การโคลนและการแสดงออกของโปรตีนผิวเซลล์ชั้นนอก LipL32 ของเชื้อเล็ปโตสไปรา

นางสาวดุจดาว บุญยอด

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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CLONING AND EXPRESSION OF AN OUTER MEMBRANE PROTEIN OF *LEPTOSPIRA*, LipL32

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ดุจดาว บุญยอด : การโคลนและการแสดงออกของโปรตีนผิวเซลล์ชั้นนอก LipL32 ของเชื้อเล็ปโตสไปรา (Cloning and Expression of an Outer Membrane Protein of *Leptospira*, LipL32) อาจารย์ที่ปรึกษา : ผู้ช่วยศาสตราจารย์ ดร. จินตนา จิรถาวร, อาจารย์ที่ปรึกษาร่วม : รองศาสตราจารย์ ดร. ภาวพันธ์ ภัทรโกศล ; 122 หน้า. ISBN : 974-17-3735-1

เล็ปโตสไปราเป็นเชื้อที่ก่อให้เกิดโรคเล็ปโตสไปโรซิส แบ่งได้เป็น 2 กลุ่ม คือ *Leptospira interrogans* ซึ่งเป็นสายพันธุ์ก่อโรค และ *Leptospira biflexa* สายพันธุ์ที่พบตามสิ่งแวดล้อมทั่วๆไป การตรวจวินิจฉัยโรคทางห้องปฏิบัติการยังคงใช้การตรวจหาแอนติบอดี เนื่องจากวิธี Microscopic Agglutination Test (MAT) ซึ่งเป็นวิธีอ้างอิงต้องใช้ตัวเชื้อเป็น และในการอ่านผลต้องอาศัยผู้มีประสบการณ์ ในปัจจุบันจึงได้มีการพัฒนาวิธีการทางน้ำเหลืองวิทยาอื่นๆ เพื่อตรวจแอนติบอดี อย่างไรก็ตามมีรายงานว่าพบผลบวกปลอมจากโรคติดเชื้ออื่นๆได้

ในปี 2543 David Haake และคณะ ได้ศึกษาคุณสมบัติของโปรตีนผิวเซลล์ชั้นนอก LipL32 ของเชื้อเล็ปโตสไปรา พบว่าโปรตีนชนิดนี้มีการแสดงออกทั้งภายในร่างกายและในหลอดทดลอง รวมทั้งพบเฉพาะในสายพันธุ์ที่ก่อโรคเท่านั้น ด้วยเหตุผลนี้โปรตีน LipL32 จึงเหมาะที่จะนำมาใช้ศึกษาการเป็นแอนติเจนสำหรับตรวจหาแอนติบอดีเพื่อช่วยวินิจฉัยโรคและการเป็นวัคซีนเพื่อใช้ในการป้องกันโรคแบบข้ามกลุ่ม

ในการศึกษาครั้งนี้ ได้ทำการโคลนยีนผิวเซลล์ชั้นนอก LipL32 จากเชื้อ *Leptospira interrogans* serovar *bratislava* ซึ่งมีรายงานว่าเป็นซีโรวาร์ที่เป็นสาเหตุการก่อโรคมากที่สุดในประเทศไทยระหว่างปี พ.ศ. 2542 ถึง 2545 โดยทำการเพิ่มจำนวนยีน LipL32 ด้วยวิธี Polymerase Chain Reaction (PCR) จากนั้นนำ DNA ของ LipL32 ที่ได้มาเชื่อมต่อเข้ากับพลาสมิด pRSETc ซึ่งมีลำดับเบสสำหรับกรดอะมิโน histidine ได้ยีน LipL32 ที่แทรกอยู่กับพลาสมิด pRSETc (pRSETc-LipL32) ทดสอบความถูกต้องการของพลาสมิดที่ได้โดยวิธี PCR และการหาลำดับเบส พบว่าพลาสมิดที่ได้มีการแทรกของชิ้น DNA ที่มีความเหมือนกับยีนของ LipL32 ที่มีการรายงานไว้ ในส่วนของการเตรียมโปรตีนที่เชื่อมต่อกับ histidine (LipL32-His fusion protein) ทำโดยกระตุ้นการแสดงออกของยีนในพลาสมิดและแยก His-LipL32 ออกมาจากโปรตีนอื่นๆโดยใช้ nickle affinity column

นำ His-LipL32 บริสุทธิ์ที่ได้ไปทดสอบหาคุณสมบัติเบื้องต้นในการเป็นแอนติเจน ด้วยวิธี Immunoblotting พบว่าโปรตีนที่ได้สามารถทำปฏิกิริยากับแอนติบอดีที่จำเพาะต่อโปรตีน LipL32 ได้ นอกจากนี้ยังนำโปรตีนไปทดสอบกับตัวอย่างน้ำเหลืองผู้ป่วย พบว่าตัวอย่างน้ำเหลืองผู้ป่วยที่ตรวจพบแอนติบอดีต่อเล็ปโตสไปราสามารถทำปฏิกิริยากับโปรตีน LipL32 ในขณะที่น้ำเหลืองของผู้ป่วยที่ตรวจพบแอนติบอดีต่อเชื้อ *T. pallidum* และน้ำเหลืองจากอาสาสมัครให้ผลลบ อย่างไรก็ตามการใช้ LipL32 เป็นแอนติเจนสามารถตรวจพบผลบวกเมื่อตรวจหาอิมมูโนโกลบูลินชนิด IgG เท่านั้น แต่ไม่พบอิมมูโกลบูลินชนิด IgM จากการนำ LipL32 ไปทดลองทำการทดสอบด้วยวิธี Dipstick โดยใช้โปรตีน LipL32 เตรียมเป็นแอนติเจนเพื่อตรวจหาแอนติบอดีชนิด IgG จากผลการทดสอบเบื้องต้นให้ผลความไวร้อยละ 100 และความจำเพาะร้อยละ 98.33 เมื่อเทียบกับวิธี MAT

ในการการศึกษาครั้งนี้นอกจากจะสามารถโคลนยีนสำหรับ LipL32 แล้วยังสามารถแสดงผลการทดสอบเบื้องต้นที่แสดงว่า LipL32 น่าจะเป็นแอนติเจนที่ดีในการตรวจหาแอนติบอดีชนิด IgG เพื่อช่วยวินิจฉัยโรคติดเชื้อเล็ปโตสไปรา

สหสาขาวิชา จุลชีววิทยาทางการแพทย์ **ลายมือชื่อนิสิต**.....

สาขาวิชา จุลชีววิทยาทางการแพทย์ **ลายมือชื่ออาจารย์ที่ปรึกษา** .....

ปีการศึกษา 2546 **ลายมือชื่ออาจารย์ที่ปรึกษาร่วม** .....

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DOOJDAO BOONYOD : THESIS TITLE : CLONING AND EXPRESSION OF AN OUTER MEMBRANE PROTEIN OF *LEPTOSPIRA*, LipL32. THESIS ADVISOR : ASSISTANT PROFESSOR CHINTANA CHIRATHAWORN, Ph.D., THESIS CO-ADVISOR : ASSOCIATE PROFESSOR PARVAPAN BHATTARAKOSOL, Ph.D., 122 pp. ISBN : 974-17-3735-1

*Leptospira,*the etiologic agent of leptospirosis, is divided into two groups, *Leptospira interrogans*, comprising all pathogenic strains, and *Leptospira biflexa*, containing the saprophytic strains. Currently, laboratory diagnosis still depends on detection of antibody. Microscopic Agglutination Test (MAT) which is a reference method, requires maintaining of live organisms and reading of results requires well trained laboratory personnel. In addition to MAT, other serological methods have been used for antibody detection, however, false positive with other infectious diseases have been reported.

David Haake *et al*., (2000) have characterized leptospiral outer membrane proteins and demonstrated that LipL32, an outer membrane protein of *leptospira* was found only in pathogenic leptospires and expressed both *in vivo* and *in vitro*. For those reasons, LipL32 was proposed to be a candidate antigen for detection of antibody to *leptospira* and to be a vaccine that may provide cross protection among pathogenic serovars.

In this study, *Leptospira interrogans* serovar *bratislava* was chosen to be a target for cloning of LipL32 gene since it was the most serovar reported to be responsible for leptospirosis cases in Thailand during 1999-2002. LipL32 gene was amplified by PCR and inserted into a plasmid, pRSETc generating recombinant construct, pRSETC-LipL32. Proper insertion was demonstrated by PCR amplification and DNA sequencing. Protein expression from this construct was a fusion protein of hisdine and LipL32 (His-LipL32) which was further purified using a nickle affinity column.

Purified His-LipL32 protein was preliminary tested for its immunogenicity. Antiserum specific for LipL32 reacted with purified LipL32 using immunoblotting. In addition, patient serum positive for antibody to *leptospira* also reacted against LipL32 protein whereas none of patient serum positive for antibody to *T. pallidum* and serum from healthy volunteer demonstrated positive reaction. However, the positive results were obtained in IgG but not IgM antibody detection. Dipstick assay using LipL32 protein as an antigen for IgG specific antibody detection was also performed. Preliminary data suggested that this protein may be a good candidate since sensitivity and specificity compared with MAT, a reference method, were 100 and 98.33 %, respectively.

In conclusion, cloning and expression of LipL32 gene were done and preliminary data demonstrating the possibility of LipL32 to be an antigen for laboratory diagnosis was obtained.

Inter-Department Medical Microbiology **Student's signature**……...

Field of study Medical Microbiology **Advisor's signature**……

Academic year 2003 **Co-advisor's signature**……...

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ABBREVIATIONS

amp^r	=	ampicillin resistant gene
bp	=	base pairs
BSA	=	bovine serum albumin
BCIP	=	5-Bromo-4-chloro-3-indolyl phosphate
$^{\circ}C$	=	degree celsius
cm	=	centrimetre
CF	=	complement fixation
DMF	=	N,N-dimethylformamide
DNA	=	deoxyribonucleic acid
dNTPs	=	deoxynucleotide triphosphates
EDTA	=	ethylenediamine tetraacetic acid
ELISA	=	enzyme-linked immunosorbent assay
EMJH	=	Johnson and Harris modification of the Ellinghausen and McCullough medium
et al.	=	et alii
etc.	=	et cetera
g	=	gram
h	=	hour
HA	=	hemagglutination
IFA	=	Immunofluorescence assay
Ig	=	Immunoglobulin
IgG	=	Immunoglobulin G
IgM	=	Immunoglobulin M
IHA	=	Indirect hemagglutination assay
IP	=	Immunoperoxidase
IPTG	=	isopropyl-D-thiogalactopyranoside
kb	=	kilobase
kDa	=	kilodalton
kg	=	kilogram

LB	=	Luria Bertani
LipL32	=	Leptospira lipoprotein 32 kDa
LPS	=	Lipopolysaccharide
M	=	molarity
mA	=	milliampare
MAT	=	Microscopic Agglutination Test
mg	=	milligram
ml	=	millilitre
mm	=	milimetre
MW	=	molecular weight
NBT	=	Nitro Blue Tetrazolium
nm	=	nanometre
N.S.S.	=	Normal saline solution
OE	=	outer envelope
OM	=	outer membrane
OMP	=	outer membrane protein
PBS	=	Phosphate Buffer Saline
PC	=	Protoplasmic Cylinder
PCR	=	Polymerase Chain Reaction
Rf	=	relative mobility
SDS-PAGE	=	Sodium Dodesyl Sulfate Polyacrylamide Gel Electrophoresis
TBS	=	Triethanolamine-Buffer Saline
TEMED	=	N,N,N,N,- Tetramethylethylenediamine
µg	=	microgram
µl	=	microlitre
µm	=	micrometre
V	=	volt
v/v	=	volume by volume
w/v	=	weight by volume
X-Gal	=	5-bromo-4-chloro-3-indolyl β-D-galactopyranoside

CHAPTER I

INTRODUCTION

Leptospirosis, a globally zoonotic disease, is caused by spirochetes of the genus *Leptospira* species *interrogans*. The disease has become an important human and veterinary health problem (1,2,3). In Thailand, a nationwide leptospirosis epidemic is ongoing and control strategies are being explored. The number of case reported each year from 1982 to 1995 ranged from 55-272 cases, with an average incidence of 0.3/100,000/year. However, the number was increased rapidly from 398 cases (incidence 0.65/100,000) in 1996 to 2,334 (incidence 3.83/100,000) in 1997. The number of cases reported each year is still high as shown in Figure 1; 2,230 (incidence 3.52/100,000), 6,080 (incidence 9.89/100,000), 14,286 (incidence 23.2/100,000). 8,611 (incidence 13.96/100,000) and 5,166 (incidence 8.35/100,000) in 1998-2002, respectively (4). The incidence peak of disease is seasoning and corresponds with rainy season, and early winter, owing to the high rainfall and stagnation (5). Water is then easily contaminated by urine from infected animals, after which flows into the rivers, canals, and contaminates animal feed stuffs on farms. Human may become an accidental host, as a result of exposure to these contaminated environments. In the past, people in occupations associated with water or sewage were particularly at risk from leptospirosis as they often worked in rat infested conditions or in water polluted with *leptospira* infected urine. The major occupational risks today are among farm workers and the people who walk in contaminated stagnant water (3, 5,6).

Clinical manifestations of leptospirosis vary from mild to severe. These include fever, myalgia, muscle tenderness, headache, injected conjunctiva, hepatomegaly, renal failure and jaundice (7,8). However, leptospirosis may remain undiagnosed since the symptoms are relatively non-specific. It may be confused with other infectious diseases such as malaria, typhoid fever, viral hepatitis and haemorrhagic fever, especially in the early stages of infections (9). Therefore, laboratory tests are frequently needed in order to confirm the diagnosis.

This may be established either by bacteriological cultivation or by demonstration of a rise in specific antibody titer (10,11,12). Since culturing requires a long incubation time (about 2-4 weeks), the immunological tests for leptospiral antibody are the most important. The microscopic agglutination test (MAT) (11,13) is the most widely employed as the standard reference test because of its high specificity. However, the MAT encounters several drawbacks including the maintenance of a large number of live stock cultures which limit its wide use (13,14,15). For that reason, many immunological tests have been developed for the determination of specific leptospiral antibody. They are the macroscopic slide agglutination test (MSAT) (16), complement fixation (CF) test (17,18), haemolysin (HL) test (19,20,21), indirect immunofluorescent (IF) test (22,23), indirect haemagglutination (IHA) test (24,25,26,27,28), enzyme-linked immunosorbent assay (ELISA), IgM specific dot ELISA (29,30,31,32,33). However, except indirect immunofluorescent test which a fluorescent microscope is required, rapid serologic tests developed so far using whole cell *leptospira*, as an antigen and most of them, the non-pathogenic *leptospires* were used. False positive results due to cross reacting serum from specimens of patients with syphilis or other diseases such as lyme disease, dengue hemorrhagic fever and hepatitis have been demonstrated (34,35,36,37,247). MAT is still required for confirmation (35,36,37).

Recombinant antigens are brought into attention because of their specificity and reproducibility of quality of prepared antigens. Recombinant antigen based serologic tests are widely used in screening for spirochetal infections such as Lyme disease and syphilis, but the use of recombinant proteins for serodiagnosis of leptospirosis has not been widely investigated (38,39,40,41). Recently, a recombinant flagella antigen immunocapture assay was described for serodiagnosis of bovine leptospirosis (42). However, the utility of recombinant antigens for the serodiagnosis of human leptospirosis has not been investigated in large validation studies.

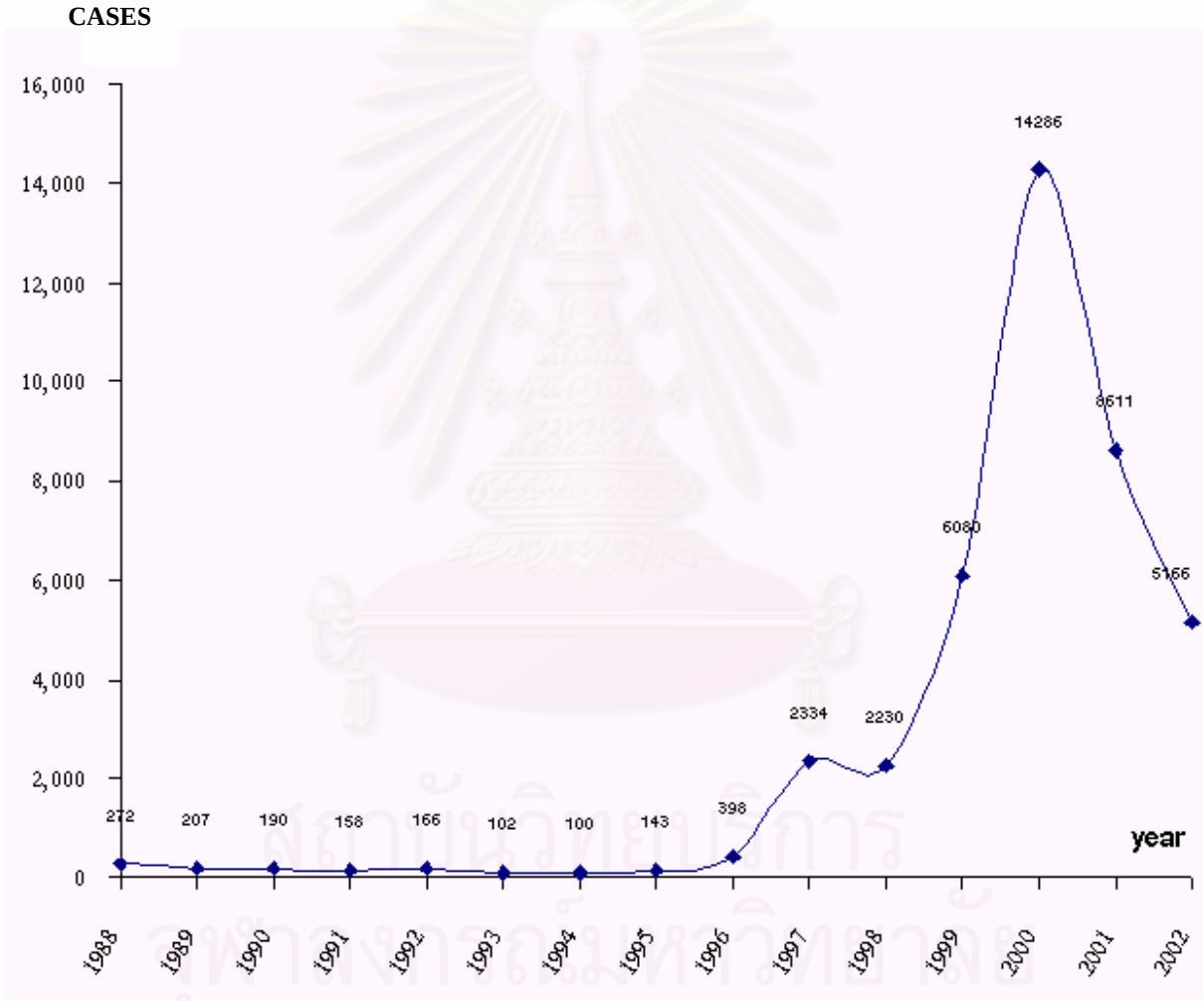
Antigenic characterization of the members of the species *Leptospira interrogans* is a necessary step not only toward understanding the interactions between leptospires and the immune system but also toward gaining information useful for antigen selection for diagnostic tests. The previous study, a global analysis of Leptospiral outer membrane proteins (OMPs), has demonstrated that

leptospiral lipopolysacharide (LPS) is a protective immunogen, however, the extensive serological diversity of LPS of *leptospires* has inspired a search for conserved outer membrane proteins (OMPs) which may stimulate heterologous immunity (43). A 32-kDa outer membrane protein was the most abundant constituent of prominent antigen in pathogenic serovars but in none of saprophytic serovars examined (43,44,45,46) so this protein may be used as antigen for detection of antibody specific to pathogenic *leptospires*. In addition, LipL32 was shown to be expressed both *in vivo* and *in vitro*. This suggests that antibody to LipL32 could be induced during infection and the preparation of this protein can be done using *leptospira* cultured *in vitro*. For those reasons, LipL32 is a candidate for using as an antigen in antibody testing.

