การศึกษาหาโปรตีนที่มีปฏิสัมพันธ์กับโปรตีน LipL32 ของเชื้อเลปโตสไปราสายพันธุ์ก่อโรคโดยใช้เทคโนโลยีการแสดงโปรตีนบนผิวฟาจ

นางสาวสุวิตตรา แช่มชื่น

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาจุลชีววิทยาทางการแพทย์ (สหสาขาวิชา)
บัณฑิตวิทยาลัย จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2552

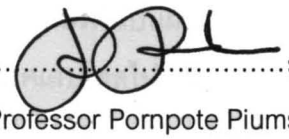
USE OF PHAGE DISPLAY TECHNOLOGY TO IDENTIFY PROTEINS THAT INTERACT WITH LIPL32, THE MAJOR OUTER MEMBRANE PROTEIN OF PATHOGENIC *LEPTOSPIRA*

Miss Suwittra Chaemchuen

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Medical Microbiology
(Interdisciplinary Program)
Graduate School
Chulalongkorn University
Academic Year 2009

Thesis Title	Use of phage display technology to identify proteins that interact with rLipL32, the major outer membrane protein of pathogenic *Leptospira*
By	Miss Suwittra Chaemchuen
Field of Study	Medical Microbiology
Thesis Advisor	Assistant Professor Kanitha Patarakul, M.D., Ph.D.
Thesis Co-Advisor	Assistant Professor Suang Rungpragayphan, Ph.D.

Accepted by the Graduate School, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree



..... Dean of the Graduate School

(Associate Professor Pornpote Piumsomboon,Ph.D.)

THESIS COMMITTEE

..... Chairman

(Assistant Professor Anan Chongthaleong, M.D.)

Kanitha Patarakul ..... Thesis Advisor

(Assistant Professor Kanitha Patarakul, M.D., Ph.D.)

..... Thesis Co-Advisor

(Assistant Professor Suang Rungpragayphan, Ph.D.)

Chanwit Tribuddharat ..... External Examiner

(Assistant Professor Chanwit Tribuddharat, M.D., Ph.D.)

สุวิตตรา แซ่มซื่น : การศึกษาหาโปรตีนที่มีปฏิสัมพันธ์กับโปรตีน LipL32 ของเชื้อเลปโตสไปราสายพันธุ์ก่อโรคโดยใช้เทคโนโลยีการแสดงโปรตีนบนผิวฟาจ (USE OF PHAGE DISPLAY TECHNOLOGY TO IDENTIFY PROTEINS THAT INTERACT WITH LIPL32, THE MAJOR OUTER MEMBRANE PROTEIN OF PATHOGENIC *LEPTOSPIRA*) อ. ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ.พญ.ดร.กนิษฐา ภัทรกุล, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ผศ.ดร.สรวง รุ่งประกายพรรณ, 140 หน้า.

โรคเลปโตสไปโรซิสเป็นโรคติดเชื้อจากสัตว์สู่คนที่พบได้ทั่วโลก สาเหตุของโรคเลปโตสไปโรซิส คือ แบคทีเรียกลุ่มสไปโรขีตสายพันธุ์ก่อโรคเรียกว่า *Leptospira interrogans* อาการของโรคเลปโตสไปโรซิสมีหลายรูปแบบ แต่อย่างไรก็ตามพยาธิกำเนิดของโรคยังไม่ทราบแน่ชัด ผนังชั้นนอกของเชื้อก่อโรคมีบทบาทสำคัญต่อปฏิสัมพันธ์ระหว่างเชื้อก่อโรคกับเจ้าบ้าน ซึ่งเป็นขั้นตอนแรกของกระบวนการติดเชื้อ ดังนั้นโปรตีนบนผนังชั้นนอกของเชื้อเลปโตสไปราอาจเป็นปัจจัยก่อโรคที่สำคัญของพยาธิกำเนิดของโรคเลปโตสไปโรซิส LipL32 เป็นโปรตีนที่พบมากที่สุดบนผนังชั้นนอกของ *L. interrogans* โดยมีการแสดงออกเฉพาะในเชื้อเลปโตสไปราสายพันธุ์ก่อโรคเท่านั้น โปรตีนแสดงออกตลอดเวลา และมีคุณสมบัติเป็นสารกระตุ้นภูมิคุ้มกันในเจ้าบ้าน นอกจากนี้ยังสามารถจับกับสารองค์ประกอบนอกเซลล์ของเซลล์สัตว์เลี้ยงลูกด้วยนม ในการศึกษานี้ได้นำเทคโนโลยีการแสดงโปรตีนบนผิวฟาจมาใช้เพื่อค้นหาโปรตีนของเจ้าบ้านที่สามารถจับกับโปรตีน LipL32 โดยใช้ recombinant LipL32 เป็นโมเลกุลเป้าหมายสำหรับคัดเลือกฟาจด้วย random heptapeptide phage library (Ph.D.-7). หลังจากนั้น สุ่มเลือก 52 plaque จากการคัดเลือกรอบที่สาม มาอ่านลำดับเบส พบว่ามีลำดับกรดอะมิโนที่แตกต่างกัน 6 แบบ โดยส่วนใหญ่มีลำดับกรดอะมิโนที่เหมือนกันคือ ทริปโตเฟน-ฮิสทีดีน-ทริปโตเฟน-ทรีโอนีน-ไทโรซีน-ไทโรซีน-ทริปโตเฟน เมื่อค้นหาโปรตีนที่สอดคล้องกับลำดับกรดอะมิโนแต่ละแบบด้วย BLASTP พบโปรตีนของเจ้าบ้านที่อาจเกี่ยวข้องกับพยาธิกำเนิดของโรคเลปโตสไปโรซิส ได้แก่ scavenger receptor class F, chloride channel assembly 2, laminin, coronin 2A, proataglandin E receptor 1 และ glycoprotein VI อย่างไรก็ตามยังต้องทดสอบการจับกันจริงของ LipL32 กับโปรตีนเหล่านี้และศึกษาบทบาทในพยาธิกำเนิดของโรคเลปโตสไปโรซิสต่อไป

สาขาวิชา จุลชีววิทยาทางการแพทย์ ลายมือชื่อนิสิต สุวิตตรา แซ่มซื่น

ปีการศึกษา 2552 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก

ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม

4989217620 : MAJOR MEDICAL MICROBIOLOGY

KEYWORDS : LipL32 / *Leptospira* / Phage display technology

SUWITTRA CHAEMCHUEN: USE OF PHAGE DISPLAY TECHNOLOGY TO IDENTIFY PROTEINS THAT INTERACT WITH LIPL32, THE MAJOR OUTER MEMBRANE PROTEIN OF PATHOGENIC *LEPTOSPIRA*

THESIS ADVISOR: ASST.PROF. KANITHA PATARAKUL, M.D., Ph.D., THESIS CO-ADVISOR: ASST.PROF. SUANG RUNGPRAGAYPHAN, Ph.D, 141 pp.

Leptospirosis is one of the most common zoonotic diseases worldwide. The causative agent of leptospirosis is pathogenic spirochetal bacteria named *Leptospira interrogans*. Leptospirosis presents broad spectrum of clinical features. However, pathogenesis remains unclear. The initial step of infection requires host-pathogen interaction. Outer membrane components including outer membrane proteins (OMPs) may play a key role in host-microbe interactions. LipL32 is the major OMP that is conserved among pathogenic leptospires, constitutively expressed, immunogenic, and capable of binding to extracellular matrix of mammalian cell. To identify the host proteins that interact with LipL32, phage display technology was employed in our study. Recombinant LipL32 was produced and used as a target molecule for biopanning with random heptapeptide phage library (Ph.D.-7). Then, 52 randomly selected plaques from the third round of panning were sequenced. The result showed 6 peptide sequence patterns. The most frequent peptide sequence is WHWTYYW. From database search, putative proteins that potentially bind to LipL32 include scavenger receptor class F, chloride channel assembly 2, laminin α-5, coronin 2A, proataglandin E receptor 1 and glycoprotein VI. However, the interactions of LipL32 with these host proteins and their roles in the pathogenesis of leptospirosis require further investigation.

Field of Study : Medical Microbiology Student's Signature .....

Academic Year : 2009 Advisor's Signature .....

Co-Advisor's Signature .....

ACKNOWLEDGEMENTS

I would like to express my thankfulness to all those who participated in the success of this thesis. I would like to thank my advisor, Assistant Professor Dr. Kanitha Patarakul for her stimulating guidance, valuable advice and supporting. Her expertise in molecular research improved my research skills and prepared me for future challenges. Especially, her kindness for giving me a good opportunity to study and inspired me to reach my goal.

I also would like to thank my co-advisor, Assistant Professor Dr. Suang Rungpragayphan, Faculty of Pharmacy, Silpakorn University for his kindness supporting and full of ideas to solve the problem in phage display. In addition, I would like to thank Assistant Professor Dr. Pokrath Hungsasuta for his kindness and assistance in protein purification.

I am grateful to Mr. Wuttisak Saksit, Mr. Jeerawat Nakkuntod for helping in molecular techniques. This work would not be accomplished without their support.

Finally, I would like to express my deepest gratitude to my parents and friends for their encouragement and understanding.

CONTENTS

PAGE

ABSTRACT (THAI)……...IV

ABSTRACT (ENGLISH).....V

ACKNOWLEGEMENTS.....VI

CONTENTS.....VII

LIST OF TABLES……X

LIST OF FIGURES……..XI

LIST OF ABBREVIATIONS……XIII

CHAPTER

I INTRODUCTION……1

II OBJECTIVE……5

III REVIEW OF RELATED LITERATURES……...6

Leptospira and Leptospirosis……6

Characteristic of *Leptospira*……..6

Cultivation Method……10

Molecular biology……11

Taxonomy and classification……..12

Epidemiology…….....14

Clinical features of leptospirosis……19

Anicteric form……19

Icterohaemorrhagic form……..19

Laboratory diagnosis……23

Microscopic visualization……..23

Cultivation……23

Serological diagnosis ……..24

Molecular diagnosis ……24

Pathogenesis……..25

PAGE

OMPs of *Leptospira*……27
LipL32……31
Phage display……33
Introduction……33
Filamentous bacteriophage biology……34
Display of peptides and proteins on phage particles……38
Phage display library……40
Screening phage display libraries……42
Application of phage display……44
Pyrosequencing……46
IV MATERIALS AND METHODS……49
Bacterial cultivation……50
Leptospira cultivation……50
Escherichia coli cultivation……50
DNA extraction……50
PCR amplification of *lipl32*……51
Plasmid extraction……51
Cloning……52
DNA sequencing……53
Recombinant LipL32 induction and expression……53
Recombinant LipL32 extraction……54
Recombinant LipL32 purification……54
Metal-affinity chromatography……54
Gel filtration chromatography……55
Western blotting……55
Protein assay……56

PAGE

Random peptide phage display screening……57
Phage titering……57
Biopaning procedure……57
Plaques amplification……59
Pyrosequencing……59
Generation of sequencing template by PCR……59
Purification and preparation of ssDNA sequencing template and pyrosequencing……60
V RESULTS……62
Cloning and DNA sequencing……62
Protein extraction and purification……65
Random peptide phage display screening……70
Pyrosequencing……71
Sequences analysis……73
VI DISCUSSION……83
VII SUMMARY……87
REFERENCES……88
APPENDICIS……122
Appendix A……123
Appendix B……137
BIOGRAPHY……140

LIST OF TABLES

TABLE		PAGE
1	Typical reservoir hosts of common leptospiral serovar	15
2	Examples of OMPs of pathogenic *Leptospira* and their functions or putative functions in pathogenesis of leptospirosis	29
3	The Ff phage genes, proteins and their properties	39
4	List of primers in experiments	61
5	Titers of input and output phage after each round of panning	70
6	The peptide sequence patterns from selected plaques obtained from third round panning	72
7	Putative proteins from BLAST analysis and their functions	74
8	The most matched putative host proteins that interacted with LipL32	82

LIST OF FIGURES

FIGURE		PAGE
1	Visualization of *Leptospira interrogans* under electron microscope and dark-field microscope	7
2	The schematic picture of leptospiral structure	8
3	The structure of leptospires membrane	9
4	Phylogenetic tree based on the sequences of leptospiral 16S rRNA sequences	13
5	Reported cases of leptospirosis per 100,000 populations, 1999-2008	16
6	Reported cases of leptospirosis per 100,000 populations by province in 2008, Thailand	17
7	Reported cases of leptospirosis per 100,000 populations by Region from 2004-2008	18
8	Target organs of *Leptospira*	21
9	The cycle of *Leptospira* infection	22
10	The dimension and architecture of filamentous bacteriophage	35
11	Genes and gene product s of filamentous phage	36
12	Life cycle of filamentous bacteriophage in *E. coli*	37
13	Phage affinity selection (bio-panning)	43
14	The enzymatic reactions in pyrosequencing	48
15	PCR amplification of *lipL32* gene and pRSET C expression vector on agarose gel	63
16	PCR amplification of *lipL32* insert in transformants	64
17	Detection of rLipL32 expression by SDS-PAGE and Western blot	65
18	Detection of purified rLipL32 from metal-chelation purification	66

PAGE

19 The schematic shows gel filtration performance monitored by UNICON 5.1 program……..67

20 Detection of purified rLipL32 from gel filtration chromatography……..68

21 Western blotting of purified rLipL32 from gel filtration chromatography……..69

22 PCR products analyzed of individual plaques amplification on agarose gel……..71

LIST OF ABBREVIATIONS

EMJH	Ellinghausen-McCullough-Johnson-Harris
LB	Luria-Bertani
°C	Degree celsius
MW	Molecular weight
kDa	Kilodalton
µl	Microliter
ml	Milliliter
µg	Microgram
ng	Nanogram
mM	Millimolar
Bp	Base pair
ssDNA	Single stranded DNA
PCR	Polymerase chain reaction
OMP	Outer membrane protein
ECM	Extra cellular matrix
LPS	Lipopolysaccharide
Lig	Leptospira Immunoglobulin-like
TLR	Toll-like receptor

NF-kB	Nuclear factor kappa B
MCP-1	Monocyte chemoattractant protein-1
RANTES	Regulated on activation normal T cell expression
iNOS	Inducible nitric oxide synthase
TNF-α	Tumor necrotic alpha
SREC-I	Scavenger receptor class F isoform I
CLCA2	Chloride channel accessory 2
EP1	Prostaglandin E receptor 1 (I)
GPVI	Glycoprotein VI
PAQR	Progestin and adipoQ receptor
MAdCAM-1	Mucosal vascular addressin cell adhesion molecule 1
IS	Insertion sequence
RF	Replicative form
PS	Packing signal
RPLs	Random peptide libraries
NPLs	Natural peptide libraries
ARF	Acute renal failure
GI	Gastrointestinal
MAT	Microscopic agglutination test

CHAPTER I

INTRODUCTION

Leptospirosis is a globally distributed zoonotic disease. The disease is endemic especially in tropical areas. However, it occurs in both industrialized and developing countries [1-4]. In Thailand, after 1996 leptospirosis cases have been dramatically increased from 400 cases to about 15,000 cases in 2000 (reported cases per 100,000 populations) [5]. Most patients are associated with working in agricultural fields. Hence, leptospirosis becomes a reemerging infectious disease in Thailand.

Clinical manifestations of leptospirosis range from a mild flu-like illness to multiorgan failure with high mortality rate [1, 2]. Severe leptospirosis, also known as Weil's disease, presents with renal and hepatic failure, pulmonary dysfunction, and hemorrhage [1, 2]. The causative agent of leptospirosis is pathogenic spirochete called *Leptospira interrogans*. Human infection is resulted from direct or indirect contact with urine of infected animal [1, 2]. Leptospires enter to the host via broken skin or mucosal membranes. The microbes disseminate through the blood stream, colonize the target organs and cause organ damage. However, the pathogenesis of leptospirosis is not well understood.

The initial step of infection involves host-pathogen interactions to gain colonization, replication, and dissemination of pathogens in the host. The outer membrane of leptospires contains adhesion molecules used to attach to the host cell surface. Moreover, the interaction may be crucial for activation of the host immune system. The ability of attachment to host cells in pathogenic *Leptospira* has been previously described. In contrast to saprophytic strains, the pathogenic leptospires were

shown to be able to adhere to cultured mammalian cells [6]. Hence, attachment ability of *Leptospira* seems to be correlated with its virulence [6, 7]. In addition, the leptospiral outer membrane components may play a key role in the first step of host-microbe interactions [8].

Outer membrane of pathogenic *Leptospira* consists of phospholipids, outer membrane proteins (OMPs) and lipopolysaccharide (LPS). Several studies demonstrated OMPs as putative virulence factors [9-16]. The leptospiral proteins were shown to be differentially expressed in response to environmental changes such as temperature and osmolarity shifts [17-21]. The results indicate that *Leptospira* responds to environmental stimuli and expression of its protein profile including OMPs should be dynamic along the step of infectious process inside the host [18, 21, 22]. However, the function of several OMPs of pathogenic *Leptospira* remains elusive.

LipL32, also known as hemolysin-associated protein 1 (Hap1), is the major constituent of pathogenic leptospires [23, 24]. LipL32 is the outer membrane lipoprotein of approximately 32 kDa of *Leptospira*. This protein is highly conserved among pathogenic serovars but absent in non-pathogenic leptospires [10, 16, 23-25]. Several reports demonstrated that LipL32 was constitutively expressed both *in vitro* and *in vivo* [16, 26]. Furthermore, its expression was shown to be up-regulated in animal models [9]. LipL32 expression was detected by immunohistochemical staining in kidney tissue of infected animal models and was also recognized by sera from leptospirosis patients [16, 27]. The immunogenicity of LipL32 had been reported. The anti-LipL32 antibody was produced during infection both in patients and animal model [14, 26, 28, 29]. Moreover, it was demonstrated that LipL32 induced tubulointerstitial nephritis via NFk-B related pathway [28, 30, 31].

Several studies revealed that LipL32 was able to bind to various extracellular matrix (ECM) proteins such as laminins, collagens, and fibronectins [32, 33]. The ECM-binding domain of LipL32 was characterized to be in the C-terminal region. Moreover, ECM-binding protein homologous to LipL32 has been identified in *Pseudoalteromonas tunicata*. These protein homologs are immunologically cross-reactive [32, 33]. The crystal structure study of LipL32 revealed acid-rich patches within its folded tertiary structure. The patches may play a role as putative binding sites for positively charged ligands such as laminin [34, 35]. In addition, it was found to have multiple surface-exposed regions that potentially mediate protein-protein interactions. However, morphology, growth rate, and virulence in animal models of the *lipL32* mutant, constructed by transposon mutagenesis, remained the same as that of the wild-type strain [36]. Hence, LipL32 may not be solely responsible for growth and pathogenicity of leptospires. It is still not known why LipL32 is highly expressed and conserved in pathogenic *Leptospira*. Therefore, the role of LipL32 in host-pathogen interaction should be further investigated.

Phage display is a powerful and high-throughput screening tool to study protein-protein interactions. The construction of phage display library is based on the basic molecular technique. The foreign DNA is inserted into phage genes that encode capsid proteins. The recombinant protein, the fusion of phage capsid proteins and foreign peptides, is expressed on the surface of phage particles providing physical link between genotype and phenotype of phages [37-40]. Phages with high affinity to the target protein are enriched by panning against immobilized target protein and washing to remove non-binding phages before amplification of bound clones. At the final round of panning, usually after 3-5 rounds, the bound clones are eluted followed by polymerase chain reaction and DNA sequencing of the inserted region to decode the peptide sequences of displayed polypeptides [37-40].

Phage display technology shows many applications in studies of infectious diseases [41], drug discovery [42], gene delivery and antibody synthesis [43]. In infectious disease studies, phage display has been used to identify vaccine candidate antigens, bacterial adhesins, epitope mapping, and host-pathogen interactions [41, 44-47]. For examples, phage display technique was used to identify adhesins in *Plasmodium falciparum* [48], Group B Streptococcus [49], *Lactobacillus reuteri* [50], *Staphylococcus aureus* [51], and hepatitis B virus [52].

In this study, we proposed to screen for host proteins that interact with LipL32 of *L. interrogans* using the random heptapeptide phage library.

CHAPER II

OBJECTIVE

Hypothesis

The phage display technique using random peptide phage library is able to identify peptide sequences that interact with LipL32.

Objective

To indentify peptides and corresponding host proteins that interact with LipL32.

CHAPTER III

REVIEW OF RELATED LITERATURES

Leptospira and leptospirosis

Characteristics of *Leptospira*

Leptospira is a genus of spirochete bacteria, including pathogenic and saprophytic species. Letospires are tightly coiled spirochetes that are about 0.1 μm in diameter and 6-20 μm in length with a wavelength of about 0.5 μm (Figure 1) [1, 53]. Leptospires are highly motile, obligate aerobic spirochetes that share features of Gram-positive and Gram-negative bacteria. One or both ends of the spirochete are usually hooked. Because they are poorly Gram stained, live *Leptospira* are best observed under the dark-field or phase-contrast microscope (Figure 1). However, the bacteria can be stained by carbon fuchsin counterstain [2, 53]

Leptospires have a double membrane structure similar to that of other spirochetes. The cell envelope consists of cytoplasmic membrane and outer membrane (Figure 2) [54]. The cytoplasmic membrane and peptidoglycan layer are closely related and are overlaid by an outer membrane [54]. The outer membrane is composed of phospholipids, outer membrane proteins (OMPs) and lipopolysaccharide (LPS) (Figure 3). LPS of leptospires has a structure similar to other Gram-negative bacteria but shows lower endotoxicity [55-57]. The two flagella extending from each end of the bacteria reside in the periplasmic space. The structure of the flagella is composed of protein complex. The flagella act as cytoskeleton element that is necessary for the motility of leptospires (Figure 2). Leptospires show two forms of movement, translational and non-translational of movement [2].

.

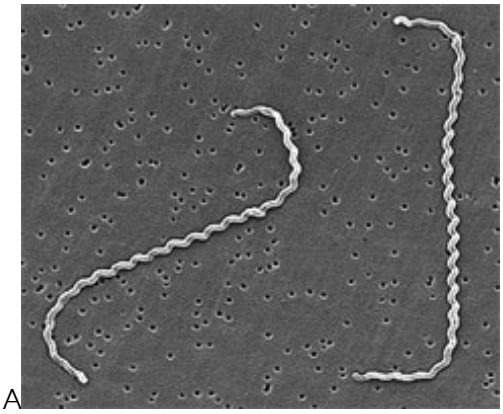
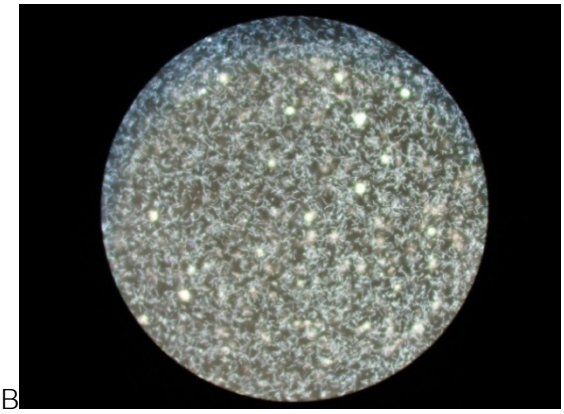
A  B 

Figure 1 Visualization of *Leptospira interrogans* under electron microscope (A) and dark-field microscope (B).

From A, the CDC Public Health Image Library; B, photo taken by bluuurgh at the dutch royal tropical institute (www.kit.nl)

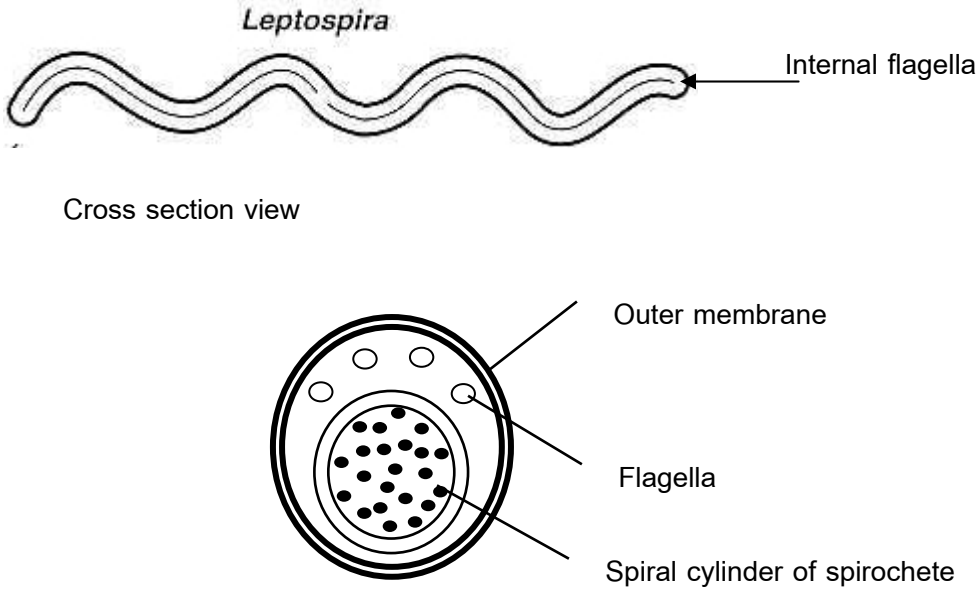


Figure 2 **The schematic picture of leptospiral structure.** Cross section view indicates the position of flagellae, outer membrane, and spiral cylinder of the spirochete.

Modified from [58]

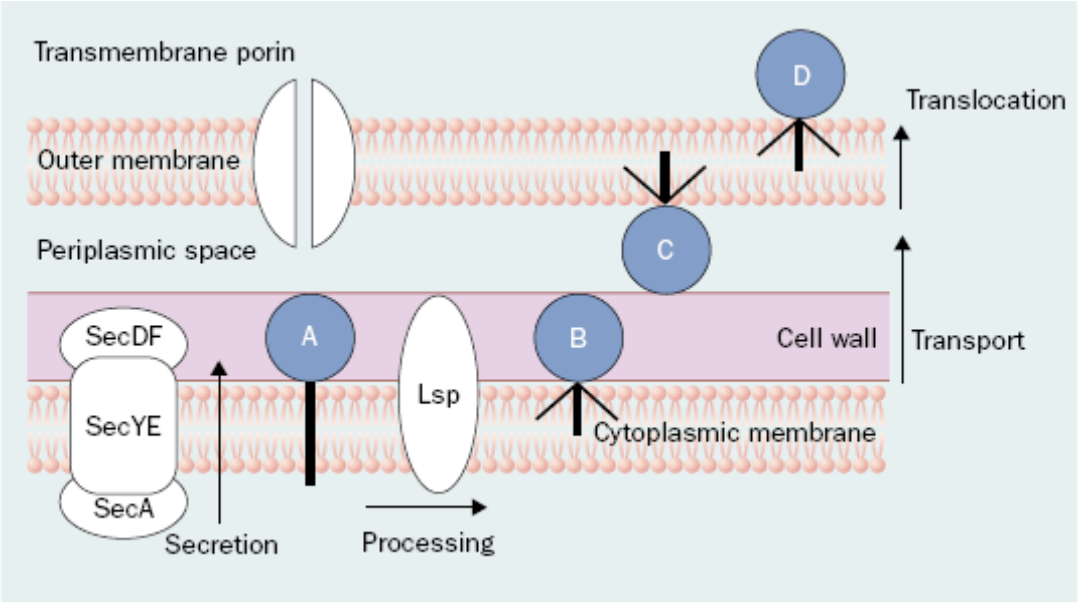


Figure 3 **The schematic picture shows the structure of leptospiral membrane.** A, prolipoprotein; B, subsurface lipoprotein in the cytoplasmic membrane; C, subsurface lipoprotein in the periplasmid space; D, surface-exposed lipoprotein (possible antigenic determinant) in the outer membrane; Lsp, prolipoprotein signal peptidase [1]

Cultivation method

Leptospires are most commonly cultivated in Ellinghausen-McCullough-Johnson-Harris (EMJH) medium. The culture medium is supplied with 10% rabbit serum or 0.2-1% bovine serum, long-chain fatty acids, vitamin B12, and ammonium salt. *Leptospira* is an aerobic bacterium that requires carbon and energy during *in vitro* growth. Long-chain fatty acids are carbon and energy sources which are metabolized by beta-oxidation. Fatty acid is usually obtained from tween. Fatty acid molecules bound to albumin are slowly released into the medium and prevent its toxic accumulation. Vitamin B2 and B12 are growth factors of leptospires [59, 60].

