-positive/IgM-ELISA-negative sera, respectively (Croda *et al.*, 2007). The specificity of our recombinant cLigA based IgM ELISA was 94% while the specificity of the cLigA based IgG ELISA was 98% of patients with clinically and serologically confirmed scrub typhus, dengue fever and melioidosis. The possibility exists that the patients might have been suffering from a mixed infection of pyrogenic pathogens, as previously demonstrated (Karande *et al.*, 2003; Saengjaruk *et al.*, 2002; Watt *et al.*, 2003). Similar to our results, N-terminus recombinant Lig based-Western blot analysis was found to have 93% specificity owing to cross-reactivity found with antibodies in sera of patients positive for Dengue fever, hepatitis, Lyme disease, and VDRL (Croda *et al.*, 2007).

Moreover, the major limitation of whole cell *Leptospira* based serologic assays is the low sensitivity (<67%) to samples collected as acute phase sera (McBride *et al.*, 2007). Whole *Leptospira* based diagnostic methods appear to detect IgM antibodies against immunodominant carbohydrate epitopes, such as broad reactive antigen (Adler and Faine, 1978; Matsuo *et al.*, 2000). In contrast, Lig proteins are hypothesized to be a virulence determinant presumably expressed in the spectrum of pathogenic *Leptospira* during host infection and elicit similar acute-phase antibody responses (Hamburger *et al.*, 1999; Luo *et al.*, 2004).

The serodiagnostic assay using a recombinant LigA protein derived from a single serovar which detects cross-reactive antibodies elicited during infection with other serovars should be improved as novel and higher specific approach. The specificities of rLigA based-ELISAs were 94% and 98% for IgM and IgG ELISAs, respectively, leading to high accuracy of the diagnostic results. The sensitivity of rLigA based-ELISAs was confirmed to the *ligA* PCR results using DNA extracted from serum samples obtained from the patients within three days after the onset of symptoms (de Abreu Fonseca *et al.*, 2006; Ooteman *et al.*, 2006).

The rLigA positive ELISA results complied with the MAT-positive results indicating the possibility of using rLigA based-ELISAs to replace MAT assay. The

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ELISA assay can be performed with simplicity, shorter turn around time, less cost, and a much lower amount of laboratory derived hazardous waste. Our finding implied that recombinant LigA-based ELISAs should be considered as a high potential approaches for early diagnosis of leptospirosis when the culture and MAT results were not available and in addition, when antimicrobial therapy provided the greatest benefit.

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CHAPTER VII
CONCLUSION

Laboratory diagnosis of leptospirosis was performed either by determining the organism or by serological tests that detect leptospiral antibodies (Levett, 2003). Culturing is difficult, requires several weeks of incubation, and has low sensitivity; the specialized culture media are stocked in few clinical laboratiories. The MAT is the reference standard test for serological diagnosis of leptospires, but requires significant expertise from its users and results inter-laboratory variations have been reported (Bharti *et al.*, 2003). One surrogate for direct demonstration of antibody to *Leptospira* in human samples is diagnosis based on ELISA, which is sensitive and has important implications for patient care because the diagnosis can be made early. The rapid diagnosis of leptospirosis is of extreme importance, because antibiotic treatment is more effective in altering the course of this disease, when initiated early. The ELISA protocol developed in this study is a low-cost and sensitive assay, and could be applied as the potential use to large-scale analysis in routine laboratories.

Leptospiral lipoproteins LA0730 and LigA were selected in this study. LA0730 gene was cloned into pCATCH expression vector to generate the recombinant plasmid. The expressions of these recombinant lipoprotein was performed in *E. coli* strain BL21 (DE3) pLysS, the recombinant fusion protein was expressed predominantly in the form of inclusion bodies (insoluble form) which determined by SDS-PAGE analysis. As the histidine fusion tag of LA0730 could not bind to Nikle column, thus purification was performed by Electro-Elution from the selected band. LigA gene was cloned into pQE30 vector and incorporated with expression host *E. coli* strain M15 [pREP4]. LigA protein was presented in soluble form and its fusion tag was able to adhere the Nikle, so this protein could use Ni-NTA affinity column for purification.