The purpose of this study was to clone and purify LipL32 from *Leptospira interrogans* serovar *bratislava* since it is responsible for most leptospirosis cases reported in Thailand during 1999-2002 (47,48). Standard recombinant DNA procedures were performed to clone LipL32 gene . The LipL32 gene was amplified from leptospiral DNA by polymerase chain reaction (PCR) using primers specific for LipL32 gene and these primers generated PCR product, which is a portion of gene encoding mature LipL32, with cut sites for designated restriction enzymes. The plasmid pRSETc containing sequence for histidine residues was used for constructing a plasmid expressing LipL32 protein fused to histidines (this constructed plasmid was called "pRSETc-LipL32" in following parts of this thesis). PCR product and pRSETc were cut with appropriate enzymes and digested products were ligated. The recombinant plasmid was transformed into *Escherichia coli* and transformed clone was selected. Successful ligation and transformation were demonstrated by PCR and DNA sequencing of the plasmid. The obtained sequence was compared with reported LipL32 sequence (Genbank accession number AF121192).

The selected clone was propagated and LipL32 protein expression was induced. LipL32 protein was purified and identified by immunoblotting using antisera specific to LipL32. For preliminary testing of the specificity of this

purified protein, immunoblotting was also performed using sera positive and negative for antibody to *leptospira* (tested by MAT and IFA) and sera positive for antibody to *Treponema pallidum*, a spirochete in another genus. Furthermore, purified LipL32 was also tested as an antigen in a dipstick assay.



Source : The Division of Epidemiology, Ministry of Public Health

Figure 1 The situation of Leptospirosis in Thailand 1988-2002.

CHAPTER II

OBJECTIVE

To clone and express LipL32 from *Leptospira interrogans* serovar *bratislava*

CHAPTER III

LITERATURE REVEIW

1. Historical Aspects

In 1886, Weil has described a severe icteric and renal syndrome which was called Weil's disease (49). Twenty years later, in 1907, Stimson demonstrated the spirochete that caused Weil's disease from a patient having died of "yellow fever" and proposed the name of this organism Spirochaete interrogans, as the organism with its hooked ends resembled a question mark (50). This same spirochete was both independently reported in 1915 in Japan and Germany. In Japan, Inada and Ido isolated spirochetes and detected specific antibodies in blood of Japanese miners with infectious jaundice (51). Two groups of German physicians studied German soldiers afflicted by "French disease" in the trenches of northeast and spirochetes were recovered from the blood of guinea pigs inoculated with blood of infected soldiers (52,53). Both Japan and German groups named the spirochete they isolated as "Spirocheta icterohaemorrhagiae". A few year later in 1917, Noguchi demonstrated that this spirochete differs from other spirochetes and he classified it in a new Genus, *Leptospira* (54). In the same year Ido and coworkers published the prevalence of *Leptospira* serovar *icterohaemorrhagiae* in rats (*Rattus norvegicus*) since then this spirochete has been recognized all the part of the world (55). For many years, the rat was considered the sole animal host of icterohaemorrhagiae, even though Randall and Cooper isolated this agent from a naturally infected dog (56).

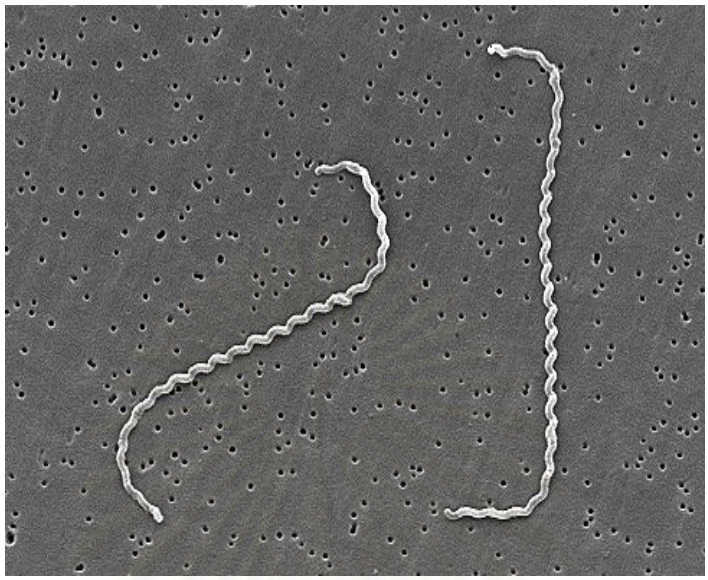
In Thailand, the first case of human leptospirosis was reported byYunibandha in 1943 (8). Subsequently icterohaemorrhagiae has been associated with many animal hosts, including cattles, swines, goats, hamsters and dogs; however the rat remains the primary reservior in most parts of the world (57,58,59,60,61). Other serovars, involving human and animals, were discovered throughout the world. They were summarized in Table 1.

Table 1 List of various serovars of *Leptospira* discovered throughout the world

Year	Serovar of *Leptospira*	Origin
1923	pyrogenes	Man; Indonesia
1926	bataviae	Man; Indonesia
1925	autumnalis	Man; Japan
1928	grippotyphosa	Man and field mice; Russia
1931	andaman A	Man; Andaman Islands
1933	canicola	Dogs; Netherlands
1937	australis A, B	Man; Australia
	pomona	Man; Australia
1939	sejroe	Man; Denmark
1944	saxkoebing	Man; Denmark
	ballum	Man; Denmark
	pomona	Swine; Switzerland
1948	hyos	Man and Swine; Switzerland
		Australia and Argentina

2. Characteristic of *Leptospira*

Leptospira differ from Treponema and Borrelia in that their spirals are very fine and close with 0.1 μm in width and 6-20 μm in length, but occational cultures may contain much longer cells. The helical amplitude is approximately 0.1 to 0.15 μm, and the wavelength is approximately 0.5 μm. Tightly coiled spirochaetes are characterized by very active motility, both rotating (spinning) and bending (62). Usually one or both ends of the cell are bent or a distinctive hooked, but the straight form also occur which rotates and travels more slowly than the hooked form. Because of narrow diameter, the leptospires are best visualized by dark-ground illumination or phase-contrast microscopy. *Leptospira* as short as 4 μm and as long as 40 μm are occasionally seen (63) (Figure 2).



www.med.sc.edu:85/*leptospira*.jpg

Figure 2 Scanning electron micrograph of *L. interrogans* serovar icterohaemorrhagiae

The genus *Leptospira* shares the following basic morphological features with other spirochaetes. Leptospires have a typical double membrane structure, refered to as the outer membrane or outer envelope (OE). The membrane is thick, almost transparent and appears in the darkground as a narrow clear zone or halo. The term protoplasmic cylinder (PC) describes the cellular components enclosed by the OE, and consists of a peptidoglycan layer and cytoplasmic membrane. Leptospiral lipopolysacharide has a composition similar to that of gram negative bacteria but has lower endotoxic activity (64,65,66). There are generally two axial filaments (periplasmic flagella) with polar insertions are located in periplasmic space (67). The structure of the flagella proteins is complex (68). They are attached to the PC in a subterminal position and the free ends extend toward the middle of the cell but do not overlap. The flagellar basal bodies resemble those of gram negative bacteria (65). During cellular reproduction, septal wall formation occurs at the middle region of the organism, which leads to transverse division. Morphologically of the free living (*L. biflexa*) and parasite leptospires (*L.interrogans*) are indistinguishable, but the morphology

of individual isolates varies with subculture in vitro and can be restored by passage in hamters (69,70). Leptospires may be stained using carbol fuchsin counterstain (71). They produce both catalase and oxidase (72).

3. Cultivation

Leptospires are obligate aerobes which can be cultivated in a suitable aerated growth temperature of 28 °C to 30 °C. The growth in simple media containing either serum or albumin plus polysorbate and in protein free synthetic media have been described (73,74). The most wildly used medium in current practice is base on the oleic acid-albumin medium EMJH (75,76). This medium is available commercially from several manufacturers and contains Tween 80 and bovine serum albumin. Some strains are more fastidious and require the addition of either pyruvate (77) or rabbit serum (78). The nonessential nutrient, pyruvate, enhances the initiation of growth of the parasitic leptospires, particularly in serovars such as hardjo and ballum. Leptospires incorporate purine but not pyrimidine bases into their nucleic acids (79). Consequently, they are resistant to the antibacterial activity of the pyrimidine analogue 5-fluorouracil leading to the use of this compound in the selective media for the isolation of leptospires from contaminated sources (80).

Vitamins B1, B12 and long-chain fatty acids are the only organic compounds that are mandatory (81,82,83,84). Fatty acids provide their major source of carbon and energy that are metabolized by beta-oxidation (72). They are also required as sources of cellular lipids since *leptospira* cannot synthesize fatty acids *de novo*. Owing to the inherent toxicity of free fatty acid, these must be supplied to the leptospires either bound to albumin or in a nontoxic esterified form. Carbohydrates are not a suitable source of energy or carbon. Amino acids are utilized to limited extent; they cannot satisfy the nitrogen requirements of these organisms and ammonium salts are effective sources of cellular nitrogen. Growth of leptospires is often slow on primary isolation, and cultures are retained for up to 13 weeks before being discarded, but pure subcultures in liquid media usually grow within 10 to 14 days. Agar may be added at a low concentrations

(0.1 to 0.2 %). In semisolid media, growth reaches a maximum density in a discrete zone beneath the surface of the medium, which becomes increasing turbid as incubation proceeds. This growth is related to the optimum oxygen tension and is known as a Dinger's ring or disk (85). The earliest report of the cultivation *in vitro* of pathogenic leptospires is that of Inada et al (51). Since then, various kinds of media including liquid, semisolid and solid media have been developed and used for diagnostic or research purposes, for instance, special media such as those enriched with rabbit serum (86,87,88), bovine serum albumin (BSA) (75,76) as EMJH medium, and protein-free medium (89,90). The optimal pH for growth and survival of the leptospires is around pH 7.4 (90). Liquid media are necessary for growing the cultures (for serological diagnosis of the infection) and for typing the isolates. Semisolid media are generally used for isolating strains and for the maintenance of stock cultures whereas solid media are useful for cloning the strains and for isolating leptospires from contaminated sources (91). Growth on media solidified with agar has been reported (91,92). Colonial morphology is dependent on agar concentration and serovars (93). Media can also be solidified using gellan gum (94). Solid media have been used for isolation of leptospires (95), to separate mixed cultures of leptospires, and for the detection of hemolysin production (96).

However, Leptospiral cultures may be maintained by repeated subculture or preferably by storage in semisolid agar containing hemoglobin (85). Long term storage by lyophilization or at -70 $^{\circ}$C (97,98) is also used.

4. Molecular Biology

Leptospires are phylogenetically related to other spirochetes (99). The leptospiral genome is approximately 5,000 kb in size, although smaller estimates have been reported (100,101,102,103). The genome is comprised of two sections, a 4,400-kb chromosome and a smaller 350-kb chromosome. Other plasmids have not been reported (104,105,106,107). Physical maps have been constructed from

serovar pomona subtype kennewicki and icterohaemorrhagiae (101,108). Leptospires contain two sets of 16S and 23S rRNA genes but only one 5S rRNA gene (109), and the rRNA genes are widely spaced (110,111,112). The study of leptospiral genetics has been slowed by the lack of a transformation system (113,114). Recently, a shuttle vector was developed using the temperate bacteriophage LE1 from *L. biflexa* (115). This advance offers the prospect of more rapid progress in the understanding of *Leptospira* at the molecular level. Several repetitive elements have been identified of which several are insertion sequences (IS) coding for transposases (116,117,118). IS1533 has a single open reading frame (119,120), while IS1500 has four. Both IS1500 and IS1533 are found in many serovar (116,120), but the copy number varies widely between different serovars and among isolates of the same serovar. A role for these insertion sequences in transposition and genomic rearrangements has been identified. Other evidence for horizontal transfer within the genus *Leptospira* has been reported (121,122). A number of leptospiral genes have been cloned and analyzed, including several for amino acid synthesis (123,124), rRNA (125,126,127), ribosomal proteins (128), RNA polymerase (129), DNA repair (130), heat shock proteins (131,132), sphingomyelinase (133,134), hemolysins (135,136), outer membrane proteins (137,138,139,140), flagellar proteins (141,142,143,144,145), and lipopolysaccharide (LPS) synthesis (146,147,148).

5. Taxonomy and Classification

Since the first isolation of the etiological agent of Weil's disease by Inada and Ido (51) in 1915, many different serovars of leptospires have been isolated all over the world. The genus *Leptospira* belongs to the family Leptospiraceae in the order Spirochaetales. Other genera within the same family are *Leptonema* and *Turneria*. In order to make the classification of leptospires conform to the international code of nomenclature of bacteria and viruses, the WHO scientific group on Leptospirosis and the Taxonomic Subcommittee on *Leptospira* have recommended base on serological classification. The genus *leptospira* was divided into two species: *L. biflexa* representing saprophytic strains that was found in the environment and *L. interrogans* representing pathogenic strains (148,149). The two species were differentiated by growth characteristics, such as growth of *L. biflexa* at 13 °C and in the presence of 8-azaguanine (225 ug/ml) and by the failure of *L. biflexa* to form spherical cells in 1 M NaCl.

Both *L. interrogans* and *L. biflexa* are divided into numerous serovars defined by agglutination after cross-absorption with homologous antigen. If more than 10% of the homologous titer remains in at least one of the two antisera on repeated testing, two strains are said to belong to different serovars . Over 60 serovars of *L. biflexa* have been recorded. Within the species *L. interrogans* over 200 serovars are recognized. Serovars that are antigenically related have traditionally been grouped into serogroups . While serogroups have no taxonomic standing, they have proved useful for epidemiological understanding.

The phenotypic classification of leptospires has been replaced by a genotypic one, in which a number of genomospecies include all serovars of both *L. interrogans* and *L. biflexa*. Genetic heterogeneity was demonstrated some time ago and DNA hybridization studies led to the definition of 10 genomospecies of *Leptospira*. After an extensive study of several hundred strains, workers at the Centers for Disease Control (CDC) more recently defined 16 genomospecies of *Leptospira*. The genotyping classification of leptospires is supported by multilocus enzyme electrophoresis data but recent studies suggest that further taxonomic revisions are likely .

The genomospecies of *Leptospira* do not correspond to the previous two species (*L. interrogans and L. biflexa*), and indeed, pathogenic and nonpathogenic serovars occur within the same species. Thus, neither serogroup nor serovar reliably predicts the species of *Leptospira*. Moreover, recent studies have included multiple strains of some serovars and demonstrated genetic heterogeneity within serovars.

The molecular classification is problematic for the clinical microbiologist, because it is clearly incompatible with the system of serogroups which has served clinicians and epidemiologists well for many years. Until simpler DNA-based identification methods are developed and validated, it will be necessary for clinical laboratories to retain the serological classification of pathogenic leptospires for the foreseeable future.

6. Characterization of Outer Membrane Proteins (OMPs)

Antigens extracted from leptospira have been characterized and classified; however, proteins located in the outer membrane are of the grate interest since their potentially expose to immune system on the leptospiral surface make them good candidates for using as an antigen for antibody detection and as a vaccine for protection. Examples of leptospiral OMPs identified were described as followed.

The porin, OmpL1

OmpL1 is the first transmembrane outer membrane proteins to have been described in a pathogenic spirochete. OmpL1 was originally isolated in surface immunoprecipitation studies intended to identify proteins exposed on the leptospiral surface (150). The N-terminal amino acid sequence was obtained from the 33-kDa surface immunoprecipitated protein and used to design oligonucleotide primers which allowed isolation of the ompL1 gene (151). Examination of the OmpL1 sequence revealed beta-sheet membrane spanning segments typical of gram negative OMPs, consistent with its property of heat modifiable electrophoretic mobility. Polyclonal antiserum generated by immunizing rabbits with a purified, recombinant His6-OmpL1 fusion protein was used to demonstrate OmpL1 surface exposure by immunoelectron microscopy. Subsequent structural studies found that OmpL1 is an integral membrane protein which is present in the native leptospiral membrane as trimers (152), another feature typical of gram-negative transmembrane OMPs. Purified, recombinant OmpL1 exhibits porin activity in planar lipid bilayer studies.

Leptospiral Lipoproteins

Like other bacterial lipoproteins, leptospiral lipoproteins appear to be anchored to membrane by fatty acids that modify the amino-terminal cysteine. The genes encoding six different leptospiral lipoproteins (Table 2) have been identified and five of which are located either exclusively or partly in the outer

membrane. The leptospiral lipoproteins were designated based upon their apparent molecular mass. For example, LipL41 has an apparent molecular mass of 41-kDa.

LipL41

LipL41 was initially identified in the same surface immunoprecipitation studies that isolated OmpL1 (150). Subsequent surface immunoprecipitation experiments confirmed that LipL41 is exposed on the leptospiral surface, while another outer membrane lipoprotein, LipL36, appears to be restricted to the periplasmic leaflet of the outer membrane (152). The most abundant proteins in spirochetes are lipoproteins, and Leptospira species are no exception to this rule. LipL32 and LipL41 appear to be expressed constitutively by all pathogenic Leptospira species under all environmental conditions, while LipL36 and LipL48, are subjected to differential expression (153). LipL36 and LipL48 are not expressed during infection, but are expressed in large amounts in culture-attenuated organisms. LipL45 is expressed in early passage cultures isolated from hamsters infected with L. kirschneri, but not in high passage cultures of the same strain. LipL45 is processed to a 31-kDa growth phase regulated peripheral membrane protein, designed P31LipL45, which is expressed in both low and high passage cultures (154). LipL53, LipL59, and LipL71, are thought to exist on the basis of intrinsic labeling of L. kirschneri with tritiated palmitate.

In each case, the deduced amino acid sequences reveal a signal peptide followed by a lipoprotein signal peptidase cleavage site (Table 2). Although some of these lipoprotein signal peptidase cleavage sites are atypical when compared to *E. coli* lipoprotein signal peptidase cleavage sites (usually L-X-Y-C), they are consistent with those of lipoproteins from other spirochetes.

Several other lines of evidence supported the conclusion that leptospiral lipoproteins are modified by lipid at an N-terminal cysteine residue. Lipid modification appears to be responsible for the hydrophobicity of these proteins. Native LipL32, LipL36, LipL41, and LipL48 partition into the Triton X-114 detergent phase, while the corresponding His6 fusion proteins partition into the Triton X-114 aqueous phase. Intrinsic labeling of *L. kirschneri* with tritiated

palmitate results in selective labeling of LipL32, LipL36, and LipL41. In the case of LipL32, acid treatment resulted in removal of the tritium label, consistent with hydrolysis of the linkage between palmitate and the amino-terminal cysteine. For LipL36 and LipL41, lipidation has been shown to be sensitive to globomycin, a selective inhibitor of lipoprotein signal peptidase.