The optimal conditions for growth of leptospires are at pH 6.8-7.4 and at temperature between 28 °C- 30°C. The minimal growth temperature of pathogenic species is 13-15 °C, while saprophyte is 5-10 °C. The ability of pathogenic species to grow at 13°C can be used to distinguish saprophytic from pathogenic *Leptospira* species [61]. The pure subcultures in liquid medium usually grow within 10-14 days. In semisolid media, containing 0.1-0.2 % agar, leptospires grow near the surface of medium. In long term storage, the cultures are lyophilized or stored at -70°C [2]. Growth of leptospires on solid medium had been reported. Colony morphology depends on agar concentration and its serovar. The solid medium has been used to isolate leptospires from mixed culture and detect activity of leptospiral hemolysins [2, 53].

Molecular biology

The whole genome sequences of *L. interrogens* serovar Lai and Copenhegeni have been reported [62, 63]. In addition, genomes of *L. borgpetersenii* [64] and the saprophyte *L. biflexa* were sequenced [65]. Leptospires contain two sets of 16S rRNA and 23S rRNA and one set of 5S rRNA [66]. Several repetitive elements including insertion sequences (IS) coding for transposases have been identified.

The genome of *Leptospira interrogans* consists of two circular chromosomes, with a genome size of 4,400 kb and 350 kb [67]. The genome of *L. borgpetersenii* is 16 % smaller than that of *L. interrogans* with a higher number of pseudogenes, gene fragments and IS. In contrast to *L. interrogans*, *L. borgpetersenii* poorly lives outside the host. This characteristic may be due to disruption of genes involved in environmental sensing and metabolite transport and utilization [64]. Comparative genomics of *L. interrogans* and *L. biflexa* showed that 627 genes of *L. interrogans* are absent in *L. biflexa* and over 500 of these genes are of unknown functions [65]. These findings may reveal novel virulence-associated genes.

The lack of efficient genetic tools for pathogenic *Leptospira* has hindered the study of the role of its putative virulence factors. Recently, mutation in *L. interrogans* using homologous recombination [68] and transposon mutagenesis [69] has been reported.

Taxonomy and classification

Leptospira is classified into kingdom Monera, phylum Spirochetes, class Spirochetes, order Spirochetales, family Leptospiraceae and genus *Leptospira*. Genus *Leptospira* is divided into two species namely *L. interrogans* which includes pathogenic strains and *L. biflexa* which are saprophytic strains living freely in the environment [2]. Both species are serologically categorized into various serovars using cross-agglutinin adsorption test (CAAT) with homologous antigens [70]. *L. biflexa* have been classified into more than 60 serovars, whereas there are over 200 serovars of *L. interrogans* [2]. The members of *Leptospira* have been broadly grouped into serogroups based on their antigenic relationship. Serological classification may be useful for epidemiological application since some serological groupings are correlated with reservoir animals [2].

The genotypic classification has been used concurrently with phenotypic or serological classification [71]. The genotypic classification was based on multilocus enzyme electrophoresis information, G+C content, DNA-DNA correlation to other *Leptospira* and 16S rRNA gene sequencing. The genus *Leptospira* is currently divided into at least 21 species based on 16S RNA sequences (Figure 4) [71-73]. However, genotypic classification is not correlated with serological classification. Some pathogenic and non-pathogenic serovars are classified into the same genospecies and some serovars are found in more than one genospecies [1].

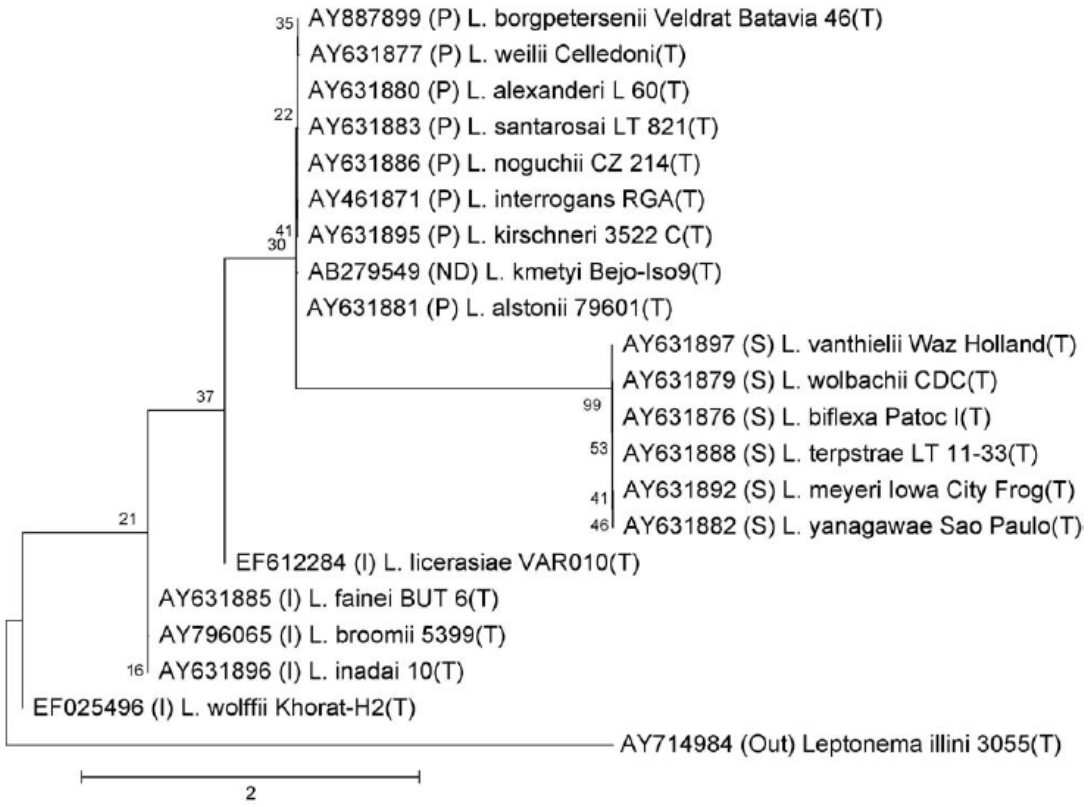


Figure 4 Phylogenetic tree based on the sequences of leptospiral 16S rRNA sequences [73].

Epidemiology

Leptospirosis is one of the most common zoonotic diseases worldwide [2]. Human infection occurs after either direct or indirect contact with urine of infected animals. The prevalence in tropical regions is significant higher than temperate regions because leptospires can live in the warm environment longer than in temperate environment. In the tropical areas, leptospirosis is associated with seasonal rainfall especially in August to September and February to March [1, 2, 74].

Leptospires is transmitted through unhealed breaks in the skin or mucous membranes by contact with urine of infected animals. Water-born transmission had been reported in several outbreaks of leptospirosis. Swallowing or splashing of contaminated water may result in infection via the mucosal membrane of respiratory tract and gastrointestinal tract. However, human to human transmission is rare due to acidic pH of human urine that restricts leptospiral survival after excretion. Infection from animal bite is uncommon [1, 2].

The habitats that expected to carry infective bacteria are muddy riverbanks, ditches, and muddy livestock areas where are regular passages of either wild or farm mammals [1, 2].

Animals involved in leptospirosis are divided into reservoir hosts (maintenance hosts), and accidental hosts. The reservoir hosts are carrier-state species that remain asymptomatic. Leptospires persistently colonize in proximal renal tubules of reservoir hosts and are shed in urine for their whole life span [1, 53]. Animals may act as reservoir hosts of some serovars but accidental hosts of others. A wide range of other mammals for examples dogs, deer, rabbits, cows, sheep and even marine mammals are also able to carry and transmit the disease as either accidental hosts or reservoir hosts [1, 2]. Some serovars are generally related to reservoir hosts (Table 1). The information

of dominant serovars and their reservoir hosts is necessary for understanding the epidemiology in each geographic area.

Table 1 Typical reservoir hosts of common leptospiral serovar

reservoir hosts	Serovar (s)
Pigs	Pomona, Tarassovi
Cattle	Hardjo, Pomona
Horses	Bratislava
Dogs	Canicola
Sheep	Hardjo, Pomona
Rats	Icterohaemorrhagiea, Copenhageni
Mice	Ballum, Arborea, Bim
Bats	Cynopteri,

Modified from [1, 2]

In Thailand, the number of leptospirosis cases reported before 1996 to the Ministry of Public Health was about 400 cases per year. After that, the number of reported cases increases (Figure 5). In year 2000, an outbreak of approximately 15,000 cases was reported. Most cases were in the Northeastern and the Northern regions. In addition, reported cases from the Southern part have been progressively increased (Figure 6, 7). Patients are predominantly male with age of between 15-65 and working in agricultural fields. Leptospirosis in Thailand is correlated with rainfall during June to October [5]. The dominant serovars in Thailand include Autumnalis, Bratislava, Bataviae, Javanica, Hebdomadis, Grippotyphosa, Bangkok, and Pyrogenes [75-77].

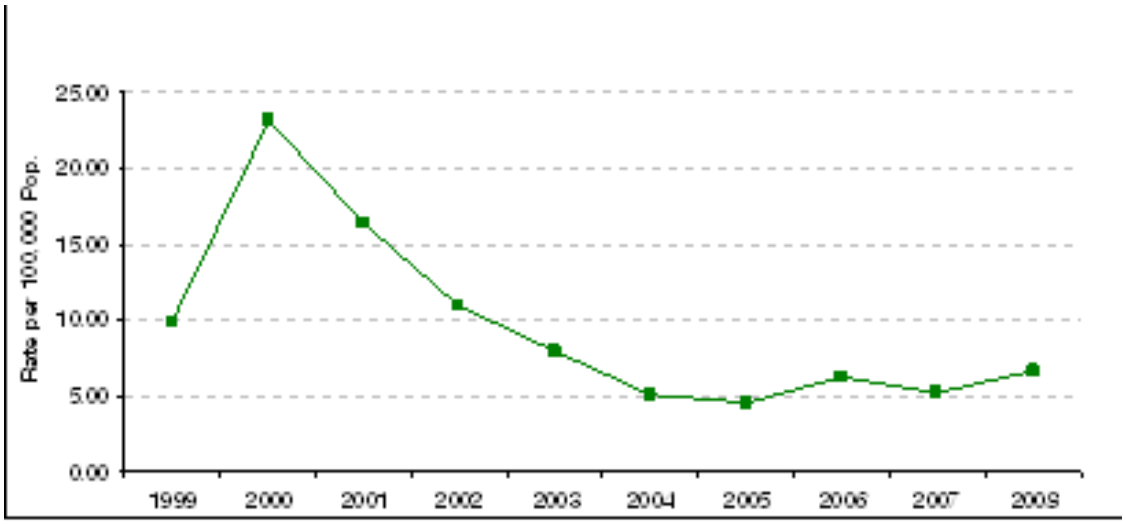


Figure 5 Reported cases of leptospirosis per 100,000 populations, 1999-2008 [5].

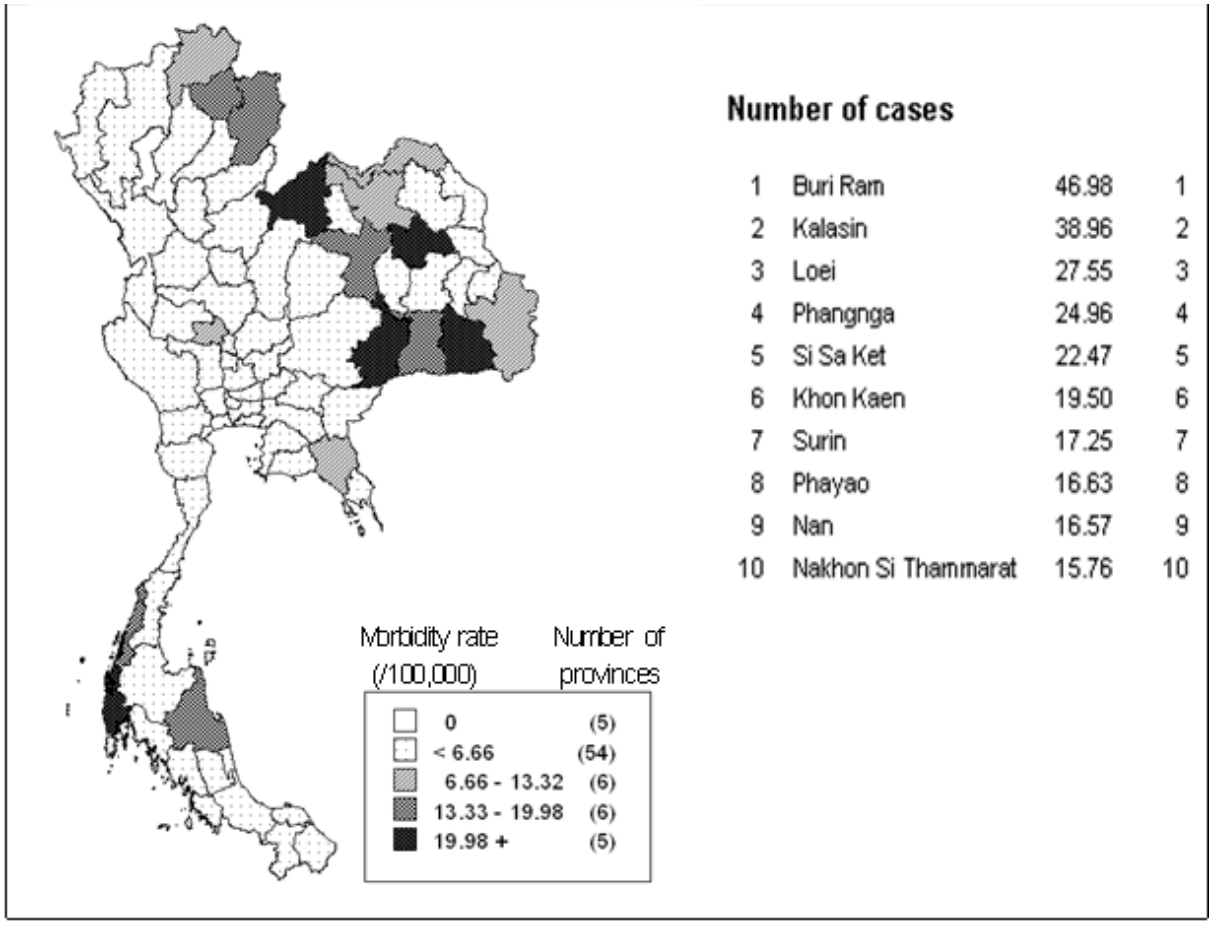


Figure 6 Reported cases of leptospirosis per 100,000 populations by province in 2008, Thailand [5].

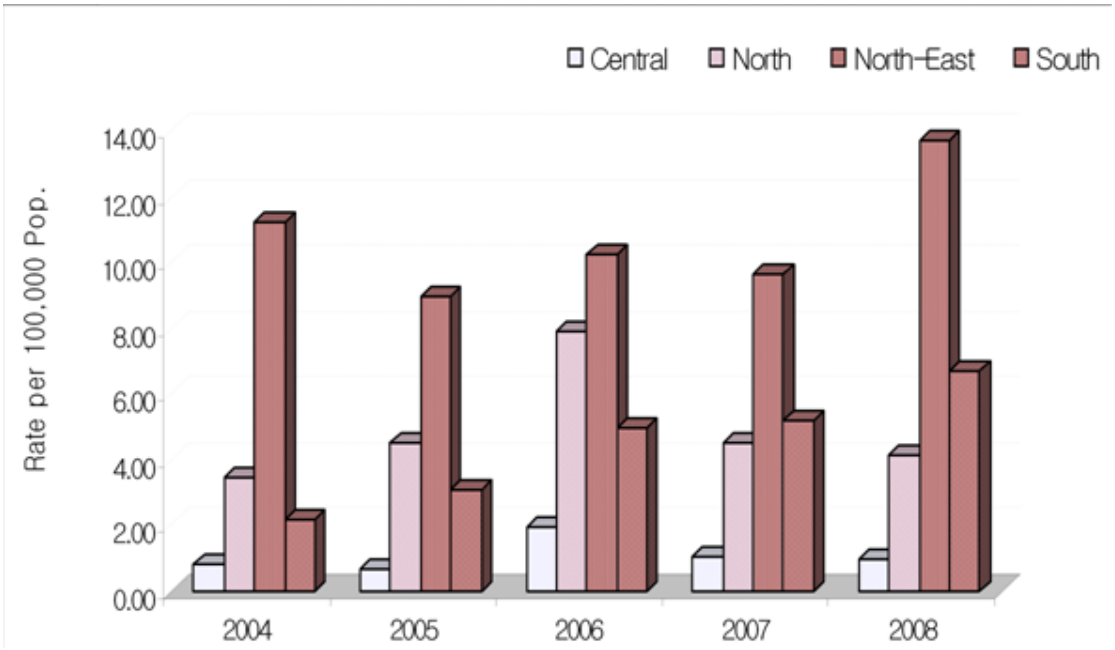


Figure 7 Reported cases of leptospirosis per 100,000 populations by Region from 2004-2008 [5].

Clinical features of leptospirosis

Clinical features of leptospirosis are diversed ranging from subclinical infection to a severe syndrome of multiorgan failure with high mortality rate [1]. Two forms of leptospirosis have been described which are anicteric form and icterohaemorrhagic form or Weil's disease. Anicteric form is a mild flu-like illness, whereas icterohaemorrhagic form is a severe infection with multi-organ involvement.

I. Anicteric form

Anicteric form is a major form of leptospirosis which is subclinical or very mild symptoms, and patients will probably not require treatment. Symptoms include chills, headache, myalgia, abdominal pain and conjunctival suffusion. The rash is rarely present or may be temporary for less than 24 hours. Anicteric syndrome usually lasts about a week and the resolution corresponds to the development of antibodies. In most cases, anicteric form is difficult to distinguish from viral infection such as influenza, HIV serocoversion and dengue.

II. Icterohaemorrhagic form or Weil's disease

Weil's disease is the most severe form of the illness and often rapidly progresses. It is described as jaundice, renal failure, and haemorrhage of target organs. This syndrome can develop after acute phase or presents alone. The icterohaemorrhagic form contributes to high fatality rate ranging from 5 to 15%. In some reports, Weil's disease was found between 5 and 10% of all leptospirosis patients [1, 2]. The jaundice is not related to hepatocellular necrosis but rather to cholestasis of sepsis [78]. Serum bilirubin may take several weeks to return to normal level. Transaminase levels moderately rise, whereas alkaline phosphatase level slightly increases.

Interstitial nephritis and tubular necrosis result in renal failure. Acute renal failure (ARF) occurs in 16-40 % of cases. ARF is a significant predictor of death with odds ratio of 9.98 [1, 2, 79].

Pulmonary involvement of leptospirosis may range from 20 to 70%. Patients present with a variety of symptoms from cough, dyspnea, hemoptysis to respiratory distress syndrome in adults [80, 81]. Severe pulmonary hemorrhage is a major cause of death in leptospirosis [82-85].

The incidence of cardiac involvement may be underreported. Electrocardiogram abnormalities may be non-specific or suggestive of myocarditis or pericarditis [86-88].

The neurological features are dominated by clouded sensorium and meningism. The features are characterized by typical neurological manifestations of meningeal irritation including headache and vomiting. Presentation as a primary neurological disease is uncommom in leptospirosis[1, 89].

Ocular involvement has been reported. Conjunctival suffusion and muscle tenderness are the key to distinguish physical findings of leptospirosis. Uveitis may be present after acute infection. Chronic visual disturbance has been reported [90, 91].

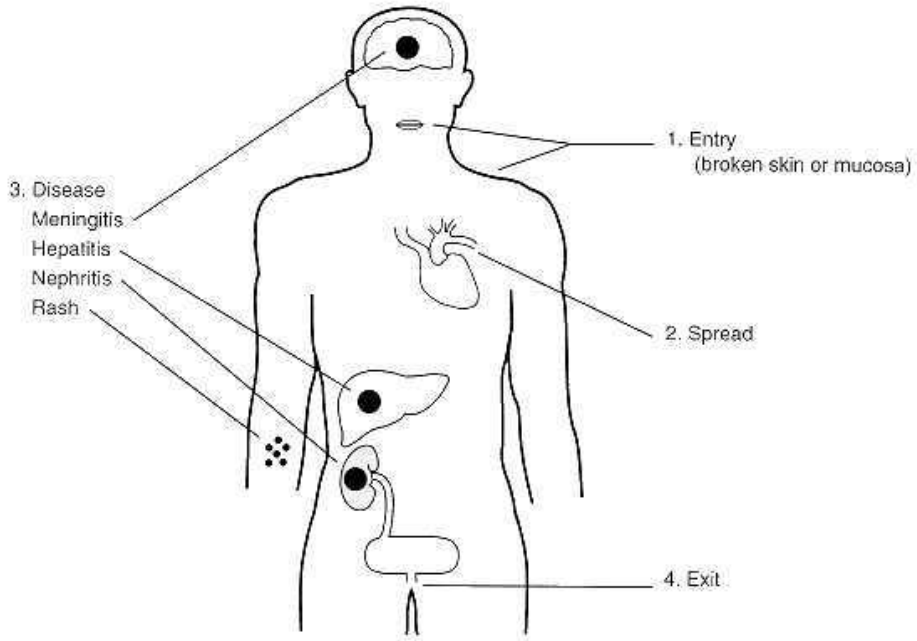


Figure 8 **The schematic picture shows target organs of *Laptospira*.** Leptospires transmit through broken skin or mucous membranes. After entry into the host, leptospires disseminate via bloodstream to target organs and excreted in urine [58].

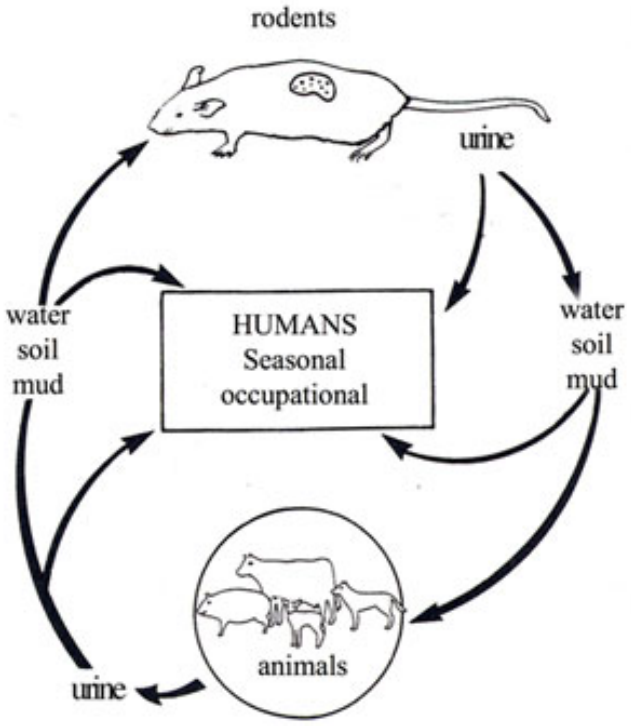


Figure 9 **The cycle of *Leptospira* infection.** Rodents are major reservoir hosts of leptospires. The spirochete is excreted in their urine. Human are usually infected through contact with contaminated water or soil [53].

Laboratory diagnosis

The clinical features of leptospirosis are non-specific. Therefore, laboratory investigation is required to confirm the diagnosis.

I. Microscopic visualization

Leptospires can be visualized under a dark-field microscope. The samples such as blood, urine, CSF or peritoneal dialysis fluid have been used. Minimal amount of leptospires that is necessary for observation by dark-field microscope is about 10^4 cells/ml. However, direct visualization under dark-field microscopy is unable to differentiate pathogenic leptospires from non-pathogenic leptospires and other spirochetes. Immunofluorescence staining and immunoperoxidase staining have been applied to increase sensitivity and specificity of direct microscopic examination. In addition, leptospires can be detected in infected tissues by silver or immunohistochemical staining [92].

II. Cultivation

It is possible to culture leptospires from blood, serum and CSF samples during the first week of illness, and from urine during the 2^{nd} and 3^{rd} week of illness [2, 93]. Cultivation method is difficult and poorly sensitive since it requires long time incubation, and special enrich medium. The growth of primary isolation may take up to 13 weeks at 30°C and the cultures can be reported as negative only after a minimum of 6–8 weeks. Therefore, cultivation method is not a useful test for routine diagnosis.

III. Serological diagnosis

Serology is the most common approach for diagnosis of leptospirosis. Antibodies are detectable in blood within 5 to 7 days after the onset of symptoms. Gold standard for serological diagnosis of leptospirosis is microscopic agglutination test (MAT). The MAT detects agglutination of serovar-specific antibodies against *Leptospira* in serum under dark-field microscope. The standard criteria for positive MAT is fourfold rising of antibody titers of pair sera or high single antibody titer above a cut-off point [1, 2].

Other serological tests are available, for examples, ELISA [94-96], dipstick ELISA [97-100], slide agglutination [101, 102], and latex agglutination [103-106].

IV. Molecular diagnosis

DNA of leptospires in human samples can be detected by polymerase chain reaction (PCR). Various primer sets have been reportedly used [107-111] such as 16S rRNA gene primer for pathogenic and non-pathogenic strains [112] and G1/G2 primer and B64-I/B64-II primer sets [113]. Currently, a real-time quantitative Taqman PCR was developed to detect 16S rRNA gene of leptospires in clinical and environmental samples [114]. The real-time SYBR green PCR is used to detect *lipL32 gene* of pathogenic strains [115].

Pathogenesis

Understanding of pathogenesis of leptospirosis is limited. After gaining entry through skin abrasions or mucous membranes, pathogenic leptospires spread hematogenously and cause systemic infection in human. Presence of leptospires does not cause inflammation at the entry site [1, 2]. Several putative virulence factors have been described but their functions remains mostly unknown.

Pathogenesis of leptospirosis may be due to two effects including direct effect by *Leptospira* and indirect effect by host immune response to infection. Motility of leptospires is an important mechanism for host invasion and dissemination to target organs. Whole genome sequence of pathogenic *Leptospira* reveals approximately 50 hypothetical genes that involve in mobility and chemotaxis [62, 116],[117].

Like other Gram-negative bacteria, endotoxin of leptospires has been reported in to be lipopolysaccharide (LPS) [118-120]. However, leptospiral LPS was shown to have less endotoxic activity than that of other Gram-negative bacteria [118, 119]. The expression of O antigen of leptospiral LPS isolated from chronically infected rat kidneys was significantly higher than that isolated from the livers of guinea pigs with acute infection, suggesting that the expression of O antigen determine acute or chronic infection of infected hosts [121].

Various hemolysins have been described in pathogenic leptospires [24, 122-127] such as sphingomyelinase C [127], sphingomyelinase H [126] and haemolysins-associated protein-1 (Hap-1, or LipL32). Sphingomyelinase H was not shown to have sphingomyelinase activity but they acted as cytotoxic pore-forming protein on several mammalian cells [126]. From genome sequence of serovar Lai, many hemolysins and sphingomyelinase-like proteins have been identified [62]. However, *in vivo* activity of these predicted virulence factors have not been established.

In contrast to non-pathogenic leptospires, pathogenic strains are able to attach to mammalian culture cells such as J929 (mouse fibroblast), J774A.1 (murine monocyte macrophage) and Vero cell (African green monkey kidney fibroblast) [6, 128]. In addition, they also adhere to renal epithelial cell *in vitro* [7]. These findings indicate that attachment ability is associated with virulence of leptospires.

. A large number of adhesins of pathogenic *Leptospira* have been described. For examples, LenA (or Lsa24), LipL32 [32], Lsa21 [129], endostatin- like family proteins [130, 131] ,Lp49 [132] and 36 kDa fibronectin- binding protein[133] were shown to be ECM binding proteins. These proteins were demonstrated to be expressed during host infection stage.

In addition, genome sequence of serovar Lai reveals genes potentially related to attachment and invasion, including homologues of mammalian cell entry gene *mce* of *Mycobacterium tuberculosis* and the invasion gene *invA* of *Rickettsia prowazeskii* [62].

Immune-mediated pathogenesis of leptospirosis has been described. Unlike LPS of other Gram-negative bacteria, LPS of leptospires activated human macrophage via Toll-like receptor (TLR) 2 instead of TLR4 [30, 53].However, in mouse macrophages, LPS activated both TLR2 and TLR4 [134]. LPS and the outer membrane protein OmpL1 are associated with interstitial nephritis in animal model [27]. OMPs of pathogenic *Leptospira* including LipL32 induced dose-dependent production of chemokines such as inducible nitric oxide mRNA increased (iNOS), monocyte chemoattractant protein-1 (MCP-1), and tumor necrosis factor-α (TNF- α) via NF-kB in renal tubular cell. Therefore, OMPs may contribute to inflammation of kidney [28, 31].