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LA0730 and LigA protein was then subjected to immunize mice. After immunization, it was likely that the LA0730 protein possessed the common epitope, thus the pre-immunized sera could react to this LA0730 protein, although more prominent in immunized serum. LigA protein was good antigenicity and immunogenicity that could stimulate the immune response in mice model. The LA0730 antiserum did not reveal a reactive protein band when reacted with native form of surface protein of leptospiral cell lysate, determined by Western blot analysis. Anti-LigA also could not react with native form of surface protein of leptospiral cell lysate by Western blot. However, we used these purified proteins to examine the antigenicity with leptospirosis patient sera. Western blot analysis revealed that the purified recombinant LA0730 protein did not react with leptospirosis patients sera. Recombinant LigA revealed positive reactive band with a few leptospirosis patient sera, so recombinant LigA protein was selected to further evaluate for leptospirosis diagnosis based on ELISA technique.

This study developed a conventional ELISA diagnostic assay based on the conserved region of LigA of pathogenic leptospires. The sensitivity, specificity and predictive values of the LigA based-IgM and IgG ELISA in conventional assay proved to be a useful tool for the early diagnosis of leptospirosis and allowing leptospirosis to be distinguished from other febrile disease.

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APPENDIX

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APPENDIX A
WHO CRITERIA

Table 16. Checklist of the WHO criteria for diagnosis of leptospirosis(Faine, 1982)

Questions	Answer	Score
Part A: Has the patient		
Headache of sudden onset?	Yes = 2	……..
	No = 0	……..
Fever?	Yes = 2	……..
	No = 0	……..
If "Yes" is the temperature 39°C or more?	Yes = 2	……..
	No = 0	……..
Conjunctival suffusion (bilateral)?	Yes = 4	……..
	No = 0	……..
Muscle pain (specially calf muscles)?	Yes = 4	……..
	No = 0	……..
Meningism?	Yes = 4	……..
	No = 0	……..
Are all 3 features (conjunctival suffusion, muscle	Yes = 10	……..
pain, meningism) present together?	No = 0	……..
Jaundice?	Yes = 1	……..
	No = 0	……..
Albuminuria or nitrogen retention?	Yes = 2	……..
	No = 0	……..

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Table 17. Checklist of the WHO criteria for diagnosis of leptospirosis (Faine,1982) (continued)

Questions	Answer	Score
Part B: Epidemiological factors		
Has there been contact with animals at home, work, leisure or in travel, or contact with known (or possible) contaminated soil or water?	Yes = 10 No = 0	…….. ……..
Part C: Bacteriological laboratory findings		
Isolation of leptospires in cultures-diagnosis certain	Yes = 25 No = 0	…….. ……..
Positive serology-leptospirosis non endemic:		
-Single positive, low titer	Yes = 2 No = 0	…….. ……..
-Single positive, high titer	Yes = 10 No = 0	…….. ……..
-Paired sera, rising titer	Yes = 25 No = 0	…….. ……..
Positive serology-leptospirosis endemic:		
-Single positive, low titer	Yes = 2 No = 0	…….. ……..
-Single positive, high titer	Yes = 10 No = 0	…….. ……..
-Paired sera, rising titer	Yes = 25 No = 0	…….. ……..
Total score		……..

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APPENDIX B

BACTERIAL MEDIA AND SOLUTION

1. Leptospiral culture media

1.1 EMJH medium

100 ml of EMJH medium consist of 90 ml of EMJH base solution and 10 ml of 10x stock, 200 ml of 10x stock are prepared by adding the following ingredients:

Bovine serum albumin	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% $ZnSO_4 \cdot 5H_2O$	2	ml
0.3% $CuSO_4 \cdot 5H_2O$	0.2	ml
0.1% vitamin B 12	0.4	ml
20% glycerol	1	ml
0.1% $MnSO_4 \cdot 4H_2O$	2	ml

The EMJH medium is stored at 4 °C

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2. *E. coli* culture media

2.1 Luria broth (LB)

Bacto Tryptone	10	g
Bacto yeast extract	5	g
NaCl	5	g
For Luria Agar	15	g
Distilled water	1,000	ml

Dissolve in distilled water and the pH was adjusted to 7.0. The final volume was adjusted to 1,000 ml with distilled water. Sterilize by autoclaving for 15 minutes at 121°C, 15 Ib/square inches.

2.2 Ampicillin stock solution

100 mg/ml in H_2O, filter sterilization, store in aliquots at -20°C.

2.3 Kanamycin stock solution (50 mg/ml)

The solution was prepared by dissolving 1 g of kanamycin powder in 21 ml of distilled water, filter sterilization and dispense the solution in aliquots. The solution was stored at -20°C.

2.4 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.