Table 2 Confirmed and potential leptospiral lipoproteins

Designation	Cleavage Site	Triton X-114 Detergent Phase	Palmitate Labeling	Membrane Location(s)	Expression During Infection
LipL31	F F A S C G D	-	?	IM	+
LipL32	S I T A C G A	+	+	OM	+
LipL36	A L T A C K S	+	+	OM	-
LipL41	F L G N C A A	+	+	OM & IM	+
LipL45	V F N A C K K	+	?	OM & IM*	+
LipL48	S F I N C K E	+	?	OM	-

*The 31-kD form of LipL45 is a peripheral membrane protein associated with both membranes.

7. Epidemiology

Leptospirosis is a widespread zoonosis in the world (156,157). The source of infection in humans is usually either direct or indirect contact with the urine of an infected animal. After leptospires were excreted in urine, this organisms may survive for weeks or months in optimum condition. Humans and animals can be divided into maintenance hosts and incidental hosts. The disease is maintained in nature by chronic infection of the renal tubules of maintenance hosts (157). A maintenance host is defined as a species in which infection is endemic and is usually transferred from animal to animal by direct contact. Infection is usually acquired at an early age, and the prevalence of chronic excretion in the urine increases with the age of the animal. Direct transmission by sexual intercourse

during convalescence has been reported in human (158,159). The indirect transmission occurs when humans contact with urine from infected host. Animals may be maintenance hosts of some serovars but incidental hosts of others, infection which may cause severe or fatal disease. The most important maintenance hosts are small mammals, which may transfer infection to domestic farm animals, dogs, and humans. Water-borne transmission has been documented; contamination of water supplies has resulted in several outbreaks of leptospirosis. Inhalation of water or aerosols also may result in infection via the mucous membranes of the respiratory tract (172). Rarely, infection may follow animal bites (160,161,162,163). Human infections may be acquired through occupational, recreational, or avocational exposures. The extent to which infection is transmitted depends on many factors, including climate, population density, and the degree of contact between maintenance and incidental hosts. Different rodent species may be reservoirs of distinct serovar, but rats are generally maintenance hosts for serovar of the serogroups icterohaemorrhagiae and ballum, and mice are the maintenance hosts for serogroup ballum (164,165,166,167). Domestic animals are also maintenance hosts; dairy cattle may harbor pomona, tarassovi, or bratislava; sheep may harbor hardjo and pomona; and dogs may harbor canicola (168). A knowledge of the prevalent serovar and their maintenance hosts is essential to understanding the epidemiology of the disease.

The prevalence of leptospirosis has been reported worldwide and the disease associated with temperature climate. The prevalence of disease is highest in the warm climate; this is due mainly to longer survival of leptospires in the environment. However, most tropical countries are also developing countries, and there are greater opportunities for exposure of the human population to infected animals, whether livestock, domestic pets, or wild or feral animals. The reported incidence of leptospirosis reflects the availability of laboratory diagnosis and the clinical index of suspicion as much as the incidence of the disease. Within the United States, the highest incidence is found in Hawaii (169). Leptospirosis caused to be a notifiable infection within the United States after December 1994 (170). 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 occurs when abrasions are likely to occur and is thus difficult to substantiate.

The disease is 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.

Occupation is a significant risk factor for humans. Direct contact with infected animals accounts for most infections in farmers, veterinarians, abattoir workers (171,172,173), meat inspectors (174), rodent control workers (175), and other occupations which require contact with animals. Indirect contact is important for sewer workers, miners, soldiers (176,177,178), septic tank cleaners, fish farmers (179,180), gamekeepers, canal workers (181), rice field workers (182,183,184), taro farmers (185), banana farmers (186), and sugar cane cutters (187). Miners were the first occupational risk group to be recognized (188). The occurrence of Weil's disease in sewer workers was first reported in the 1930s (189,190,191,192). Serovar icterohaemorrhagiae was isolated by guinea pig inoculation from patients, from rats trapped in sewers and from the slime lining the sewers (193). In Glasgow, Scotland, a seroprevalence among sewer workers of 17% was reported (192). The recognition of this important risk activity led to the adoption of rodent control programs and the use of protective clothing, resulting in a significant reduction in cases associated with this occupation. The presence in waste water of detergents is also thought to have reduced the survival of leptospires in sewers (194), since leptospires are inhibited at low detergent concentrations (195). Fish workers were another occupational group whose risk of contacting leptospirosis was recognized early. Between 1934 and 1948, 86% of all cases in the northeast of Scotland occurred in fish workers in Aberdeen (196). Recognition of risk factors and adoption of both preventive measures and rodent control have reduced the incidence of these occupational infections greatly. From 1933 to 1948 in the British Isles, there were 139 cases in coal miners, 79 in sewer workers, and 216 in fish workers. However, in the period from 1978 to 1983, there were nine cases in these three occupations combined (194). More recently, fish farmers have been shown to be at risk, particularly for infection with serovar

icterohaemorrhagiae (197), presumed to be derived from rat infestation of premises. Because of the high mortality rate associated with icterohaemorrhagiae infections, this was considered an important occupational risk group despite the very small absolute number of workers affected (211). Livestock farming is a major occupational risk factor throughout the world. The highest risk is associated with dairy farming and is associated with serovar hardjo (198,199,200,201,202), in particular with milking of dairy cattle(203,204). Human cases can be associated with clinical disease in cattle, but are not invariably so (205). Cattle are maintenance hosts of serovar hardjo (206), and infection with this serovar occurs throughout the world (207,208). Many animals are seronegative carriers. After infection, leptospires localize in the kidneys and are excreted intermittently in the urine (209). Serovar hardjo causes outbreaks of mastitis and abortion. Serovar hardjo is found in aborted fetuses and in premature calves. In addition, hardjo has been isolated from normal fetuses (230), the genital tracts of pregnant cattle (209), vaginal discharge after calving, and the genital tract and urinary tract of 50% of cows and bulls (210,211,212). In Australia, both serovar hardjo and pomona were demonstrated in bovine abortions, but serological evidence suggested that the incidence of hardjo infection was much higher (213,214). In Scotland, 42% of cattle were seropositive for hardjo, representing 85% of all seropositive animals. In the United States, serovar hardjo is the most commonly isolated serovar in cattle (215), but pomona also occurs. There is a significant risk associated with recreational exposures occurring in water sports (216), including swimming, canoeing (217,218), white water rafting (219,220), fresh water fishing, and other sports where exposure is common, such as potholing and caving (221). The potential for exposure of large numbers of individuals occurs during competitive events.

Several outbreaks of leptospirosis associated with water have been reported. Many of these outbreaks have followed extended periods of hot, dry weather, when pathogenic leptospires presumably have multiplied in freshwater ponds or rivers. Cases of leptospirosis also follow extensive flooding (221,222,223,224,225,226,227, 228). Pathogenic serovars have been isolated from water in tropical regions (229) and in the United States, where serovar

icterohaemorrhagiae, dakota, ballum, pomona, and grippotyphosa have been recovered. Many sporadic cases of leptospirosis in tropical regions are acquired following avocational exposures that occur during the activities of daily life. Many infections result from barefooted walking in damp conditions or gardening with bare hands (230). Dogs are a significant reservoir for human infection in many tropical countries (231) and may be an important source of outbreaks. A number of outbreaks of leptospirosis have resulted from contamination of drinking water and from handling rodents (232).

Survival of pathogenic leptospires in the environment is dependent on several factors, including pH, temperature, and the presence of inhibitory compounds. Most studies have used single serovar and quite different methodologies, but some broad conclusions may be drawn. Under laboratory conditions, leptospires in water at room temperature remain viable for several months at pH 7.2 to 8.0 (233,234), but in river water survival is shorter and is prolonged at lower temperatures. The presence of domestic sewage decreases the survival time to a matter of hours (235), but in an oxidation ditch filled with cattle slurry, viable leptospires were detected for several weeks. In acidic soil (pH 6.2) taken from canefields in Australia, serovar australis survived for up to 7 weeks, and in rainwater- flooded soil it survived for at least 3 weeks (236). When soil was contaminated with urine from infected rats or voles, leptospires survived for approximately 2 weeks (237). In slightly different soil, serovar pomona survived for up to 7 weeks under conditions approximating the New Zealand winter (238).

In 1994, Faine described the three patterns of epidemiology of leptospirosis (70). The first occurs in temperate climates where few serovars are involved and human infection almost invariably occurs by direct contact with infected animals though farming of cattle and pigs. Control by immunization of animals and/or humans is potentially possible. The second occurs in tropical wet areas, within which there are many more serovars infecting humans and animals and larger numbers of reservoir species, including rodents, farm animals, and dogs. Human exposure is not limited by occupation but results more often from the widespread environmental contamination, particularly during the rainy season. Control of rodent populations, drainage of wet areas, and occupational hygiene are

all necessary for prevention of human leptospirosis. These are also the areas where large outbreaks of leptospirosis are most likely to occur following floods, hurricanes, or other disasters (161,221,223,224,225,226,227). The third pattern comprises rodent borne infection in the urban environment. While this is of lesser significance throughout most of the world.

The situation of Leptospirosis in Thailand has also been investigated. In 1966, Sundharagiati et al., (239) reported an incidence of 23-35% leptospiral seropositive agglutination reactions in Thailand with the highest incidence occurring in the southern region. The prevalence of *Leptospira* among rats and dogs in the provinces of Thailand including Bangkok was also fairly high (240,241) and several serovars of *Leptospira* have been isolated from man and animals. In 1983, a seroepidemiological study of leptospirosis in man and rodents in the north and northeast of Thailand revealed 0.27% serological positive cases of human population tested (242). The number of cases reported from 1982 to 1995 ranged from 55-272 cases per years, with an average incidence of 0.3/100,000/year. However, the number was increased rapidly from 398 cases (incidence 0.65/100,000) in 1996 to 2,334 (incidence 3.83/100,000) in 1997. The number of cases reported each year is still high as shown in Figure 1; 2,230 (incidence 3.52/100,000), 6,080 (incidence 9.89/100,000), 14,286 (incidence 23.2/100,000), 8,611 (incidence 13.96/100,000) and 5,166 (incidence 8.35/100,000) in 1998-2002, respectively (4). The large outbreaks typically in Thailand involved a group of people who have been immersed in flood or who have gathered together to engage in a common activity such as harvesting crops or swimming. For example, in 1999, an outbreak of *leptospira* was reported in the Northeast of Thailand. The incidence of leptospirosis increased between November and December concomitant with seasonal heavy rainfalls and floods (243). However, it seems that the number of cases reported is decreasing. The reasons may be that there are not only the disease surveillance but also more diagnostic tests available for differentiating leptospirosis from other infectious diseases with similar clinical manifestations. Clinical manifestations without laboratory diagnosis supported may not provide accurate diagnosis in some cases.

8. Clinical Features of Leptospirosis

The spectrum of symptoms of leptospirosis is extremely broad; the classical syndrome of Weil's disease represents only the most severe presentation. Formerly, it was considered that distinct clinical syndromes were associated with specific serogroups (244). However, this view was questioned by some authors and more intense study over the past 30 years has refuted this hypothesis. An explanation for many of the observed associations may be found in the ecology of the maintenance animal hosts in a geographic region. A region with a richly varied fauna will support a greater variety of serogroups than will a region with few animal hosts. In humans, severe leptospirosis is frequently but not invariably caused by serovar of the icterohaemorrhagiae serogroup. The specific serovar involved depend largely on the geographic location and the ecology of local maintenance hosts. Thus in Europe, serovar copenhageni and icterohaemorrhagiae, carried by rats, are usually responsible for infection, while in Southeast Asia, serovar lai is common. The clinical presentation of leptospirosis is biphasic (Figure 3), with the acute or septicemia phase lasting about a week, followed by the immune phase, characterized by antibody production and excretion of leptospires in the urine (245,246,247). Most of the complications of leptospirosis are associated with localization of leptospires within the tissues during the immune phase and thus occur during the second week of the illness.

Anicteric Leptospirosis

The great majority of infections caused by leptospires are either subclinical or of very mild severity, and patients will probably not seek medical attention. A smaller proportion of infections, but the overwhelming majority of the recognized cases, present with a febrile illness of sudden onset. Other symptoms include chills, headache, myalgia, abdominal pain, conjunctival suffusion, and less often a skin rash. If present, the rash is often transient, lasting less than 24 h. This anicteric syndrome usually lasts for about a week, and its resolution coincides with the appearance of antibodies. The fever may be biphasic and may recur after a remission of 3 to 4 days. The headache is often severe, resembling that occurring in dengue, with retro-orbital pain and photophobia. Myalgia affecting the lower back,

thighs, and calves is often intense (248). Aseptic meningitis may be found in ≤25% of all leptospirosis cases and may account for a significant minority of all causes of aseptic meningitis (249,250,251,252). Patients with aseptic meningitis have tended to be younger than those with icteric leptospirosis. In their series of 616 cases, Alston and Broom (86) noted that 62% of children ≤14 years old presented with aseptic meningitis, whereas only 31% of patients aged 15 to 29 years did so and only 10% of those over 30 years of age. Mortality is almost nil in anicteric leptospirosis, but death resulting from massive pulmonary hemorrhage occurred in 2.4% of the anicteric patients in a Chinese outbreak (299). The differential diagnosis must include common viral infections, such as influenza, human immunodeficiency virus seroconversion , and, in the tropics, dengue, in addition to the bacterial causes of fever of unknown origin, such as typhoid. Turner provided a comprehensive list of other conditions that may be mimicked by leptospirosis, including encephalitis, poliomyelitis, rickettsiosis, glandular fever (infectious mononucleosis), brucellosis, malaria, viral hepatitis, and pneumonitis (34). Hantavirus infections must also be considered in the differential diagnosis for patients with pulmonary involvement (253). Petechial or purpuric lesions may occur (254), and recently, cases of leptospirosis resembling viral hemorrhagic fevers have been reported in travelers returning from Africa (255,256).

Icteric Leptospirosis

Icteric leptospirosis is a much more severe disease in which the clinical course is often very rapidly progressive. Severe cases often present late in the course of the disease, and this contributes to the high mortality rate, which ranges between 5 and 15%. (257). The jaundice occurring in leptospirosis is not associated with hepatocellular necrosis,and liver function returns to normal after recovery (258). The complications of severe leptospirosis emphasize the multisystemic nature of the disease. Leptospirosis is a common cause of acute renal failure (ARF), which occurs in 16 to 40% of cases (259,260).

The occurrence of pulmonary symptoms in cases of leptospirosis was first noted by Silverstein (261). Subsequent reports have shown that pulmonary involvement may be the major manifestation of leptospirosis in some clusters of cases and in some sporadic cases. The severity of respiratory disease is unrelated to the presence of jaundice. Patients may present with a spectrum of symptoms, ranging from cough, dyspnea, and hemoptysis (which may be mild or severe) to adult respiratory distress syndrome. Intra-alveolar hemorrhage was detected in the majority of patients, even in the absence of overt pulmonary symptoms. Pulmonary hemorrhage may be severe enough to cause death.

Cardiac involvement in leptospirosis is common but may be underestimated. Fatal myocarditis was first described in 1935. Clinical evidence of myocardial involvement, including abnormal T waves, was detected in 10% of 80 severe icteric cases in Louisiana while similar electrocardiographic (ECG) abnormalities were detected in over 40% of patients in China, India, Sri Lanka, and the Philippines, including both icteric and nonicteric cases. However, in a prospective study in Malaysia, identical ECG changes were found in patients with either leptospirosis or malaria and it was concluded that such ECG changes were nonspecific. Other ECG abnormalities have been reported less frequently. The presence of myocarditis was strongly associated with the severity of pulmonary symptoms in anicteric Chinese patients. A mortality rate of 54% was reported in severe leptospirosis cases with myocarditis. Repolarization abnormalities on ECG were considered a poor prognostic indicator (OR 5.9) in severe leptospirosis cases as were arrhythmias (OR 2.83) in a Brazilian series (262).

Ocular Involvement

Ocular manifestations of severe leptospirosis were noted in early reports. Anterior uveitis, either unilateral or bilateral, occurs after recovery from the acute illness in a minority of cases. Uveitis may present weeks, months, or occasionally years after the acute stage. Chronic visual disturbance, persisting 20 years or more after the acute illness, has been reported. The incidence of ocular complications is variable, but this probably reflects the long time scale over which they may occur. In the United States the incidence was estimated at 3% (257) while in Romania an

incidence of 2% was estimated between 1979 and 1985. However, in abattoir workers with evidence of recent leptospirosis, the latter authors reported an incidence of 40%. In most cases uveitis is presumed to be an immune phenomenon, but leptospires have been isolated from human and equine eyes and more recently, leptospiral DNA has been demonstrated in aqueous humor by PCR. Late-onset uveitis may result from an autoimmune reaction to subsequent exposure. Recently, a large cluster of cases of uveitis was reported from Madurai in southern India following an outbreak of leptospirosis which occurred after heavy flooding. The majority of affected patients were males, with a mean age of 35 years. Eyes were involved bilaterally in 38 patients (52%), and panuveitis was present in 96% of eyes. Other significant ocular findings included anterior chamber cells, vitreous opacities, and vasculitis in the absence of visual deficit.

Other Complications

Acute infection in pregnancy has been reported to cause abortion and fetal death (264) but not invariably so. In one of the cases reported by Chung (265) leptospires were isolated from amniotic fluid, placenta, and cord blood; the infant was mildly ill and was discharged at 2 weeks of age. In another case, a neonate developed jaundice and died 2 days after birth. Leptospires were demonstrated in the liver and kidneys by silver staining, but serological evidence of leptospiral infection in the mother was only obtained 2 weeks after delivery. Leptospires have been isolated from human breast milk and in one case serovar hardjo was probably transmitted from an infected mother to her infant by breast feeding (266). Rare complications include cerebrovascular accidents rhabdomyolysis thrombotic thrombocytopenic purpura acute acalculous cholecystitis erythema nodosum aortic stenosis Kawasaki syndrome reactive arthritis epididymitis nerve palsy male hypogonadism and Guillain-Barre´ syndrome. Cerebral arteritis, resembling Moyamoya disease, has been reported in a series of patients from China.

Chronic or Latent Infection

Leptospirosis may induce chronic symptoms analogous to those produced by other spirochetal infections, such as Lyme disease. However, there is very little objective evidence to support or disprove this hypothesis. The possibility of chronic human infection was suggested, without evidence of infection other than serology (267). A single case of late-onset meningitis following icteric leptospirosis has been described, in which leptospires were isolated from both cerebrospinal fluid (CSF) and urine. This patient exhibited a negligible antibody response to the infecting strain, suggesting the presence of an immune defect. Of the sequelae of acute leptospirosis described above, uveitis is a potentially chronic condition and is a recognized chronic sequel of leptospirosis in humans and horses. Equine recurrent uveitis appears to be an autoimmune disease, and Faine (78) suggested that late-onset uveitis in humans may result from an autoimmune reaction to subsequent exposure. Immune involvement in retinal pathology has been demonstrated in horses with spontaneous uveitis. Leptospires have been isolated from the human eye, and more recently, leptospiral DNA has been amplified from aqueous humor of patients with uveitis (267). In these cases, uveitis has occurred relatively soon after the acute illness. One follow-up study of 11 patients with a mean time of 22 years (range, 6 to 34 years) after recovery from acute leptospirosis has been reported. Four patients complained of persistent headaches since their acute illness. Two patients complained of visual disturbances; both had evidence of past bilateral anterior uveitis. No biochemical or hematologic abnormalities were detected to suggest continuing liver or renal impairment. No studies to date have attempted to confirm the persistence of leptospires in the tissues of patients who have subsequently died of other causes.