The mechanism of host immunity to pathogenic leptospires remains unclear. However, the humoral immunity is the primary response mechanism to *Leptospira* [135]. Protective immunity can be achieved by passive transfer of convalescent serum [136]. LPS activates innate immunity and induces antibodies productions that are a serovar-

specific antibody [137, 138]. Moreover, anti-LPS antibody showed protective activity in animal model such as guinea pigs, hamsters and dogs [139-142]. OMPs of pathogenic *Leptospira*, for examples, combination of OmpL1 and LipL41 [137, 143], LipL32 [144, 145], LipL21 and Lig protein were shown to induce partially protective immunity in many animal models, In contrast to other hosts, cell-mediated immunity plays a crucial role in protective immunity in cattles [146].

OMPs of *Leptospira interrogans*

In contrast to saprophytic strains, pathogenic *Leptospira* were demonstrated their ability to attach cultured mammalian cells indicating that attachment ability of pathogenic *Leptospira* is correlated with virulence of organisms [6, 7]. Initiation step of infection is host-pathogen interaction. The spirochete is able to colonize, adhere and invade host tissue. Therefore, host-pathogen interaction is a key for understanding pathogenesis of leptospirosis. OMPs of *Leptospira* are the cell components that contact directly with environment, host tissue and immune system. Hence, OMPs may play an important role in pathogenesis of leptospirosis [8].

OMPs of *Leptospira* have been defined in three classes based on their locations and fractionation in detergents [147]; (i) lipoprotein is the most abundant class, such as LipL32, LipL41 and LipL21. (ii) transmembrane protein, such as OmpL1. (iii) peripheral membrane protein such as LipL45. Most OMPs described to be virulence factors are up regulated *in vivo*, expressed only in pathogenic strains, and can stimulate protective host immune responses. However, functions of OMPs in pathogenic *Leptospira* are slightly verified. The properties of some OMPs are described in Table 2.

Expression of OMPs depends on environmental conditions, such as osmolarity and temperature to survive in the host [18, 21, 22, 147, 148]. In addition, OMPs are able to induce host immune responses [31]. Recently, genetic tool has been employed to reveal the role of putative virulence factor of pathogenic *Leptospira* [68, 69, 149]. The first genetically proved virulence factor, Loa22, has been generated using transposon mutagenesis. The Loa22 mutant became attenuated in virulence in animal models [13]. In addition, Murray et.al. generated mutants of *L. interrogens* by transposon mutagenesis. They found two attenuated mutants whose transposon insertion sites were located in hypothetical genes [69]. In addition, LigB and LipL32 mutants remained virulence in animal models [36]. However, their functions should be further elucidated. The genetic tool will provide the essential information to understanding biology of *L. interrogens* and pathogenesis of leptospirosis.

Table 2 Examples of OMPs of pathogenic *Leptospira* and their functions or putative functions in pathogenesis of leptospirosis

Proteins	Properties	References
Loa22	● the first genetically defined virulence factor of *Leptospira* ● conserved among pathogenic leptospires ● strongly recognized by leptospirosis patients sera ● mutant *loa22* leptospires loss virulence ability in animal model ● not essential for *in vitro* growth	[9, 13, 14, 150, 151]
LipL32	● the major OMP ● conserved among pathogenic strains ● up-regulated expression in animal model ● detectable in kidney tissue of animal model by immunohistochemistry ● able to bind to ECM ● homolog with a protein of *Pseudoalteromonas tunicate*	[10, 14, 16, 25, 26, 30-33, 152]
LipL21	● the second most abundant OMP ● conserved among pathogenic strains ● recognized by infected hamster sera ● expression not affected by environmental conditions ● DNA vaccine shows protective effect in animal model	[25, 153, 154]
Lp49	● recognized by leptospirosis patient sera ● protein with putative binding site (*in silico*), tertiary structure belongs to the all-beta-proteins class ● N-terminal contains Ig-like region	[132, 155]

Table 2 (continue)

Proteins	Properties	References
Lsa24 (or LenA or LfhA)	● endostatin-like structure ● able to attach mammalial ECM, especially laminin ● acts as factor H-binding protein ● expression level is not affected by environment conditions	[130, 131, 156]
Endostatin-like family; LenA, LenB,LenC, LenD, LenE, LenF	● LenB able to bind human factor H ● all Len proteins are able to bind laminin ● LenB, LenC, LenD, LenE and LenF display fibronectin binding ability	[131]
Lsa21	● able to bind ECM, especially laminin ● unique and conserved in pathogenic *Leptospira* ● protein *expression* is not affected by environmental factors ● detectable in liver and kidney tissues of patients	[148]
Leptospira immunoglobulin like (Lig) family; LigA, LigB and LigC	● contains immunoglobulin-like domain ● able to be recognized by patient sera ● act as protective antigen in animal model ● up-regulated expression in physiological osmolarity ● *ligB* mutant remained virulent	[12, 68, 157-162]

LipL32

LipL32, 32 kDa lipoprotein, or hemolysin associated protein 1 (hap-1) [24] is the major constituent of OM of pathogenic *Leptospira* [10, 16, 25]. In contrast to non-pathogenic leptospires, *lipL32 gene* is highly conserved among pathogenic strains [16].

LipL32 is expressed not only *in vitro* but also during mammalian infection [16, 27]. The temperature changeover does not affect LipL32 expression [21]. LipL32 was expressed both *in vitro* and during acute infection of guinea pig. Expression of LipL32 was detected by sera from infected animal model and patients [14, 26, 29].

The association of LipL32 and host immune response has been reported. LipL32 triggered tubulointerstitial nephritis via NFk-B related pathway. LipL32 preparation from *L. shermani* caused dose-dependent expression of MCP-1, RANTES, iNOS, TNF-α, NFk-B and AP1 transcription factors in proximal tubule cells [28].

LipL32 has been demonstrated as an ECM binding protein [32, 33]. The protein is able to bind laminin, plasma fibronectin, collagen I, collagen IV and collagen V in a dose-dependent manner [32, 33]. Moreover, the C-terminal region of LipL32 is immunogenic and able to bind to collagen IV and plasma fibronectin [33]. In addition, its orthologous protein in *Pseudoalteromonas tunicata* was shown to have ECM binding function and be immunologically cross-reactive [32].

Crystal structure of LipL32 showed compact, globular and jelly-roll fold regions [34, 35]. Two monomers of LipL32 formed a dimer with about 2-fold rotation. Putative binding sites for hydrophilic and hydrophobic ligands were identified suggesting that LipL32 may plays an important role in protein interaction. In addition, LipL32 thermostability was increased in the presence of calcium [35].

Murray and colleagues constructed *lipL32* mutant in *L. interrogans* by transposon mutagenesis. Morphology and growth rate of wild type and mutant were not different. LipL32 mutant remained virulence in hamsters and rats, which acted as acute and chronic infection model, respectively. Therefore, LipL32 is not essential for both acute and chronic infections [36]. However, these findings cannot exclude the role of LipL32 in host-pathogen interaction which should be further investigated.

Phage display

Introduction

Phage display is an effective technique for protein-protein interactions study, established by Smith in 1985 [37]. Phage display is described as a technique that recombinant peptides or proteins are expressed on filamentous phage particles. The phage display library is constructed by standard recombinant technology. The foreign genes are inserted into the phage genome and fused to the gene encoding one of phage capsid proteins. Therefore, the recombinant peptides are displayed on the surface of phage particles. This provides physical link between a displayed peptides and its DNA encoding region [37, 40, 163]. Affinity selection (bio-panning) is performed several rounds to identify the polypeptides with desired properties from a large libraries (Figure 15). At the final step, sequencing of the insert in obtained clones provides corresponding amino acid sequences of peptides responsible for protein-protein interactions [37, 38, 40, 164].

The advantages of phage display technology include (i) large phage libraries can be constructed (ii) high selectivity because affinity selection can be performed at high concentration of phage (iii) direct link between genotype and phenotype provides amino acid sequence data (ix) the stringency of washing step during bio-panning can be varied to get the specific bound phages. Moreover, the technique is simple, cheap, and rapid to set up and no special equipment is required. However, disadvantages of this technology include the size limitation of displayed proteins and the usage of bacteria as host strain which may result in incorrect folding and modification of displayed peptides [37, 38, 40, 164].

Filamentous bacteriophage biology

Filamentous bacteriophage is a group of viruses that contain a circular single-stranded DNA genome. The genome of virus is encapsulated in a protein capsid cylinder. The bacteriophage is able to infect a wide variety of Gram-negative bacteria, including *Escherichia coli*, *Xanthomonas*, *Thermus*, *Pseudomonas*, *Salmonella* and *Vibrio*. The Ff class of filamentous phage is the most studied phage. Bacteriophage uses the tip of F conjugative pilus as a receptor, thus phage is specific for F plasmid containing *E. coli*. The Ff class is consisted of M13, fd and f1 [38, 40, 163]. M13 is the most commonly used for phage display technology. Life cycle of filamentous phage and its gene products are shown in Figure 12 and 11.

The Ff phage particle is a flexible tube, about 6.5 nm in diameter and 1 μm in length (Figure 10). The engineered viruses are longer than wild-type strains because the length of phage particles depends on the length of encapsulated genome. The Ff phages are produced and secreted from infected bacteria without lysis, thus called non lytic phage [38, 40, 163].

Phage particle is composed of five capsid proteins (Figure 11). The phage contains approximate 2,700 copies of the 50 amino acid-major capsid protein, pVIII. At one end of the particle contains minor capsid proteins, about five molecules each of pVII and acid pIX. pVII and pIX are required for initiation and maintenance of phage assembly in the host bacteria. The other end contains minor capsid protein, about five molecules each of pIII and pVI. pVI and pIII involve in bacterial cell binding and the termination step of phage particle assembly. The orientation of phage genome is determined by the packing signal (PS), a 76 nucleotide hair pin region. The PS is located at the end of the particle that contains pVII and pIX proteins [38, 40, 163].

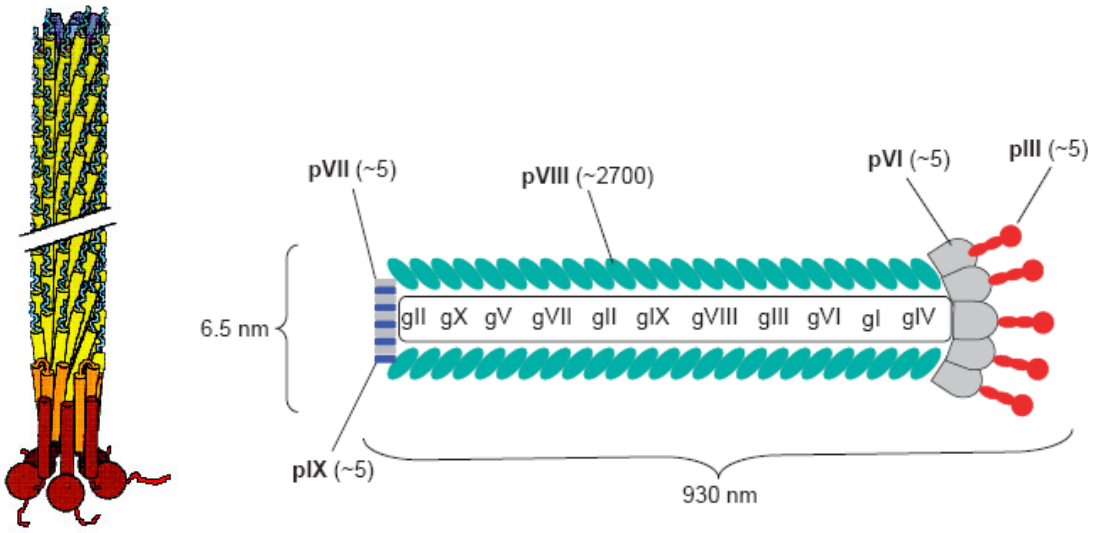


Figure 10 **The dimension and architecture of filamentous bacteriophage.** The copy numbers of each protein are shown in brackets [41].

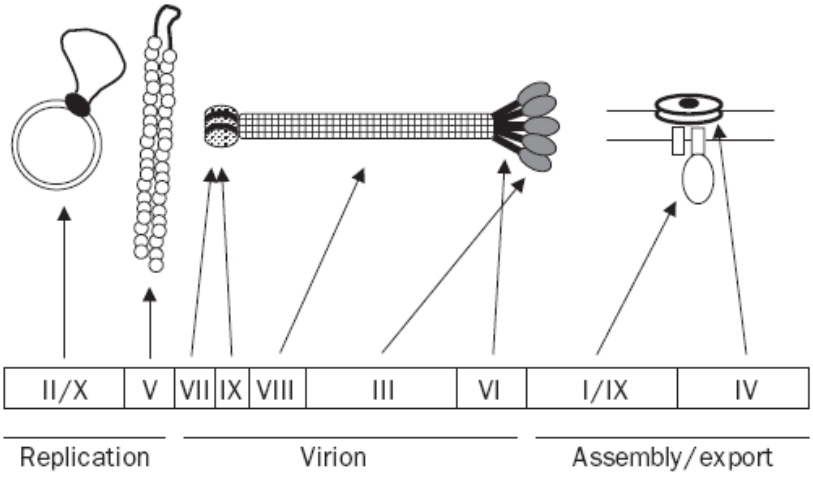


Figure 11 The genes and gene products of filamentous phage. pII binds to IG region of ds DNA and make a nick in (+) strand, initiating replication. pX is required for regulation of RF DNA synthesis. pV is the ssDNA binding protein. pVII and pIX are small proteins located at the tip of virus. pVIII is a phage major capsid protein. pIII and pVI are a minor capsid protein which are located at the tip of phage particle. Moreover, pIII and pVI mediate assembly termination, release of the virion and infection. *Gene I* encodes pI and pXI which are cytoplasmic membrane protein. *Gene IV* encodes pIV, which forms oligomer channels residing in the outer membrane. This channel becomes phage exit pore [38].

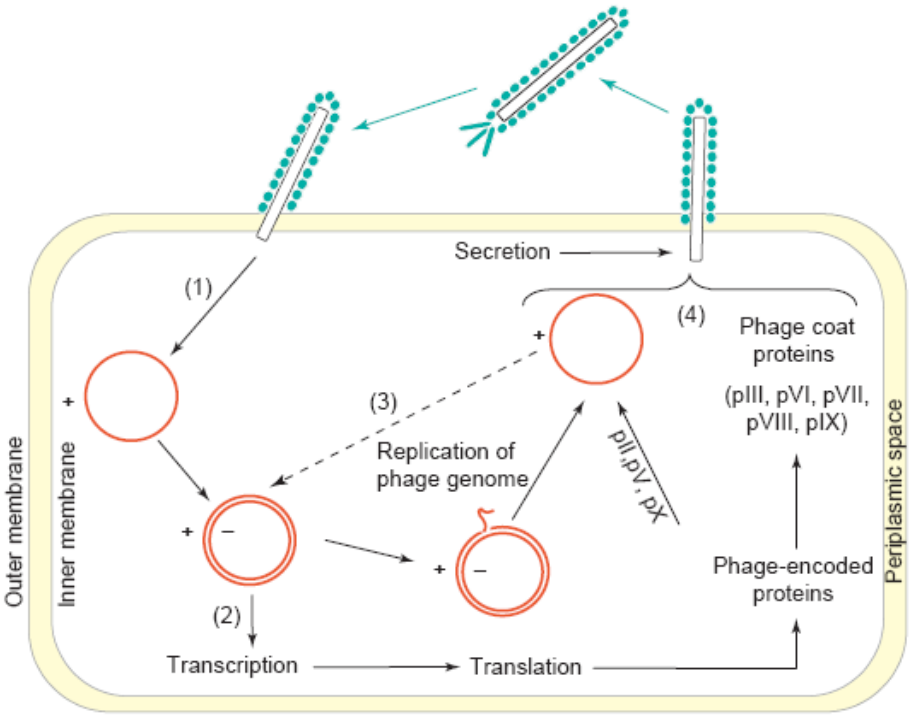


Figure 12 **The life cycle of filamentous bacteriophage in *E. coli*.**

A. Phage binds to the *E. coli* cell through the pIII capsid protein. The single-stranded viral genome (+strand, single circle) is injected into the cell and a complementary strand (- strand) is synthesized to form a double-stranded phage genome (RF).

B. Subsequently, all ten phage-encoded proteins are produced by host-mediated protein synthesis, including capsid proteins (pIII, pVI, pVII, pVIII and pIX), proteins for replication (pII, pV and pX) and proteins involved in assembly and export (pI and pIV).

C. The phage genome is replicated using the (+)-strand as a primer and the (-)-strand as a template.

D. Virions are assembled and exported across the bacterial membranes.

From [41]

Display of peptides and proteins on phage particle

All five capsid proteins have been used to display peptides or proteins as described in Table 3. The pIII and pVIII are the most commonly used for protein display [37, 39, 40]. The foreign DNA must be inserted correctly to the reading frame of the capsid protein [38]. Moreover, pVI has been used for display. The foreign DNA is fused to the C-terminus so that the inclusion of stop codon will not prevent peptide display. Hence, pVI is suitable for expression of cDNA libraries [38].

The DNA sequence encoding the peptide to display on pVIII is generally inserted between the signal sequence and the N-terminal of coding mature capsid protein. This insertion leads to the production of phage particles with recombinant proteins on their surface. However, only 6-8 amino acid peptides can be displayed on every copy of pVIII in a virion. pVIII containing large size of insert can be less efficiently packaged into phage particles resulting in failure to form observable plaques [38, 40, 164].

The minor capsid protein pIII is the most popular for phage display. The disadvantage of pIII is that only 5 molecules can be displayed per phage particles. The advantage is its tolerance for large insert. In most cases, foreign DNA is inserted between the signal sequence and the beginning of the first domain (N1) of pIII. Foreign DNA can also be inserted between the N1 and N2 domains as well as N2 and C-terminal domains. However, large insertions may reduce phage infectivity. This limits the ability to retrieve displayed proteins of a particular size [38, 40, 164].

Filamentous phage is a non lytic phage. This characteristic prevents transportation of long recombinant proteins across the inner membrane of *E. coli*. To overcome this limitation, alternative bacteriophage display systems have been developed using lytic bacteriophages such as T4, T7 and lambda [165].

Table 3 The Ff phage genes, proteins and their properties

Gene	Function	Location	Size(amino acid)	Use for Displays?
I	I	Assembly	384	
	XI	Assembly	108	
II	II	DNA replication	409	
	X	DNA replication	111	
III	III	Minor capsid protein	406[a]	Yes
IV	IV	Assembly	405[a]	
V	V	Binding ssDNA	87	
VI	VI	Minor capsid protein	112	Yes
VII	VII	Minor capsid protein	33	Yes
VIII	VIII	Minor capsid protein	50[a]	Yes
IX	IX	Minor capsid protein	32	Yes

[a]Mature protein without signal sequence

Modified from [38]

Phage display Library

Display libraries are produced by cloning a large number of DNA into the phage genome. Phage libraries thus contain billions of unique peptides and proteins. The recombinant proteins are constructed either in phage vector or phagemid [38, 40, 163].

Phage vectors are derived from the natural Ff phage sequence which is modified to carry restriction sites for cloning. The foreign DNA is inserted directly into the gene encoding phage capsid protein. After vectors are introduced into *E.coli* host cells, all copies of phage capsid proteins are produced followed by display of foreign peptides. Examples of phage display vectors include the FUSE, the M13KE vector and the derivatives of M13KE vector [38, 40, 163].

Phagemid vector is a plasmid containing phage origin of replication. The foreign DNA is fused into a plasmid and displayed protein is expressed under control of a weak promoter. The wild-type phage capsid proteins are supplied by the helper phage. The helper phage is an Ff phage with defective Ff origin of replication resulting in unable to assemble phage particles. *E. coli* expresses wild-type phage capsid proteins from helper phage genome and a small amount of foreign proteins from phagemid. Thus, the phage particles contain both proteins, typically with excess of wild-type proteins. Phagemid has been commonly used for pIII fusion. Moreover, pVIII and pVI are also displayed by phagemid. The advantages of phagemid are its small size and easily cloning [38, 40, 163].

There are two types of phage display library including random peptide libraries (RPLs) and natural peptide libraries (NPLs). RPLs are the most common type of displayed libraries. The peptides that displayed in RPLs are encoded by synthetic random oligonucleotides which are derived from degenerate oligonucleotides [38, 40, 166]. The oliginucleotides are synthesized chemically by adding nucleotide mixtures to construct nucleotide chains [38, 40, 41]. In general, RPLs contain about a billion phage clones. The advantage of this library is their universal nature. However, the disadvantage of RPLs is peptide sequences that may not be found in natural proteins [40, 41]. In contrast, NPLs are constructed from random fragments of genomic DNA or cDNA from a selected organism. Therefore, the libraries display fragments of natural proteins. The genomic DNA libraries represent all coding sequences which mostly encode nonfunctional proteins, whereas the cDNA libraries contain only the functional coding regions of a genome [38, 40, 41].

Screening phage display libraries

Phage display selection can be performed both *in vivo* and *in vitro* [38, 164, 167]. *In vivo* screening was performed to identify organ-specific molecules [168, 169]. Examples of *in vivo* targets are vascular endothelium cells [170-172] and mosquito organs [173]. *In vitro* target molecules can be not only biological targets but also inorganic targets [174]. The target molecules are immobilized on a solid support. A wide variety of solid supports have been used for bio-panning including polystyrene (plastic) plate, magnetic particles, plastic beads, nitrocellulose membrane, and agarose beads. The most commonly used solid support is polystyrene. Non-covalent adsorption is a common method for coating targets on a hydrophobic plastic surface. However, the covalent attachment method is used in the case of highly hydrophilic or low molecular weighed target molecules [164].

Bio-panning procedure is involved the following steps; (i) library amplification (ii) exposure of the library to target molecules (iii) removal of unbound phages by washing (ix) elution and amplification of bound phages (Figure 15). The bio-panning process is repeated, typically 2-6 times. Plaques from the final eluate are individually characterized [38, 40].

The concept of washing step is to remove unbound phages to select and enrich bound phages. The desired clones should be clones with high affinity and specificity. However, the library contains a large number of clones with various affinity and specificity. The affinity and specificity of obtained clones depend on stringency in washing step adjusted by washing times, detergent concentration, and progressively increased the washing stringent. A number of elution conditions have been used, for examples, competitive elution, extremes pH, ionic strength and enzymatic cleavage [38, 40].

After a single round of bio-panning, bound phages can be enriched at least 10 fold over unbound phages. The enrichment is monitored by tittering of input and output phages to determine sufficient rounds of panning.

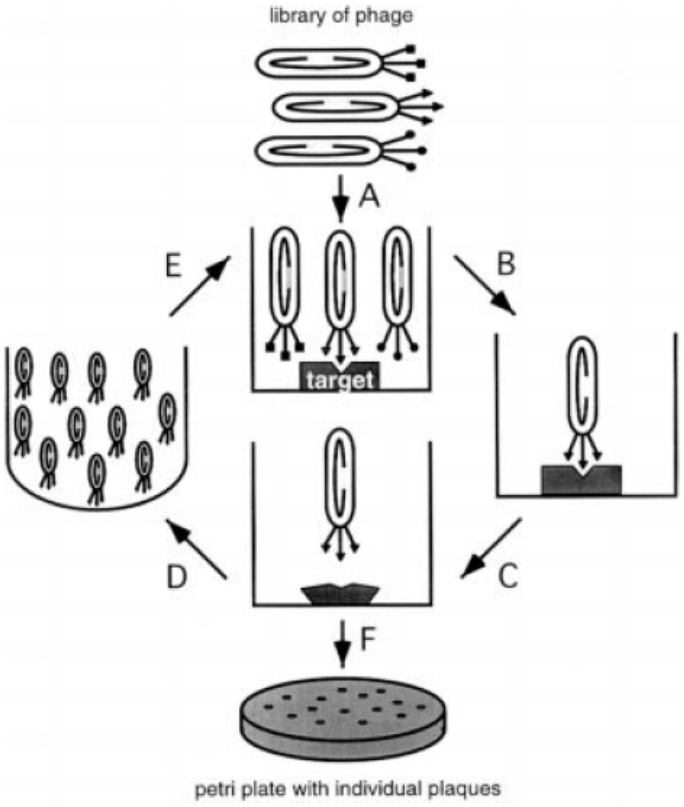


Figure 13 **The phage affinity selection (bio-panning).**

A. The phage display library, each displaying a different peptide sequences, is exposed to immobilized target molecules.
B. Unbound phages are washed away.
C. Bound phages are eluted.
D. Eluted phages are amplified and the panning is repeated.
E. Individual clone is isolated and sequenced.

From; [175]

Application of phage display

Phage display technology has been used in many applications including [39, 164] the identification of a new receptor and natural ligands [46, 176-179], epitope mapping [180-184], vaccine development [185], identification of peptides drug candidates and the isolation and engineering of recombinant antibodies [44, 186, 187].

Phage display is a useful tool for investigation of host-pathogen interactions [179, 188], identification of bacterial adhesins, epitope mapping and identification of vaccine candidate antigens. The technique provides essential information in infectious disease studies such as fibronectin binding protein of Group B Streptococci [49, 189], IgG and albumin binding domain of Group C Streptococci [190], platelet-binding domain within fibronectin binding protein of *Staphylococcus aureus* [51, 191], laminin binding site mapping of *Yersinia pestis* plasminogen activator (PLA) [192], the *Plasmodium falciparum* protein that involved in entrance and exit from human erythrocyte [173] , and the salivary gland and midgut peptide 1 (SM1) of *P. faliciparum*, associated with *Plasmodium* invasion of salivary gland and midgut in mosquito [48]

Phage display is a successful method for epitope mapping in many pathogens such as protease epitopes of *Burkholderia pseudomallei* [193], mimotopes of capsule of *Streptococcus pneumoniae* [194], antigenic mimotopes of B-cell epitopes of *Mycoplasma hyopneumoniae* [195], and putative epitopes within the nucleocapsid protein of Nipah virus [196].

. In leptospirosis study, phage display technology was used for epitope mapping. Tungtrakanpong and colleagues used random heptapeptide phage library to identify mimotopes of monoclonal antibodies (mAb) against pathogenic *Leptospira* and leptospirosis patient sera. The mimotopes were matched with leptospiral putative outer membrane proteins, thermolysin precursor protein, and hypothetical protein LIC 12228

[197]. They also mapped epitopes of five monoclonal antibodies. The antibodies are specific to serovars Australis, Bangkok, and Bratislava. Amino acid sequences of obtained clones are corresponded to a segment of hypothetical proteins of *Leptospira*. However, host-pathogen interaction study using phage display technology in leptospirosis has never been reported.

In our study, the random peptide phage library was used for screening host proteins that interact with LipL32.

Pyrosequencing

Pyrosequencing is the method of DNA sequencing based on DNA synthesis. This technique requires a biotin-tagged primer and labeled nucleic acids. The pyrosequencing procedure relies on detection of pyrophosphate (PPi) released during DNA synthesis. The visible light is generated in proportion to the number of incorporated nucleotides [198].

The sequencing primer is hybridized to biotin labeled, single-stranded DNA template. The reaction requires adenosine 5' phosphosulfate (APS), luciferin, and enzymes including DNA polymerase, ATP sulfurylase, and luciferase (Figure 14). The deoxribonucleotide triphosphate (dNTP) is sequentially added to the reaction mixture. After addition of nucleotides, DNA polymerase catalyzed the incorporated dNTPs followed by release of PPi in proportion to a number of incorporated nucleotides. The released PPi is subsequently converted to ATP by ATP sulfurylase in the presence of APS. The generated ATP drives luciferin converted to oxyluciferin by luciferase. The light in luciferase catalytic reaction is detected which is shown as a peak signal in raw data output. Unincorporated nucleotides are removed. When nucleotide removal is completed, another nucleotide is sequentially added. During the process of DNA synthesis, complementary DNA is extended and nucleotide sequence is monitored by the signal peak. The height of each peak (light signal) is proportional to the number of nucleotides incorporated.

Curently, two different pyrosequencing approaches are available, solid phase pyrosequencing and liquid phase pyrosequencing. Washing step of solid phase system is performed to remove excessive nucleotides to allow repeated addition. In contrast, excess nucleotide removal of liquid phase pyrosequencing is achived by apyrase, nucleotide-degrading enzyme.

The advantages of pyrosequencing are rapid, simple, cheap, and high throughput method. Pyrosequencing can read sequence signal immediately downstream of sequencing primer. However, its drawback is that optimal length of readable sequence limits to about 20-30 bases.

Pyrosequencing has been utilized in many applications such as single-nucleotide polymorphism (SNP) genotyping [199-201] and microbial typing [202-205]. Moreover, this technology is applied for analysis of difficult secondary structures [206], mutation detection [207] and clone checking [208].