2.4 IPTG (1 M)

The solution was prepared by dissolving 5 g of IPTG (MW 238.3) in 21 ml of distilled water, filter sterilization. Dispense the solution into 1 ml aliquots and stored at -20°C.

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2.5 10x PBS pH 7.2

Na_2HPO_4	141.96 g
$NaH_2PO_4.2H_2O$	156.01 g

Dissolve in distilled water and the pH was adjusted to 7.2. The final volume was adjusted to 1,000 ml with distilled water. Sterilize by autoclaving for 15 minutes at 121°C, 15 Ib/square inches and store at 4°C.

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APPENDIX C
REAGENTS AND BUFFERS FOR PURIFICATION UNDER NATIVE CONDITION

1. Lysis buffer 500 ml

50 mM Na_2HPO_4	3.45 g Na_2HPO_4 (MW 137.99)
300 mM NaCl	30 ml of 5 M NaCl
10 mM Imidazole	5 ml of 1M Imidazole

2. 1M Imidazole

100 ml Na_2HPO_4 plus NaCl buffer pH 8.0

6.808 g Imidazole (MW 68.08)

3. Wash buffer

50 mM Na_2HPO_4	250 ml lysis buffer
300 mM NaCl	
20 mM Imidazole	2.5 ml 1M Imidazole

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APPENDIX D
BUFFERS FOR PURIFICATION UNDER DENATURING CONDITION

1. Base buffer:

100 mM NaH_2PO_4	13.8 g $NaH_2PO_4H_2O$ (MW 137.99 g/mol)
10 mM Tris HCl	1.2 g Tris base (MW 121.1 g/mol)

Dissolve in distilled water to the final volume of 1,000 ml. Sterilized by autoclaving for 15 minutes at 121°C, 15 Ib/square inches. The solution was stored at 4°C.

Buffer B:

The buffer was prepared by adding 14.4 g of urea (8 M urea, MW 60.06 g/mol) in base buffer and the pH was adjusted to 8.0 using 1 N NaOH. The final volume was adjusted to 30 ml (should prepare freshly before each usage).

2. Wash buffer

Buffer C:

The buffer was prepared by adding 14.4 g of urea (8 M urea, MW 60.06 g/mol) in base buffer and the pH was adjusted to 6.3 using 1 N HCl. The final volume was adjusted to 30 ml (should prepare freshly before each usage).

3. Elution buffers

Buffer D:

The buffer was prepared by adding 14.4 g of urea (8 M urea, MW 60.06 g/mol) in base buffer and the pH was adjusted to 5.9 using 1 N HCl. The final volume was adjusted to 30 ml (should prepare freshly before each usage).

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Buffer E:

The buffer was prepared by adding 14.4 g of urea (8 M urea, MW 60.06 g/mol) in base buffer and the pH was adjusted to 4.5 using 1 N HCl. The final volume was adjusted to 30 ml (should prepare freshly before each usage).

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APPENDIX E
REAGENTS AND BUFFERS FOR SDS-PAGE GEL ELECTROPHORESIS

1. 2x Sample buffer (SDS reducing buffer)

The sample buffer was prepared as a stock solution by combining the following ingredients:

0.5 M Tris-HCl pH 6.8 with 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

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

2. 40% stock Acrylamide separating gel solution

Acrylamide	40	g
Bis	0.32	g
Distilled water	100	ml

This mixture is stored in dark bottle at 4°C

3. 30% stock Acrylamide stacking gel solution

40% Acrylamide	75	ml
Bis	0.8	g
Distilled water	25	ml

This mixture is stored in dark bottle at 4°C.

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4. 2x Separating gel buffer

Tris	36.32	g
SDS	0.8	g (or 8 ml of 10% SDS)
Distilled water	400	ml

Dissolve in distilled water, adjust volume with 1 N HCl to pH 8.8 and adjust to 400 ml final volume with distilled water. This mixture was stored at 4°C.

5. 2x Stacking gel buffer

Tris	6.04	g
SDS	0.4	g (or 4 ml of 10% SDS)
Distilled water	200	ml

Dissolve in distilled water, adjust volume with 1 N HCl to pH 6.8 and adjust to 200 ml final volume with distilled water. This mixture was stored at 4°C.

6. Ammonium persulfate (10% w/v)

This solution was prepared just before use by dissolving 50 mg of ammonium persulfate (Bio-Rad, USA) in 0.5 ml of distilled water.