9. Immunological Response

The agglutinating antibody response in human leptospirosis has been extensively studied and used for diagnosis. In general, leptospiral agglutinating antibodies appear 7-8 days after the onset of the disease (268) ; reach their peak after 15-20 days and remain stable for a most variable period, ranging from a few

months to as much as 20 years (269) ; these are followed by a gradual decline with low levels persisting for an indefinite period.

Studies to evaluate the increase of the agglutinating antibodies and their relationships with several immunoglobulins have been undertaken since circulating antibodies seem to play an important role in the defense against leptospirosis. In 1965, Pike *et al*., (270) found that the predominant homologous agglutinins in four patients for whom serological test had indicated infection with serovar canicola, grippotyphosa or pomona, were IgM. IgM antibodies appear first, and remain for several weeks before IgG antibodies can be demostrated. Antibodies of both Ig classes, derived from either human and cattle sera, have been shown to be protective in passive protection tests.

In 1964, Faine demonstrated the major role of fixed phagocytes of the reticuloendothelial system in clearing pathogenic leptospires from the blood of mice and the opsonizing role of specific antibody. This was also confirmed in the study of macrophages and polymorphonuclear neutrophils by Faine et al (271) although, no *in vitro* opsonizing effect of antiserum on the phagocytosis of leptospires by human monocytes was observed by Rose (272). In 1984, Wang *et al.*, (184) revealed that human neutrophils were able to ingest and kill nonpathogenic *L. biflexa* but not pathogenic *L. interrogans* even in the presence of normal serum. Only when the organisms have been opsonized by specific antibodies did human monocytes, macrophages and neutrophils possess the capacity to ingest and kill pathogenic *L. interrogans* serovar *icterohaemorrhagiae.* These results indicate that the opsonizing effect of specific antibody may play an important role in the mechanism of host defense against leptospirosis.

Apart from the humoral immune response, the cell mediated immune response (CMIR) to leptospirosis was shown to appear earlier and might be useful in the early detection of the disease (273).

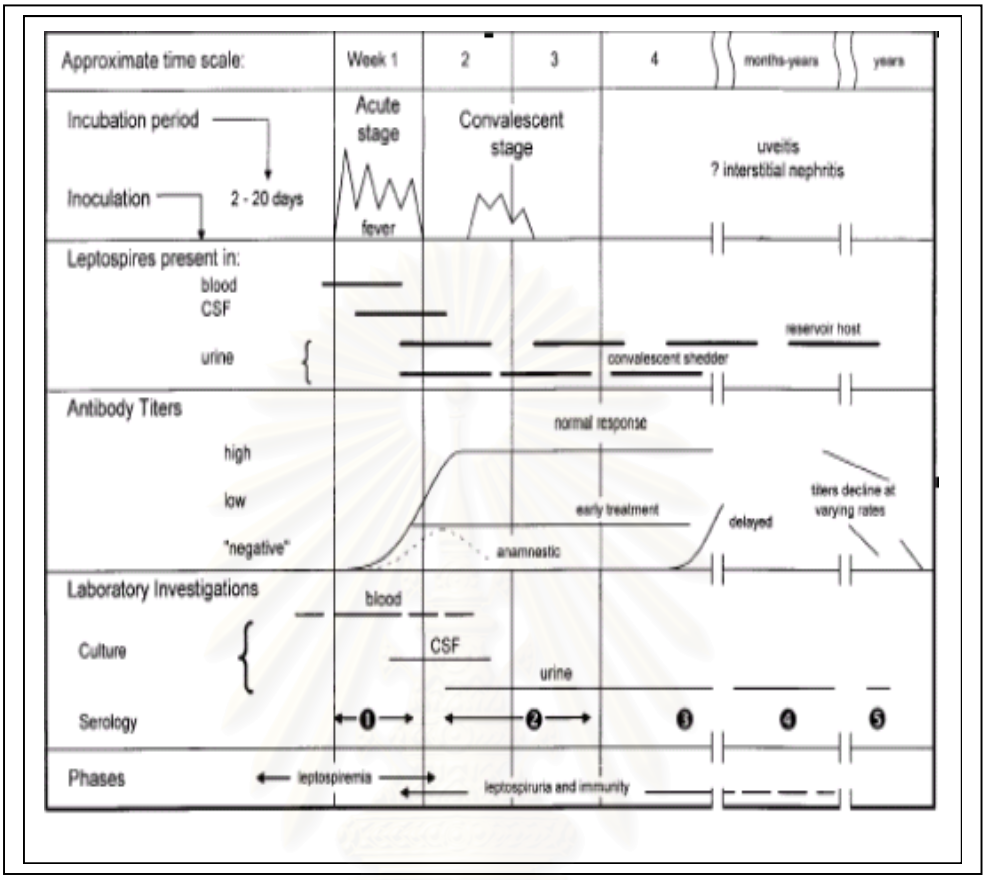


Figure 3 Biphasic nature of leptospirosis and relavant investigations at different stages of disease. Specimens 1 and 2 for serology are acute-phase specimens, 3 is a convalescent-phase samples which may facilitate detection of a delayed immune response, and 4 and 5 are follow-up samples which can provide epidemiological information, such as the presumtive infecting serogroup.

10. Laboratory Diagnosis

Isolation of *Leptospira*

Leptospires can be isolated from the blood, cerebrospinal fluid, urine or organs of dead or sacrificed animals. Generally the organisms are present in the blood and cerebrospinal fluid only during the first 10 days of clinical illness, although isolation has occasionally been made from samples obtained as late as

the 19th day (274). Leptospires usually appear in the urine at the beginning of the second week of the disease and may persist for 30 days or longer.

Many different media have been developed for the cultivation of leptospires (5). For routine use, Fletcher's semisolid medium or EMJH semisolid medium is recommended. For blood culture, the specimens should be collected during the leptospiremic stage, prior to treatment with antimicrobial therapy. It is advisable to vary the number of drops of blood inoculated in each tube of cultivation medium since an excessive amount of blood may inhibit growth of leptospires. If the seeding cannot be affected immediately after taking the sample, it is preferable to add the blood to an anticoagulant buffer solution. Cultures are then incubated at 28 °C to 30 °C in the dark for 5 to 6 weeks or longer, and examined by dark-field microscopy. Cerebrospinal fluid culture is carried out in the same manner.

Serological Tests

Since cultures may be positive only during the first 10 days of illness and may require several weeks to cultivate, laboratory diagnosis often depends on the demonstration of antibodies to leptospires in the sera. Thus, a variety of tests have been developed (Table 3). At first, the agglutination tests were performed. They are classed as microscopic (read by a low power dark field microscope) or macroscopic (read by naked eyes) agglutination. The microscopic agglutination test requires multiple serovar of live *leptospira* responsible for the infection as the antigens. The maintenance of a battery of live cultures to provide the antigens are necessary. Thus, the risk of infection and time-consumption limit the usefulness of this test for routine application in diagnostic laboratories. However, the microscopic agglutination test is still the standard reference test because of its high specificity and sensitivity (275,276).

Table 3 Serological tests for determination of leptospiral antibodies

Tests	Antigen	Comments
1. Microscopic Agglutination	live leptospires	- high specificity and sensitivity - time consuming
2. Macroscopic Agglutination Test Galton (16) and Stonner (17)	pooled formalinized leptospires	- stability of antigen - simplicity of test to perform - less sensitive and difficult to read results in same cases - difficult to look at
3. Complement fixation (CF) test	sonicated leptospiral antigen	
Randall (277) Schneider (278)	serologically active "fraction I" (heat stable an non-protein antigen)	- technical complexity of test - instability of reagents
Ezell (279)	ethanol soluble antigen (type-specific, heat stable and non-protein antigen)	
Terzin (18)	acetone extracted antigen	

4. Hemolysis (HL) test.	ethanol-extracted antigen	- high sensitivity, broad specificity and simplicity of test
Cox (19, 280)	erythrocyte sensitizing substance	- useful in epidemiological survey - need complement activity and serum absorption with sheep erythrocyte antigen
5. Heamagglutination (HA) test	ethanol-extracted antigen sensitized on fresh human erythrocytes	- sensitivity and reliability of test - showing genus-specific reaction
Chang and McComb (21)		- stability of sensitized erythrocytes
Baker (287)	ethanol-extracted antigen sensitized on glutaraldehyde fixed erythrocytes	- easy of performance - suggesting that only IgM antibodies took part in the reaction
Palit (24)	ethanol-extracted antigen sensitized on fresh sheep erythrocytes and formalinized sheep erythrocytes	

6. Latex agglutination (LA) test Murachi (282) Kelen (283) Pimjai (281)	formalinized leptospires pyridine-extracted antigen	- non infective antigen - high sensitivity, specifficity, and rapidity of test - easily employing - stability of reagents
7. Microcapsule agglutination test (MCAT) Yoshiko (284)	sonicated leptsopiral antigen	
8. Indirect immunoflu-orescent assay (IFA) Torten (22)	fixed-whole cell leptospires	- showing genus-specific reaction - the simplicity and rapidity of assay - suitable for antibody screening - need of fluorescent microscope and specific training
9. Enzyme-linked immunosorbent assay (ELISA) Adler (31)	sonicated leptospiral antigen	- highly sensitive and specific - detecting of both IgM and IgG antibodies
Hartman (201)	outer envelop antigen	- showing genus-specific reaction
Terpstras (29)	heat-extracted antigen	- stability of antigen - highly sensitive and specific - detecting of both IgM and IgG antibodies

The antibody response is only detectable after the first week of leptospiremia (235,16) or it may remain negative even in fatal case. This poses problems in diagnosis. Consequently, Adler et al in 1982 attempted to develop tests for the detection of leptospiral antigen (*L. interrogans* serovar *hardjo*) in which the best sensitivity obtained by ELISA was 10^5 leptospires per ml, and 10^4 to 10^5 organisms per ml by radioimmunoassay (RIA). Nonetheless, this has not been performed in the clinical specimen.

In 1985 and 1988, a newly IgM-specific dot ELISA test using ethanol-extracted leptospiral antigen was shown to be comparable to the classic MA test in its ability to detect antibody against leptospires. It appeared to be sensitive and specific for the serodiagnosis of acute leptospirosis. In addition, it is inexpensive, simple to perform, utilizes only small volumes of killed antigen and easily adapted to field use.

Since leptospiral antigens have been more widely characterized as described earlier, purified or recombinant antigens have been proposed to used as antigens for development of assays for leptospiral laboratory diagnosis. However, such assay is not available or validated.

CHAPTER IV

Materials and Methods

1. Bacterial Strains and Serum Samples

All leptospire used in this study (as shown in Table 4) were obtained from the Department of Medical Science, Ministry of Public Health, Thailand. The bacteria were cultivated in Johnson- Harris bovine serum albumin- Tween 80 (EMJH) medium. Stock laboratory cultures were maintained by regular subculture into fresh medium. *Leptospira interrogans* serovar *bratislava* was used for cloning and expression of LipL32 gene and all serovars of leptospires listed in Table 4 were used in MAT.

All serum samples used for antibody testing were obtained from Department of Microbiology, King Chulalongkorn Memorial Hospital. They were 20 sera whose leptospirosis was suspected and positive for antibody to *leptospira* antibody by MAT and IFA, 20 sera positive for antibody to *T. pallidum* tested by IFA Test using *T. pallidum* as an antigen (the test was done at Immunology Unit, Department of Microbiology, King Chulalongkorn Memorial Hospital), and 20 sera from healthy volunteers.

2. Microscopic Agglutination Test (MAT)

Since MAT is the reference method for determining of antibody to *Leptospira*, it was used for determining antibody in samples used in this study. All live leptospires serovars listed in Table 4 were tested with serum samples. Leptospires were grown in EMJH medium and checked for viability and non-autoagglutination under a dark-field microscope before use. The antigen concentrations of *leptospires* were adjusted to McFarland 0.5 units or 10^8 cells/ml. Serum samples were mixed for first screening against all serovars at a

1:100 dilution in normal saline solution (NSS). The mixture was left at room temperature for 2 hours, after which 10 ul from each dilution was examined for agglutination using dark-field microscope. Sera showing positive reaction at 1:100 were than retested against the respective serovar(s) to determine the endpoint agglutination titer. Thus patient's sera were diluted from 1:100 to 1:30,000 in a dilution plate. To each dilution was added an equal volume (3 droplets) of viable *Leptospira* culture. The dilution plate was incubated at room temperature for 2 hours, after which a loopful from each well was removed to examine for agglutination under dark-field microscope. Positive controls (wells containing serum positive for antibody to *leptospira*) and antigen controls (wells containing *leptospires* without serum) were also included. The endpoint agglutination titer was the highest dilution giving ≥ 50 % agglutination (2+) of leptospires. The antibody titer over or equal to 1:100 was considered positive.

Table 4 *Leptospira* serovars tested in this study.

No.	**Serogroup**	**Serovar**	**Strain**
1	Autumnalis	autumnalis	Akiyami
2	Ballum	ballum	Mus 127
3	Bataviae	bataviae	Van Tienam
4	Australis	bratislava	Jez Bratislava
5	Canicola	canicola	Hond Utrecht IV
6	Celledoni	celledoni	Celledoni
7	Cynopteri	cynopteri	3522 C
8	Djasiman	djasiman	Djasiman
9	Grippotyphosa	grippotyphosa	Moskva V

No.	Serogroup	Serovar	Strain
10	Hebdomadis	hebdomadis	Hebdomadis
11	Icterohaemorrhagiae	icterohaemorrhagiae	RGA
12	Javanica	javanica	Veldrat Bat. 46
13	Louisiana	saigon	L 79
14	Panama	panama	Panama
15	Pomona	pomona	Pomona
16	Pyrogenes	pyrogenes	Salinem
17	Sejroe	sejroe	M 84
18*	Samaranga	patoc	Patoc I

* non-pathogenic strain

3. Indirect Immunofluorescence Assay (IFA)

In addition to MAT, IFA was also used for detection of specific IgG and IgM antibodies to *leptospira*. The *L. interrogans* serovar *bratislava* was cultivated and prepared as an antigen for IFA. This strain was grown in EMJH medium and incubated at 28-30 °C for seven days. The bacteria were counted under dark-field microscope and centrifuged at 5,000 rpm for 10 minutes. Then the sediment was washed with 0.85 percent saline three times. After that, bacterial suspension was adjusted to approximately 10^8 cells per ml with distilled water and was dropped onto wells of IFA slide and air dried. The slide was then fixed with acetone for 10 minutes and left to air dry at room temperature. Ten ul of each serial dilution of sera, starting with 1:100 up to 1:3,200 was added to antigen on the slide and was incubated in moist chamber at 37 °C for 30 minutes. The slide was then washed with phosphate buffer saline (PBS pH 7.2-7.4) three times. A drop of 10 ul of 1:40 dilution of rabbit anti-human immunoglobulin conjugated to FITC was added onto each well and

the slide was incubated at 37 °C for 30 minutes. The slide was washed with PBS and mounted with glycerol buffer. Under the fluorescence microscope, a positive reaction was indicated by a green fluorescent color of the antigen. Antibody titer over or equal to 1:100 is considered postive.

4. Cloning of LipL32

4.1 PCR amplification of LipL32 gene

DNA of *Leptospira interrogans* serovar *bratislava* was extracted as previously described. Briefly, organisms were counted under dark-field microscope and adjusted concentration to approximately 10^6-10^8 cells per ml. One ml of culture was transferred to a sterile eppendorf tube and centrifuged at 13,000 x g for 30 minutes at 4 °C. The pellets were washed twice with PBS (pH 7.2-7.4) and resuspended in 100 ul of 0.1 mM Tris-HCl (pH 7.0) and boiled for 10 minutes at 100 °C. The DNA concentrations were measured by spectrophotometry (OD_{260}/OD_{280}) and stored at – 20 °C until processed.

Amplification of LipL32 gene of *L. interrogans* serovar *bratislarva* was performed using primers as previously reported (293). DNA of a non-pathogenic strain, *Leptospira biflexa* serovar *patoc I* was used as a negative control.

Extracted leptospiral DNA (200 ng) was used as the template for PCR amplification of the portion of the LipL32 gene encoding the mature protein, beginning with the first residue after the amino-terminal cysteine. The 5' oligonucleotide contained the nucleotide sequence encoding for the six amino acids after the amino-terminal cysteine of mature LipL32, including a *XhoI* restriction endonuclease site: 5'-TTA CCG CTC GAG GTG CTT TCG GTG GTC TGC-3'. The 3' oligonucleotide contained the nucleotide sequence encoding for the five carboxy-terminal amino acids and the LipL32 stop codon,

including a *SmaI* restriction endonuclease site: 5'-TGT TAA CCC GGG TTA CTT AGT CGC GTC AGA-3'.

The PCR reactions were carried out in sterile 0.2 ml tubes which contained 25 ul of the mixture as followed: 1x PCR buffer, 200 uM dNTPs, 2 mM $MgCl_2$, 100 nmole of each primer, and 0.5 U Taq DNA polymerase. The amplification was performed using the following conditions: heat denaturation at 94 °C for 3 minutes, and then 20 cycles of heat denaturation at 94 °C for 1 minute, primer annealing at 59 °C for 1 minute, and DNA extension at 72 °C for 1 minute. After the last cycle, elongation step was extended for 10 minutes. The amplification products were analyzed by electrophoresis in 1.5% agarose gel stained with ethidium bromide. With this protocol, the presence of a 782-bp band indicated the successful amplification of the LipL32 gene.

4.2 PCR amplification of 16s rRNA

Amplification of 16s rRNA from *leptospira* DNA was performed as an internal control. The 16s rRNA primers was kindly provided by Dr. Kanitha Patarakul, Department of Bacteriology, Faculty of Medicine, Chulalongkorn University.

The sequences of primers are as followed : 5'-GGT AAA GAT TTA TTG CTC GG-3' and 5'-AAA TAA GCA GCA ATG AGA TG-3'.

The PCR reactions using 200 ng of DNA were carried out in sterile 0.2 ml tubes which contained 25 ul of the mixture as followed: 1x PCR buffer, 200 uM dNTPs, 2 mM $MgCl_2$, 100 nmole of each primer, and 0.5 U Taq DNA polymerase. The amplification was performed using the same condition of LipL32 gene : heat denaturation at 94 °C for 3 minutes, and then 20 cycles of heat denaturation at 94 °C for 1 minute, primer annealing at 59 °C for 1 minute,

and DNA extension at 72 °C for 1 minute. After the last cycle, elongation step was extended for 10 minutes. The amplification products were analyzed by electrophoresis in 1.5% agarose gel stained with ethidium bromide. With this protocol, the presence of a 285-bp band indicated the successful amplification of the 16s rRNA.

4.3 Construction of pRSETc-LipL32 plasmid

The 782 bp band obtained from amplification of DNA of *Leptospira interrogans* serovar *bratislava* was cut from gel and purified by nucleospin kit according to the manufacturer's instruction as followed. The target fragment was cut from gel with a clean scalpel, transferred to a sterile tube and melted with NT1 buffer (100 mg gel per 300 ml of NT1 buffer) by incubating at 50 °C for 5-10 minutes. The samples were loaded onto a column and spin down at 8,000 x g for 1 minute. Then, the column was washed twice with 200 ul of NT3 buffer and spin down again. DNA was eluted from the column by 20 ul of elution buffer and spinning at 11,000 for 1 minute. Purified PCR product was used for construction of a plasmid expressing LipL32 protein as mentioned later.

The purified 782-bp fragment of LipL32 gene was digested with *XhoI* and *SmaI* and ligated into pRSETc (generously provided by Professor Ming-Jeng Pan, Gradute Institute of Veterinary Medicine, National Taiwan University) digested with *XhoI* and *PvuII*. One microgram of both 782-bp fragment and pRSETc were digested with appropriate restriction enzymes (as shown in Table 5) using buffers and conditions as manufacture's suggestion.