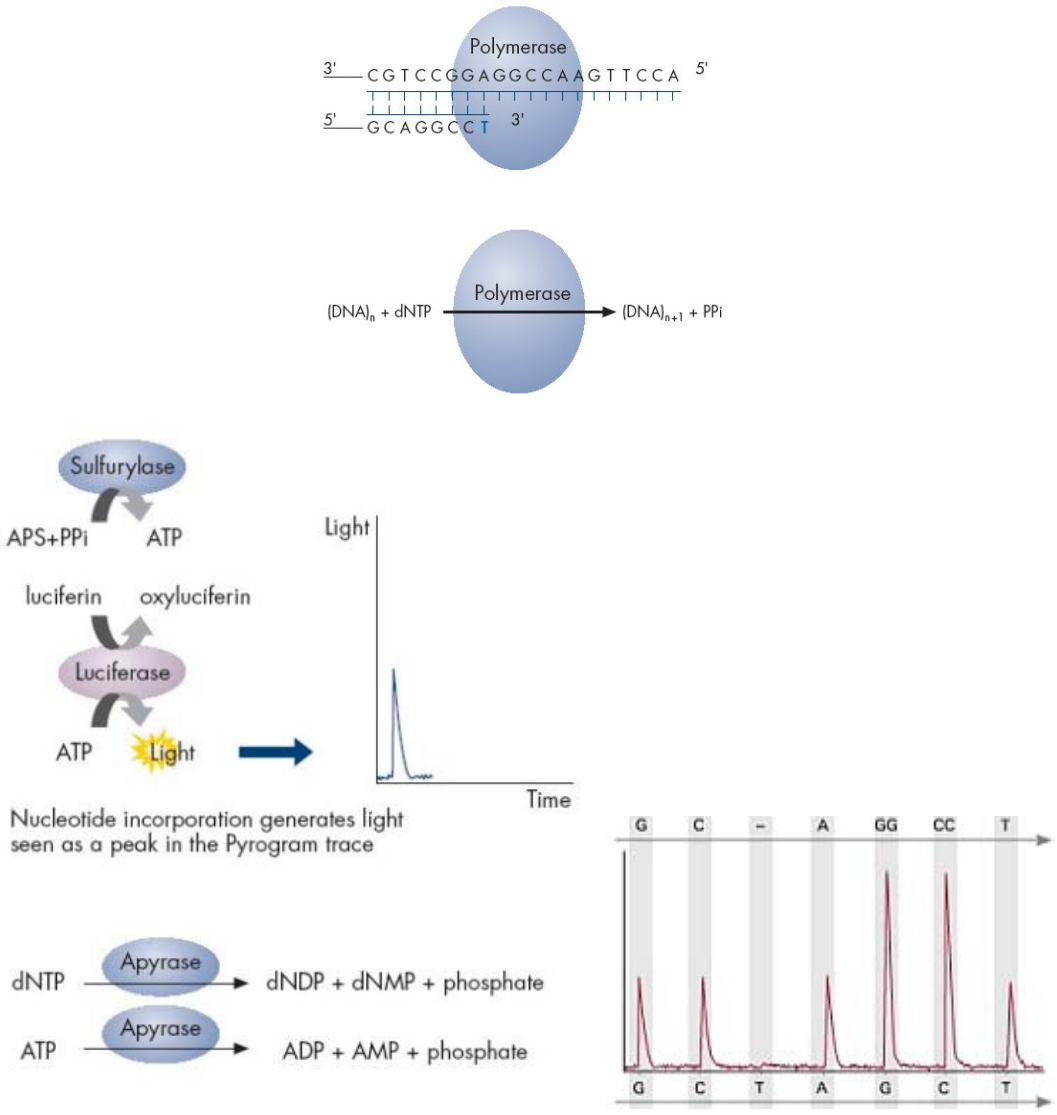
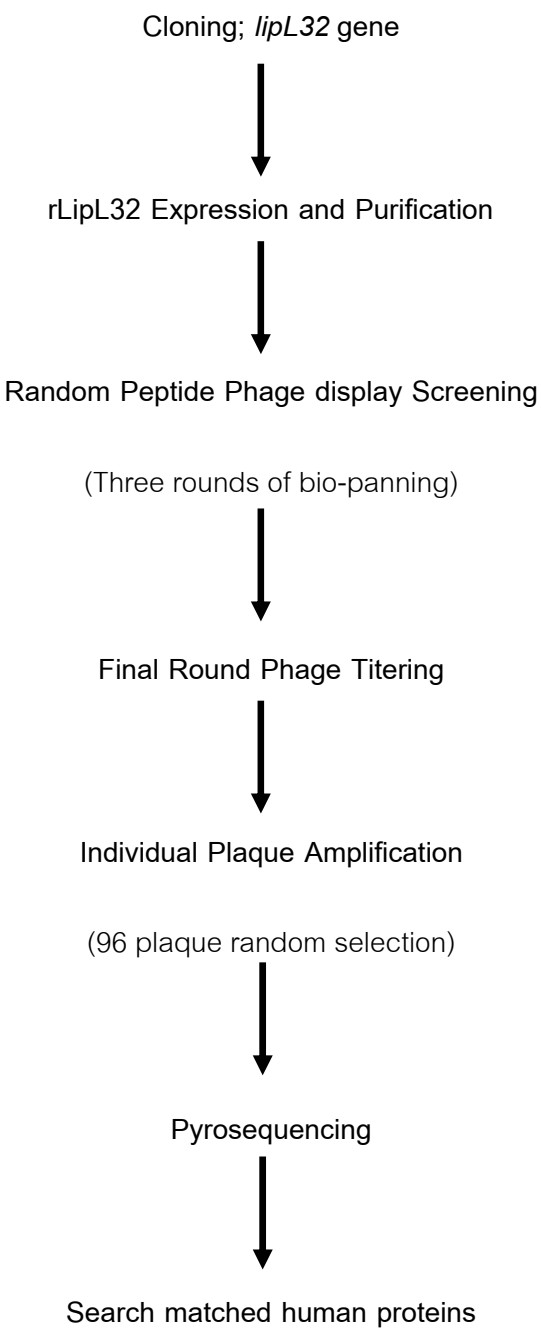


Figure 14 **The enzymatic reactions in pyrosequencing.** The sequencing primer hybridized to a template, single-stranded PCR product. The four dNTPs are added stepwise and incorporated, if it is complementary to the base in the template strand, PPi will be releases. ATP sulfurylase converts PPi to ATP in the presence of APS. This ATP is provided the energy to luciferase to oxidize luciferin and generate light in proportional to the amount of ATP. The light produced in the luciferase-catalyzed reaction is detected and seen as a peak in the raw data output. The nucleotides are removed allowing addition of subsequent nucleotide.

From ***www.pyrosequencing.com*** *(September, 2009)*

CHAPTER IV

MATERIALS AND METHODS



Bacterial cultivation

Leptospira cultivation

L. interrogans serovar Pomona was obtained from Khon Kaen University. *Leptospira* was cultivated in EMJH leptospiral enrichment culture media at 30°C for 5 to 7 days until cell density reached 10^8 cell/ml which was counted under dark-filed microscopy using Petroff Hausser counting chamber (Miller JN spirochetes in body fluids) before harvested by centrifugation.

Escherichia coli

E. coli strain BL21(DE3) pLysS and *E. coli* strain DH5α were cultivated in Luria-Bertani broth under shaking condition or Luria-Bertani agar with appropriate antibiotic at in incubator chamber. Both of them were incubated at 37°C.

DNA extraction

The genomic DNA of *L. interrogans* serovar Pomona was prepared by phenol chloroform method. A 10 ml of 10^8 cell/ml *L. interrogans* serovar Pomona were harvested by centrifugation at 8,000xg for 15 minutes. The pellet was resuspended with 378 µl of TE buffer, added 20 µl of 10% SDS and 2 µl of Proteinase K (20 mg/ml) then suspended the solution by vortex mix and incubated at 37 °C for 1 hour. After incubation, 200 µl of 5 M NaCl was added to the solution, vortex mix, and 500 µl of phenol: chloroform: isoamyl alcohol (25:24:1) was added, mixed by inverting a tube and centrifuged at 12,000 rpm for 5 minutes. Upper aqueous phase was transferred to a new tube and added 2 volume of chloroform: isoamyl alcohol (24:1), inverted mix and centrifuged at 12,000 rpm for 5 minutes. Upper phase was transferred to a new tube and added 2 volume of cool absolute ethanol, inverted mix and incubated at -20°C for 1 hour then centrifuged at 12,000 rpm for 10 minutes. DNA pellet was washed with 70%

Ethanol and spun for 5 minutes. The pellet was let dry at RT before resuspended in sterilized distilled water.

PCR amplification of *lipL32*

The extracted DNA of *L. interrogans* serovar Pomona was used as PCR template for amplification of *lipL32* gene encoding mature protein without lipoprotein signal peptide, from amino acid 21 to 252, with primer 5' TTA CCG CTC GAG GTG CTT TCGGTG GTC TGC 3' and 5' TGT TAA CCC GGG TTA CTT AGT CGC GTC AGA 3' (underlined restriction sites of *Xho*I and *Sma*I, respectively). The PCR amplification was performed in total volume for 50 µl reaction mixture in 1X DyNazyme EXT Mg $^{2+}$ free buffer (FINNZYMES) and 50 ng of extracted DNA. PCR amplification was performed using the following condition; Primary denaturation at 94 °C for 3 minutes, followed by 30 cycles of denaturation at 94 °C for 1 minute, annealing at 60° C for 1 minute, extension at 72 °C for 1 minute and final extension at 72 °C for 7 minutes. After the final reaction, PCR products were analyzed using 1.5 % agarose gel electrophoresis.

Plasmid extraction

E. coli strain DH5α which contains pRSET C vector was cultivated in LB broth with 100 µg/ml ampicillin at 37 °C under shaking condition overnight. The overnight culture was harvested by centrifugation at 8,000 rpm for 2 minutes followed by plasmid extraction using Nucleospin®Plasmid kit (Macherey-Nagel) according to the manufacturer's instruction as briefly described; cell pellets were suspended in 250 µl buffer A1 by vortex, added 250 µl buffer A2 and gently mixed by inverting the tube for 8 times, added 300 µl buffer A3 and mixed gently by inverting the tube for 8 times followed by centrifuged for 10 minutes at 11,000xg. The supernatant was transferred to Nucleospin®Plasmid column, centrifuged for 1 minute at 11,000xg, and discarded flow through. After that, column was washed with 600 µl of buffer A4 then centrifuged for 1 minute at 11,000xg, discarded flow through and respun for 2 minutes. Finally, the

column was placed in a 1.5 ml microcentrifuge tube and added 20 µl buffer AE, incubated at room temperature for 5 minutes then centrifuged at 11,000xg for 2 minutes. The extracted plasmid was analyzed using 0.8 % agarose gel electrophoresis.

Cloning

The *lipL32* gene PCR products and extracted pRSET C vector were digested by *Xho*I and *Sma*I restriction enzymes (New England Biolabs) under this condition; 1µg of PCR products or 2 µg pRSET C was suspended in NEB buffer 4 (New England Biolabs) with 1X BSA, 5 units of *Xho*I was added then incubated at 25°C for 3 hours then added 5 units of *Sma*I, incubated 37°C for 3 hours. After incubation, enzyme was heat inactivated at 65°C for 10 minutes. Digested DNA was purified with Nucleospin®Extract II (Macherey-Nagel) according to manufacturer's protocol as followed; mixed 2 volume of NT buffer with 1 volume of digestion products, loaded the solution onto Nucleospin®Extract II column, centrifuged at 11,000xg for 1 minute, and discarded flow through. A 700 µl of buffer NT3 was loaded onto the column, centrifuged at 11,000xg for 1 minute, re-spun for 2 minutes, and discarded flow through. Finally, the column was placed in a 1.5 ml microcentrifuge tube, added 20 µl buffer NE, incubated at room temperature for 5 minutes and then centrifuged at 11,000xg for 2 minutes.

Ligation reaction was performed by T4 DNA ligase (fermentus) under following condition; 5 units of T4 ligase, 200 ng of each digested *lipL32* and digested pRSET C then incubated overnight at 22 °C followed by enzyme heat inactivated at 65°C for 10 minutes. After incubation, ligation mixture was transformed into expression host, *E. coli* strain BL21 (pLys), by heat shock as described; added 10 µl of ligation mixture to microcentrifuge tube which contained 100 µl of BL21(DE3)pLysS competent cells and incubated on ice for 30 minutes. After incubation, the solution was placed in to 42°C water bath for 90 seconds and incubated on ice for 2 minutes. A 900 µl of SOC was

added to the solution, mixed by pipetting and incubated for 1.5 hours at 37°C under shaking condition. After incubation, the transformants were plated onto LB agar containing 100 µg/ml ampicillin and 35 µg/ml chloramphenicol, and incubated at 37 °C overnight.

DNA sequencing

The positive colonies were selected and confirmed by colony PCR under similar condition as *lipL32* gene amplification but primary denaturation step was changed to 94 °C for 10 minutes. PCR products were analyzed using 1.5 % gel electrophoresis. Positive colonies were cultured in LB broth containing antibiotics overnight at 37°C. Plasmid was extracted using Nucleospin®Plasmid kit. The plasmid sequences were determined using T7 promoter and T7 terminator universal primesr (First BASE Laboratories, Malaysia). DNA sequencing results were compared to *lipL32* gene of *L. interrogans* serovar Pomona in GenBank database.

Recombinant LipL32 induction and expression

The obtained BL21 clones were cultivated in LB broth with 35 µg/ml chloramphenicol and 100 µg/ml ampicillin at 250 rpm, 37°C overnight. Next morning, overnight culture was added to fresh media until OD_{600} reached 0.4 followed by addition of isopropyl-β-D thiogalactopyranoside (IPTG) at a final concentration of 0.1 mM. The culture was incubated at 30°C with shaking at 200 rpm for 16 hours before harvested by centrifugation at 8000xg 15 minutes.

Recombinant LipL32 extraction

The harvested cells were resuspended in BugBuster reagent (Novagen) which acted as lysis buffer under following condition; 5 ml BugBuster reagent per gram of wet cell paste, 1ml Protease inhibitor (Calbiochem) per 20 grams of wet cell paste, 25 units of Bensonase Nuclease (Novagen) per ml of BugBuster, and 10 units of lysozyme (BioBasic INC.) per gram of wet cell paste followed by incubated at room temperature for 20 minutes. Then, the solution was centrifuged at 16000xg for 20 min at 4°C. Supernatant was transferred to a fresh tube. Supernatant containg soluble proteins and pellet containing inclusion body or insoluble proteins were analyzed using 15% SDS-PAGE.

Recombinant LipL32 purification

Metal-affinity chromatography

Supernatant from extraction step was centrifuged at 10000xg for 15 minutes at 4°C before added 2 M imidazole at a final concentration of 20 mM which same concentration as in binding buffer. Nickel-sepharose column (GraviTrap, GE healthcare) was prepared as following described; loaded 2 ml sepharose slurry onto 15 ml tube then centrifuged at 500xg for 5 minutes, discarded supernatant and replaced with 5 column volumes (CV) of distilled water, shook for 3 minutes and centrifuged at 500xg for 5 minutes, repeated this step. Next, repeated latest step but change distilled water to binding buffer. Finally, binding buffer was added to make 50 % slurry before loaded onto PD-10 column which contained filter and allowed slurry packed in the column. After column preparation, the supernatant was loaded onto the column and incubated at room temperature on shaker at low speed for 1 hour then discarded flow through. Next, 10 ml (5 CV) of binding buffer was loaded onto column, discarded flow through followed

by loaded 5 ml elution buffer onto column and collected the eluate. The eluate was analyzed using 15% SDS-PAGE.

Gel filtration chromatography

The rLipL32 elutates from nickel column purification step were pooled and concentrated by Vivaspin20 (GE healthcare). The eluate was loaded onto Vivaspin20 and then centrifuged at 4500xg 4°C for 20 minutes, and collected flow through. 1M Dithiothreitol (DTT) was added to elute rLipL32 to make a final concentration of 0.1 mM prior to injecting into chromatography column. The rLipL32 was purified using Hiload™ 16/60 Seperdex™ 200 pg (GE healthcare) column. Purification process was performed using AKTA FPLC system (GE healthcare) which controlled and monitored by UNICORN 5.10 program. The procedure was performed as described; washed column with 60 ml (0.5 CV) of filtrated Milli-Q water then equilibrated with 240 ml (2 CV) of filtrated 1xPBS pH 7.4. Afterward, 5 ml of sample was injected into column and then washed with 120 ml (1 CV) of filtrated 1xPBS pH 7.4. Finally, column was washed with 120 ml (1 CV) of filtrated 20% ethanol. Fractions of 1 ml each were collected and analyzed by 15% SDS-PAGE and verified by Western blot.

Western blotting

After transfer protein from SDS-PAGE to nitrocellulose membrane, the membrane was incubated with blocking buffer at room temperature for 1 hour followed by wash membrane 10 minutes for 3 times with TBS-Tween buffer. After that, membrane was incubated in mouse anti-6His antiserum (1:3000 in blocking buffer) (KPL) or rabbit anti-rLipL32 antiserum (1:3000 in blocking buffer) (Monash University, Australia) at room temperature for 1 hour followed by washing 10 minutes for 3 times with TBS-Tween buffer. After that, membrane was incubated in horseradish peroxidase-conjugated goat anti-mouse antiserum (1:3000 in blocking buffer) (KPL) or horseradish peroxidase-

conjugated goat anti-rabbit (1:3000 in blocking buffer) (KPL) at room temperature for 1 hour and then washed 10 minutes for 3 times with TBS-Tween buffer. After washing step, membrane was soaked with alkaline phosphate buffer twice. Finally, immunoblot was developed by colorimetric detection using BCIP/NBT Phosphatase substrate (KPL).

Protein assay

Protein concentration was measured by *RC DC* protein assay (Bio-Rad), which is based on Lowry assay. The measurement was performed according to manufacturer's protocol as followed; add 5 µl of *DC* Reagent S to each 250 µl of *DC* Reagent A. This solution is referred to as Reagent A. Each standard or sample requires 127 µl of Reagent A. Then, 5 dilutions of BSA were prepared; 0.2, 0.5, 0.75, 1.0 and 1.5 mg/ml. Added 25 µl of standards or samples into microcentrifuge tubes followed by adding 125 µl *RC* Reagent I into each tube, vortex. Incubate the tubes for 1 minute at room temperature. After incubation, 125 µl *RC* Reagent II was added into each tube, vortex. Centrifuge the tubes at 15,000xg for 5 minutes, discarded the supernatant and allowed the liquid to drain completely from the tubes. Add 127 µl Reagent A´ to each tube, vortex and incubated at room temperature for 5 minutes. Add 1 ml of *DC* Reagent B to each tube and vortex immediately. Incubate at room temperature for 15 minutes. After incubation, the absorbance was read using spectrophotometer at 750 nm.

Random peptide phage display screening

Phage titering

Single colony of ER2738 was inoculated in 5–10 ml of LB and incubated with shaking until mid-log phase (OD_{600} ~ 0.5). While cells are growing, agarose top was melted in microwave and aliquot 3 ml into sterile culture tubes, one per expected phage dilution, and then stored at 45°C until ready for use. 10-fold serial dilutions of phage were prepared in LB. Dilution ranges were prepared under following recommended; amplified phage culture supernatants, 10^{8}–10^{11} and unamplified panning eluates, 10^{1}–10^{4}. When the culture reached mid-log phase, 200 µl of culture was dispensed into microcentrifuge tubes, 1 for each phage dilution. A 10 µl of each phage dilution was added to each tube which contained 200 µl of bacteria, vortexed quickly, and incubated at room temperature for 5 minutes. One at a time, transferred the infected cells to a tube which contained preheated agarose top, immediately mixed by vortex and poured onto a pre-warmed LB/IPTG/Xgal plate. Make sure that the agarose top was evenly spreaded and allowed plates to cool, and incubated overnight at 37°C. After incubation period, the titer of phage was calculated by counting the blue colonies on each plate and multiplying this number by dilution factor.

Bio-panning procedure

The Ph.D.-7 phage display peptide library kit (New England Biolabs) was used for affinity selection. This library was a construct of displayed random 7-mer peptides fused to a minor coat protein (pIII) of M13 filamentous phage and contained $2.0x10^{11}$ pfu/10 µl. Three rounds of biopanning were undertaken according to the manufacturer's protocol with some modifications. First of all, a 96-well microtiter plate (Greiner bio-one) was coated with 100 µl purified rLipL32 (100 µg/ml in 0.1 M $NaHCO_3$ pH 8.6) and incubated overnight at 4 °C in a humidified container. The wells were washed 10 times with 0.1% TBST, filled each well fully with Blocking Buffer and incubated at 4°C for 1

hour, discarded the blocking solution and washed each well with TBST for 6 times. In the first round of bio-panning, 10 µl of original library (2 x 10^{11}phage) was diluted in 100 µl TBST+ 5 mg/ml skim milk and loaded onto coated plate and gently rocked for 1 hour at room temperature and then discarded nonbinding phage by poured off and slapped plate face-down onto a clean paper towel and washed each well with TBST for 10 times. Bound phages were eluted from the well with 100 µl of elution buffer and gently rocked for 7 minutes. After that, the eluate was removed into a microcentrifuge tube and neutralized with 15 µl 1 M Tris-HCl (pH 9.1). A 10 µl of phage solution was taken for dilution with LB medium and the titer of phages was determined. The remaining eluate was amplified by infecting 20 ml of a 1:100 of *E. coli* ER 2783 overnight culture and incubated at 37°C 250 rpm for 4.5 hours. The culture was transferred to centrifuge tube and spun 10 minutes at 10,000 rpm at 4 °C. After centrifugation, the supernatant was transferred to a fresh tube and re-spun. The upper 80% of the supernatant was transferred to a fresh tube and added 1/6 volume of PEG/NaCl. Phages were allowed to precipitate at 4°C overnight. Next day, the solution was spun 15 minutes at 10,000 rpm, at 4°C then discarded supernatant, re-spun briefly, and removed residual supernatant. The pellet was suspended with 1 ml TBS then transferred to microcentrifuge tube and spun for 5 minutes at 4°C. Afterward, re-precipitate with 1/6 volume of PEG/NaCl, incubated on ice for 1 hour. After incubation, centrifugation was performed at 10,000 rpm at 4°C for 10 minutes, discarded supernatant, re-spun briefly, and removed remained supernatant with pipet. The phages pellet was suspended with 200 µl TBS+0.02% NaN_3 then incubated on ice for 1 hour followed by microcentrifuged 1 minute, transferred supernatant to fresh tube. The amplified eluate was taken for phage titering. In the second and the third rounds of biopanning, the amplified phages were incubated with purified rLipL32 as described above. The procedures were the same as in the first round except the washing steps were performed with 0.5% TBST. In the third rounds, the unamplified bound phage was taken for phage titering and plaques from this titering can be used for sequencing.

Plaques amplification

Overnight culture of *E. coli* ER 2783 was diluted 1:100 in LB and aliquot to 96-well plate, 200 µl/ well. A sterile wooden stick was used to pick a blue plaque and transfer to each well containing diluted culture. The culture then incubated at 37°C with shaking for 4.5 hour. After incubation period, the 96-well plate was centrifuged at 5000xg for 20 minutes at 4°C then transferred upper 80% supernatant to a new plate. For long-term storage, the supernatant was diluted 1:1 with sterile glycerol and store at – 20°C.

Pyrosequencing

Pyrosequencing was used to analyzed the sequence of random 21-bp from phage genome encoding 7 amino acid peptides. The procedures were performed using Biotag AB pyrosequencer model according to manufacturer's instruction.

Generation of sequencing template by PCR

The PCR reaction was performed as previously described [209] in 0.5 ml autoclaved PCR tubes which contained the following mixture in a total of 50 µl: 1X PCR TrueStart™ *Taq*, 2 m*M* $MgCl_2$, 0.2 mM dNTP mix, 0.2 pmol forward primer, 0.2 pmol reverse primer, 1.25 units TrueStartTaq and 1 µl of an amplified preparation of an individual phage, as explained previously. The PCR reactions were carried out using the following condition; Primary denaturation at 95 °C for 5 minutes, followed by 45 cycle of denaturation at 94 °C for 30 second, annealing at 54° C for 30 second, extension at 72 °C for 1 minute and final extension at 72 °C for 10 minutes. PCR products were analyzed using 2.5 % agarose gel electrophoresis.

Purification and preparation of single-stranded sequencing template and pyrosequencing

A 40 µl volume of each PCR products were transferred to 96-well plate. The streptavidin-coated beads are completely suspended by vortexing, mixed 384 µl of beads with 3,840 µl of binding buffer followed by transferred 40 µl of the solution to each well of 96-well plate and incubated at room temperature under shaking condition for 10 minutes. After incubation, 96-well palted was placed on a vacuum manifold, vacuum was applied until all the liquid passed through the membrane then washed stepwise with denaturing solution, 70 % ethanol and washing solution, while applying vacuum. The beads were transferred to PSQ 96 Plate Low (Biotage AB) containing 50 µl of annealing buffer per well and 5 µl volume of 3 pmol sequencing primer was added. The beads and primer were gently mixed by pipetting. The plate was heated at 80 °C for 2 minutes and then left at room temperature for 10 minutes. A dispensation cartridge (Biotage AB) for a PSQ 96MA System (Biotage AB) was prepared and filled with PSQ SNP 96 reagent kit (Biotage AB) according to the manufacturer's protocol and inserted into the sequencer. The plate containing the samples was placed inside the sequencer where the directed sequencing reaction occurred. The peptide sequences from pyrosequencing were analyzed and used to search matched host proteins in protein databases using the BLASTP program from National Center for Biotechnology Information (NCBI).

Table 4 List of Primers in experiments

Primer name	Sequences	Product size (bp)
lipL32 gene Forward	5’ TTA CCG CTC GAG GTG CTT TCG GTG GTC TGC 3’	759
lipL32 gene Reverse	5’ TGT TAA CCC GGG TTA CTT AGT CGC GTC AGA 3’	
Ph.D.7 Amplify Forward [209]	5'-ATTCGCAATTCCTTTAGTGGTA-3'	107
Ph.D.7 Amplify Reverse [209]	5'-biotin-GGGATTTTGCTAAACAACTTT-3'	
Ph.D.7 Sequencing Primer [209]	5'-TGGTACCTTTCTATTCTCAC-3'	

CHAPTER IV

RESULTS

Cloning of *lipL32* and expression in *E. coli*

PCR amplification of *lipL32* gene was performed using genomic DNA of pathogenic *Leptospira* serovar Pomona as a template. Primers were designed to give a full-length *lipL32* gene without its signal sequence. The forward and reverse primers contained *Xho* I and *Sma* I-restriction sites, respectively. The amplified product and pRSET C vector were digested with *Xho* I and *Sma* I, purified, and analyzed on agarose gel (Figure 15). To produce recombinant protein of LipL32 (rLipL32), digested amplicons of *lipL32* gene were cloned into the pRSET C expression vector and then transformed into *E. coli* host strain BL21(DE3) pLysS before plating onto LB agar with antibiotic selection.

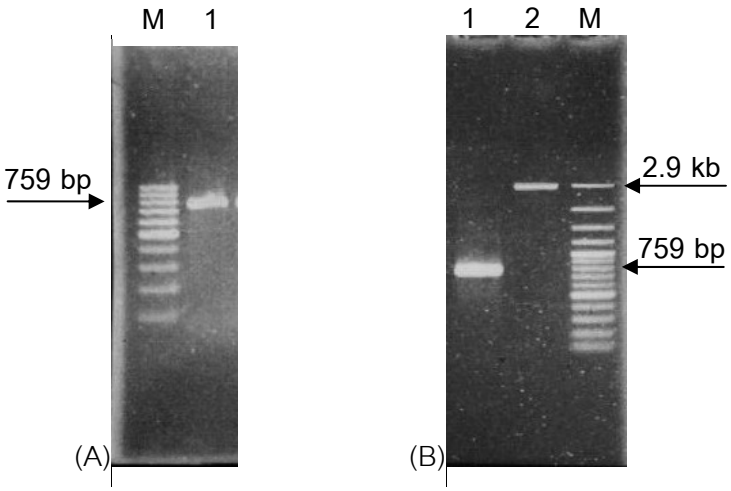


Figure 15 PCR amplification of *lipL32* gene and pRSET C expression vector on agarose gel. *(A) lipL32* gene amplicon of *L. interrogans* serovar Pomona. Lane M, 100 bp DNA ladder; lane 1 is shown single band PCR products (759 bp) (B) Digestion products of *lipL32* PCR product and pRSET C vector that digested by *Xho*I and *Sma*I. Lane M, 1 kb DNA ladder; lane1, digested *lipL32* gene PCR amplicon (759 bp); lane 2, digested pRSET C vector (2.9 kb).

Transformants on antibiotic selected agar plate were screened for *lipL32* gene insertion by PCR. The positive clones were selected for DNA sequencing of the inserted gene (Figure 16). Clones containing *lipL32* with correct sequence and in frame with the expression vector were further used for induction of protein expression.