7. Separating gel (13%)

Distilled water	1.67	ml
2x separating buffer	5	ml
40% Acrylamide	3.3	ml

The reagents are gently mixed, and the polymerization is initiated by adding 40 µl of the 10% ammonium persulfate (freshly prepared) and 14 µl of TEMED (Bio Basic Inc.). The gel is poured on the vertical gel electrophoresis and overlay with isopropanol. Then the gel solution is allowed to polymerize for 30 minutes.

8. Stacking gel (5%)

Distilled water	0.9	ml
2x stacking buffer	1.5	ml
30% Acrylamide	0.6	ml
10% ammonium persulfate	18	µl

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TEMED	3.3	µl

The gel solution was immediately loaded on the vertical gel electrophoresis. The comb was gently inserted on the top of the gel to make well for sample application. Then the gel solution was allowed to polymerize for 45 minutes.

9. 10x Electrode buffer

Tris base	24.25	g
Glycine	157.5	g
SDS	10	g (or 100 ml of 10% SDS)

Make 1,000 ml final volume with UDW. Dissolve glycine first then adjusted to pH 8.3 with Tris powder. The solution was stored at 4°C and warm to room temperature before use if precipitation occurs.

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APPENDIX F
REAGENTS FOR GEL STAINING

1. Coomassie brilliant blue R-250 (Protein staining buffer)

Coomassie brilliant blue R-250	1	g
Methanol	250	ml
Glacial acetic acid	100	ml

Dissolve Coomassie brilliant blue R-250 with distilled water first and then adjusted the volume to 1,000 ml. Filter the solution through a Whatman No. 1 paper to remove any particular matter and store at room temperature.

2. Destaining solution

Glacial acetic acid	100	ml
Isopropanol	100	ml
Distilled water	800	ml

Dissolve and adjust the volume to 1,000 ml with distilled water. The solution was stored at room temperature.

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APPENDIX G
REAGENTS AND BUFFERS FOR PREPARATION OF WHOLE CELL LYSATE

1. Lysis buffer

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

Mixed the above reagents and adjust the volume to 200 ml with distilled water.

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APPENDIX H
REAGENTS AND BUFFERS
FOR IMMUNOBLOT ANALYSIS

1. Towbin's buffer

Tris base	0.3	g
Glycine	1.44	g
SDS	0.01	g
Methanol	15	ml

Dissolve and adjust the volume to 100 ml with distilled water (should prepare freshly before each usage).

2. 0.15 M Tris buffer saline (TBS) pH 7.4

The buffer was prepared by dissolving 18.18 g of Tris base in 1,000 ml of distilled water. The pH of this buffer was adjusted to 7.4 with 1N HCl.

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

The solution was prepared by adding 0.5 ml of Tween-20 in one liter of TBS pH 7.4 and mixed well.

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

The solution was freshly prepared by dissolving 2.5 g of skim milk powder in 50 ml of TBS-Tween by heating on a hot plate. The solution was cooled down to room temperature before used.

5. Conjugate solution

This solution was prepared by diluting goat anti-mouse Ig-horse radish peroxidase (or alkaline phosphatase) conjugate with blocking buffer to make the desire dilution.

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6. Substrate solution for peroxidase

Part A:

4-Chloro-1-naphthol (Merck)	15	mg
Methanol	5	ml

Part B:

30% (v/v) H_2O_2	20	µl
TBS	25	ml

Part A and B was freshly prepared and combined immediately before use. Part A is light sensitive and should be prepared in a shielded container.

7. Substrate solution for alkaline phosphatase

1x buffer III	10	ml
NBT	45	µl
BCIP	35	µl

This solution was freshly prepared and incubated with membrane in the dark at room temperature until the bands can be visualized. The reaction will be stopped by washing with distilled water.

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APPENDIX I
REAGENTS AND BUFFERS FOR ELISA

1. 0.05 M Carbonate-bicarbonate 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. Blocking solution

0.2% of BSA and gelatin in PBS-T

3. 3 M NaOH (Stop reaction)

NaOH	120	g
Distilled water	1000	ml

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APPENDIX J
REAGENTS AND BUFFERS FOR PURIFICATION BY ELECTRO-ELUTER

1. 0.25 M KCl

The solution was prepared by dissolving 18.64 g of KCl in 1,000 ml of distilled water. The solution was stored at 4°C.

2. Protein Elution Buffer

Tris base	3.0	g
Glycine	14.4	g
SDS	1.0	g

The buffer was prepared by dissolving all of the above reagents in a final volume of 1,000 ml with distilled water. The solution was stored at 4°C and warm to room temperature before used if precipitation occurs.

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