In order to construct a plasmid expressing His-LipL32 protein, digested LipL32 gene and pRSETc plasmid were ligated with T4 DNA ligase. The mixture for ligation was set up so that the molar ratio of insert : plasmid was

5:1. The amounts of insert and plasmid used were calculated using the following formular.

$$\text{ng of insert} = \frac{\text{ng of vector x kb size of insert}}{\text{kb size of vector}} \times \text{molar ratio of insert/vector}$$

In this experiment, 100 ng of vector was used, the size of insert was 0.782 kb, the size of cut vector was 2.895 kb and molar ratio of insert : vector 5:1 was used. The calculation of amount of insert needed for 100 ng of pRSETc was 135.06 ng which was obtained by formulation as below.

$$\frac{100 \text{ ng} \times 0.782 \text{ kb}}{2.895 \text{ kb}} \times 5/1 = 135.06 \text{ ng of insert}$$

DNA ligation was performed by incubating both cut pRSETc and LipL32 fragment with 0.1 U of T4 DNA ligase at 15–20 °C for 4 hours.

Table 5 Restriction enzymes used in this study.

Restriction Enzyme	**Source**	**Restriction site**
PvuII	*Proteus vulgaris*	5'-CAG ↓CTG-3' 3'-GTC ↑GAC-5'
SmaI	*Serrata marcescens*	5'-CCC ↓GGG-3' 3'-GGG ↑CCC-5'
XhoI	*Xanthomonas compestis* *(X. holicola)*	5'-C ↓TCGAG-3' 3'-GAGC T↑C-5'

4.4 Bacterial transformation

The resulting construct, pRSETc-LipL32,was transformed into competent *Escherichia coli* BL21 (DE3) for expression.

4.4.1 Preparation of competent *Escherichia coli* BL21 (DE3)

The *E. coli* strain BL21 (DE3) for transformation was generously provided by Assistant Professor Wimon Chancham, Department of Microbiology, Faculty of Science, Ramkhamhang Univeristy. The competent *E. coli* was prepared as followed. A single bacterial colony was inoculated into 5 ml of Luria Bertani (LB) broth and incubated for 16-20 hours at 37 °C. One ml of culture was transferred into 100 ml of LB broth and incubated with shaking at 37 °C approximately for 3 to 5 hours, and the growth was monitored by reading absorbance at 500 nm. When the absorbance was about 0.35 – 0.4, the bacterial cells were transferred to cold 50-ml polypropylene tubes and chilled on ice for 10 minutes. The bacterial cells were recovered by centrifugation at 2,700 x g for 10 minutes at 4 °C. Afterward, the cultures were decanted and pellets were reconstituted with 30 ml ice cold $MgCl_2$-$CaCl_2$ solution (80 mM $MgCl_2$, 20 mM $CaCl_2$). Then, the suspensions were centrifugated at 2,700 x g for another 10 minutes at 4 °C. The cultures were decanted and reconstituted with 2 ml of ice cold 0.1 M $CaCl_2$ solution for each 50 ml of original culture. Competent cells were aliquoted and kept at -70 °C until processed.

4.4.2 Bacterial transformation

A tube of competent E. coli BL21(DE3) cells was thawed on ice and dispensed 0.1 ml into each prechilled eppendorf tubes. Three tubes of competent cells were needed for transformation. The first tube, 10 µl of ligation

mixture was added, the second, for 50 ng of empty pRSETc plasmid and the last tube was competent cell control (no plasmid added). DNA and competent cell mixtures were left on ice for 30 minutes before being transferred to a 42 °C waterbath for exactly 90 seconds. Then all tube were placed on ice for 1 minute and 0.8 ml of SOC was added into each tubes before incubating in a 37 °C shaking incubator for 45 minutes to allow the bacteria to recover and to express the antibiotic resistance markers. After that, 50 μl of bacterial suspension was plated and spread onto LB plates containing 50 ug/ml ampicillin, 50 ug/ml 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal), and 1 mM isopropyl-D-thiogalactopyranoside (IPTG). Plates were incubated at 37 °C overnight.

IPTG induced *lacZ* gene expression and the hydrolysis of X-Gal by *lacZ* gene product resulted in blue product production. In the presence of an insert in the plasmid, *lacZ* gene was disrupted. For those reasons, white colonies on LB plate suspected transformed pRSETc-LipL32 gene were selected and further tested.

4.5 Testing of transformed cells

Transformed *E.coli* were tested whether they contained pRSETc-LipL32 plasmid by PCR and DNA sequencing.

4.5.1 PCR amplification

White colonies obtained from transformation were amplified by PCR using specific primers for LipL32 and pRSETc (as shown in Table 6). Briefly, a single white colony was inoculated into 5 ml LB broth containing 50 ug/ml ampicillin and incubated at 37 °C overnight. The plasmids in overnight

cultures were purified with minipreparation (Appendix II). The purified plasmids were used for PCR and DNA sequencing as mentioned followed.

PCR reactions using 200 ng of purified plasmid were carried out using the same condition as for amplification of LipL32 gene from DNA extracted from *Leptospira* as mentioned earlier. The presence of a 782-bp band from the selected transformant using primers specific for LipL32 gene indicated that transformant contained plasmid with LipL32 gene insert. In addition, the 961-bp PCR product had to be obtained if primers specific for pRSETc were used.

4.5.2 DNA sequencing

The purified plasmid was sequenced using primers LipL32-F, pRSETc-F, and pRSETc-R (the primers as shown in Table 6), ABI Prism Bigdye Terminator Cycle Sequencing Ready Reaction kit version 3.1 and the Perkin Elmer 9600 automated nucleic acid sequencer as followed. PCR amplification was done in total 20 ul reaction containing 0.8 ug of plasmid, 4 ul of Big dye terminator, and 3.2 pmole of each primer in kit buffer. PCR was performed for 25 cycles in the following condition : rapid heat 96 °C, heat denaturation at 96 °C for 10 seconds, and primer annealing at 50 °C for 5 seconds, and DNA extension at 60 °C for 4 minutes. The product was centrifuged and DNA was precipitated with glycogen and 75% isopropanol 80 ul and left at room temperature for 15 minutes to precipitate the extension products. The mixtures were centrifuged at 12,000 x g for 5 minutes and the supernatants were aspirated. The pellets were washed with 250 ul 70% ethanol, and precipitated with 100% ethanol. Samples were dried in a vacuum

centrifuge for 10-15 minutes. Samples were transferred to sequencing tubes and run on automated nucleic acid sequencer. The sequence obtained was alignment with Clustal X program and homology Blast search in Genbank database.

Table 6 The primers used for PCR and DNA sequencing of plasmid from transformed cells.

Primers*	Sequences
1. pRSETc-F	**5’- TAG CAT GAC TGG TGG ACA GC-3’**
2. pRSETc-R	**5’- CCC CAA GGG GTT ATG CTA GT-3’**
3. LipL32 –F	**5'-TTA CCG CTC GAG GTG CTT TCG GTG GTC TGC-3'**
4. LipL32-R	**5'-TGT TAA CCC GGG TTA CTT AGT CGC GTC AGA-3'.**

* For sequencing, only primers 1-3 were used.

5. Protein expression

5.1 Kinetic of protein expression

The transformant proven to contain pRSETc-LipL32 plasmid was used for protein expression. A colony was inoculated into 1 ml of LB broth containing 50 ug/ml of ampicillin and incubated overnight at 37 °C with shaking incubator. The culture was transferred to 6 ml broth at 37 °C in a shaking incubator until cells reached mid-log growth (OD_{550} ~ 0.5 to 1). The culture was dispensed into 6 aliquots (1 ml each). One tube (zero-time aliquots) was centrifuged for 1 minute at 12,000 rpm and supernate was collected and left on ice. IPTG was added to 5 tubes of culture at the final concentration 1 mM and incubation was continued. Each tube of culture was collected by centrifugation at 1, 2, 4, and 6 hours following IPTG addition. Supernates were collected and left on ice until all of the samples are collected. Samples were mixed with sample loading dye and heated at 100 °C for 3 minutes. The samples (0.15 OD_{550} units of each original culture) were subjected to 10% SDS-polyacrylamide gel electrophoresis, at 8-15 V/cm until the bromphenol blue reached the bottom of resolving gel. Gel was stained with Coomassie Brilliant Blue and molecular weight of protein was estimated using the standard curve from molecular weight markers. The induction time that gave highest production of the 32 kDa protein was selected.

5.2 LipL32 purification

The pRSETc plasmid contains the sequence for 6 histidine residues so LipL32 expressed from recombinant plasmid was fused with 6 histidine residues generating His-LipL32 protein (as shown in Figure 4). In order to purify His-LipL32, Nickle-nitrilotriacetic acid (Ni-NTA) spin column

was used. Ni-NTA spin column is metal–affinity chromatography matrices which selectively bind to biomolecules tagged with 6 consecutive histidine residues (6xHis tag). Ni-NTA silica was packaged in ready to use spin column in which crude cell lysates can be passed and purified.

For large scale expression and purification of the target protein, a colony of *E. coli* containing the pRSETc-LipL32 recombinant construct was inoculated into 10 ml of LB broth containing 50 ug/ml ampicillin and incubated overnight at 37 °C. Afterward, 5 ml of overnight culture was transferred to 500 ml of LB containing 50 ug/ml ampicillin and incubated in shaking incubator until mid-log phase (OD_{550} 0.5). The target protein was induced by IPTG for an optimal time obtained from previous experiment.

Following induction, culture was harvested by centrifugation for 15 minutes at 6,000 rpm at 4 °C. Cell pellets were resuspended in lysis buffer contain 1 mg/ml of lysozyme and incubated on ice for 30 minutes. The samples were sonicated for six times ,10 seconds each with 5-second pause between). For protein purification, Ni-NTA spin column was equilibrated with 600 ul lysis buffer and centrifuged at 2,000 rpm for 2 minutes. The sample, upto 600 ul, was loaded onto the pre-equilibrated Ni-NTA spin column and column was centrifuged at 2,000 rpm for 2 minutes. The flow through of cultures were saved. The column was washed twice with 600 ul washing buffer and washed fractions were collected. Bound protein was eluted with 400 ul of elution buffer. The eluate was collected and protein concentration was determined by Bradford method (Appendix II). All the fractions collected were subjected to 10% SDS-PAGE and gel was stained with Coomasie blue to demonstrate the purifed protein.

6. Immunoblotting

The purified protein was tested for antigenicity against immune sera by immunblotting. His-LipL32 protein (500 ng) was subjected onto 10% SDS-

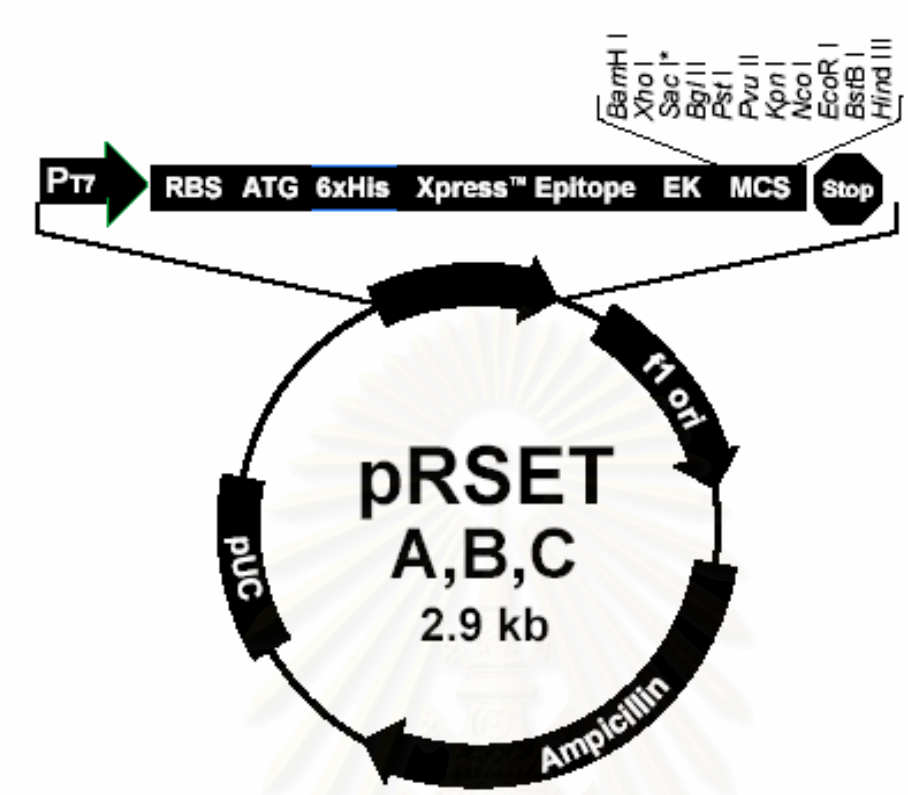
PAGE at 100 volt for 1 hour and 20 minutes. The gel was transferred to nitrocellulose membrane by at 70 volt for 1 hour. The blot was blocked with blocking solution containing 5% non fat dry milk in TBS–0.1% Tween 20 (TBS-T), for 60 minutes and then probed with rabbit anti-LipL32 antibody (kindly provided by Professor Ming-Jeng Pan, Graduate Institute of Veterinary Medicine, National Taiwan University, Republic of China), human sera positive for antibody to *leptospira*, positive for antibody to *T. pallidum* and from healthy volunteer for 90 minutes. Following washing with PBS-T and the blot was incubated with appropriate secondary antibody conjugated with alkaline phosphatase for 60 minutes and colorimetric detection was done using appropriate substrate. 5-Bromo-4-chloro-3-indolyl phosphate (BCIP) and Nitro Blue Tetrazolium (NBT) were used as the substrate for the alkaline phosphatase in order to visualize the antigen-antibody complexes.

7. Dipstick assay

Since the rapid and simple test will be very useful for laboratory diagnosis of leptospirosis, we attempted to establish a dipstick assay using LipL32 for antibody detection. For checkerboard titration, serum positive for antibody to *leptospira*, serum positive for anti- *T. pallidum* and serum from healthy volunteer were used. Various amounts of His-LipL32 were applied on 5-mm nitrocellulose disks, the disks were air dried for 30 minutes, blocked with 5% non fat dry milk in TBS for one hour. The disks were washed three times with PBS containing 0.1% Tween (PBST), then incubated with various dilutions of sera for 90 minutes and secondary antibody conjugated with alkaline phosphatase for one hour. Washing for three times with PBST was followed each step. Color was developed by adding NBT/BCIP solution (Appendix II) for 20-30 minutes followed by washing with distilled water. Blue-purple dots indicated the positive results. Optimal amounts of antigen, serum and conjugate were selected, and a dipstick assay for detection of antibody in patient

sera was performed by applying antigen on nitrocellulose membrane fixed on the plastic support. The following steps were the same as mentioned above. Anti-IgG or IgM (20 ng) was used as an internal control by applying as a dot above the dot of antigen.

Figure 4 Schematic diagram of pRSETc plasmid mapping



* Version C does not contain Sec I

Properties of pRSETc plasmid

T7 promoter base : bases 20-39
6xHis tag : bases 112-29
T7 gene 10 leader : bases 133-62
Multiple cloning site : bases 202-48
pRSET reverse primimg site : bases 295-314
bla promotor : bases 943-1047
ampicillin resistance gene : bases 1042-1902
pUC origin : bases 916-2852

CHAPTER V

RESULTS

1. Detection of LipL32 gene in *L. bratislava* and other serovars by PCR

A 782 bp fragment of LipL32 gene was amplified from *Leptospira interrogans* serovar *bratislava*, the seorvar that most reported in Thailand, as mentioned in Materials and Methods. In addition, other 17 pathogenic serovars of *L. interrogans* (Table 4) and a non-pathogenic serovar, *L. biflexa patoc I* were also investigated for this gene.

As shown in Figure 5, the 782 bp fragment PCR product was obtained from all pathogenic serovars used in this experiment (Lane 1-17). There was no band detected from DNA of the non-pathogenic strain (Lane 18). These results suggested that LipL32 gene was highly conserve only in pathogenic leptospires, at least the serovars tested in our study (Figure 5).

In order to confirm the absence of LipL32 gene in non-pathogenic *leptospires*, primers specific for 16S rRNA of *leptospires* were also used. The 285-bp PCR product from amplification of 16S rRNA was obtained from all serovars tested including *blifexa*, *patoc I* (Figure 6) demonstrating the exist of *leptospira* DNA and absence of LipL32 gene.

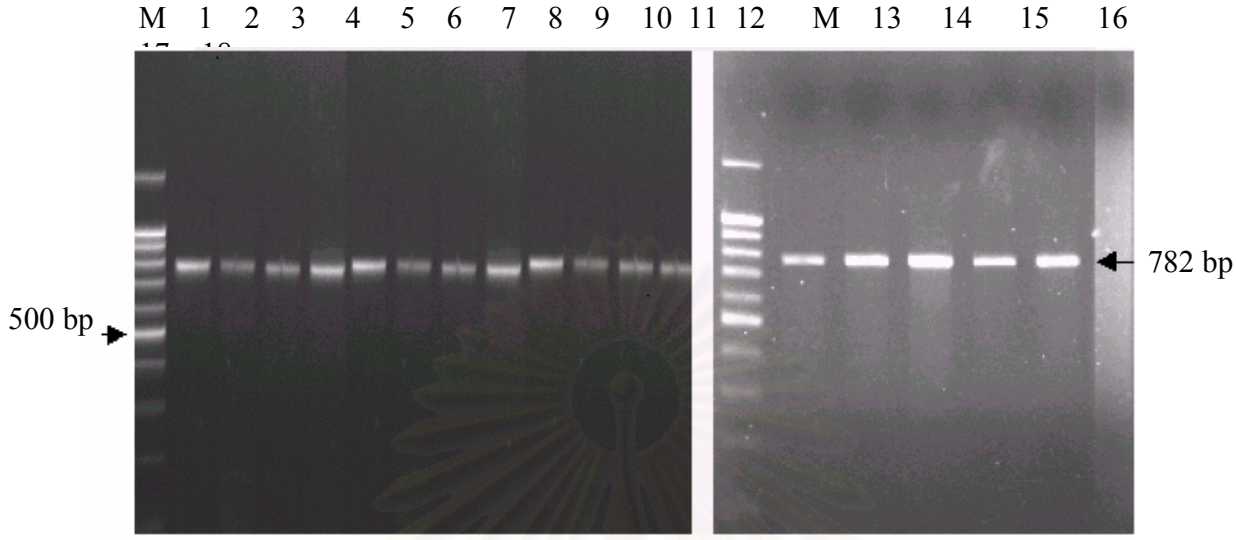


Figure 5 Agarose gel electrophoresis of PCR products using primers specific for LipL32 gene. DNA from pathogenic *leptospira* serovars *bratislava* (Lane 4) and other serovars : *L. autumnalis* (Lane 1), *L. ballum* (Lane 2), *L. bataviae* (Lane 3), *L. canicola* (Lane 5), *L. celledoni* (Lane 6), *L. cynopteri* (Lane 7), *L. djasiman* (Lane 8), *L.grippotyphosa* (Lane 9), *L. hebdomadis* (Lane 10), *L. icterohaemorrhagiae* (Lane 11), *L. javanica* (Lane 12), *L. louisiana* (Lane 13), *L. panama* (Lane 14), *L. pomona* (Lane 15), *L. pyrogenes* (Lane 16), *L. sejroe* (Lane 17) *and* non-pathogenic *L. biflexa patoc* I (Lane 18) were amplified. Arrow indicated the 782 bp PCR product. M = 100 bp marker.