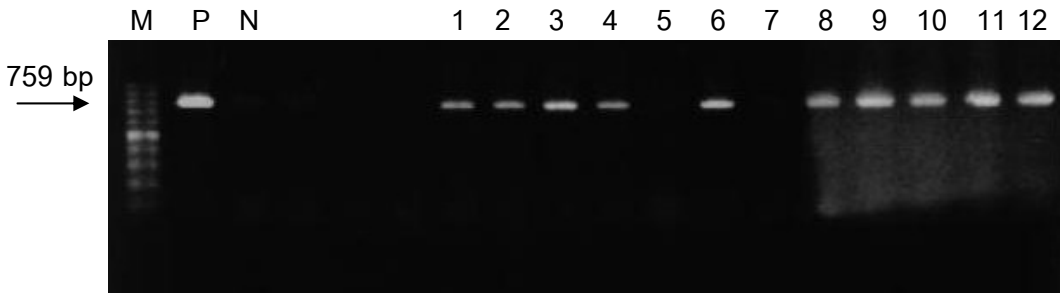


Figure 16 PCR amplification of *lipL32* insert in transformants. Gel electrophoresis shows single band PCR products of 759 bp from colony PCR. M, 100 bp DNA ladder; P, positive control (genomic DNA of *Leptospira* serovar Pomona); N, negative control, Lane 1-12 are PCR products of selected colony.

We randomly selected two positive clones for sequencing. Both sequences are the same as *lipL32* gene of *L. interrogans* serovar Pomona as reported in GenBank database. LipL32 is conserved among pathogenic leptospires. Hence, LipL32 from serovar Pomona can represent the protein from other serovars.

Protein Extraction and Purification

Expression of LipL32 was induced by IPTG in *E. coli* strain BL21 (DE3) pLysS. The rLipL32 was expressed with predicted size 32.2 kDa in both soluble and insoluble parts as shown on SDS-PAGE and Western blot (Figure 17).

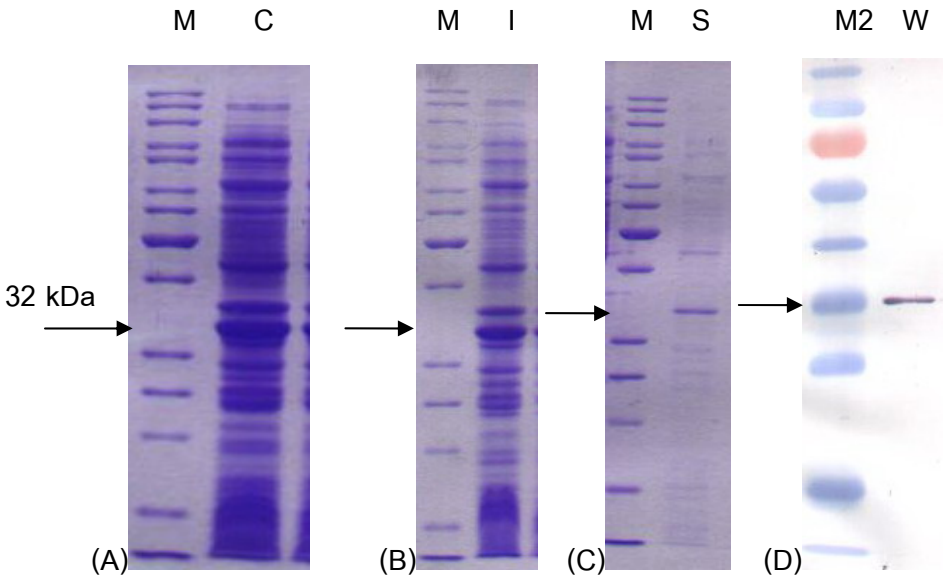


Figure 17 Detection of rLipL32 expression by SDS-PAGE and Western blot. The rLipL32 expression was detected by Coomassie blue staining of SDS-PAGE and verified by Western blot using anti-His antibody. (A) Crude proteins from *E. coli* extraction (C, crude protein). (B) Proteins from insoluble part (I, insoluble). (C) Proteins from soluble part (S, soluble). (D) Western blot detected LipL32 expression in *E. coli*. M, unstained protein ladder; M2, pre-stained protein ladder.

The soluble fraction of LipL32 was purified by Ni^{2+}-chelating sepharose. rLipL32 appeared as the major band on SDS-PAGE and Western blot (Figure 18). However, contaminants were observed indicating that the protein was not pure enough for phage display screening. Since the contaminated protein may cause false positive results, i.e. enrichment of phages that bind to contaminants instead of LipL32, gel filtration chromatography was performed.

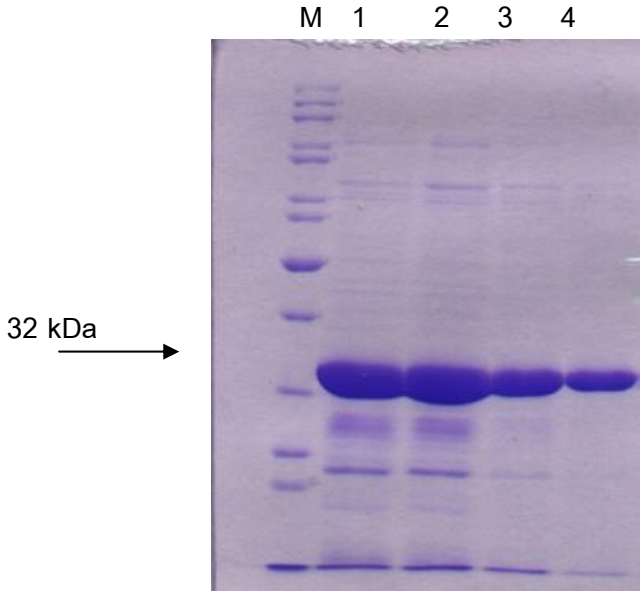


Figure 18 Detection of purified rLipL32 from metal-chelation purification. The purify of rLipL32 was analyzed by Coomassie blue staining of SDS-PAGE. The purified rLipL32 shows as a major band with some contaminants. M, unstained protein ladder; Lanes 1-4 present rLipL32 eluted fractions from Ni^{2+} column. Protein concentration in lane 1, 42.4 µg; lane 2, 29.9 µg; lane 3, 17.3 µg and lane 4, 7.2 µg.

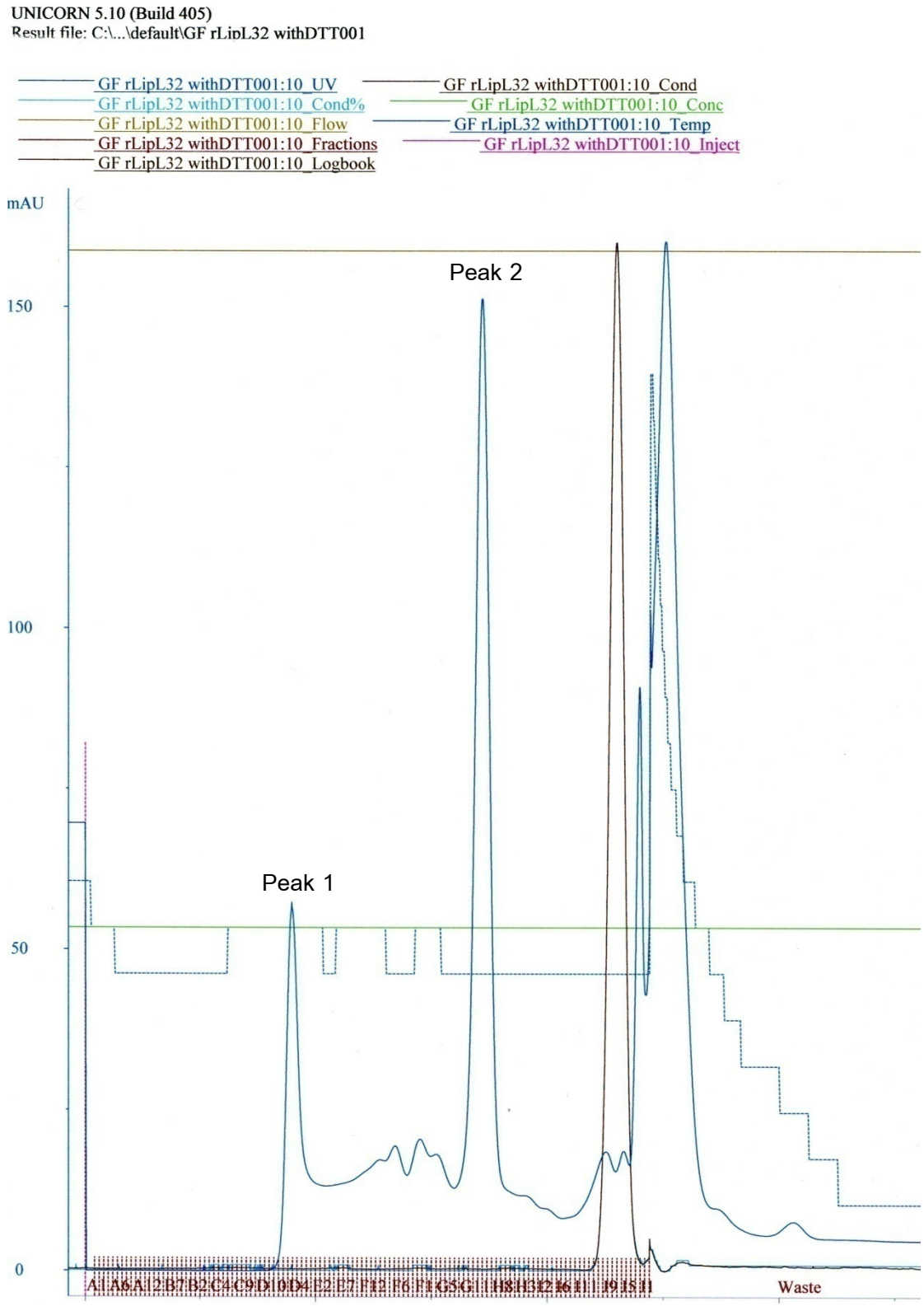


Figure 19 The schematic shows gel filtration performance monitored by UNICON 5.1 program. The rLipL32 presented in first and second peaks.

After purification with gel filtration chromatography, rLipL32 appeared in the first and second peaks of the eluate. Since gel filtration separates proteins based on molecular size, it is possible that rLipL32 from the first peak was in a dimer form (Figure 19). This explanation is supported by the study of crystal structure of LipL32 showing that two monomers of LipL32 tended to form a dimer. The purity of rLipL32 was verified by SDS-PAGE (Figure 20) and Western blot using anti-His or anti-LipL32 antibody (Figure 21). The result showed only single band protein of LipL32, indicating that all contaminants were removed. Therefore, after two steps purification the purity of rLipL32 was suitable for random peptide phage library screening.

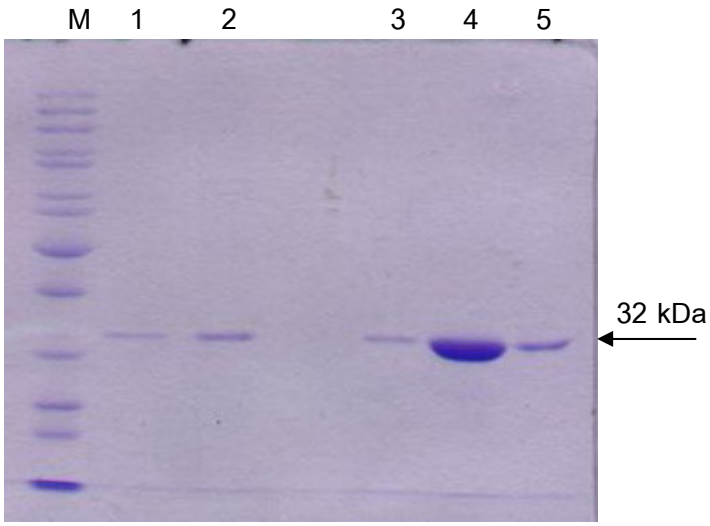


Figure 20 Detection of purified rLipL32 from gel filtration chromatography. The Gel shows single band protein with expected size, approximate 32 kDa. M, unstained protein ladder. Lane 1-2 are eluted fractions from first peak; lane 1, 0.07 ng; lane 2, 0.22 µg. Lane 3-5 are eluted fractions from second peak; lane 3, 0.53 µg; lane 4, 7.39 µg; lane 5, 0.85 µg.

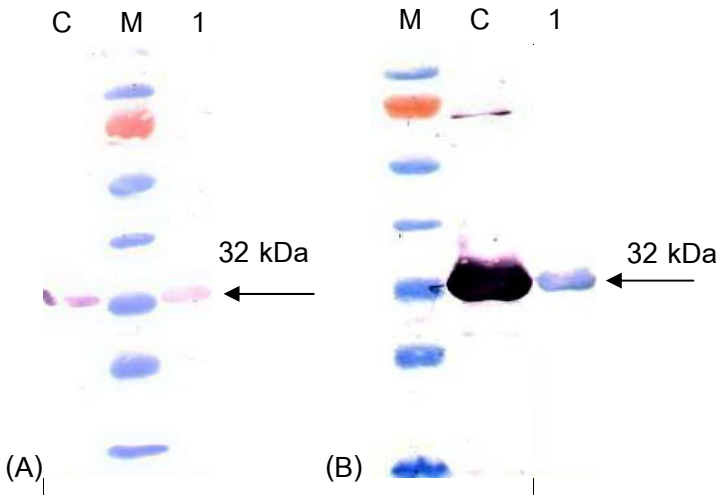


Figure 21 **Western blotting of purified rLipL32 from gel filtration chromatography.** The proteins example sample are from second peak of gel filtration. (A) Detection of purified rLipL32 by anti-His antibody. The purified rLipL32 presents in lane 1 (3.67 µg), C, crude protein extracted from *E. coli* (3.35 µg). (B) Detection of purified rLipL32 by anti-LipL32 antibody. The protein presents in lane 1 (3.67 µg), C, crude protein extracted from *E. coli* (19.56 µg). M, Prestained Protein Ladder.

Phage display screening

In order to identify proteins that interact with LipL32, three rounds of bio-panning were performed using Ph.D.-7 random peptide phage library. The titer of eluted phage from each round was calculated and measured by plaque forming unit (pfu) assay. The titers of phages that bound to rLipL32 increased after each round of panning indicating that clones with higher affinity were enriched (Table 5).

Table 5 **Titers of input and output phage after each round of panning.**

Round	rLipL32 concentration (μg)	Input phage (pfu)	Output phage (pfu)	Recovery Rate*
1	10	$2.0x10^{11}$	$4x10^{4}$	$2x10^{-7}$
2	10	$1.05x10^{11}$	$6.8x10^{5}$	$6.48x10^{-6}$
3	10	$1.19x10^{11}$	$2.6x10^{8}$	$2.18x10^{-3}$

Recovery rate is the proportion of output to input phage titer*

Ninety-six plaques were randomly selected from the third round output phage and were subjected to pyrosequencing.

Pyrosequencing

To determine the random 21-bp inserted sequence of individual plaques, the sequencing should be performed and pyrosequencing is appropriate for high-throughput sequencing. In order to generate the sequencing template, PCR was performed to amplified inserted sequence of 96 plaques and PCR products were analyzed as showed in Figure 22. From gel electrophoresis, 3 clones from 96 clones presented PCR products with larger than expected size, approximately 200 bp.

After amplification, 52 plaques with corrected PCR product size were sequenced by pyrosequencing. From pyrosequencing, 8 plaques from 52 plaques sequencing were failed. We found 6 different peptide sequence patterns from 44 plaques. The most common sequence: WHWTYYW was obtained 39 plaques out of total 44 plaques (88.64 %). The 6 peptide sequence patterns are shown in Table 6.

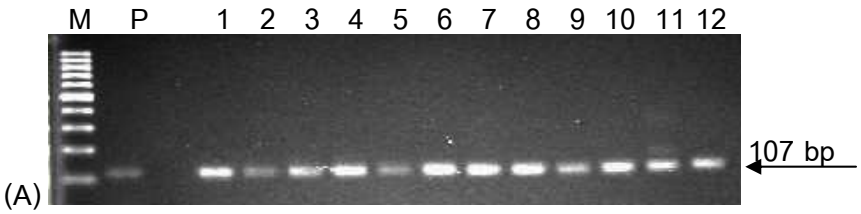


Figure 22 PCR products analyzed of individual plaques amplification on agarose gel. The figure represents the PCR product from individual plaques. The inserted sequence of each plaque was amplified. M, 100 bp DNA Ladder; P, positive control; lane 1-12 represents PCR product with 107 bp.

Table 6 The peptide sequence patterns from selected plaques obtained from third round panning.

Pattern	Peptide sequences	Frequency (of total 44 clones)	Percentage of total clones
1	WHWTYYW	39	88.64
2	HLPPNHT	1	2.27
3	WHWLWLQ	1	2.27
4	SSLRLLP	1	2.27
5	THKFPWI	1	2.27
6	KLWTIPM	1	2.27

Protein sequence analysis

After sequencing of the inserted region of selected plaques, 6 peptide sequence patterns were obtained and then used to search for matched human proteins in the database. We retrieved only sequences of membrane proteins with at least 4 exact amino acid matches for further analysis (Table 7). Next, we compared the property of amino acid residues of peptide sequences with the selected proteins. We found that only one protein, a scavenger receptor class F, contained 7 amino acids with similar property to the THKFPWI peptide sequences. In addition, seven proteins contained 6 amino acids similarity to one of six peptide sequence patterns (Table 8).

Table 7 Putative proteins from BLAST analysis and their functions.

Peptide sequences	Matched proteins	Reported function	Expression cell	References
WHWTYYW	Chloride channel accessory 2 WHWTYYW GHWTYTL	The ion transport channel shows broad range functions; regulation of pH, volume homeostasis, organic solute transport, cell migration, cell proliferation and differentiation Act as adhesion molecule for lung metastatic cancer cells, mediating vascular arrest and colonization. Act as a tumor suppressor gene for breast cancer The chloride channel involved in *Vibrio cholera* toxin uptake	Expressed in various species and human tissues: highly in bronchia epithelium cell, skin, tongue, adipocyte and tonsil.	[210-217]

75

Table 7 (Continue)

Peptide sequences	Matched proteins	Reported function	Expression cell	References
	Glycoprotein VI WHWTYYW PGWTTYW	Act as platelet collagen receptor; collagen-induced activation and aggregation of platelets Able to interact with LYN (Src protein kinase family)	Expressed in platelet membrane Moderately expressed in lung	[218-225]
	G-protein couple receptor TYMSTR WHWTYYW THWEYYA	Integral membrane receptor Orphan chemokine and HIV/SIV co-receptor receptor	Activated T cell lymphocyte	[226, 227]
	Transmembrane serine protease 2 isoform 2 WHWTYYW WHWTAFA	Contains several domains; a protease domain, type II transmembrane domain, receptor class A domain, scavenger receptor cysteine-rich domain	The luminal epithelial cells of the mouse and human prostate	[228]

76

Table 7 (Continue)

Peptide sequences	Matched proteins	Reported function	Expression cell	References
	Tight junction protein WHWTYYW EQWSYYD	Located in cytoplasmic membrane May involved in signal transduction at cell-cell junctions	Expressed in various tissue; highly expression in lung	[229, 230]
HLPPNHT	Collagen XX HLPPNHT PLPPEHT Matched in laminin G-like domain. Over 90% of the collagens in the body are of type I, II, III, and IV.	Membrane component	Moderately expresses in lung, liver and kidney	[231]
	Tyrosine kinase HLPPNHT WLPPNHI	A member of JAKs kinase family Associated with cytokine receptor	Lymphoid cell	[232]

Table 7 (Continue)

Peptide sequences	Matched proteins	Reported function	Expression cell	References
	Cell adhesion molecule, homolog to L1CAM HLPPNHT NLQPNHT	Involved in signal transduction pathways	Neuron cells	[233-236]
	catenin (cadherin-associated protein), delta 2 (neural plakophilin-related arm-repeat protein) HLPPNHT PLPPAHT	The protein active in the nervous system, help cell to cell attachment. Plays a role in cell movement	Expressed in various tissues; highly expressed in neuron cell.	[237-239]
	Nemo like kinase HLPPNHT HLPPPHL	Involved in cell signaling, Wnt pathway		[240, 241]

Table 7 (Continue)

Peptide sequences	Matched proteins	Reported function	Expression cell	References
	tetratricopeptide repeat protein isoform 3 HLPPNHT TLPHNHT	A minor histocompatibility antigen which may induce graft rejection of male stem cell grafts		[242]
	Gap junction HLPPNHT HLPPPHA	Intercellular connection between two cell, composed of two connexons Play a role in cell-cell communication and secretion both in normal and cancer cell	Expressed in various tissue: moderately expressed in lung	[243, 244]
WHWLWLQ	Laminin alpha 5 WHWLWLQ GRWLWLD	The major component at basement membrane A member of alpha subfamily of laminin chains It's an extracellular glycoprotein Able to interacted with many pathogens such as *L.interrogans*, *Treponema pallidum*	Expressed in various tissues Highly expressed in heart, lung, and kidney	[245-250]

Table 7 (Continue)

Peptide sequences	Matched proteins	Reported function	Expression cell	References
	progestin and adipoQ receptor (PAQR) family member VI isoform 1 W-HWLWLQ WPHWSWLQ	Membrane progestin, steroid or adiponectin receptor (in eukaryote) Founds in both prokaryote and eukaryote.	Expressed only in the brain	[251]
SSLRLLP	mucosal vascular addressin cell adhesion molecule 1 SSLRLLP ASLRLLP	A member of immuloglobulin family A membrane-bound leukocyte receptor Involve in mucosal immunity, reposed to pro-inflammatory cytokines. Act as lymphocyte homing to lymphoid tissue via lymphocyte integrin $\alpha 4\beta 7$, the MAdCAM-1 receptor Involve in gastrointestinal tract diseases	Highly expressed on high endothelial venules (HEV) of gut-associated lymphoid tissues, and on venules at chronically Inflammation sites.	[245, 248, 249, 252-254]

Table 7 (Continue)

Peptide sequences	Matched proteins	Reported function	Expression cell	References
	prostaglandin E receptor 1 SSLRLLP QLLRLLP	One of four receptors for prostaglandin E2 A member of G-protein coupled family The protein may mediate adrenocorticotropic hormone response to bacterial endotoxin Correlated with colon carcinogenesis, calcium mobility and constitute a contractile receptor group	Expressed in various tissues: highly expressed in kidney, lung, stomach	[255-258]
	Protocadherin 20 SSLRLLP EDLRLLP	A member of protocadherin gene family, a subfamily of the cadherin superfamily Play a role in tissue morphogenesis and neuron cell formation	Highly expressed in central nerve system	[259, 260]

81

Table 7 (Continue)

Peptide sequences	Matched proteins	Reported function	Expression cell	References
THKFPWI	scavenger receptor class F THKFPWI SHKLPWV	LPS is able to activate SREC expression Recognized modified lipoprotein Associated with atherosclerosis Acts as receptors mediating host cell entry of *Neisseria gonorrhoeae* Function in APCs	Expressed in various tissues: highly expressed in cardiac myocytes, endothelium cell	[261-264]
	FRAS1 related extracellular matrix 1 precursor THKFPWI RHKFPIN	Involved in skin epithelial morphogenesis during early development Link with Fraser syndrome (autosomal recessive disorder disease)	Expressed in embryonic epithelial cell.	[265]
KLWTIPM	Coronin, actin binding protein, 2A KLWTIPM KIWSIPK	Associated with the phagocytic apparatus Involved in actin –mediated processes: cytokinase, cell locomotion	Expressed in various tissues: highly expressed in spleen, lung, liver, kidney	[266-268]

Under line amino acid is matched with peptide sequences

Table 8 The most matched putative host proteins that interacted with LipL32.

Number	Candidate proteins	Matched position
1	scavenger receptor class F isoform I (SREC-I)	7
2	Chloride channel accessory 2 (CLCA2)	6
3	Coronin, actin binding protein, 2A	6
4	Prostaglandin E receptor 1 (EP1)	6
5	Glycoprotein VI (GPVI)	6
6	Progestin and adipoQ receptor (PAQR) family member VI isoform 1	6
7	Mucosal vascular addressin cell adhesion molecule 1 (MAdCAM-1)	6
8	Laminin alpha5	5

CHAPTER VI

DISCUSSION

The initial step of infectious processes involves host-pathogen interaction followed by colonization, replication and dissemination of pathogens in the host. Previous reports showed that outer membrane components including outer membrane proteins (OMPs) of several pathogens play a key role in the first step of host-microbe interactions [8]. However, host membrane proteins that interact with leptoapiral OMPs have never been addressed.

Lipl32 is the major OMP of leptospires with highly conserved among pathogenic strain and not found in non-pathogenic leptospires [16, 23-25]. This protein is constitutively expressed both *in vitro* and *in vivo* and highly immunogenic [16, 26, 28, 31]. LipL32 was expressed in kidney of animal model [16] and induced an inflammatory response in kidney cell culture [28, 269, 270]. LipL32 was also recognized by sera from leptrospirosis patients [16, 33]. The anti-LipL32 antibody was produced during infection and showed partial protection in animal model [144, 271]. Moreover, the C-terminus of LipL32 had binding ability to ECM proteins [32, 33]. Surprisingly, *lipL32* mutant generated by transposon mutagenesis showed no difference in virulence compared to wild type strain [272]. It is possible that LipL32 is required but not essential for leptospiral infection. Alternatively, other proteins may confer redundant function. However, considerable evidence, as mentioned above, suggested the importance of LipL32 in pathogenesis of leptospirosis. Therefore, the role of LipL32 in host-pathogen interaction cannot be excluded.

In the present study, we screened for proteins that interacted with LipL32 using phage display technology. The recombinant protein of LipL32 was used as a target molecule for bio-panning with random heptapeptide phage library. After three rounds of panning, six peptide sequence patterns were identified. Here we will discuss about proteins which are expressed in target organs or potentially involved in pathogenesis of leptospirosis.

After searching the database and sequence comparison, the highest matched protein with 7 amino acid similarity to the peptide THKFPWI is a scavenger receptor class F or scavenger receptor expressed by endothelial cell (SREC). The SREC is a membrane protein that belongs to a scavenger receptor family [262, 264, 273, 274]. An SREC-I was demonstrated to bind to modified lipoproteins and many ligands [262]. The protein also mediated cell morphological change [263]. Berwin and colleagues showed that overexpressed SREC-I in macrophages increased endocytosis of chaperon, which is required for receptor-mediated endocytosis pathway of the antigen presenting cells (APCs) [264]. SRECs was demonstrated as a receptor of the major outer membrane pore PorB ($PorB_{IA}$) of *Neisseria gonorrhoeae* and involved in host cell invasion [261]. In contrast to non pathogenic *Leptospira*, pathogenic leptospires were reported to invade mammalian cell lines including canine kidney cells [275]. Hence, pathogenic *Leptospira* may employ LipL32 to invade host cell via SREC.

The WHWLWLQ peptide sequences obtained from our phage display screening was matched with laminin alpha 5. Laminins are glycoprotein with heterotrimeric (α, β, γ) [276, 277]. Laminins are major component of basement membrane [278, 279]. Whereas, Laminin α 5 is a component of laminin-511 ($\alpha5\beta1\gamma1$) and laminin-521 ($\alpha5\beta2\gamma1$), which are widely expressed in adult tissues and may be a major component of laminin chain in basement membrane [245, 249]. This finding is consistent with the results in previous studies demonstrating that LipL32 was able to bind to laminin [32, 33]. Furthermore, the crystal structure revealed the putative binding site for positively

charged proteins such as laminin [34, 35]. Therefore, phage display technique is a valid tool for screening of host-microbe protein interactions.

The peptide sequences KLWTIPM matched with coronin type II isoform A (coronin 2A). Coronin is the actin binding protein that plays a role in cell motility and actin-mediated processes [268, 280]. The human coronin type II expressed in several cell types [280]. Function of coronin 2A remains unclear. The recently reported suggested that coronin 2A may be important in whole-cell motility and internal focal-adhesion turnover [267]. In addition, other coronins play a role in endocytosis mechanism [268]. *Mycobacterium tuberculosis* utilized coronin 1A binding protein to retain coronin 1A on the phagosome leading to inhibition of phagolysosome fusion in macrophages and thereby evading host immune response [266]. Previous studies showed that pathogenic leptospires were able to be uptaken by macrophages [128, 281, 282]. In contrast to murine macrophages, leptospires survived freely and replicated in the cytosol of human macrophages [283]. Although the function of coronin 2A in bacterial infection has never been documented, it should be tested as a receptor for LipL32 and its role in intracellular survival of pathogenic leptospires in human macrophages.

The sequence WHWTYYW matched with chloride channel accessory 2 (CLCA 2). The CLCA2 is a transporter of anions across cellular membranes [215]. The protein is expressed in various tissues, especially in bronchial epithelial cells and mammary glands [214, 215]. The channel showed broad ranged function, for examples regulation of pH, organic solute transport, and cell migration [214-216]. In addition, chloride transport plays an important role in the lungs and involves in pulmonary diseases such as asthma and pulmonary edema [216, 284]. Previous studies reported that leptospires induced pulmonary edema and hemorrhage [285-287]. Morover, the sodium transport ability of alveolar epithelial cells was affected in leptospirosis leading to lung injury [288]. In the lungs of hamster infected with leptospires, epithelial sodium channels were down-regulated whereas Na^{+}-K^{+}-Cl^{-} co-transporters were up-regulated [288]. Generlly,

Na^+-K^+-Cl^- co-transporter work in concert with CLCA2 [217]. It will be interesting to study the effect LipL32 on CLCA2 in leptospirosis.

The sequence SSLRLLP matched with prostaglandin E receptor 1 (EP1), one of four prostaglandin E receptor subtypes (EP1-4) [256, 257, 289]. EP1 mRNA is expressed in various tissues including papillary collecting ducts of the kidney [290]. The EP1 had been reported to mediate adrenocorticotropic hormone response to bacterial endotoxin [291]. In addition, tissue damage can induce production of PGs, which activates EP1 receptor expression [292]. Previous study reported that EP1 level in injured human sensory neurons was induced by cyclooxygenase-2 (COX-2) [293]. In addition, EP1 and PGE2 play a role in inflammation of neurons, and colon cancer [258, 293, 294]. Acute renal failure manifests in severe form of leptospirosis. It was shown that COX-2 in medullar thick ascending limb (mTAL) cells was not induced by OMPs of *Leptospira* [31]. LipL32 was shown to induce tubulointerstitial nephritis [28]. However, the effect of EP1 and LipL32 on inflammatory response in the infected kidney is not known.

Glycoprotein VI (GPVI) that matched with the sequence WHWTYYW is a platelet membrane protein and had been described as collagen receptor [219, 223]. GPVI plays a role in the collagen induced activation and aggregation of platelets [219-221, 225]. Thrombocytopenia is a common manifestation of severe leptospirosis. In guinea pig model of leptospirosis, it was suggested that thrombocytopenia was the result of platelet aggregation but not the disseminated intravascular coagulation (DIC) [295]. Hence, the interaction of LipL32 with GPVI and it relation to platelet aggregation should be elucidated.

CHAPTER VII

SUMMARY

The aim of this study is to identify host proteins that interact with LipL32 using phage display technology. We obtained 6 patterns of peptide sequences from enrichment of bound phages. The highest frequency of peptide sequences is WHWTYYW. The BLAST program search provides putative proteins that bind to LipL32.

Host proteins with 6 to 7 amino acids matched to random heptapeptide sequences and have potential role in pathogenesis of leptospirosis are SREC-I, coronin 2A, laminin, CLCA2, EP1 and GPVI.

Further investigation is required to confirm the interactions of these proteins to LipL32 *in vitro* and *in vivo.* The knowledge obtained from the study may elucidate the novel function of LipL32 in pathogenesis of leptospirosis.

.

REFERENCES

[1] Bharti AR, Nally JE, Ricaldi JN, Matthias MA, Diaz MM, Lovett MA, Levett PN, Gilman RH, Willig MR, Gotuzzo E, Vinetz JM. 2003. Leptospirosis: a zoonotic disease of global importance. Lancet Infect Dis 3(12):757-71.

[2] Levett PN. 2001. Leptospirosis. Clinical microbiology reviews 14(2):296-326.

[3] Cruz LS, Vargas R, Lopes AA. 2009. Leptospirosis: a worldwide resurgent zoonosis and important cause of acute renal failure and death in developing nations. Ethn Dis 19(1 Suppl 1):S137-41.

[4] Adler B, de la Pena Moctezuma A. 2009. Leptospira and leptospirosis. Vet Microbiol.

[5] สำนักระบาดวิทยา. 2551. สรุปรายงานการเฝ้าระวังโรคเลปโตสไปโรซิส 2551. สำนักระบาดวิทยา, กระทรวงสาธารณสุข.

[6] Liu Y, Zheng W, Li L, Mao Y, Yan J. 2007. Pathogenesis of leptospirosis: interaction of *Leptospira interrogans* with in vitro cultured mammalian cells. Med Microbiol Immunol. 196(4): 233-239

[7] Ballard SA, Williamson M, Adler B, Vinh T, Faine S. 1986. Interactions of virulent and avirulent leptospires with primary cultures of renal epithelial cells. J Med Microbiol 21(1):59-67.

[8] Kuehn MJ, Kesty NC. 2005. Bacterial outer membrane vesicles and the host-pathogen interaction. Genes Dev 19(22):2645-55.

[9] Nally JE, Whitelegge JP, Bassilian S, Blanco DR, Lovett MA. 2007. Characterization of the outer membrane proteome of *Leptospira interrogans* expressed during acute lethal infection. Infect Immun 75(2):766-73.

[10] Nally JE, Whitelegge JP, Aguilera R, Pereira MM, Blanco DR, Lovett MA. 2005. Purification and proteomic analysis of outer membrane vesicles from a clinical isolate of *Leptospira interrogans* serovar Copenhageni. Proteomics 5(1):144-52.

[11] Cullen PA, Haake DA, Adler B. 2004. Outer membrane proteins of pathogenic spirochetes. FEMS Microbiol Rev 28(3):291-318.

[12] Matsunaga J, Barocchi MA, Croda J, Young TA, Sanchez Y, Siqueira I, Bolin CA, Reis MG, Riley LW, Haake DA, Ko AI. 2003. Pathogenic *Leptospira* species express surface-exposed proteins belonging to the bacterial immunoglobulin superfamily. Mol Microbiol 49(4):929-45.

[13] Ristow P, Bourhy P, McBride FW, Figueira CP, Huerre M, Ave P, Girons IS, Ko AI, Picardeau M. 2007. The OmpA-Like Protein Loa22 Is Essential for Leptospiral Virulence. PLoS Pathog 3(7):e97.

[14] Gamberini M, Gomez RM, Atzingen MV, Martins EA, Vasconcellos SA, Romero EC, Leite LC, Ho PL, Nascimento AL. 2005. Whole-genome analysis of *Leptospira interrogans* to identify potential vaccine candidates against leptospirosis. FEMS Microbiol Lett 244(2):305-13.

[15] Choy HA, Kelley MM, Chen TL, Moller AK, Matsunaga J, Haake DA. 2007. Physiological osmotic induction of Leptospira interrogans adhesion: LigA and LigB bind extracellular matrix proteins and fibrinogen. Infect Immun 75(5):2441-50.

[16] Haake DA, Chao G, Zuerner RL, Barnett JK, Barnett D, Mazel M, Matsunaga J, Levett PN, Bolin CA. 2000. The leptospiral major outer membrane protein LipL32 is a lipoprotein expressed during mammalian infection. Infect Immun 68(4):2276-85.

[17] Nally JE, Timoney JF, Stevenson B. 2001. Temperature-regulated protein synthesis by *Leptospira interrogans*. Infect Immun 69(1):400-4.

[18] Matsunaga J, Sanchez Y, Xu X, Haake DA. 2005. Osmolarity, a key environmental signal controlling expression of leptospiral proteins LigA and LigB and the extracellular release of LigA. Infect Immun 73(1):70-8.

[19] Matsunaga J, Lo M, Bulach DM, Zuerner RL, Adler B, Haake DA. 2007. Response of *Leptospira interrogans* to Physiologic Osmolarity: Relevance in Signaling the Environment-to-Host Transition. Infect Immun 75(6):2864-74.

[20] Nally JE, Artiushin S, Timoney JF. 2001. Molecular characterization of thermoinduced immunogenic proteins Q1p42 and Hsp15 of *Leptospira interrogans.* Infect Immun 69(12):7616-24.

[21] Lo M, Bulach DM, Powell DR, Haake DA, Matsunaga J, Paustian ML, Zuerner RL, Adler B. 2006. Effects of temperature on gene expression patterns in *Leptospira interrogans* serovar Lai as assessed by whole-genome microarrays. Infection and immunity 74(10):5848-59.

[22] Matsunaga J, Medeiros MA, Sanchez Y, Werneid KF, Ko AI. 2007. Osmotic regulation of expression of two extracellular matrix-binding proteins and a haemolysin of *Leptospira interrogans*: differential effects on LigA and Sph2 extracellular release. Microbiology 153(Pt 10):3390-8.

[23] Cullen PA, Cordwell SJ, Bulach DM, Haake DA, Adler B. 2002. Global analysis of outer membrane proteins from *Leptospira interrogans* serovar Lai. Infect Immun 70(5):2311-8.

[24] Lee SH, Kim KA, Park YG, Seong IW, Kim MJ, Lee YJ. 2000. Identification and partial characterization of a novel hemolysin from *Leptospira interrogans* serovar Lai. Gene 254(1-2):19-28.

[25] Cullen PA, Xu X, Matsunaga J, Sanchez Y, Ko AI, Haake DA, Adler B. 2005. Surfaceome of *Leptospira* spp. Infect Immun 73(8):4853-63.

[26] Guerreiro H, Croda J, Flannery B, Mazel M, Matsunaga J, Galvao Reis M, Levett PN, Ko AI, Haake DA. 2001. Leptospiral proteins recognized during the humoral immune response to leptospirosis in humans. Infect Immun 69(8):4958-68.

[27] Barnett JK, Barnett D, Bolin CA, Summers TA, Wagar EA, Cheville NF, Hartskeerl RA, Haake DA. 1999. Expression and distribution of leptospiral outer membrane components during renal infection of hamsters. Infect Immun 67(2):853-61.

[28] Yang CW, Wu MS, Pan MJ, Hsieh WJ, Vandewalle A, Huang CC. 2002. The *Leptospira* outer membrane protein LipL32 induces tubulointerstitial nephritis-mediated gene expression in mouse proximal tubule cells. J Am Soc Nephrol 13(8):2037-45.

[29] Nally JE, Whitelegge JP, Bassilian S, Blanco DR, Lovett MA. 2006. Characterization of the outer membrane proteome of *Leptospira interrogans* expressed during acute lethal infection. Infect Immun. 75(2):766-73

[30] Werts C, Tapping RI, Mathison JC, Chuang TH, Kravchenko V, Saint Girons I, Haake DA, Godowski PJ, Hayashi F, Ozinsky A, Underhill DM, Kirschning CJ, Wagner H, Aderem A, Tobias PS, Ulevitch RJ. 2001. Leptospiral lipopolysaccharide activates cells through a TLR2-dependent mechanism. Nature immunology 2(4):346-52.

[31] Yang CW, Wu MS, Pan MJ, Hong JJ, Yu CC, Vandewalle A, Huang CC. 2000. *Leptospira* outer membrane protein activates NF-kappaB and downstream genes expressed in medullary thick ascending limb cells. J Am Soc Nephrol 11(11):2017-26.

[32] Hoke DE, Egan S, Cullen PA, Adler B. 2008. LipL32 is an extracellular matrix-interacting protein of *Leptospira* spp. and Pseudoalteromonas tunicata. Infect Immun 76(5):2063-9.

[33] Hauk P, Macedo F, Romero EC, Vasconcellos SA, de Morais ZM, Barbosa AS, Ho PL. 2008. In LipL32, the major leptospiral lipoprotein, the C terminus is the primary immunogenic domain and mediates interaction with collagen IV and plasma fibronectin. Infect Immun 76(6):2642-50.

[34] Vivian JP, Beddoe T, McAlister AD, Wilce MC, Zaker-Tabrizi L, Troy S, Byres E, Hoke DE, Cullen PA, Lo M, Murray GL, Adler B, Rossjohn J. 2009. Crystal structure of LipL32, the most abundant surface protein of pathogenic *Leptospira* spp. J Mol Biol 387(5):1229-38.

[35] Hauk P, Guzzo CR, Roman Ramos H, Ho PL, Farah CS. 2009. Structure and calcium-binding activity of LipL32, the major surface antigen of pathogenic *Leptospira* sp. J Mol Biol 390(4):722-36.

[36] Murray GL, Srikram A, Hoke DE, Wunder EA, Jr., Henry R, Lo M, Zhang K, Sermswan RW, Ko AI, Adler B. 2009. Major surface protein LipL32 is not required for either acute or chronic infection with *Leptospira interrogans*. Infect Immun 77(3):952-8.

[37] Smith GP. 1985. Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surface. Science 228(4705):1315-7.

[38] Tim Clackson, Henry BL. 2004. Phage display. New York: Oxford university press.

[39] Kehoe JW, Kay BK. 2005. Filamentous phage display in the new millennium. Chem Rev 105(11):4056-72.

[40] Carlos F. Barbas, Dennis R. Burton, Jamie K. Scott, Gregg J. Silverman. 2001. Phage Display A Laboratory manual. New York: Cold spring Harbor Laboratory Press..

[41] Mullen LM, Nair SP, Ward JM, Rycroft AN, Henderson B. 2006. Phage display in the study of infectious diseases. Trends Microbiol 14(3):141-7.

[42] Rothe A, Hosse RJ, Power BE. 2006. Ribosome display for improved biotherapeutic molecules. Expert Opin Biol Ther 6(2):177-87.

[43] Ladner RC, Sato AK, Gorzelany J, de Souza M. 2004. Phage display-derived peptides as therapeutic alternatives to antibodies. Drug Discov Today 9(12):525-9.

[44] Benhar I. 2001. Biotechnological applications of phage and cell display. Biotechnol Adv 19(1):1-33.

[45] Rosander A, Bjerketorp J, Frykberg L, Jacobsson K. 2002. Phage display as a novel screening method to identify extracellular proteins. J Microbiol Methods 51(1):43-55.

[46] Rowley MJ, O'Connor K, Wijeyewickrema L. 2004. Phage display for epitope determination: a paradigm for identifying receptor-ligand interactions. Biotechnology annual review 10:151-88.

[47] Sidhu SS, Fairbrother WJ, Deshayes K. 2003. Exploring protein-protein interactions with phage display. Chembiochem 4(1):14-25.

[48] Ghosh AK, Ribolla PE, Jacobs-Lorena M. 2001. Targeting *Plasmodium* ligands on mosquito salivary glands and midgut with a phage display peptide library. Proc Natl Acad Sci U S A 98(23):13278-81.

[49] Tamura GS, Hull JR, Oberg MD, Castner DG. 2006. High-affinity interaction between fibronectin and the group B streptococcal C5a peptidase is unaffected by a naturally occurring four-amino-acid deletion that eliminates peptidase activity. Infect Immun 74(10):5739-46.

[50] Wall T, Roos S, Jacobsson K, Rosander A, Jonsson H. 2003. Phage display reveals 52 novel extracellular and transmembrane proteins from *Lactobacillus reuteri* DSM 20016(T). Microbiology (Reading, England) 149(Pt 12):3493-505.

[51] Heilmann C, Herrmann M, Kehrel BE, Peters G. 2002. Platelet-binding domains in 2 fibrinogen-binding proteins of *Staphylococcus aureus* identified by phage display. The Journal of infectious diseases 186(1):32-9.

[52] Deng Q, Zhai JW, Michel ML, Zhang J, Qin J, Kong YY, Zhang XX, Budkowska A, Tiollais P, Wang Y, Xie YH. 2007. Identification and characterization of peptides that interact with hepatitis B virus via the putative receptor binding site. Journal of virology 81(8):4244-54.

[53] Faine S AB, Bolin C, Petrolat P. 1999. *Leptospira* and leptospirosis. 2nd edition. Melbourne, Australia: Medisci.

[54] Haake DA. 2000. Spirochaetal lipoproteins and pathogenesis. Microbiology 146 (Pt 7):1491-504.

[55] Vinh T, Adler B, Faine S. 1986. Ultrastructure and chemical composition of lipopolysaccharide extracted from *Leptospira interrogans* serovar copenhageni. J Gen Microbiol 132 (Pt 1):103-9.

[56] Shimizu T, Matsusaka E, Takayanagi K, Masuzawa T, Iwamoto Y, Morita T, Mifuchi I, Yanagihara Y. 1987. Biological activities of lipopolysaccharide-like substance (LLS) extracted from *Leptospira interrogans* serovar Canicola strain Moulton. Microbiol Immunol 31(8):727-35.

[57] Vinh T, Adler B, Faine S. 1986. Glycolipoprotein cytotoxin from *Leptospira interrogans* serovar Copenhageni. J Gen Microbiol 132 (Pt 1):111-23.

[58] Patrick R. Murray, Michael A. Pfaller, Ken S. Rosenthal, Patrick R. Murray, Ken S. Rosenthal. 1996. Medical microbiology. 4th Edition. Texas: The University of Texas Medical Branch at Galveston.

[59] Johnson RC, Gary ND. 1963. Nutrition of Leptospira Pomona. Ii. Fatty Acid Requirements. J Bacteriol 85:976-82.

[60] Henneberry RC, Cox CD. 1970. Beta-oxidation of fatty acids by Leptospira. Can J Microbiol 16(1):41-5.

[61] Johnson RC, Harris VG. 1967. Differentiation of pathogenic and saprophytic letospires. I. Growth at low temperatures. J Bacteriol 94(1):27-31.

[62] Ren SX, Fu G, Jiang XG, Zeng R, Miao YG, Xu H, Zhang YX, Xiong H, Lu G, Lu LF, Jiang HQ, Jia J, Tu YF, Jiang JX, Gu WY, Zhang YQ, Cai Z, Sheng HH, Yin HF, Zhang Y, Zhu GF, Wan M, Huang HL, Qian Z, Wang SY, Ma W, Yao ZJ, Shen Y, Qiang BQ, Xia QC, Guo XK, Danchin A, Saint Girons I, Somerville RL, Wen YM, Shi MH, Chen Z, Xu JG, Zhao GP. 2003. Unique physiological and pathogenic features of *Leptospira interrogans* revealed by whole-genome sequencing. Nature 422(6934):888-93.

[63] Nascimento AL, Ko AI, Martins EA, Monteiro-Vitorello CB, Ho PL, Haake DA, Verjovski-Almeida S, Hartskeerl RA, Marques MV, Oliveira MC, Menck CF, Leite LC, Carrer H, Coutinho LL, Degrave WM, Dellagostin OA, El-Dorry H, Ferro ES, Ferro MI, Furlan LR, Gamberini M, Giglioti EA, Goes-Neto A, Goldman GH, Goldman MH, Harakava R, Jeronimo SM, Junqueira-de-Azevedo IL, Kimura ET, Kuramae EE, Lemos EG, Lemos MV, Marino CL, Nunes LR, de Oliveira RC, Pereira GG, Reis MS, Schriefer A, Siqueira WJ, Sommer P, Tsai SM, Simpson AJ, Ferro JA,

Camargo LE, Kitajima JP, Setubal JC, Van Sluys MA. 2004. Comparative genomics of two *Leptospira interrogans* serovars reveals novel insights into physiology and pathogenesis. J Bacteriol 186(7):2164-72.

[64] Bulach DM, Zuerner RL, Wilson P, Seemann T, McGrath A, Cullen PA, Davis J, Johnson M, Kuczek E, Alt DP, Peterson-Burch B, Coppel RL, Rood JI, Davies JK, Adler B. 2006. Genome reduction in *Leptospira borgpetersenii* reflects limited transmission potential. Proc Natl Acad Sci U S A 103(39):14560-5.

[65] Picardeau M, Bulach DM, Bouchier C, Zuerner RL, Zidane N, Wilson PJ, Creno S, Kuczek ES, Bommezzadri S, Davis JC, McGrath A, Johnson MJ, Boursaux-Eude C, Seemann T, Rouy Z, Coppel RL, Rood JI, Lajus A, Davies JK, Medigue C, Adler B. 2008. Genome sequence of the saprophyte *Leptospira biflexa* provides insights into the evolution of Leptospira and the pathogenesis of leptospirosis. PLoS ONE 3(2):e1607.

[66] Feresu SB, Ann Bolin C, van de Kemp H, Korver H. 1999. Identification of a serogroup bataviae *Leptospira* strain isolated from an ox in Zimbabwe. Zentralbl Bakteriol 289(1):19-29.

[67] Boursaux-Eude C, Saint Girons I, Zuerner R. 1998. *Leptospira* genomics. Electrophoresis 19(4):589-92.

[68] Croda J, Figueira CP, Wunder EA, Jr., Santos CS, Reis MG, Ko AI, Picardeau M. 2008. Targeted mutagenesis in pathogenic *Leptospira* species: disruption of the LigB gene does not affect virulence in animal models of leptospirosis. Infect Immun 76(12):5826-33.

[69] Murray GL, Morel V, Cerqueira GM, Croda J, Srikram A, Henry R, Ko AI, Dellagostin OA, Bulach DM, Sermswan RW, Adler B, Picardeau M. 2009. Genome-wide transposon mutagenesis in pathogenic *Leptospira* species. Infect Immun 77(2):810-6.

[70] Terpstra WJ. 1992. Typing *Leptospira* from the perspective of a reference laboratory. Acta Leiden 60(2):79-87.

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

[72] Ramadass P, Jarvis BD, Corner RJ, Penny D, Marshall RB. 1992. Genetic characterization of pathogenic *Leptospira* species by DNA hybridization. Int J Syst Bacteriol 42(2):215-9.

[73] Cerqueira GM, Picardeau M. 2009. A century of *Leptospira* strain typing. Infect Genet Evol 9(5):760-8.

[74] Langston CE, Heuter KJ. 2003. Leptospirosis. A re-emerging zoonotic disease. Vet Clin North Am Small Anim Pract 33(4):791-807.

[75] ขจรศักดิ์ ศิลปโภชากุล ชส, รุ่งเรือง ลิ้มไพบูลย์, ประวิทย์ ชุมเกษียร, พิมพ์ใจ นัยโกวิทย์, ดาริกา กิ่งเนตร, ดวงพร พูลสุขสมบัติ, วราลักษณ์ ตังคณะกุล, สุขุม เสมา, สุรพล แสงรัตนชัย, พวงทอง บุทรง, เสริมศักดิ์ หงส์นาค, ยุวลักษณ์ ขอประเสริฐ, ประจง สุดโต. คู่มือวิชาการโรคเลปโตสไปโรซิส Leptospirosis. กองโรคติดต่อทั่วไป กรมควบคุมโรคติดต่อ กระทรวงสาธารณสุข.

[76] Laras K, Cao BV, Bounlu K, Nguyen TK, Olson JG, Thongchanh S, Tran NV, Hoang KL, Punjabi N, Ha BK, Ung SA, Insisiengmay S, Watts DM, Beecham HJ, Corwin AL. 2002. The importance of leptospirosis in Southeast Asia. Am J Trop Med Hyg 67(3):278-86.

[77] Wuthiekanun V, Sirisukkarn N, Daengsupa P, Sakaraserane P, Sangkakam A, Chierakul W, Smythe LD, Symonds ML, Dohnt MF, Slack AT, Day NP, Peacock SJ. 2007. Clinical diagnosis and geographic distribution of leptospirosis, Thailand. Emerg Infect Dis 13(1):124-6.

[78] Moseley RH. 1997. Sepsis-associated cholestasis. Gastroenterology 112(1):302-6.

[79] Daher E, Zanetta DM, Cavalcante MB, Abdulkader RC. 1999. Risk factors for death and changing patterns in leptospirosis acute renal failure. Am J Trop Med Hyg 61(4):630-4.

[80] Hill MK, Sanders CV. 1997. Leptospiral pneumonia. Semin Respir Infect 12(1):44-9.

[81] Dursun B, Bostan F, Artac M, Varan HI, Suleymanlar G. 2007. Severe pulmonary haemorrhage accompanying hepatorenal failure in fulminant leptospirosis. Int J Clin Pract 61(1):164-7.

[82] Yersin C, Bovet P, Merien F, Clement J, Laille M, Van Ranst M, Perolat P. 2000. Pulmonary haemorrhage as a predominant cause of death in leptospirosis in Seychelles. Trans R Soc Trop Med Hyg 94(1):71-6.

[83] Salavert M, Roig P, Nieto A, Navarro V. 1991. [Weil's disease with pulmonary, visceral and cutaneous manifestations]. Med Clin (Barc) 96(13):518.

[84] Papa A, Theoharidou D, Antoniadis A. 2009. Pulmonary involvement and leptospirosis, Greece. Emerg Infect Dis 15(5):834-5.

[85] Budiono E, Sumardi, Riyanto BS, Hisyam B, Hartopo AB. 2009. Pulmonary involvement predicts mortality in severe leptospirosis patients. Acta Med Indones 41(1):11-4.

[86] Sodeman WA, Killough JH. 1951. The cardiac manifestations of Weil's disease. Am J Trop Med Hyg 31(4):479-88.

[87] Ramachandran S, Perera MV. 1977. Cardiac and pulmonary involvement in leptospirosis. Trans R Soc Trop Med Hyg 71(1):56-9.

[88] Lee MG, Char G, Dianzumba S, Prussia P. 1986. Cardiac involvement in severe leptospirosis. West Indian Med J 35(4):295-300.

[89] Rugiero HR, Chilabert E, Castiglioni E. 1950. [Weil's disease; neurologic forms.]. Prensa Med Argent 37(42):2516-8.

[90] Rathinam SR. 2002. Ocular leptospirosis. Curr Opin Ophthalmol 13(6):381-6.

[91] Rathinam SR. 2005. Ocular manifestations of leptospirosis. J Postgrad Med 51(3):189-94.

[92] Vijayachari P, Sugunan AP, Umapathi T, Sehgal SC. 2001. Evaluation of darkground microscopy as a rapid diagnostic procedure in leptospirosis. Indian J Med Res 114:54-8.

[93] Levett PN. 2003. Usefulness of serologic analysis as a predictor of the infecting serovar in patients with severe leptospirosis. Clin Infect Dis 36(4): 447-52.

[94] Surujballi O, Elmgren C. 2000. Monoclonal antibodies suitable for incorporation into a competitive enzyme-linked immunosorbent assay (ELISA) for detection of specific antibodies to *Leptospira interrogans* serovar Pomona. Vet Microbiol 71(1-2):149-59.

[95] Sharma R, Tuteja U, Khushiramani R, Shukla J, Batra HV. 2007. Application of rapid dot-ELISA for antibody detection of leptospirosis. J Med Microbiol 56(Pt 6):873-4.

[96] Vitale G, La Russa C, Galioto A, Chifari N, Mocciaro C, Caruso R, Micalizzi A, Mansueto P, Di Rosa S, Mansueto S. 2004. Evaluation of an IgM-ELISA test for the diagnosis of human leptospirosis. New Microbiol 27(2): 149-54.

[97] Dey S, Mohan CM, Ramadass P, Nachimuthu K. 2007. Recombinant antigen-based dipstick ELISA for the diagnosis of leptospirosis in dogs. Vet Rec 160(6):186-8.

[98] Boonyod D, Poovorawan Y, Bhattarakosol P, Chirathaworn C. 2005. LipL32, an outer membrane protein of *Leptospira*, as an antigen in a dipstick assay for diagnosis of leptospirosis. Asian Pacific journal of allergy and immunology / launched by the Allergy and Immunology Society of Thailand 23(2-3):133-41.

[99] Smits HL, Ananyina YV, Chereshsky A, Dancel L, Lai AFRF, Chee HD, Levett PN, Masuzawa T, Yanagihara Y, Muthusethupathi MA, Sanders EJ, Sasaki DM, Domen H, Yersin C, Aye T, Bragg SL, Gussenhoven GC, Goris MG, Terpstra WJ, Hartskeerl RA. 1999. International multicenter evaluation of the clinical utility of a dipstick assay for detection of *Leptospira*-specific

immunoglobulin M antibodies in human serum specimens. J Clin Microbiol 37(9):2904-9.

[100] Gussenhoven GC, van der Hoorn MA, Goris MG, Terpstra WJ, Hartskeerl RA, Mol BW, van Ingen CW, Smits HL. 1997. LEPTO dipstick, a dipstick assay for detection of *Leptospira*-specific immunoglobulin M antibodies in human sera. J Clin Microbiol 35(1):92-7.

[101] Andreescu N. 1990. A new prepatory method of thermically inactivated *Leptospira* Patoc antigen for rapid slide agglutination used as serosurvey test for human leptospiroses. Arch Roum Pathol Exp Microbiol 49(3):223-7.

[102] Lilenbaum W, Ristow P, Fraguas SA, da Silva ED. 2002. Evaluation of a rapid slide agglutination test for the diagnosis of acute canine leptospirosis. Rev Latinoam Microbiol 44(3-4):124-8.