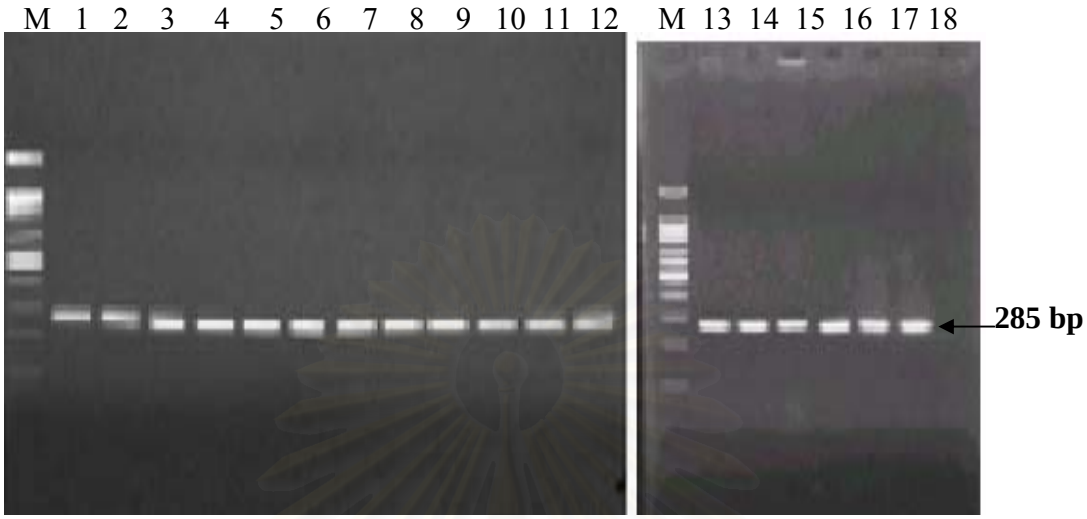


Figure 6 Agarose gel electrophoresis of PCR products using primers specific for 16S rRNA. DNA from pathogenic *leptospira* serovar *bratislava* (Lane 4) and other serovar : *L. autumnalis* (Lane 1), *L. ballum* (Lane 2), *L. bataviae* (Lane 3), *L. canicola* (Lane 5), *L. celledoni* (Lane 6), *L. cynopteri* (Lane 7), *L. djasiman* (Lane 8), *L. grippotyphosa* (Lane 9), *L. hebdomadis* (Lane 10), *L. icterohaemorrhagiae* (Lane 11), *L. javanica* (Lane 12), *L. Louisiana* (Lane 13), *L. panama* (Lane 14), *L. pomona* (Lane 15), *L. pyrogenes* (Lane 16), *L. sejroe* (Lane 17) and non-pathogenic *L. biflexa patoc* I (Lane 18) were amplified. Arrow indicated the 285-bp PCR product. M = 100 bp marker.

2. Cloning of LipL32 gene

The 782 bp PCR product from DNA of *Leptospira interrogans* serovar *bratislava* was cut and purified from gel. Both purified product and pRSETc plasmid were cut with appropriate restriction enzymes and ligated with T4 DNA ligase. The ligation mixture was transformed into *E. coli* BL21(DE). Three white colonies were selected and the correct insert was demonstrated by PCR of selected clones using primers specific for LipL32 gene and pRSETc plasmid as shown in Materials and Methods. The expected product sizes for colonies with the desired insert using primers for LipL32 and pRSETc were 782 and 961 bp, respectively. As shown in Figure 7, the sizes of PCR products obtained from a selected colony were about the expected sizes (782 bp in Figure 7A, Lane 2 and 961 bp in Figure 7B, Lanes 1 and 2). In addition, for empty plasmid, the PCR product using primers for pRSETc was about the size of 179 bp as (Figure 7B, Lane 3). This suggested that the selected clone contained a plasmid pRSETc with 782 bp insert. All three colonies selected gave the same result (data not shown).

In addition to PCR, the correct insert was also demonstrated by sequencing (Figure 8). Plasmid was purified from the selected clone and sequenced as described in Materials and Methods. The obtained sequence was compared with the previously submitted sequences. A Blast search of the GenBank database revealed homology of our constructed plasmid with the major outer membrane protein, LipL32, of *Leptospira* (GenBank accession number AF111292).

The selected colony was used for further protein expression and also stored as a glycerol stock and kept at –70 °C.

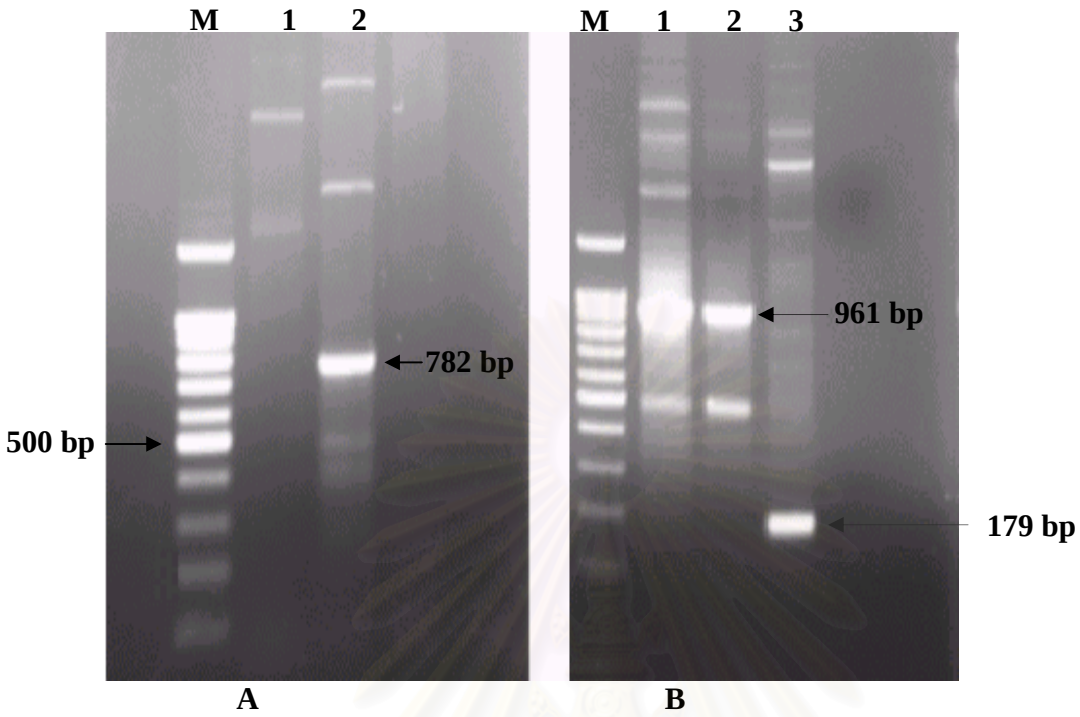


Figure 7 Agarose gel electrophoresis of PCR products using primers for LipL32 gene (A) or for pRSETc plasmid (B). Figure 8A, Lane 1 and Figure 8B, Lane 3 were from amplification of empty pRSETc plasmid. Figure 8A, Lane2 and Figure 8B, Lanes 1 and 2 were from amplification of the constructed plasmid, pRSETc-LipL32. M = 100 bp markers, arrows indicate PCR products.

5’ TTACCGCTCGAGGTGCTTTCGGTGGTCTGCCAAACGGAAGCAGCT

CGTTAGTTCAGAGCCAGGACCAATCCAACCGTGGCGAACCATGGAA

CGAACCGTAAACGTTCTTCCTCGCATTGTGACAGGTATTCGGATCGT

AAAGCAGGACAAGCGCCGGACGGTTTAGTCGATGGAAACGAAAAA

GCATACTATATCTATCTTTGGACTCCTGCCGTAATCGCAGAAATGGG

AGTTCGTATGATTTCCCCAACAGGCGAAATCGGTGAACCAAGCGAT

GGAGACTTAGTAAGCGACGCTTACAAAGAGGCTACGGCAGACGAA

ATTTTCATGCCACCCTGGTTTGATACATGAATCCGTGTAGAAAGAAT

GTCGGCGATTATGCCTGACCAAATCGCCAAAGCTGCGAAAGCAAAA

CCCGTTCAAAACTTGGACGATGATGATACTGGTGCCGATACTTATAT

AGAAGACAGACGTAATAACTACAACTCTCTTACTAGAATCAAGATC

CCTAATCCTCCAGAATCATTTGACGACCTGAAAATCATCGATACTAA

AAGACTTTTAGTAAGAGGTCATTGCAGAATTTCT TTCCCTACCTACA

AACCAGGTGAAGTGACTGGATCTTTCGTTGCATCTGT TGGTCTGCTT

TTCCCACCAGGTATTCCAGAGGTGAGCCCGCTGATCCACTAAATCAT

GAAAATTGCAATTTCAAGCTAACGCTGCTGAAGCCTCATTGAAATAG

CGTGCTTCAAACGCGACTAAGTAATCTGTTTTAACAATTT-3’

Figure 8 Sequence of 782-bp inserted in pRSETc plasmid.

3. LipL32 protein expression and purification

To optimize the expression of protein from our construct, a time course analysis of the level of protein expression was done. The selected clone was grown and protein expression was induced by 1 mM IPTG for 0, 1, 2, 4, and 6 hours. Bacterial lysates were collected and subjected to 10% SDS-PAGE. As shown in Figure 9, the protein at apparent molecular weight 32 kDa was constitutively expressed (Figure 9A;Lane 1-5). Following IPTG induction, the induction time that gave highest expression of this protein was 2 hours and the expression was declined at 4 hours. There was no protein at this apparent molecular weight observed when bacteria containing empty vector were tested (Figure 9B;Lanes 1-5).

The results suggested that the LipL32 protein was constitutively expressed and the optimal time for IPTG induction was 2 hours which was used for LipL32 preparation in the following experiment.

After the optimal time for IPTG induction was obtained, 50 ml of culture of bacteria containing pRSETc-LipL32 was grown and LipL32 was purified using Ni-NTA spin column kit as mentioned in Materials and Methods. Successful purification was demonstrated by subjecting samples into 10% SDS-PAGE. As shown in Figure 10, the purified protein at apparent molecular weight about 32 kDa was observed.

The large scale preparation of His-LipL32 was performed using 500 ml bacterial culture and 412 ug of protein measured by Bradford method (Appendix II) was obtained for further used.

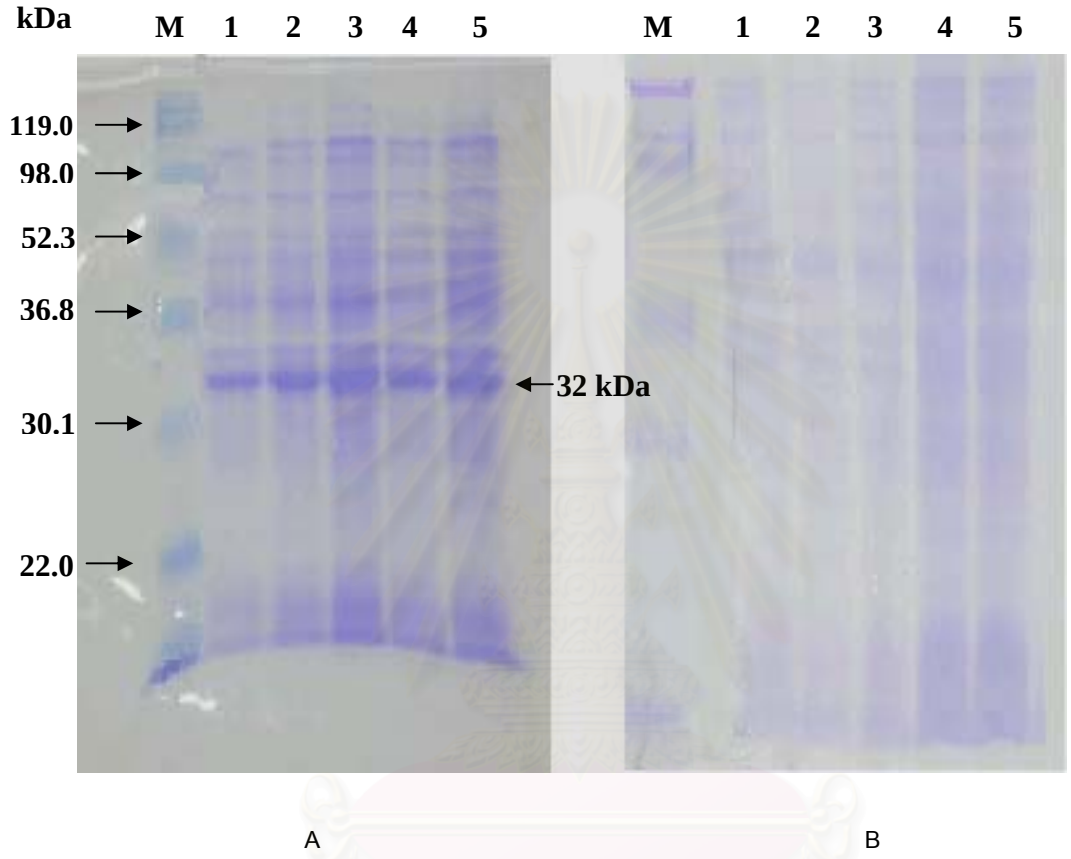


Figure 9 Kinetics of 32 kDa protein expression following IPTG induction. The bacterial lysates were resolved on a 10% SDS-PAGE. Forty micrograms of crude cell lysate was added to each lane. Gels were run at 100 volt for approximately 1 hour and twenty minutes and proteins bands were visualized by Coomassie staining. Figure 9A was from bacteria containing pRSETC-LipL32 and Figure 9B was from bacteria containing empty pRSETc. Lanes 1-5 of both gels were from bacteria induced with IPTG for 0, 1, 2, 4, and 6 hours, respectively. Lane M is protein molecular weight markers. Arrow indicates the protein at expected size (about 32 kDa).

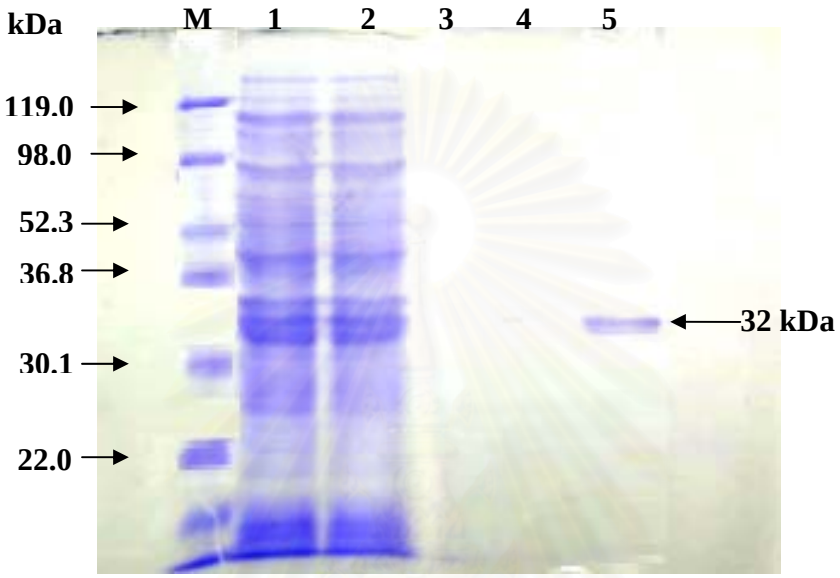


Figure 10 Purification of His-LipL32 protein. The recombinant His-LipL32 protein was expressed in *E. coli* and purified by affinity chromatography as described in Materials and Methods. The molecular mass of the His-LipL32 was estimated to be approximately 32 KDa by SDS-PAGE. Each lane was loaded with 40 ug of proteins. Lane M is protein molecular weight markers. Lanes 1 and 2 are crude cell lysate, Lanes 3 and 4 are flow through and Lane 5 is the eluate.

4. Immunological reactivity of LipL32

In order to confirm that our purified protein obtained was LipL32, immunoblotting using rabbit antisera against LipL32 was performed. As shown in Figure 11, the band with apparent molecular weight 32 kDa was visualized when antisera to LipL32 (dilution 1:1000) was used (Strip 1). There was no band detected with normal rabbit serum (Strip 2). This suggested that our purified protein is LipL32.

Since one of our goal for cloning and expressing of LipL32 gene is to use LipL32 as an antigen for detection of *leptospira* antibody, in addition to antisera specific to LipL32, we also tested this purified protein with patient serum positive for antibody to *leptospira*, patient serum positive for antibody to *T. pallidum* (but negative for antibody to *leptospira*) and healthy volunteer serum negative for antibody to *leptospira*. Secondary antibody used in this experiment was rabbit anti-human IgG conjugated with alkaline phosphatase. The results of testing of antibody to *leptospira* of sera used in this experiment were shown in Table 7.

Immunoblotting using 500 ng/lane of LipL32 protein was performed using sera mentioned above. The band with apparent molecular weight about 32 kDa was detected only when serum positive for antibody to *leptospira* was used (Figure 12, Strip 1). However, no reactivity with LipL32 protein detected with sera positive for antibody to *T. pallidum* and serum from healthy volunteer (Figure 12, Strips 2 and 3, respectively). All sera used were diluted at 1:200 and secondary antibody was used at dilution 1:2,000.

In addition, anti-human IgM conjugate (1:2,000) was also used to detect IgM antibody to LipL32. However, there was no band detected even when serum positive for *leptospira* was used (data not shown).

Table 7 Results of leptospiral antibodies detected by using Immunofluorescent Assay (IFA), and Microscopic Agglutination Test (MAT)

Serum no.	**Results**		
	IFA		**MAT**
	IgG	**IgM**	
A	Positive, titer 1:400	Positive, titer 1:800	Positive for *L. bratislava,* titer 1:1600
B	negative	negative	negative
C	negative	negative	negative

A = serum from a patient whose leptospirosis was suspected.

B = serum positive for antibody to *T. pallidum*

C = serum from healthy volunteer

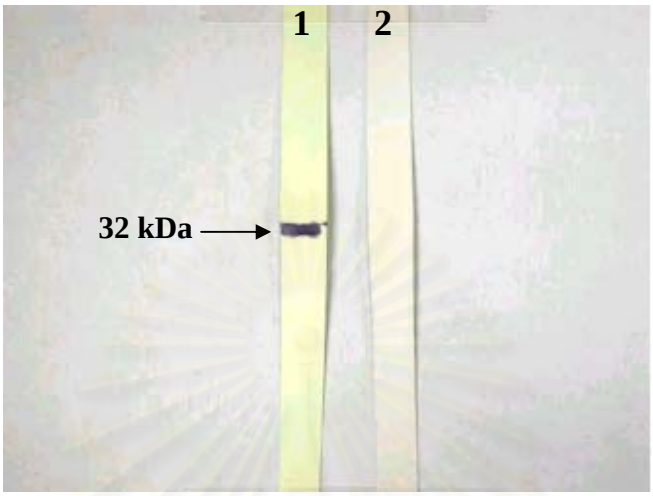


Figure 11 Immunoblotting of purified LipL32 protein. Purified His-LipL32 (500 ng/lane) was subjected to 10% SDS-PAGE and transferred to nitrocellulose membrane. Blots were probed with 1 :1,000 dilution of rabbit antisera to LipL32 (Strip 1) or 1:1,000 dilution of normal rabbit serum (Strip 2) followed by 1:2,000 Dilution of goat anti-rabbit Ig conjugated with alkaline peroxidase .