[103] Senthilkumar T, Subathra M, Phil M, Ramadass P, Ramaswamy V. 2008. Rapid serodiagnosis of leptospirosis by latex agglutination test and flow-through assay. Indian J Med Microbiol 26(1):45-9.

[104] Dey S, Madhan Mohan C, Ramadass P, Nachimuthu K. 2007. Recombinant antigen-based latex agglutination test for rapid serodiagnosis of leptospirosis. Vet Res Commun 31(1):9-15.

[105] Hull-Jackson C, Glass MB, Ari MD, Bragg SL, Branch SL, Whittington CU, Edwards CN, Levett PN. 2006. Evaluation of a commercial latex agglutination assay for serological diagnosis of leptospirosis. J Clin Microbiol 44(5):1853-5.

[106] Pradutkanchana S, Nakarin J. 2005. The use of latex agglutination for the diagnosis of acute human leptospirosis. J Med Assoc Thai 88(10): 1395-400.

[107] Ooteman MC, Vago AR, Koury MC. 2006. Evaluation of MAT, IgM ELISA and PCR methods for the diagnosis of human leptospirosis. J Microbiol Methods 65(2):247-57.

[108] Ooteman MC, Vago AR, Koury MC. 2005. Evaluation of MAT, IgM ELISA and PCR methods for the diagnosis of human leptospirosis. J Microbiol Methods. 65: 247-57.

[109] Ooteman MC, Vago AR, Koury MC. 2004. Potential application of low-stringency single specific primer-PCR in the identification of *Leptospira* in the serum of patients with suspected leptospirosis. Can J Microbiol 50(12):1073-9.

[110] Oliveira MA, Caballero OL, Vago AR, Harskeerl RA, Romanha AJ, Pena SD, Simpson AJ, Koury MC. 2003. Low-stringency single specific primer PCR for identification of *Leptospira*. J Med Microbiol 52(Pt 2):127-35.

[111] Bal AE, Gravekamp C, Hartskeerl RA, De Meza-Brewster J, Korver H, Terpstra WJ. 1994. Detection of leptospires in urine by PCR for early diagnosis of leptospirosis. J Clin Microbiol 32(8):1894-8.

[112] Merien F, Amouriaux P, Perolat P, Baranton G, Saint Girons I. 1992. Polymerase chain reaction for detection of *Leptospira* spp. in clinical samples. J Clin Microbiol 30(9):2219-24.

[113] Gravekamp C, Van de Kemp H, Franzen M, Carrington D, Schoone GJ, Van Eys GJ, Everard CO, Hartskeerl RA, Terpstra WJ. 1993. Detection of seven species of pathogenic leptospires by PCR using two sets of primers. J Gen Microbiol 139 (Pt 8):1691-700.

[114] Smythe LD, Smith IL, Smith GA, Dohnt MF, Symonds ML, Barnett LJ, McKay DB. 2002. A quantitative PCR (TaqMan) assay for pathogenic *Leptospira* spp. BMC Infect Dis 2(1):13.

[115] Levett PN, Morey RE, Galloway RL, Turner DE, Steigerwalt AG, Mayer LW. 2005. Detection of pathogenic leptospires by real-time quantitative PCR. J Med Microbiol 54(Pt 1):45-9.

[116] Lux R, Moter A, Shi W. 2000. Chemotaxis in pathogenic spirochetes: directed movement toward targeting tissues? J Mol Microbiol Biotechnol 2(4):355-64.

[117] Yuri K, Takamoto Y, Okada M, Hiramune T, Kikuchi N, Yanagawa R. 1993. Chemotaxis of leptospires to hemoglobin in relation to virulence. Infect Immun 61(5):2270-2.

[118] De-Souza L, Koury MC. 1992. Chemical and biological properties of endotoxin from *Leptospira interrogans* serovars Canicola and Icterohaemorrhagiae. Braz J Med Biol Res 25(5):467-75.

[119] Isogai E, Isogai H, Kurebayashi Y, Ito N. 1986. Biological activities of leptospiral lipopolysaccharide. Zentralbl Bakteriol Mikrobiol Hyg A 261(1):53-64.

[120] Masuzawa T, Nakamura R, Shimizu T, Yanagihara Y. 1990. Biological activities and endotoxic activities of protective antigens (PAgs) of *Leptospira interrogans*. Zentralbl Bakteriol 274(1):109-17.

[121] Nally JE, Chow E, Fishbein MC, Blanco DR, Lovett MA. 2005. Changes in lipopolysaccharide O antigen distinguish acute versus chronic *Leptospira interrogans* infections. Infect Immun 73(6):3251-60.

[122] Bernheimer AW, Bey RF. 1986. Copurification of *Leptospira interrogans* serovar Pomona hemolysin and sphingomyelinase C. Infect Immun 54(1):262-4.

[123] del Real G, Segers RP, van der Zeijst BA, Gaastra W. 1989. Cloning of a hemolysin gene from *Leptospira interrogans* serovar Hardjo. Infect Immun 57(8):2588-90.

[124] Zhang YX, Geng Y, Bi B, He JY, Wu CF, Guo XK, Zhao GP. 2005. Identification and classification of all potential hemolysin encoding genes and their products from *Leptospira interrogans* serogroup Icterohae-morrhagiae serovar Lai. Acta Pharmacol Sin 26(4):453-61.

[125] Hauk P, Negrotto S, Romero EC, Vasconcellos SA, Genovez ME, Ward RJ, Schattner M, Gomez RM, Ho PL. 2005. Expression and characterization of HlyX hemolysin from *Leptospira interrogans* serovar Copenhageni: potentiation of hemolytic activity by LipL32. Biochem Biophys Res Commun 333(4):1341-7.

[126] Lee SH, Kim S, Park SC, Kim MJ. 2002. Cytotoxic activities of *Leptospira interrogans* hemolysin SphH as a pore-forming protein on mammalian cells. Infect Immun 70(1):315-22.

[127] Segers RP, van der Drift A, de Nijs A, Corcione P, van der Zeijst BA, Gaastra W. 1990. Molecular analysis of a sphingomyelinase C gene from *Leptospira interrogans* serovar Hardjo. Infect Immun 58(7):2177-85.

[128] Li L, Ojcius DM, Yan J. 2007. Comparison of invasion of fibroblasts and macrophages by high- and low-virulence *Leptospira* strains: colonization of the host-cell nucleus and induction of necrosis by the virulent strain. Archives of microbiology 188(6):591-8.

[129] Luo DJ, Hu Y, Dennin RH, Yan J. 2007. [Reconstruction of *Leptospira interrogans* lipL21 gene and characteristics of its expression product]. Zhejiang da xue xue bao. Yi xue ban = Journal of Zhejiang University 36(5):458-64.

[130] Barbosa AS, Abreu PA, Neves FO, Atzingen MV, Watanabe MM, Vieira ML, Morais ZM, Vasconcellos SA, Nascimento AL. 2006. A newly identified leptospiral adhesin mediates attachment to laminin. Infect Immun 74(11):6356-64.

[131] Stevenson B, Choy HA, Pinne M, Rotondi ML, Miller MC, Demoll E, Kraiczy P, Cooley AE, Creamer TP, Suchard MA, Brissette CA, Verma A, Haake DA. 2007. *Leptospira interrogans* endostatin-like outer membrane proteins bind host fibronectin, laminin and regulators of complement. PLoS ONE 2(11):e1188.

[132] Giuseppe PO, Neves FO, Nascimento AL, Guimaraes BG. 2008. The leptospiral antigen Lp49 is a two-domain protein with putative protein binding function. J Struct Biol 163(1):53-60.

[133] Merien F, Truccolo J, Baranton G, Perolat P. 2000. Identification of a 36-kDa fibronectin-binding protein expressed by a virulent variant of *Leptospira interrogans* serovar Icterohaemorrhagiae. FEMS microbiology letters 185(1):17-22.

[134] Nahori MA, Fournie-Amazouz E, Que-Gewirth NS, Balloy V, Chignard M, Raetz CR, Saint Girons I, Werts C. 2005. Differential TLR recognition of leptospiral lipid A and lipopolysaccharide in murine and human cells. J Immunol 175(9):6022-31.

[135] Adler B, Faine S. 1977. Host immunological mechanisms in the resistance of mice to leptospiral infections. Infect Immun 17(1):67-72.

[136] Adler B, Faine S. 1978. The antibodies involved in the human immune response to leptospiral infection. J Med Microbiol 11(4):387-400.

[137] Sonrier C, Branger C, Michel V, Ruvoen-Clouet N, Ganiere JP, Andre-Fontaine G. 2000. Evidence of cross-protection within *Leptospira interrogans* in an experimental model. Vaccine 19(1):86-94.

[138] Chapman AJ, Adler B, Faine S. 1988. Antigens recognised by the human immune response to infection with *Leptospira interrogans* serovar Hardjo. J Med Microbiol 25(4):269-78.

[139] Jost BH, Adler B, Faine S. 1989. Experimental immunisation of hamsters with lipopolysaccharide antigens of *Leptospira interrogans*. J Med Microbiol 29(2):115-20.

[140] Jost BH, Adler B, Vinh T, Faine S. 1986. A monoclonal antibody reacting with a determinant on leptospiral lipopolysaccharide protects guinea pigs against leptospirosis. J Med Microbiol 22(3):269-75.

[141] Midwinter A, Faine S, Adler B. 1990. Vaccination of mice with lipopolysaccharide (LPS) and LPS-derived immuno-conjugates from *Leptospira interrogans*. J Med Microbiol 33(3):199-204.

[142] Masuzawa T, Nakamura R, Beppu Y, Yanagihara Y. 1996. Immunochemical characteristics and localization on cells of protective antigen (PAg) prepared from *Leptospira interrogans* serovar Lai. Microbiol Immunol 40(3):237-41.

[143] Haake DA, Mazel MK, McCoy AM, Milward F, Chao G, Matsunaga J, Wagar EA. 1999. Leptospiral outer membrane proteins OmpL1 and LipL41 exhibit synergistic immunoprotection. Infect Immun 67(12):6572-82.

[144] Branger C, Chatrenet B, Gauvrit A, Aviat F, Aubert A, Bach JM, Andre-Fontaine G. 2005. Protection against *Leptospira interrogans* sensu lato challenge by DNA immunization with the gene encoding hemolysin-associated protein 1. Infect Immun 73(7):4062-9.

[145] Seixas FK, da Silva EF, Hartwig DD, Cerqueira GM, Amaral M, Fagundes MQ, Dossa RG, Dellagostin OA. 2007. Recombinant *Mycobacterium bovis* BCG expressing the LipL32 antigen of *Leptospira interrogans* protects hamsters from challenge. Vaccine 26(1):88-95.

[146] Klimpel GR, Matthias MA, Vinetz JM. 2003. *Leptospira interrogans* activation of human peripheral blood mononuclear cells: preferential expansion of TCR gamma delta+ T cells vs TCR alpha beta+ T cells. J Immunol 171(3):1447-55.

[147] Feng CY, Li QT, Zhang XY, Dong K, Hu BY, Guo XK. 2009. Immune strategies using single-component LipL32 and multi-component recombinant LipL32-41-OmpL1 vaccines against *Leptospira*. Braz J Med Biol Res.

[148] Atzingen MV, Barbosa AS, De Brito T, Vasconcellos SA, de Morais ZM, Lima DM, Abreu PA, Nascimento AL. 2008. Lsa21, a novel leptospiral protein binding adhesive matrix molecules and present during human infection. BMC microbiology 8:70.

[149] Bourhy P, Louvel H, Saint Girons I, Picardeau M. 2005. Random insertional mutagenesis of *Leptospira interrogans*, the agent of leptospirosis, using a mariner transposon. J Bacteriol 187(9):3255-8.

[150] Koizumi N, Watanabe H. 2003. Molecular cloning and characterization of a novel leptospiral lipoprotein with OmpA domain. FEMS Microbiol Lett 226(2):215-9.

[151] Yang HL, Zhu YZ, Qin JH, He P, Jiang XC, Zhao GP, Guo XK. 2006. In silico and microarray-based genomic approaches to identifying potential vaccine candidates against *Leptospira interrogans*. BMC Genomics 7:293.

[152] Zuerner RL, Knudtson W, Bolin CA, Trueba G. 1991. Characterization of outer membrane and secreted proteins of *Leptospira interrogans* serovar Pomona. Microb Pathog 10(4):311-22.

[153] Cullen PA, Haake DA, Bulach DM, Zuerner RL, Adler B. 2003. LipL21 is a novel surface-exposed lipoprotein of pathogenic *Leptospira* species. Infect Immun 71(5):2414-21.

[154] He HJ, Wang WY, Wu ZD, Lv ZY, Li J, Tan LZ. 2008. Protection of guinea pigs against *Leptospira interrogans* serovar Lai by LipL21 DNA vaccine. Cell Mol Immunol 5(5):385-91.

[155] Neves FO, Abreu PA, Vasconcellos SA, de Morais ZM, Romero EC, Nascimento AL. 2007. Identification of a novel potential antigen for early-phase serodiagnosis of leptospirosis. Archives of microbiology 188(5):523-32.

[156] Verma A, Hellwage J, Artiushin S, Zipfel PF, Kraiczy P, Timoney JF, Stevenson B. 2006. LfhA, a novel factor H-binding protein of Leptospira interrogans. Infect Immun 74(5):2659-66.

[157] Koizumi N, Watanabe H. 2004. Leptospiral immunoglobulin-like proteins elicit protective immunity. Vaccine 22(11-12):1545-52.

[158] Palaniappan RU, Chang YF, Jusuf SS, Artiushin S, Timoney JF, McDonough SP, Barr SC, Divers TJ, Simpson KW, McDonough PL, Mohammed HO. 2002. Cloning and molecular characterization of an immunogenic LigA protein of *Leptospira interrogans*. Infect Immun 70(11):5924-30.

[159] Palaniappan RU, McDonough SP, Divers TJ, Chen CS, Pan MJ, Matsumoto M, Chang YF. 2006. Immunoprotection of recombinant leptospiral immunoglobulin-like protein A against *Leptospira interrogans* serovar Pomona infection. Infect Immun 74(3):1745-50.

[160] Srimanote P, Wongdeethai N, Jieanampunkul P, Samonkiert S, Leepiyasakulchai C, Kalambaheti T, Prachayasittikul V. 2008. Recombinant ligA for leptospirosis diagnosis and ligA among the *Leptospira* spp. clinical isolates. J Microbiol Methods 72(1):73-81.

[161] Faisal SM, Yan W, Chen CS, Palaniappan RU, McDonough SP, Chang YF. 2008. Evaluation of protective immunity of *Leptospira* immunoglobulin like protein A (LigA) DNA vaccine against challenge in hamsters. Vaccine 26(2):277-87.

[162] Silva EF, Medeiros MA, McBride AJ, Matsunaga J, Esteves GS, Ramos JG, Santos CS, Croda J, Homma A, Dellagostin OA, Haake DA, Reis MG, Ko AI. 2007. The terminal portion of leptospiral immunoglobulin-like protein LigA confers protective immunity against lethal infection in the hamster model of leptospirosis. Vaccine 25(33):6277-86.

[163] Sidhu SS. 2005. Phage display in biotechnology and drug discovery. Florida: CRC Press Taylor & Francis Group.

[164] Smith GP, Petrenko VA. 1997. Phage Display. Chem Rev 97(2):391-410.

[165] Castagnoli L, Zucconi A, Quondam M, Rossi M, Vaccaro P, Panni S, Paoluzi S, Santonico E, Dente L, Cesareni G. 2001. Alternative bacteriophage display systems. Comb Chem High Throughput Screen 4(2):121-33.

[166] Scott JK, Smith GP. 1990. Searching for peptide ligands with an epitope library. Science 249(4967):386-90.

[167] Antonara S, Chafel RM, Lafrance M, Coburn J. 2007. *Borrelia burgdorferi* adhesins identified using in vivo phage display. Mol Microbiol 66(1): 262-76.

[168] Rahim AA. 2007. Pyrosequencing of phage display libraries for the identification of cell-specific targeting ligands. Methods Mol Biol 373:135-46.

[169] Valadon P, Garnett JD, Testa JE, Bauerle M, Oh P, Schnitzer JE. 2006. Screening phage display libraries for organ-specific vascular immunotargeting in vivo. Proc Natl Acad Sci U S A 103(2):407-12.

[170] El-Mousawi M, Tchistiakova L, Yurchenko L, Pietrzynski G, Moreno M, Stanimirovic D, Ahmad D, Alakhov V. 2003. A vascular endothelial growth factor high affinity receptor 1-specific peptide with antiangiogenic activity identified using a phage display peptide library. J Biol Chem 278(47):46681-91.

[171] Hetian L, Ping A, Shumei S, Xiaoying L, Luowen H, Jian W, Lin M, Meisheng L, Junshan Y, Chengchao S. 2002. A novel peptide isolated from a phage display library inhibits tumor growth and metastasis by blocking the binding of vascular endothelial growth factor to its kinase domain receptor. J Biol Chem 277(45):43137-42.

[172] Zhu Z, Rockwell P, Lu D, Kotanides H, Pytowski B, Hicklin DJ, Bohlen P, Witte L. 1998. Inhibition of vascular endothelial growth factor-induced receptor activation with anti-kinase insert domain-containing receptor single-chain antibodies from a phage display library. Cancer Res 58(15):3209-14.

[173] Lauterbach SB, Lanzillotti R, Coetzer TL. 2003. Construction and use of *Plasmodium falciparum* phage display libraries to identify host parasite interactions. Malar J 2(1):47.

[174] Whaley SR, English DS, Hu EL, Barbara PF, Belcher AM. 2000. Selection of peptides with semiconductor binding specificity for directed nanocrystal assembly. Nature 405(6787):665-8.

[175] Kay BK, Kasanov J, Yamabhai M. 2001. Screening phage-displayed combinatorial peptide libraries. Methods (San Diego, Calif 24(3):240-6.

[176] Mongiovi AM, Romano PR, Panni S, Mendoza M, Wong WT, Musacchio A, Cesareni G, Di Fiore PP. 1999. A novel peptide-SH3 interaction. Embo J 18(19):5300-9.

[177] Pillutla RC, Hsiao KC, Beasley JR, Brandt J, Ostergaard S, Hansen PH, Spetzler JC, Danielsen GM, Andersen AS, Brissette RE, Lennick M, Fletcher PW, Blume AJ, Schaffer L, Goldstein NI. 2002. Peptides identify the critical hotspots involved in the biological activation of the insulin receptor. J Biol Chem 277(25):22590-4.

[178] Binetruy-Tournaire R, Demangel C, Malavaud B, Vassy R, Rouyre S, Kraemer M, Plouet J, Derbin C, Perret G, Mazie JC. 2000. Identification of a peptide blocking vascular endothelial growth factor (VEGF)-mediated angiogenesis. Embo J 19(7):1525-33.

[179] Sidhu SS, Koide S. 2007. Phage display for engineering and analyzing protein interaction interfaces. Curr Opin Struct Biol 17(4):481-7.

[180] Mayrose I, Shlomi T, Rubinstein ND, Gershoni JM, Ruppin E, Sharan R, Pupko T. 2007. Epitope mapping using combinatorial phage-display libraries: a graph-based algorithm. Nucleic Acids Res 35(1):69-78.

[181] Kola A, Baensch M, Bautsch W, Hennecke M, Klos A, Casaretto M, Kohl J. 1996. Epitope mapping of a C5a neutralizing mAb using a combined approach of phage display, synthetic peptides and site-directed mutagenesis. Immunotechnology 2(2):115-26.

[182] Ramasoota P, Tungtrakanpoung R, Pitaksajjakul P, Ekpo P, Froman G, Chaicumpa W. 2005. Epitope mapping of monoclonal antibodies specific to serovar of *Leptospira*, using phage display technique. Southeast Asian J Trop Med Public Health 36 Suppl 4:206-12.

[183] Oleksiewicz MB, Botner A, Toft P, Normann P, Storgaard T. 2001. Epitope mapping porcine reproductive and respiratory syndrome virus by phage display: the nsp2 fragment of the replicase polyprotein contains a cluster of B-cell epitopes. J Virol 75(7):3277-90.

[184] Mullaney BP, Pallavicini MG, Marks JD. 2001. Epitope mapping of neutralizing botulinum neurotoxin A antibodies by phage display. Infect Immun 69(10):6511-4.

[185] Wang LF, Yu M. 2004. Epitope identification and discovery using phage display libraries: applications in vaccine development and diagnostics. Curr Drug Targets 5(1):1-15.

[186] Schmitz U, Versmold A, Kaufmann P, Frank HG. 2000. Phage display: a molecular tool for the generation of antibodies--a review. Placenta 21 Suppl A:S106-12.

[187] Sergeeva A, Kolonin MG, Molldrem JJ, Pasqualini R, Arap W. 2006. Display technologies: application for the discovery of drug and gene delivery agents. Adv Drug Deliv Rev 58(15):1622-54.

[188] Mullen LM, Nair SP, Ward JM, Rycroft AN, Williams RJ, Henderson B. 2007. Comparative functional genomic analysis of *Pasteurellaceae* adhesins using phage display. Vet Microbiol 122(1-2):123-34.

[189] Beckmann C, Waggoner JD, Harris TO, Tamura GS, Rubens CE. 2002. Identification of novel adhesins from Group B streptococci by use of phage display reveals that C5a peptidase mediates fibronectin binding. Infect Immun 70(6):2869-76.

[190] Jacobsson K, Jonsson H, Lindmark H, Guss B, Lindberg M, Frykberg L. 1997. Shot-gun phage display mapping of two streptococcal cell-surface proteins. Microbiol Res 152(2):121-8.

[191] Heilmann C, Niemann S, Sinha B, Herrmann M, Kehrel BE, Peters G. 2004. *Staphylococcus aureus* fibronectin-binding protein (FnBP)-mediated adherence to platelets, and aggregation of platelets induced by FnBPA but not by FnBPB. J Infect Dis 190(2):321-9.

[192] Benedek O, Khan AS, Schneider G, Nagy G, Autar R, Pieters RJ, Emody L. 2005. Identification of laminin-binding motifs of *Yersinia* pestis plasminogen activator by phage display. Int J Med Microbiol 295(2): 87-98.

[193] Chan SW, Nathan S. 2005. Epitope mapping of *Burkholderia pseudomallei* serine metalloprotease: identification of serine protease epitope mimics. FEMS Immunol Med Microbiol 43(1):37-44.

[194] Buchwald UK, Lees A, Steinitz M, Pirofski LA. 2005. A peptide mimotope of type 8 pneumococcal capsular polysaccharide induces a protective immune response in mice. Infect Immun 73(1):325-33.

[195] Yang WJ, Lai JF, Peng KC, Chiang HJ, Weng CN, Shiuan D. 2005. Epitope mapping of *Mycoplasma hyopneumoniae* using phage displayed peptide libraries and the immune responses of the selected phagotopes. J Immunol Methods 304(1-2):15-29.

[196] Eshaghi M, Tan WS, Yusoff K. 2005. Identification of epitopes in the nucleocapsid protein of Nipah virus using a linear phage-displayed random peptide library. J Med Virol 75(1):147-52.

[197] Tungtrakanpoung R, Pitaksajjakul P, Na-Ngarm N, Chaicumpa W, Ekpo P, Saengjaruk P, Froman G, Ramasoota P. 2006. Mimotope of *Leptospira* from phage-displayed random peptide library is reactive with both monoclonal antibodies and patients' sera. Vet Microbiol 115(1-3):54-63.

[198] Ronaghi M. 2001. Pyrosequencing sheds light on DNA sequencing. Genome Research 11(1):3-11.

[199] Ahmadian A, Gharizadeh B, Gustafsson AC, Sterky F, Nyren P, Uhlen M, Lundeberg J. 2000. Single-nucleotide polymorphism analysis by pyrosequencing. Anal Biochem 280(1):103-10.

[200] Nordstrom T, Ronaghi M, Forsberg L, de Faire U, Morgenstern R, Nyren P. 2000. Direct analysis of single-nucleotide polymorphism on double-stranded DNA by pyrosequencing. Biotechnology and Applied Biochemistry 31:107-12.

[201] Neve B, Froguel P, Corset L, Vaillant E, Vatin V, Boutin P. 2002. Rapid SNP allele frequency determination in enomic DNA pools by Pyrosequencing (TM). Biotechniques 32(5):1138-42.

[202] Gharizadeh B, Kalantari M, Garcia CA, Johansson B, Nyren P. 2001. Typing of human papillomavirus by pyrosequencing. Laboratory Investigation 81(5):673-79.

[203] Gharizadeh B, Norberg E, Loffler J, Jalal S, Tollemar J, Einsele H, Klingspor L, Nyren P. 2004. Identification of medically important fungi by the Pyrosequencing (TM) technology. Mycoses 47(1-2):29-33.

[204] Grahn N, Hmani-Aifa M, Fransen K, Soderkvist P, Monstein HJ. 2005. Molecular identification of *Helicobacter* DNA present in human colorectal adenocarcinomas by 16S rDNA PCR amplification and pyrosequencing analysis. Journal of Medical Microbiology 54(11):1031-35.

[205] Ronaghi M, Elahi E. 2002. Pyrosequencing for microbial typing. Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences 782(1-2):67-72.

[206] Ronaghi M, Nygren M, Lundeberg J, Nyren P. 1999. Analyses of secondary structures in DNA by pyrosequencing. Analytical Biochemistry 267(1):65-71.

[207] Ahmadian A, Lundeberg J, Nyren P, Uhlen M, Ronaghi M. 2000. Analysis of the p53 tumor suppressor gene by pyrosequencing. Biotechniques 28(1):140-147.

[208] Rahim A, Coutelle C, Harbottle R. 2003. High-throughput Pyrosequencing of a phage display library for the identification of enriched target-specific peptides. Biotechniques 35(2):317-20, 22, 24.

[209] Rahim M, Coutelle C, Harbottle R. 2003. High-throughput Pyrosequencing (TM) of a phage display library for the identification of enriched target-specific peptides. Biotechniques 35(2):317-+.

[210] Walia V, Ding M, Kumar S, Nie D, Premkumar LS, Elble RC. 2009. hCLCA2 Is a p53-Inducible Inhibitor of Breast Cancer Cell Proliferation. Cancer Res 69(16):6624-32.

[211] Elble RC, Walia V, Cheng HC, Connon CJ, Mundhenk L, Gruber AD, Pauli BU. 2006. The putative chloride channel hCLCA2 has a single C-terminal transmembrane segment. J Biol Chem 281(40):29448-54.

[212] Connon CJ, Kawasaki S, Yamasaki K, Quantock AJ, Kinoshita S. 2005. The quantification of hCLCA2 and colocalisation with integrin beta4 in stratified human epithelia. Acta Histochem 106(6):421-5.

[213] Abdel-Ghany M, Cheng HC, Elble RC, Pauli BU. 2001. The breast cancer beta 4 integrin and endothelial human CLCA2 mediate lung metastasis. J Biol Chem 276(27):25438-46.

[214] Gruber AD, Schreur KD, Ji HL, Fuller CM, Pauli BU. 1999. Molecular cloning and transmembrane structure of hCLCA2 from human lung, trachea, and mammary gland. Am J Physiol 276(6 Pt 1):C1261-70.

[215] Cuppoletti J, Malinowska DH. 1999. Ca2+-activated Cl- channels: focus on "molecular cloning and transmembrane structure of hCLCA2 from human lung, trachea, and mammary gland". Am J Physiol 276(6 Pt 1):C1259-60.

[216] Suzuki M, Morita T, Iwamoto T. 2006. Diversity of Cl(-) channels. Cell Mol Life Sci 63(1):12-24.

[217] Loewen ME, Forsyth GW. 2005. Structure and function of CLCA proteins. Physiol Rev 85(3):1061-92.

[218] Suzuki-Inoue K, Tulasne D, Shen Y, Bori-Sanz T, Inoue O, Jung SM, Moroi M, Andrews RK, Berndt MC, Watson SP. 2002. Association of Fyn and Lyn with the proline-rich domain of glycoprotein VI regulates intracellular signaling. J Biol Chem 277(24):21561-6.

[219] Jandrot-Perrus M, Busfield S, Lagrue AH, Xiong X, Debili N, Chickering T, Le Couedic JP, Goodearl A, Dussault B, Fraser C, Vainchenker W, Villeval JL. 2000. Cloning, characterization, and functional studies of human and mouse glycoprotein VI: a platelet-specific collagen receptor from the immunoglobulin superfamily. Blood 96(5):1798-807.

[220] Ezumi Y, Uchiyama T, Takayama H. 2000. Molecular cloning, genomic structure, chromosomal localization, and alternative splice forms of the platelet collagen receptor glycoprotein VI. Biochem Biophys Res Commun 277(1):27-36.

[221] Moroi M, Jung SM. 2004. Platelet glycoprotein VI: its structure and function. Thromb Res 114(4):221-33.