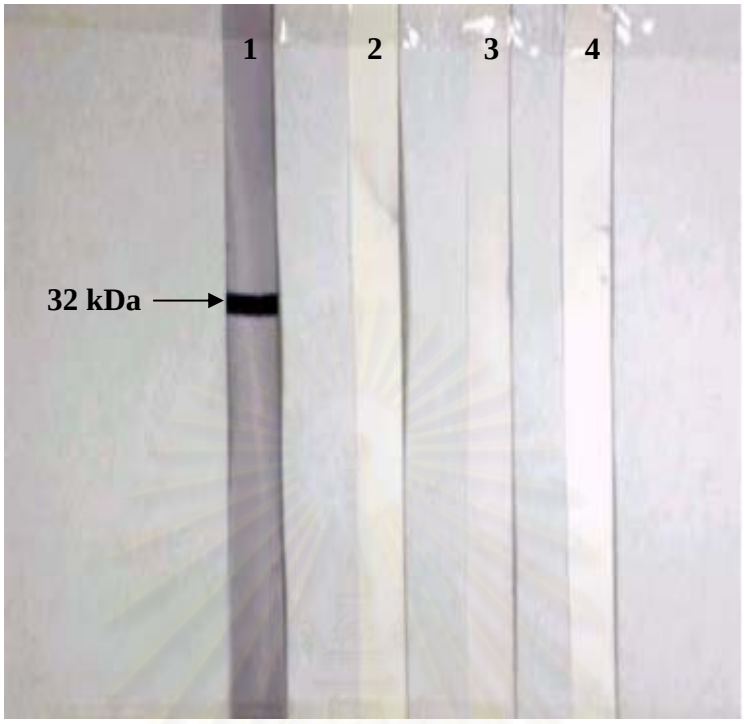


Figure 12 Immunoblotting of His-LipL32 using human sera. Each strip contained 0.5 ug of His-LipL32 and were probed with serum positive for antibody to *leptospira* (strip 1), serum positive for antibody to *T. pallidum* (Strip 2), and serum from negative *leptospira* antibody (Strip 3). All sera were diluted at 1:200. Strip 4 were probed with secondary antibody only.

5. Dipstick assay

As mentioned earlier that LipL32 is a candidate antigen for detection of leptospira antibody, additional work for demonstrating LipL32 antigenicity has been done. Checkerboard titration was performed as followed using the same sera as in the immunoblotting experiment described above. Various amounts of His-LipL32 (25, 50,100, and 200 ng) were dotted on nitrocellulose membrane and probed with 25, 50,100, and 200–fold dilutions of serum and 500 ,1000, and 2000–fold dilutions of secondary antibodies. Both anti-human IgG and anti-human IgM conjugates were used in this experiment.

According to checkerboard titration results (Figure 13 and data not shown), when IgG antibody was detected, 50 ng of LipL32 antigen, 1:50 dilution of serum and 1:2,000 dilution of conjugates were used since at this condition gave visible spots with least background and non-specific result. When higher amount of antigen and lower serum dilution were used, weakly positive result was obtained from serum positive for antibody to *T. pallidum*.

Checkboard titration for detection of IgM antibody specific to *leptospira* was also performed in the same manner for detection of IgG antibody. There was no visible spots detected in all sera tested. The concentration of anti-human IgM conjugate and sera were increased in order to see whether the positive reactions could be observed. The weakly reactions were obtained with serum positive for antibody to *leptospira* when the dilution of conjugate was 1:500 and the dilutions of sera were 1:25, 1:50 and 1:100. However, the positive reactions were also observed when sera positive for antibody to *T. pallidum* was tested. This indicated that the increase in the amount of conjugate also increased non-specific reaction.

Since a rapid and easy laboratory assay is needed for diagnosis of leptospirosis, we are interested in investigating whether LipL32 can be used as an antigen in a dipstick assay. From the data shown in Figure 13, 50 ng of antigen, 1:50 dilution of serum and 1:2,000 dilution of anti-human IgG conjugate were used

for preliminary establishing a dipstick assay for detection of *leptospira* antibody. An upper dot was an internal control (20 ng of anti-IgG) which should give positive result by all samples tested to ensure that proper reaction occurred. Figure 14-16 were examples of the results of dipsticks probed with sera positive for antibody to *leptospira* antibody (by MAT and IFA), sera positive for antibody to *T. pallidum* and sera from donors (negative for antibody to *leptospira*), respectively. Twenty sera of each group were used. All samples positive for *leptospira* antibody gave positive results with the dipstick assay, 1 out of 20 samples positive for *T. pallidum* antibody gave positive result and none of samples from donors used in this experiments reacted with LipL32 (Figure 14-16 and Table 8). This preliminary data suggested that sensitivity and specificity of our IgG-dipstick assay compared with MAT and IFA were 100% and 97.5%, respectively.

The same conditions for the IgG dipstick assay were also applied for testing IgM antibody. As shown in Table 8, 17 out of 20 samples positive for antibody to leptospira by MAT, IFA and IgG-dipstick assay gave negative results and 3 out of 20 sample showed weakly positive result. None of sera positive for antibody to T. pallidum and sera from donors gave positive results.

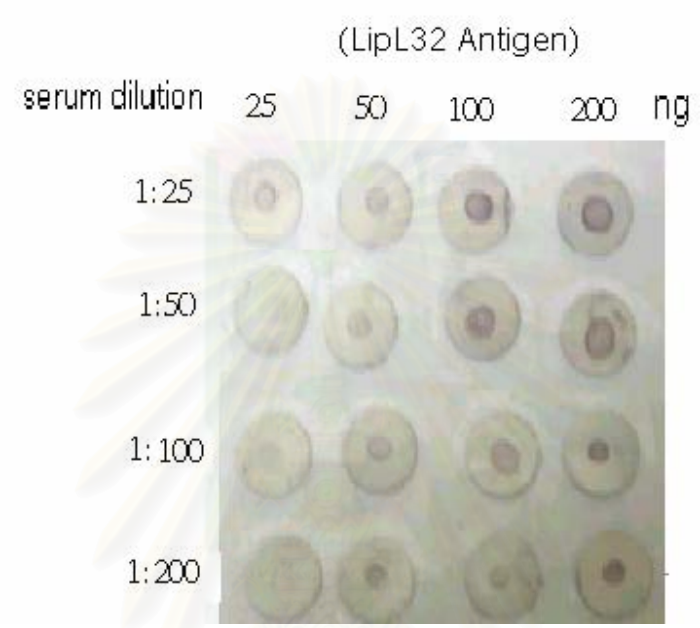


Figure 13 Dot-ELISA of checkerboard titration for detection of antibody to leptospira. Various amount of His-LipL32 (25,50,100,and 200 ng) and various dilutions (1:25, 1;50, 1:100, and 1:200) of serum positive for antibody to *leptospira* (tested by MAT and IFA) were used. For the experiment shown in this Figure, anti-human IgG conjugated with alkaline phosphatase at dilution 1:2,000 was used. Serum positive for antibody to *T. pallidum* and serum from a volunteer negative for antibody to *leptopsira* were also tested in the same manner (data not shown).

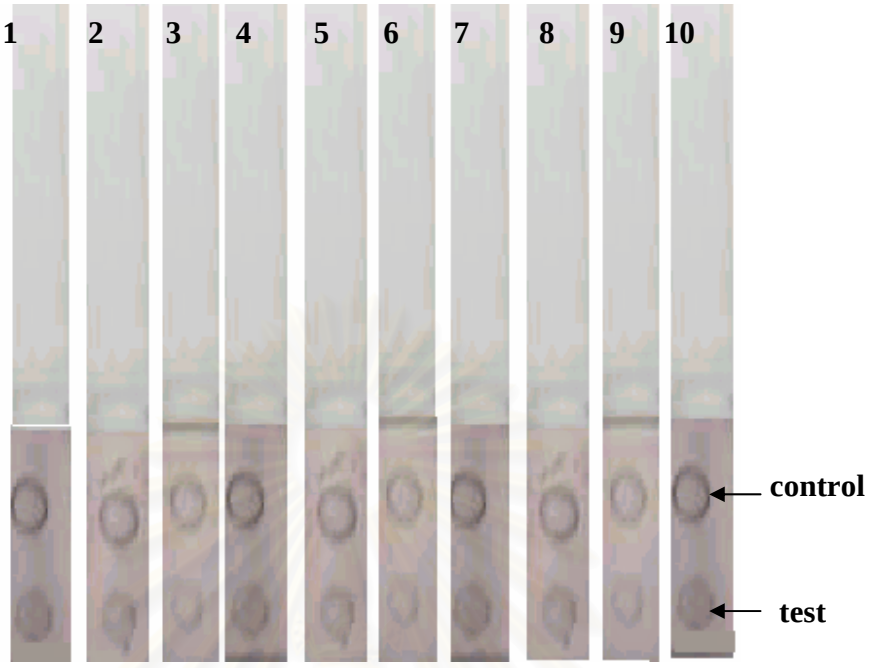


Figure 14 IgG dipstick assay using antibody to *Leptospira*. Strips were coated with 50 ng of His-LipL32 , probed with 1:50 dilution of sera and followed by anti-human IgG antibodies conjugate alkaline phosphatase at 1:2,000 dilution. For internal control, the strip was also coated with 20 ng of anti-IgG (an upper dot). Sera samples used in this Figure were positive for antibody to *leptospira*.

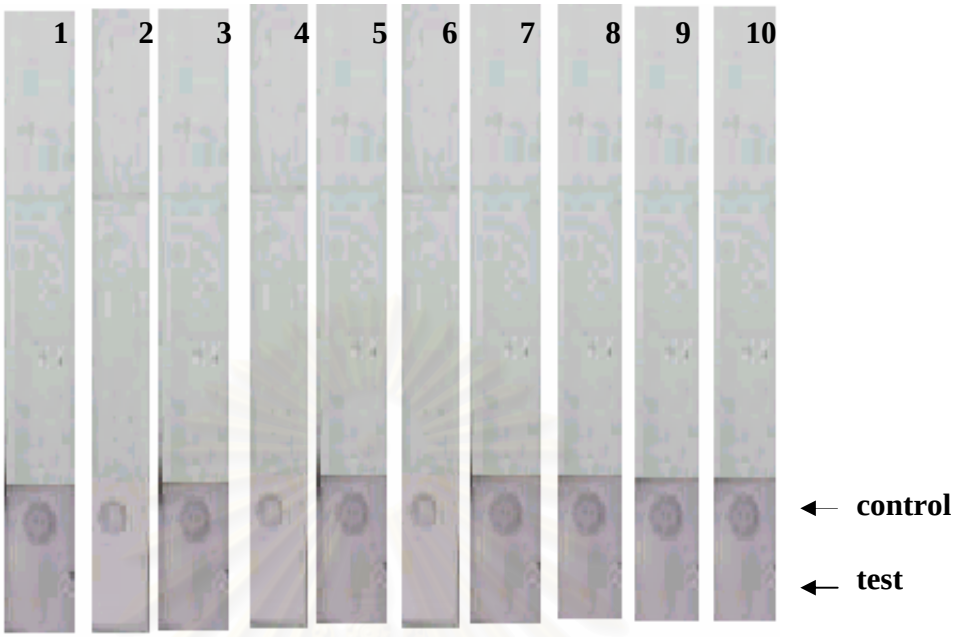


Figure 15 IgG dipstick assay using antibody to *T. pallidum*. Strips were coated with 50 ng of His-LipL32, probed with 1:50 dilution of sera and followed by anti-human IgG antibodies conjugate alkaline phosphatase at 1:2,000 dilution. For internal control, the strip was also coated with 20 ng of anti-IgG (an upper dot). Sera samples used in this figure were positive for antibody to *T. pallidum.*

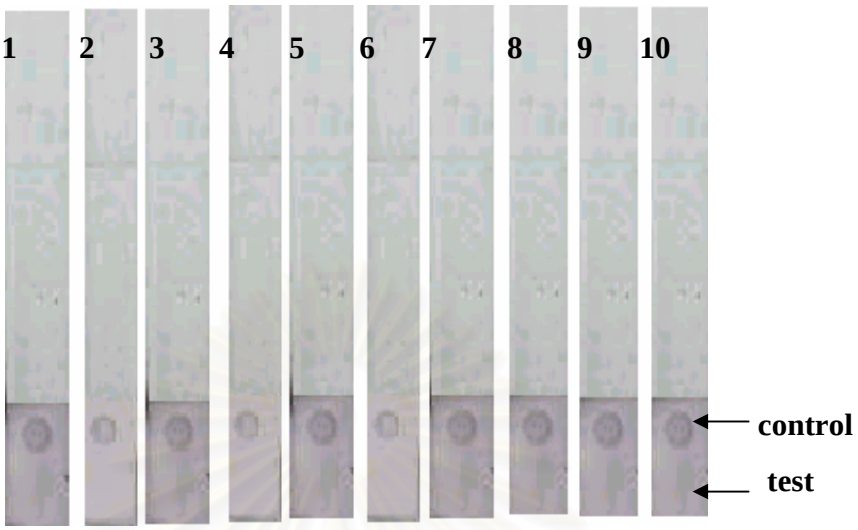


Figure 16 IgG dipstick assay using serum negative for *leptospira* antibody. Strips were coated with 50 ng of His-LipL32 , probed with 1:50 dilution of sera and followed by anti-human IgG antibodies conjugate alkaline phosphatase at 1:2,000 dilution. For internal control, the strip was also coated with 20 ng of anti-IgG (an upper dot). Sera samples used in this Figure were from donors and negative for antibody to *leptospira*.

Table 8 Comparison of results from MAT, IFA, Dipstick assays for detection of antibody to *leptospira*.

Serum samples	**Number of samples**	**MAT**	**IFA**		**Dipstick (His-LipL32 Ag)**	
			IgG	**IgM**	**IgG**	**IgM**
A	20	P	P	P	P	W=3, N=17
B	20	N	N	N	N=19 P=1	N
C	20	N	N	N	N	N

A = sera positive for antibody to *leptospira*

B = sera positive for antibody to *T. pallidum*

C = sera negative for antibody to *leptospira*

N = negative, P = positive, W= weakly positive

CHAPTER VI

DISCUSSION

Leptospirosis is considered a reemerging infectious disease, not only for the increasing in its incidence during the past recent years but also for the increased severity of the illness which frequently leads to mortality, especially in patients with delayed diagnosis and/or patients improperly treated. The disease is not readily distinguishable, based on the clinical presentation. Improved diagnostic tests for leptospirosis are needed to aid clinical diagnosis. In Thailand, most reported cases were from rural areas so the diagnostic test should be not only specific but also rapid to be performed for timely administration of antibiotics or other treatments can be done (3,7,285,286,288,289)

Because the reference method, MAT, is time consuming, requires live stock of organisms and is difficult to be done in laboratories in remote areas, many diagnostic tests using a variety of immunological principles have been established. However, most tests utilized whole-cell extracts as antigens and the immunodominant moiety in whole–cell preparations appears to be a broadly reactive antigen (33,84) that is a disaccharide epitope present in nonpathogenic leptospires as well as a diverse group of nonleptospiral species (290,291). False positive reactions obtained from those assays have been reported when some of samples from patients with other diseases such as syphilis, dengue, hepatitis, cytomegalovirus, rhumatoid, pyraxia of unknown origin, mycoplasma, Epstein-Barr virus, infection, (34,35,36,37,293,294,295,296,297,298) were tested.

Because the requirement of a more specific test for laboratory diagnosis, purified antigens have been the next research step in diagnostic assays. Gene and protein expression in bacteria changes in the response to environment in order to adapt and interact with their environment. For this reason, the candidate antigen for antibody detection should be expressed during infection and able to induce immune response. In addition, in order to be able to prepare the antigen *in vitro*, the protein of

interest should be expressed in organisms cultured in laboratory. Outer membrane proteins are selected since their surface exposure makes them accessible to immune recognition and attack. Outer membrane proteins of *leptospira* have been characterized by both biochemical and molecular biology methods. They include OmpL1, LipL32, LipL36, LipL41 and LipL48 and other less well-characterized outer membrane proteins such as proteins at molecular masses of 16, 21, 21.5, 22, 31, 36, 44, 48, 90 and 116 kDa (150,151,152,153,154, 155).

LipL32 is an outer membrane protein which is highly conserved among pathogenic leptospires and was found to be expressed in proximal tubules of infected animals. In addition, it is a prominent immunogen during leptospirosis. From immunoblotting experiments, acute and convalescent phase sera of human leptospirosis showed great reactivity against LipL32 protein (45). Expression of this protein in laboratory culture has also been demonstrated. For those reasons, LipL32 was chosen to be studied in this work.

Although, LipL32 gene has been cloned and protein expression has been done from *L* serovar *kirschneri* and *shermani* (44,293) (the serovar most commonly reported in Thailand, *L. bratislava* has not been investigated. This study successfully cloned and expressed LipL32 *in vitro* as demonstrated earlier. The cloned gene has sequence homology to LipL32 from other serovars reported which provided additional information that LipL32 is conserved. As mentioned earlier that there are more than 200 serovars of leptospires reported throughout the world, only a few serovars have been demonstrated the existence of LipL32 gene. In addition to *L. bratislava*, LipL32 gene of other 16 pathogenic serovars reported in Thailand was also demonstrated. This study added up the list of pathogenic serovars reported to possess LipL32 gene (44).

Since we considered that a rapid test is needed for leptospirosis diagnosis, establishing a dipstick assay using purified His-LipL32 was initiated. We attempted to detect antibodies both specific IgM and IgG subtype. However, only specific IgG antibody was detected. Sensitivity and specificity for IgG detection were quite high (100 and 98.33%, respectively) when compared with MAT. IFA was also done to demonstrate the subtypes of antibodies. Sera samples positive for antibody to *T.*

pallidum were included for non-specificity testing since bacteria in Genus Treponema is also spirochete which cross-reactivity has been demonstrated when some sera from syphilis patients were tested with leptospiral antigens (34,39,294). In our study, there were only one out of 20 samples gave false positive results. Moreover, all sera from healthy volunteer gave quite clear negative result. Although the results from IgG detection is promising for further study, specific IgM antibody was weakly detected in only 3 out of 20 samples that were proved to be positive for antibody to *leptospira*. This may be due to the characteristic of an antigen that could induce certain subtype of immunoglobulin. It has been shown that most IgM antibodies were specific to LPS whereas most IgG antibodies were for protein fractions. Chapman *et al.*; 1991 (137) demonstrated that when leptospiral antigens were treated with proteinase K, the pattern of IgM antibody recognition was unchanged whereas most bands obtained from IgG antibody were lost when compared with non-treated antigens. However, larger and more varieties groups of samples and more experiments are needed to be done in order to clearly explain the observed results.

In addition to the advantage in diagnostic assay, LipL32 protein is also proposed to be a candidate antigen for immunoprotection. Vaccines currently used are combination of various serovars of inactivated whole leptospires. Variations in carbohydrate side chains of LPS are responsible for the antigenic diversity observed among leptospiral serovars. It has been shown that LPS of leptospires or whole cell vaccine were not able to provide cross-protection against serovars not contained in vaccine preparation. Moreover, some serovars could not even protect animals from challenging with the homologous serovar (292). Hunting for candidate antigens that can induce cross protection is challenging and antigen which is highly conserved among pathogenic serovars should be selected. It has been shown that leptospiral protein extracts can induce protection against heterogous serovars in an experimental animal model (155,292,293). Besides the reasons that this protein is expressed during infection and induced immune response *in vivo*, LipL32 is expressed by all pathogenic serovars investigated in this study and previous; (44,293) but not by non-pathogenic one, make it a good candidate for vaccination that induces cross -serovar protection.

Finally, LipL32 protein has been shown to involve in pathogenesis of leptospirosis. The main renal manifestation caused by pathogenic leptospires is tubulointestinal nephritis. The information on components of organisms crucial for induction of inflammatory response will certainly shed light on mechanisms of pathogenicity of *leptospira*. It has been shown that LipL32 involved in pathogenicity of leptospirosis leading to tubulointestinal nephritis. LipL32 induced expression of chemokines/cytokines, enzymes and transcription factors involving inflammatory response such as, MCP-1, nitric oxide synthase (iNOS), RANTES, TNF-alpha□, NF-kB and AP-1 in proximal tubule cells. These effects were inhibited by antibody to LipL32 which suggested that LipL32 may induce inflammatory response leading to tubulointestinal nephritis (293). According to all reports mentioned , LipL32 protein is one of *leptospira* proteins that may not be only useful for laboratory diagnosis, vaccination but also for understanding of pathogenicity of this infectious disease which is still remained unclear.

In conclusion, in addition to cloning and expression of LipL32 protein, preliminary data providing information on this antigen for a rapid assay was also demonstrated. Testing this antigen with larger groups of samples should be done to support our data. Further investigating the role of this protein in cross-immunoprotection and pathogenicity are also our interest.

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APPENDICES

Appendix I

Chemicals Agents and Instruments

A. Chemical substances

Acrylamide gel (Pharmacia biotech, USA)
Agarose (Gibco BRL, USA)
Ammonium persulfate (Sigma, USA)
Ampicillin (Sigma, USA)
Anti-human IgM conjugate with
alkaline phosphatase (Dako, Denmark)
Anti-human IgG conjugate with
alkaline phosphatase (Dako, Denmark)
Bacto agar (Difco, USA)
Bacto- tryptone (Difco, USA)
5-bromo-4-chloro-3-indolyl β-D-
galactopyranoside (Bio-Basic, Canada)
Bovine serum albumin (Sigma, USA)
Calcium chloride (Merck, Germany)
4-chloro-1-napthol (Sigma, USA)
Dimethylformamide (Sigma, USA)
Disodium hydrogen phosphate (Merck, Germany)
Dye chain terminator (Applies Biosystems, USA)
Ethanol (Merck, Germany)
Ethidium Bromide (Sigma ,USA)
Ethylenediaminetetra-acetic acid (BDH, England)
Glacial acetic acid (Merck, Germany)
Glucose (Difco, USA)
Glycerine (Sigma,USA)
Glycine (Sigma, USA)
His-tag purification kit (Qiagen, USA)
Hydrochloric acid (Merck, Germany)

isopropyl- D-thiogalactopyranoside (Bio-Basic, Canada)

Leptospira EMJH base (Difco, USA)

2-mercaptoethanol (Sigma, USA)

Methanol (Merck, Germany)

Nitrocellulose paper (Bio-Rad, USA)

Non fat dry milk (Carnation, USA)

N,N,- Methylene-bis acrylamide (Pharmacia biotech, USA)

Nucleospin kit (Macherey-Nagel , Germany)

PCR reagents (Promega, USA)

Phenol (Sigma, USA)

Chloroform (Merck, Germany)

Potassium chloride (Merck, Germany)

Potassium dihydrogen phosphate (Merck, Germany)

Potassium acetate (Merck, Germany)

Protein molecular weight markers (Bio-Rad, USA)

Restriction enzyme

- *PvuII* (Biolabs, USA)
- *SmaI* (Biolabs, USA)
- *XhoI* (Biolabs, USA)

Sodium acetate (Sigma ,USA)

Sodium dodecyl sulfate (Sigma, USA)

Sodium chloride (Merck, Germany)

Sodium hydroxide (Merck, Germany)

T4 ligase (Promega, USA)

TEMED (N',N',N',N'–tetrametylenediamine) (Sigma, USA)

Trisma–base (Sigma, USA)

Tryptone (Difco, USA)

Tween 20 (Merck, Germany)

Yeast extract (Difco, USA)

B. Glasswares

Beaker (Pyrex, USA)

Cylinder (Witeg, Germany)

Erlenmeyer flask (Pyrex, USA)

Glass tube (Pyrex, USA)

Petridish (Pyrex, USA)

Screw-cap erlenmayer flask (Pyrex, USA)

Serological pipette (Pyrex, USA)

C. Instruments

Analytical balance (OHAUS, Switzerland)

Dark-field microscope (Olympus, Japan)

Eppendorf microfuge model 5412 (Beckman Instrument, USA)

Incubator (Memmert, Germany)

Mixer Vortex-Genie (Scientific industries, USA)

PHM 83 Auto cal pH meter (Radiometer , Denmark)

Pipette Washer (Scientific Apparatus, USA)

Power supply (LKB 2197 , Sweden)

Refrigerated centrifuge (Beckman Instrument, USA)

Spectrophometer (Bio-Rad, USA)

Trans-Blot cell (Bio-Rad, USA)

Mini Protean cell (Bio-Rad, USA)

Water bath (Precision , USA)

Appendix II

MEDIA, REAGENTS AND PREPARATIONS

A. MEDIA

1. *Leptospira* EMJH medium

Leptospira EMJH Base	2.3	g
Deionized distilled water	900	ml

The pH of this base was adjusted to 7.4 before autoclaving at 15 psi for 15 minutes. When the temperature of EMJH has decreased, 100 ml of *Leptospira* EMJH enrichment media was added. Store the medium in a refrigerature.

2. Luria Bertani (LB) medium

Per Liter:

To 950 ml of deionized distilled water , add:

tryptone	10.0	g
yeast extract	5.0	g
NaCl	10.0	g

Shake until the solutes have dissolved. Adjust the pH to 7.0 with 5N NaOH (~ 0.2 ml). Adjust the volume of the solution to 1 liter with deionized water. Sterilized by autoclaving for 15 minutes at 15 psi. Store the medium / plates in plastic sleeve in a refrigerator.

For preparation of LB agar plates: add 15 g of bacto agar per liter and autoclave.

For ampicillin indicator : allow media to cool and add amplicillin 0.5 ml of 100 mg/ml per liter before pour plates. This should result in a final concentration of 50 ug/ml ampicillin in LB plates.

For X-Gal and IPTG indicator: allow media to cool before adding solution. (Add X-Gal 2 ml of 20 mg/ml and 0.2 ml of 1M IPTG solution to the cooled media). In general, transformants that are white contain an insert in many of the standard cloning vectors.

3. SOB medium

Per liter

To 950 ml of Deionized distilled water , add:

tryptone	20.0	g
yeast extract	5.0	g
NaCl	0.5	g

Shake until the solutes have dissolved. Add 10 ml of 250 mM solution of KCl. (This solution is made by dissolving 1.86 g of KCl in 100 ml of deionized distilled water). Adjust the pH to 7.0 with 5N NaOH (~0.2 ml). Adjust the volume of solution to 1 liter with deionized distilled water. Sterilize by autoclaving for 15 minutes at 15 psi. Just before use , add 5 ml of sterile solution of 2 M $MgCl_2$. (this solution is made by dissolving 19 g of $MgCl_2$ in 90 ml of deionized distilled water. Adjust the volume of the solution to 100 ml with deionized distilled water and sterilized by autoclaving.

4. SOC medium

SOC medium is identical to SOB medium, except that it contains 20 mM glucose. After the SOB medium has been autoclaved, allow it cool to 60 $^{\circ}C$ or less. Add 20 ml of a sterile 1 M glucose (This solution is made by dissolving 18 g of glucose in 90 ml of deionized distilled water and sterilize by passing it through a 0.22 um filter).

B. REAGENTS

1. Alkaline lysis for minipreparation

1.1 Alkaline lysis solution I

50 mM glucose

25 mM Tris-HCl pH 8.0

10 mM EDTA

1.2 Alkaline lysis solution II

0.2 N NaOH (freshly diluted from a 10 N NaOH)

1% SDS

1.3 Alkaline lysis solution III

5 M potassium acetate	60.0	ml
glacial acetic acid	11.5	ml
distilled water	28.5	ml

2. Reagent for competent cell preparation

80 mM $MgCl_2$

20 mM $CaCl_2$

3. Reagents for SDS-PAGE

3.1 Stock acrylamide, 30%

Acrylamide	30.0	g
N, N – methylene bisacrylamide	0.8	g
Deionized distilled water	100	ml

3.2 Separating gel, 10%

Stock acrylamide	16.65	ml
1.5 M. Tris-HCI pH 8.8	12.50	ml

10% SDS	0.50	ml
0.2 M. EDTA	0.50	ml
Deionized distilled water	19.25	ml
TEMED	0.025	ml
10% ammonium per sulfate	0.25	ml

3.3 Stacking gel, 4%

Stock, acrylmaide	0.67	ml
0.5 M, Tris-HCI pH 6.8	1.25	ml
20% SDS	0.025	ml
0.2 M, EDTA	0.1	ml
Deionized distilled water	3.075	ml
TEMED	0.005	ml
10% ammonium persulfate	0.025	ml

3.4 Sample buffer pH 6.8 (5X)

Tris	0.3784	g
SDS	0.5	g
Glycerol	5.0	ml
2-mercaptoethanol	2.5	ml
Bromphenol blue	0.0005	%
Deionized distilled water to	10	ml
Adjust pH to 6.8		

3.5 Running buffer, pH 8.3

Tris	3.0	g
Glycine	14.4	g
SDS	1.0	g
Dieonized distilled water to	1,000	ml
Adjust pH to 8.3		

3.6 Protein Molecular Weight : Individual proteins included in the

	Approx.MW (dalton)
Lysozyme	22,000

Soybean trypsin inhibitor	30,100
Carbonic anhydrase	36,800
Ovalbumin	52,300
Bovine serum albumin	98,000
B-galactosidase	119,000

3.7 Coomassie brilliant blue stain

Coomassie brilliant blue R-250	0.25	g
Methanol	40	ml
Glacial acetic acid	10	ml
Dieonized distilled water	454	ml

Dissolve the dye in methanol: deionized distilled water (1:1 v/v), then add glacial acetic acid 10 ml. Remove insoluble material by filtration through filter paper. The dye solution can be stored for months at room temperature but any precipitate formed should be removed before use.

3.8 Destaining solution

Methanol	250	ml
Glacial acetic acid	100	ml
Deionized distilled water	650	ml

4. Reagents for immunoblotting

4.1 Transfer buffer, pH 8.3

Tris	3.03	g
Glycine	14.4	g
Methanol	800	ml
Add deionized distilled water to	1,000	ml
Adjust pH to 8.3		

4.2 PBS 0.15 M pH 7.4 containing 0.1% Tween 20 (Washing buffer)

NaCI	8.0	g
KCI	0.2	g

$Na_2 HPO_4$	1.15	g
$KH_2 PO_4$	0.2	g
Tween-20	1.0	ml
Add deionized distilled water to	1,000	ml

4.3 Substrate solution for immunoblot

BCIP/NBT (For alkaline phosphatase conjugate)

Stock Nitro Blue tetrazolium (NBT) : dissolve 0.5 g in 10 ml of 70% (v/v) dimethylformamide

Stock Bromochloroindolyl phosphate (BCIP) : dissolve 0.5 g in 10 ml of 100% (v/v) dimethylformamide

Working NBT/BCIP (freshly prepare)

Combine 100 ul of stock NBT and 50 ul of stock BCIP to TBS 15 ml

5. Reagent for Ni-NTA spin kit (under native condition)

5.1 lysis buffer pH 8.0

dilute from stock solution kit

50 M Na_2HPO_4 (pH 8.0)

50 M NaCl (pH 8.0)

50 M imidazole (pH 8.0)

5.2 lysozyme (10 mg/ml)

Dissolve solid lysozyme at concentration of 10 mg/ml in 10 mM Tris-Cl (pH 8.0) immediately before use. Make sure that the pH of Tris solution is 8.0 before dissolving the protein.

5.3 Washing buffer pH 8.0

dilute from stock solution kit

50 mM MNa_2HPO_4 (pH 8.0)

300 mM NaCl (pH 8.0)

20 mM imidazole (pH 8.0)

5.4 Elution buffer pH 8.0

50 mM MNa_2HPO_4

300 mM NaCl

250 mM imidazole

6. STOCK REAGENTS

6.1 0.5 M EDTA (pH 8.0)

18.61 g for 100 ml

EDTA will not dissolve until pH reaches 8. To adjust to pH 8, add approx. 80 ml 10 N NaOH

6.2 2 M Glucose

36 g of glucose dissolved in 100 ml of distilled water

6.3 20% Glucose

20 g of glucose dissolved in 100 ml of distilled water

6.4 10 N NaOH

40 g of NaOH dissolved in 100 ml of distilled water

6.5 1 N NaOH

10 N NaOH dissolved in distilled water to 100 ml

6.6 5 M Sodium Acetate (pH 5.2)

NaOAc 408 g dissolved in 800 ml of distilled water

Adjust pH to 5.2 with glacial acetic acid

Add distilled water to 1,000 ml

6.7 1 M Tris-HCl (pH 7.4 , 7.6, 8.0, 8.5, 9.0, 9.5)

Tris base 121.1 g

Adjust to desired pH with concentrated HCl and then

add distilled water to 1,000 ml

6.8 10% Ammonium persulfate (APS)

1 g APS dissolved in distilled water to 10 ml

6.9 Stock of 100 ug/ul ampicillin in sterile distilled water

1 g ampicillin dissolved in distilled water to 10 ml

6.10 1 mg/ml BSA

5 mg BSA dissolved in distilled water to 5 ml

6.11 10 mg/ml ethidium bromide

500 mg EtBr dissolved in distilled water to 50 ml

6.12 1 M KCl

7.5 g KCl dissolved in distilled water to 100 ml

6.13 1 M $MgCl_2$

20.33 g $MgCl_2$-$6H_2O$ dissolved in distilled water to 100 ml

6.14 1 M $MgSO_4$

12.04 g $MgSO_4$ dissolved in distilled water to 100 ml

6.15 1 M $MnCl_2$

1.98 g $MnCl_2$ dissolved in distilled water to 10 ml

6.16 50X TAE buffer:

Tris base	424.0	g
Glacial acetic acid	57.1	g
0.5 M EDTA pH 8.0	100	ml

Adjust volume to 1,000 ml with distilled water. The solution was mixed and sterilzes by autoclaving at 15 psi 15 minutes.

7. Nucleospin *(Macherey-Nagel, Germany)*

7.1 The Principle of Nucleospin

With the Nucleospin extract, DNA binds in the presence of chaotropic salts (buffer NT1 and NT2) to silica membrane. Buffer NT1 contains additional conponents in order to dissolve agarose gel slices. Afterwards, binding mixtures are loaded directly onto nucleospin extract columns. Contaminations like salts and soluble macromolecular components are removed by a simple washing step with ethanolic buffer NT3. Pure DNA is finally eluted under low ionic strength conditions with slightly alkaline buffer NE (5 mM Tris-Cl, pH 8.5).

7.2 Protocol for DNA extraction from agarose gels

The DNA fragment in gel was cut with a clean scalpel and transfer to a sterile tube. A gel was melt by buffer NT1 (100 mg gel per 300 ml of NT1) and incubate at 50 $^{\circ}$C for 5-10 minutes. The samples were loaded onto column and spin down at 8,000 x g 1 minute. Afterward this columns were washed twice with NT3 buffer 200 ul and spin down again. The DNA was eluted from column by elution buffer 20 ul and spin at 11,000 for 1 minute.

8. Determination Molecular Weight of Protein

The molecular weight of unknown protein can be determine by comparing its electrophoretic mobility with standard protein markers. A linear relationship was obtained by plotting the molecular weight of the standard polypeptide chains logarithimically against their respective electrophoretic relative mobilities (Rf).

After elctrophoresis or immunoblotting, the dried gel and immunostained nitrocellulose membrane were photographed. The migration distance of each protein was measured from the top of the gel and the relative mobility was determined.

$$Rf = \frac{\text{Distance of protein migration}}{\text{Distance of tracking dye migration}}$$

9. Determination Protein Concentration by Bradford Method

Purification His6-LipL32 protein by Ni-NTA spin column were determined with Bradford methods according to the manufacturer's instructions (Bio-Rad, California , USA) with bovine serum albumin as the standard solutions. Preparation of standard curve with BSA (50,100,200,400 and 800 ug/ml). Add 0.1 ml each BSA standard in assay reagent 5 ml (diluted Bio-Rad dye concentration 4 volume with distilled water before use) mix well and incubate at room temperature for 5 minutes. The protein concentration was measured at absorbance 595 nm. Prepare a graph of absorbance at 595 nm vs [Protein] for the protein standards. Repeat the steps above for each samples to be assayed.

10. Minipreparation of plasmid

10.1 Incoculation 2 ml of LB medium containing the appropriate antibiotic with a single colony of transformed bacteria. Incubate the culture overnight at 37 °C with vigorous shaking.

10.2 Pour 1.5 ml of the culture into a microfuge tube. Centrifuge at maximum speed for 30 seconds at 4 °C in a microfuge. Store the unused portion of the original culture at 4 °C.

10.3 When centrifugation is complete, remove the medium by aspiration, leaving the bacterial pellet as dry as possible.

10.4 Lysis of cells, resuspend the bactrial pellet in 100 µl of ice-cold Alkaline lysis solution I by vigorous vortexing.

10.5 Add 200 µl of freshly prepared Alkaline lysis solution II to each bacterial suspension. Close to tube tightly, and mix the contents by inverting the tube rapidly five times. Store the tube on ice.

10.6 Add 150 µl of ice-cold Alkaline lysis solution III. Close the

lution III through the viscous

bacterial lysate by inverting the tube several times. Store the tube on ice for 3-5 minutes.

10.7 Centrifuge the bacterial lysate at maximum speed for 5 minutes at 4 °C in a microfuge. Transfer the supernatant to a fresh tube.

10.8 Add an equal volume of phenal:chloroform. Mix the organic and aqueous phase by vortexing and then centrifuge the emulsion at maximum speed for 2 minutes at 4 °C in a microfuge. Transfer the eqeous upper layer to a fresh tube.

10.9 Precipitate nucleic acids from the supernatant by adding 2 volumes of ethanol at room temperature. Mix the solution by vortexing and then allow the mixture to stand for 2 minutes at room temperature.

10.10 Collect the precipitated nucleic acids by centrifugation at maximum speed for 5 minutes at 4 °C in a microfuge.

10.11 Remove the supernatant by gentle aspiration as described in Step 3 above. Stand the tube in an inverted position on a paper towel to allow all of the fluid to drain away.

10.12 Add 1 ml of 70% ethanol to the pellet and invert the closed tube several times. Recover the DNA by centrifugatuion at maximum speed for 2 minutes at 4 °C in a microfuge.

10.13 Again remove all of the supernatant by gentle aspiration.

10.14 Remove any beads of ethanol that form on the sides of the tube. Store the open tube at room temperature untill the ethanol has evaporated and no fluid is visible in the tube (5 –10 minutes)

10.15 Dissolve the nucleic acids in 50 ul of TE (pH 8.0) containing 20 ug/ml DNase-free Rnase A . Vortex the solution gently for a few seconds. Store the DNA solution at –20 °C.

BIOGRAPHY

Miss Doojdao Boonyod was born on March 19, 1971 in Phitsanulok. Thailand. She graduated with the Bachelor degree of Science in Medical Technology from Faculty of Medical Technology, Rangsit University in 1993. Currently, she is a member of the Pathology Division, Regional Medical Sciences Center, Phitsanulok, Department of Medical Sciences, Thailand.