[222] Lecut C, Arocas V, Ulrichts H, Elbaz A, Villeval JL, Lacapere JJ, Deckmyn H, Jandrot-Perrus M. 2004. Identification of residues within human glycoprotein VI involved in the binding to collagen: evidence for the existence of distinct binding sites. J Biol Chem 279(50):52293-9.

[223] Miura Y, Ohnuma M, Jung SM, Moroi M. 2000. Cloning and expression of the platelet-specific collagen receptor glycoprotein VI. Thromb Res 98(4):301-9.

[224] Ezumi Y, Shindoh K, Tsuji M, Takayama H. 1998. Physical and functional association of the Src family kinases Fyn and Lyn with the collagen receptor glycoprotein VI-Fc receptor gamma chain complex on human platelets. J Exp Med 188(2):267-76.

[225] Varga-Szabo D, Pleines I, Nieswandt B. 2008. Cell adhesion mechanisms in platelets. Arterioscler Thromb Vasc Biol 28(3):403-12.

[226] Attwood TK, Findlay JB. 1994. Fingerprinting G-protein-coupled receptors. Protein Eng 7(2):195-203.

[227] Ferguson SS. 2001. Evolving concepts in G protein-coupled receptor endocytosis: the role in receptor desensitization and signaling. Pharmacol Rev 53(1):1-24.

[228] Vaarala MH, Porvari KS, Kellokumpu S, Kyllonen AP, Vihko PT. 2001. Expression of transmembrane serine protease TMPRSS2 in mouse and human tissues. J Pathol 193(1):134-40.

[229] Su AI, Wiltshire T, Batalov S, Lapp H, Ching KA, Block D, Zhang J, Soden R, Hayakawa M, Kreiman G, Cooke MP, Walker JR, Hogenesch JB. 2004. A gene atlas of the mouse and human protein-encoding transcriptomes. Proc Natl Acad Sci U S A 101(16):6062-7.

[230] Tafazoli F, Holmstrom A, Forsberg A, Magnusson KE. 2000. Apically exposed, tight junction-associated beta1-integrins allow binding and YopE-mediated perturbation of epithelial barriers by wild-type *Yersinia* bacteria. Infect Immun 68(9):5335-43.

[231] Koch M, Foley JE, Hahn R, Zhou P, Burgeson RE, Gerecke DR, Gordon MK. 2001. alpha 1(Xx) collagen, a new member of the collagen subfamily, fibril-associated collagens with interrupted triple helices. J Biol Chem 276(25):23120-6.

[232] Krolewski JJ, Lee R, Eddy R, Shows TB, Dalla-Favera R. 1990. Identification and chromosomal mapping of new human tyrosine kinase genes. Oncogene 5(3):277-82.

[233] Haspel J, Grumet M. 2003. The L1CAM extracellular region: a multi-domain protein with modular and cooperative binding modes. Front Biosci 8:s1210-25.

[234] Gast D, Riedle S, Issa Y, Pfeifer M, Beckhove P, Sanderson MP, Arlt M, Moldenhauer G, Fogel M, Kruger A, Altevogt P. 2008. The cytoplasmic part of L1-CAM controls growth and gene expression in human tumors that is reversed by therapeutic antibodies. Oncogene 27(9):1281-9.

[235] Runyan SA, Roy R, Zhong H, Phelps PE. 2005. L1 CAM expression in the superficial dorsal horn is derived from the dorsal root ganglion. J Comp Neurol 485(4):267-79.

[236] Kobayashi M, Miura M, Asou H, Uyemura K. 1991. Molecular cloning of cell adhesion molecule L1 from human nervous tissue: a comparison of the primary sequences of L1 molecules of different origin. Biochim Biophys Acta 1090(2):238-40.

[237] Israely I, Costa RM, Xie CW, Silva AJ, Kosik KS, Liu X. 2004. Deletion of the neuron-specific protein delta-catenin leads to severe cognitive and synaptic dysfunction. Curr Biol 14(18):1657-63.

[238] Kosik KS, Donahue CP, Israely I, Liu X, Ochiishi T. 2005. Delta-catenin at the synaptic-adherens junction. Trends Cell Biol 15(3):172-8.

[239] Medina M, Marinescu RC, Overhauser J, Kosik KS. 2000. Hemizygosity of delta-catenin (CTNND2) is associated with severe mental retardation in cri-du-chat syndrome. Genomics 63(2):157-64.

[240] Harada H, Yoshida S, Nobe Y, Ezura Y, Atake T, Koguchi T, Emi M. 2002. Genomic structure of the human NLK (nemo-like kinase) gene and analysis of its promoter region. Gene 285(1-2):175-82.

[241] Rottinger E, Croce J, Lhomond G, Besnardeau L, Gache C, Lepage T. 2006. Nemo-like kinase (NLK) acts downstream of Notch/Delta signalling to downregulate TCF during mesoderm induction in the sea urchin embryo. Development 133(21):4341-53.

[242] Warren EH, Gavin MA, Simpson E, Chandler P, Page DC, Disteche C, Stankey KA, Greenberg PD, Riddell SR. 2000. The human UTY gene encodes a novel HLA-B8-restricted H-Y antigen. J Immunol 164(5):2807-14.

[243] Michon L, Nlend Nlend R, Bavamian S, Bischoff L, Boucard N, Caille D, Cancela J, Charollais A, Charpantier E, Klee P, Peyrou M, Populaire C, Zulianello L, Meda P. 2005. Involvement of gap junctional communication in secretion. Biochim Biophys Acta 1719(1-2):82-101.

[244] Munari-Silem Y, Rousset B. 1996. Gap junction-mediated cell-to-cell communication in endocrine glands--molecular and functional aspects: a review. Eur J Endocrinol 135(3):251-64.

[245] Durkin ME, Loechel F, Mattei MG, Gilpin BJ, Albrechtsen R, Wewer UM. 1997. Tissue-specific expression of the human laminin alpha5-chain, and mapping of the gene to human chromosome 20q13.2-13.3 and to distal mouse chromosome 2 near the locus for the ragged (Ra) mutation. FEBS Lett 411(2-3):296-300.

[246] Gorfu G, Virtanen I, Hukkanen M, Lehto VP, Rousselle P, Kenne E, Lindbom L, Kramer R, Tryggvason K, Patarroyo M. 2008. Laminin isoforms of lymph nodes and predominant role of alpha5-laminin(s) in adhesion and migration of blood lymphocytes. J Leukoc Biol 84(3):701-12.

[247] Webb AE, Sanderford J, Frank D, Talbot WS, Driever W, Kimelman D. 2007. Laminin alpha5 is essential for the formation of the zebrafish fins. Dev Biol 311(2):369-82.

[248] Longhi MT, Oliveira TR, Romero EC, Goncales AP, Morais ZM, Vasconcellos SA, Nascimento AL. 2009. A novel identified protein of *Leptospira interrogans* mediates binding to laminin. J Med Microbiol.

[249] Miner JH, Lewis RM, Sanes JR. 1995. Molecular cloning of a novel laminin chain, alpha 5, and widespread expression in adult mouse tissues. J Biol Chem 270(48):28523-6.

[250] Cameron CE. 2003. Identification of a *Treponema pallidum* laminin-binding protein. Infect Immun 71(5):2525-33.

[251] Tang YT, Hu T, Arterburn M, Boyle B, Bright JM, Emtage PC, Funk WD. 2005. PAQR proteins: a novel membrane receptor family defined by an ancient 7-transmembrane pass motif. J Mol Evol 61(3):372-80.

[252] Leung E, Kanwar RK, Kanwar JR, Krissansen GW. 2003. Mucosal vascular addressin cell adhesion molecule-1 is expressed outside the endothelial lineage on fibroblasts and melanoma cells. Immunol Cell Biol 81(4): 320-7.

[253] Briskin M, Winsor-Hines D, Shyjan A, Cochran N, Bloom S, Wilson J, McEvoy LM, Butcher EC, Kassam N, Mackay CR, Newman W, Ringler DJ. 1997. Human mucosal addressin cell adhesion molecule-1 is preferentially expressed in intestinal tract and associated lymphoid tissue. Am J Pathol 151(1):97-110.

[254] Shyjan AM, Bertagnolli M, Kenney CJ, Briskin MJ. 1996. Human mucosal addressin cell adhesion molecule-1 (MAdCAM-1) demonstrates structural and functional similarities to the alpha 4 beta 7-integrin binding domains of murine MAdCAM-1, but extreme divergence of mucin-like sequences. J Immunol 156(8):2851-7.

[255] Sugimoto Y, Narumiya S. 2007. Prostaglandin E receptors. J Biol Chem 282(16):11613-7.

[256] Watabe A, Sugimoto Y, Honda A, Irie A, Namba T, Negishi M, Ito S, Narumiya S, Ichikawa A. 1993. Cloning and expression of cDNA for a mouse EP1 subtype of prostaglandin E receptor. J Biol Chem 268(27):20175-8.

[257] Funk CD, Furci L, FitzGerald GA, Grygorczyk R, Rochette C, Bayne MA, Abramovitz M, Adam M, Metters KM. 1993. Cloning and expression of a cDNA for the human prostaglandin E receptor EP1 subtype. J Biol Chem 268(35):26767-72.

[258] Mutoh M, Watanabe K, Kitamura T, Shoji Y, Takahashi M, Kawamori T, Tani K, Kobayashi M, Maruyama T, Kobayashi K, Ohuchida S, Sugimoto Y, Narumiya S, Sugimura T, Wakabayashi K. 2002. Involvement of prostaglandin E receptor subtype EP(4) in colon carcinogenesis. Cancer Res 62(1):28-32.

[259] Frank M, Kemler R. 2002. Protocadherins. Curr Opin Cell Biol 14(5):557-62.

[260] Suzuki ST. 2000. Recent progress in protocadherin research. Exp Cell Res 261(1):13-8.

[261] Rechner C, Kuhlewein C, Muller A, Schild H, Rudel T. 2007. Host glycoprotein Gp96 and scavenger receptor SREC interact with PorB of disseminating *Neisseria gonorrhoeae* in an epithelial invasion pathway. Cell Host Microbe 2(6):393-403.

[262] Tamura Y, Osuga J, Adachi H, Tozawa R, Takanezawa Y, Ohashi K, Yahagi N, Sekiya M, Okazaki H, Tomita S, Iizuka Y, Koizumi H, Inaba T, Yagyu H, Kamada N, Suzuki H, Shimano H, Kadowaki T, Tsujimoto M, Arai H, Yamada N, Ishibashi S. 2004. Scavenger receptor expressed by endothelial cells I (SREC-I) mediates the uptake of acetylated low density lipoproteins by macrophages stimulated with lipopolysaccharide. J Biol Chem 279(30):30938-44.

[263] Shibata M, Ishii J, Koizumi H, Shibata N, Dohmae N, Takio K, Adachi H, Tsujimoto M, Arai H. 2004. Type F scavenger receptor SREC-I interacts with advillin, a member of the gelsolin/villin family, and induces neurite-like outgrowth. J Biol Chem 279(38):40084-90.

[264] Berwin B, Delneste Y, Lovingood RV, Post SR, Pizzo SV. 2004. SREC-I, a type F scavenger receptor, is an endocytic receptor for calreticulin. J Biol Chem 279(49):51250-7.

[265] Pitera JE, Scambler PJ, Woolf AS. 2008. Fras1, a basement membrane-associated protein mutated in Fraser syndrome, mediates both the initiation of the mammalian kidney and the integrity of renal glomeruli. Hum Mol Genet 17(24):3953-64.

[266] Schuller S, Neefjes J, Ottenhoff T, Thole J, Young D. 2001. Coronin is involved in uptake of *Mycobacterium bovis* BCG in human macrophages but not in phagosome maintenance. Cell Microbiol 3(12):785-93.

[267] Marshall TW, Aloor HL, Bear JE. 2009. Coronin 2A regulates a subset of focal-adhesion-turnover events through the cofilin pathway. J Cell Sci 122 (Pt 17):3061-9.

[268] Maniak M, Rauchenberger R, Albrecht R, Murphy J, Gerisch G. 1995. Coronin involved in phagocytosis: dynamics of particle-induced relocalization visualized by a green fluorescent protein Tag. Cell 83(6):915-24.

[269] Naiman BM, Alt D, Bolin CA, Zuerner R, Baldwin CL. 2001. Protective killed *Leptospira borgpetersenii* vaccine induces potent Th1 immunity comprising responses by CD4 and gammadelta T lymphocytes. Infect Immun 69(12):7550-8.

[270] Naiman BM, Blumerman S, Alt D, Bolin CA, Brown R, Zuerner R, Baldwin CL. 2002. Evaluation of type 1 immune response in naive and vaccinated animals following challenge with *Leptospira borgpetersenii* serovar Hardjo: involvement of WC1(+) gammadelta and CD4 T cells. Infect Immun 70(11):6147-57.

[271] Seixas FK, Fernandes CH, Hartwig DD, Conceicao FR, Aleixo JA, Dellagostin OA. 2007. Evaluation of different ways of presenting LipL32 to the immune system with the aim of developing a recombinant vaccine against leptospirosis. Can J Microbiol 53(4):472-9.

[272] Murray GL, Srikram A, Hoke DE, Wunder EA, Jr., Henry R, Lo M, Zhang K, Sermswan RW, Ko AI, Adler B. 2008. The major surface protein LipL32 is not required for either acute or chronic infection with *Leptospira interrogans.* Infect Immun. 46: 792-799

[273] Zhou Z, Hartwieg E, Horvitz HR. 2001. CED-1 is a transmembrane receptor that mediates cell corpse engulfment in C. elegans. Cell 104(1):43-56.

[274] Adachi H, Tsujimoto M, Arai H, Inoue K. 1997. Expression cloning of a novel scavenger receptor from human endothelial cells. J Biol Chem 272(50):31217-20.

[275] Barocchi MA, Ko AI, Reis MG, McDonald KL, Riley LW. 2002. Rapid translocation of polarized MDCK cell monolayers by *Leptospira interrogans*, an invasive but nonintracellular pathogen. Infect Immun 70(12):6926-32.

[276] Aumailley M, Bruckner-Tuderman L, Carter WG, Deutzmann R, Edgar D, Ekblom P, Engel J, Engvall E, Hohenester E, Jones JC, Kleinman HK, Marinkovich MP, Martin GR, Mayer U, Meneguzzi G, Miner JH, Miyazaki K, Patarroyo M, Paulsson M, Quaranta V, Sanes JR, Sasaki T, Sekiguchi K, Sorokin LM, Talts JF, Tryggvason K, Uitto J, Virtanen I, von der Mark K, Wewer UM, Yamada Y, Yurchenco PD. 2005. A simplified laminin nomenclature. Matrix Biol 24(5):326-32.

[277] Ekblom M, Falk M, Salmivirta K, Durbeej M, Ekblom P. 1998. Laminin isoforms and epithelial development. Ann N Y Acad Sci 857:194-211.

[278] Miner JH, Yurchenco PD. 2004. Laminin functions in tissue morphogenesis. Annu Rev Cell Dev Biol 20:255-84.

[279] Sasaki T, Fassler R, Hohenester E. 2004. Laminin: the crux of basement membrane assembly. J Cell Biol 164(7):959-63.

[280] de Hostos EL. 1999. The coronin family of actin-associated proteins. Trends Cell Biol 9(9):345-50.

[281] Merien F, Baranton G, Perolat P. 1997. Invasion of Vero cells and induction of apoptosis in macrophages by pathogenic *Leptospira interrogans* are correlated with virulence. Infect Immun 65(2):729-38.

[282] Liu Y, Zheng W, Li L, Mao Y, Yan J. 2007. Pathogenesis of leptospirosis: interaction of *Leptospira interrogans* with in vitro cultured mammalian cells. Med Microbiol Immunol 196(4):233-9.

[283] Li S, Ojcius DM, Liao S, Li L, Xue F, Dong H, Yan J. 2009. Replication or death: distinct fates of pathogenic *Leptospira* strain Lai within macrophages of human or mouse origin. Innate Immun.

[284] Fortner CN, Lorenz JN, Paul RJ. 2001. Chloride channel function is linked to epithelium-dependent airway relaxation. Am J Physiol Lung Cell Mol Physiol 280(2):L334-41.

[285] Nally JE, Chantranuwat C, Wu XY, Fishbein MC, Pereira MM, Da Silva JJ, Blanco DR, Lovett MA. 2004. Alveolar septal deposition of immunoglobulin and complement parallels pulmonary hemorrhage in a guinea pig model of severe pulmonary leptospirosis. Am J Pathol 164(3):1115-27.

[286] Nicodemo AC, Duarte MI, Alves VA, Takakura CF, Santos RT, Nicodemo EL. 1997. Lung lesions in human leptospirosis: microscopic, immunohistochemical, and ultrastructural features related to thrombocytopenia. Am J Trop Med Hyg 56(2):181-7.

[287] Bethlem EP, Carvalho CR. 2000. Pulmonary leptospirosis. Curr Opin Pulm Med 6(5):436-41.

[288] Andrade L, Rodrigues AC, Jr., Sanches TR, Souza RB, Seguro AC. 2007. Leptospirosis leads to dysregulation of sodium transporters in the kidney and lung. Am J Physiol Renal Physiol 292(2):F586-92.

[289] Narumiya S, Sugimoto Y, Ushikubi F. 1999. Prostanoid receptors: structures, properties, and functions. Physiol Rev 79(4):1193-226.

[290] Sugimoto Y, Namba T, Shigemoto R, Negishi M, Ichikawa A, Narumiya S. 1994. Distinct cellular localization of mRNAs for three subtypes of prostaglandin E receptor in kidney. Am J Physiol 266(5 Pt 2):F823-8.

[291] Matsuoka Y, Furuyashiki T, Bito H, Ushikubi F, Tanaka Y, Kobayashi T, Muro S, Satoh N, Kayahara T, Higashi M, Mizoguchi A, Shichi H, Fukuda Y, Nakao K, Narumiya S. 2003. Impaired adrenocorticotropic hormone response to bacterial endotoxin in mice deficient in prostaglandin E receptor EP1 and EP3 subtypes. Proc Natl Acad Sci U S A 100(7): 4132-7.

[292] Stock JL, Shinjo K, Burkhardt J, Roach M, Taniguchi K, Ishikawa T, Kim HS, Flannery PJ, Coffman TM, McNeish JD, Audoly LP. 2001. The prostaglandin E2 EP1 receptor mediates pain perception and regulates blood pressure. J Clin Invest 107(3):325-31.

[293] Durrenberger PF, Facer P, Casula MA, Yiangou Y, Gray RA, Chessell IP, Day NC, Collins SD, Bingham S, Wilson AW, Elliot D, Birch R, Anand P. 2006. Prostanoid receptor EP1 and Cox-2 in injured human nerves and a rat model of nerve injury: a time-course study. BMC Neurol 6:1.

[294] Han C, Wu T. 2005. Cyclooxygenase-2-derived prostaglandin E2 promotes human cholangiocarcinoma cell growth and invasion through EP1 receptor-mediated activation of the epidermal growth factor receptor and Akt. J Biol Chem 280(25):24053-63.

[295] Yang HL, Jiang XC, Zhang XY, Li WJ, Hu BY, Zhao GP, Guo XK. 2006. Thrombocytopenia in the experimental leptospirosis of guinea pig is not related to disseminated intravascular coagulation. BMC Infect Dis 6(1):19.

APPENDICES

APPENDIX A

BUFFER AND REAGENT

Reagents for EMJH media

1. **Albummin fatty acid supplement stock solution**

$CaCl_2$ + $MgCl_2 \cdot 6H_2O$	0.076	g	store at -20°C
$ZnSO_4 \cdot 7H_2O$	0.04	g	store at -20°C
$CuSO_4 \cdot H_2O$	0.03	g	store at 4°C
Vit B12	0.002	g	store at -20°C
Tween 80	1	g	store at -20°C
Glycerol	1	g	store at -20°C

Dissolve each reagent by separately in 10 ml of distilled water.

2. **Albumin fatty acid supplement solution, ready to use (50 ml)**

BSA	5	g
$CaCl_2$ + $MgCl_2 \cdot 6H_2O$	750	µl
$ZnSO_4 \cdot 7H_2O$	500	µl
$CuSO_4 \cdot 5H_2O$	50	µl
$FeSO_4$	0.025	g
Sodium pyruvate	0.02	g
Vitamin B12	500	µl
Tween 80	6.25	ml
glycerol stock	500	µl

Dissolve in distilled water and adjust pH 7.4-7.6 with HCl (conc). Adjust volume with distilled water to make 50 ml. Sterilize the solution by filtration. Store at -20°C.

3. **Basal Media (90 ml)**

Bacto Leptospira Media Base EMJH dehydrated	0.23	g

Dissolve in distilled water and adjust volume to 90 ml. Sterilize the solution by autoclaving at 121°C for 15 minutes.

4. **EMJH media**

Basal Media	90	ml
Albumin fatty acid supplement solution	10	ml

Mix the solution and store at 4°C.

Reagents for DNA Extraction

1. **0.5 M EDTA pH 8.0**

Disodium ethylenediamine tetraacetate	18.66	g

Dissolve in distilled water and adjust pH 8.0 with HCl (conc). Adjust volume with distilled water to make 100 ml. Sterilize the solution by autoclaving at 121°C for 15 minute.

2. **TE buffer**

1 M Tris-HCl pH 8.0	1	ml
0.5 M EDTA pH 8.0	200	µl

Dissolve in distilled water and adjust volume to 100 ml. Sterilize the solution by autoclaving at 121°C for 15 minutes.

3. **10% Sodium lauryl sarcosine (SDS)**

Sodium lauryl sarcosine (SDS)	1	g
Distilled water	10	ml

Mix the solution and store at room temperature.

4. **5 M NaCl (100 ml)**

NaCl	14.61	g

Dissolve in distilled water and adjust volume to 50 ml. Sterilize the solution by autoclaving at 121°C for 15 minutes.

5. **25:24:1 (v/v) phenol: chloroform: isoamyl alcohol**

Saturated phenol	50	ml
Chloroform	48	ml
Isoamyl alcohol	2	ml

Mix the reagent vigorously, store at 4 °C in dark.

6. **24:1 (v/v)chloroform: isoamyl alcohol**

Chloroform	48	ml
Isoamyl alcohol	2	ml

Mix the reagent vigorously, store at 4 °C in dark.

Reagents for agarose gel electrophoresis

1. **50x Tris-Acetate buffer (TAE)**

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

Dissolve in distilled water and adjust volume to 1 liter. Sterilize the solution by autoclaving at 121°C for 15 minutes.

2. **10 mg/ml Ethidium bromide**

Ethidium bromide	1.0	g
Distilled water	100	ml

Mix the solution and store in the dark at 4°C.

3. **Agarose gel**

Agarose	0.5	g (2.5 % gel)

(0.3 g for 1.5 % gel and 0.16 g for 0.8 % gel)

1xTAE	20	ml

The solution was dissolved by heating in microwave oven and occasional mix until no granules of agarose are present.

Reagents for Cloning

1. **1 M Glucose (10 ml)**

Glucose	1.8	g

Dissolve in distilled water and adjust volume to 10 ml. Sterilize the solution by filtration.

2. **2 M $MgCl_2$ (10 ml)**

$MgCl_2$	1.9	g

Dissolve in distilled water and adjust volume to 10 ml. Sterilize the solution by autoclaving at 121°C for 15 minutes.

3. **SOB (100 ml)**

Tryptone	2	g
Yeast Extract	0.5	g
NaCl	0.05	g
KCl	18.6	mg

Dissolve in distilled water and adjust volume to 500 ml. Sterilize the solution by autoclaving at 121°C for 15 minutes.

4. **SOC (10 ml)**

SOB	10	ml
2 M $MgCl_2$	50	µl
1 M Glucose	200	µl

Mix the solution and store at 4°C.

5. **Ampicilin stock (100 mg/ml)**

Ampicilin	1	g

Dissolve in 10 ml of distilled water. Store at -20°C.

6. **Chloramphenicol stock (35 mg/ml)**

Chloramphenicol	140	mg

Dissolve in 4 ml of absolute ethanol. Store at -20°C.

7. **LB Medium (1Litter)**

Bacto-Tryptone	10	g
Yeast Extract	5	g
NaCl	5	g

Dissolve in distilled water and adjust volume to 1 liter. Sterilize the solution by autoclaving at 121°C for 15 minutes.

8. **LB- Ampicillin plates**

Bacto-Tryptone	10	g
Yeast Extract	5	g
NaCl	5	g
Agar	15	g

Dissolve in distilled water and adjust volume to 1 liter. Sterilize the solution by autoclaving at 121°C for 15 minutes. After autoclaved, allowed media cool down, added 1 ml ampicilin stock, poured and stored plates at 4°C in the dark.

9. **LB- Ampicillin-Chloramphenicol plates**

Bacto-Tryptone	10	g
Yeast Extract	5	g
NaCl	5	g
Agar	15	g

Dissolve in distilled water and adjust volume to 1 liter. Sterilize the solution by autoclaving at 121°C for 15 minutes. After autoclaved, allowed media cool down, added 1 ml ampicilin stock and 1 ml chloramphenicol stock, poured and stored plates at 4°C in the dark.

Reagents for SDS- Polyacrylamide Gel Electrophoresis (SDS-PAGE)

1. **1 M Tris-HCl pH 8.8**

Tris base	12.11	g

Dissolve in distilled water and adjust pH to 8.8 with HCl (conc). Adjust volume with distilled water to make 100 ml. Sterilize the solution by autoclaving at 121°C for 15 minutes.

2. **0.5 M Tris-HCl pH 6.8**

Tris base	6.055	g

Dissolve in distilled water and adjust pH to 6.8 with HCl (conc). Adjust volume with distilled water to make 100 ml. Sterilize the solution by autoclaving at 121°C for 15 minutes.

3. **4xTris HCl/SDS pH 8.8 (100 ml)**

Tris base	18.21	g
SDS	0.4	g

Dissolve in distilled water and adjust pH to 8.8 with HCl (conc).Store at 4 °C

4. **Running Buffer (1 liter)**

Tris base	15.1	g
Glycine	72	g
SDS	5.0	g

Dissolve in distilled water and adjust volume to 1000 ml. Store at room temperature.

5. **6x sample buffer with DTT (10 ml)**

4xTris HCl/SDS pH 8.8	7	ml
Glycerol	3	ml
SDS	1	g
DTT	0.93	g
Bromphenol Blue	1.2	mg

Dissolve the solution and adjust volume to 10 ml. Store at room temperature.

6. **10% Ammonium Persulfate (APS)**

APS	1	g
Distilled water	10	ml

Mix the solution and store at -20 °C.

7. **10% Sodium lauryl sarcosine (SDS)**

Sodium lauryl sarcosine (SDS)	1	g
Distilled water	10	ml

Mix the solution and store at room temperature.

8. **30% Acrylamide/0.8% Bisacrylamide (100 ml)**

Acrylamide	30	g
Bisacrylamide	0.8	g

Dissolve the solution in distilled water and adjust volume to 100 ml. Sterilize the solution by filtration. Store in dark at room temperature.

9. **15% SDS-PAGE**

Separating gel (15 ml)

Acrylamide/bis	6.0	ml
1 M Tris-HCl pH 8.8	3.75	ml
10% SDS	0.15	ml
10% APS	75	µl
TEMED	7.5	µl
Distilled water	4.0	ml

Stacking gel (5 ml)

Acrylamide/bis	0.67	ml
0.5 M Tris-HCl pH 6.8	0.5	ml
10% SDS	40	µl
10% APS	40	µl
TEMED	4.0	µl
Distilled water	2.7	ml

Reagents for Colony blot and Western blot

1. **Denaturing Solution (500 ml)**

NaCl	10	g
NaOH	43.83	g

Dissolve in distilled water and adjust volume to 500 ml.

2. **Neutralization Solution (500 ml)**

NaCl	10	g
Tris base	30.275	g

Dissolve in distilled water and adjust volume to 500 ml.

3. **20x SSC (500 ml)**

NaCl	87.65	g
Trisodium citrate	50.25	g

Dissolve in distilled water and adjust volume to 500 ml.

4. **TBS (1 liter)**

1 M Tris base pH 7.5	20	ml
NaCl	29.22	g

Dissolve in distilled water and adjust volume to 1 liter. Sterilize the solution by autoclaving at 121°C for 15 minutes.

5. **TBS- 0.1 % (v/v)Tween (500 ml)**

TBS	500	ml
Tween-20	500	µl

Mix the solution and store at room temperature.

6. **Blotting Buffer (1 Litter)**

Tris base	2.42	g
Glycine	11.24	g
Distilled water	800	ml

Dissolve in distilled water and add 200 ml methanol. Store at room temperature.

7. **Alkaline Phosphate buffer (1 Litter)**

1 M Tris base pH 9.5	50	ml
NaCl	2.922	g
2 M $MgCl_2$	625	µl

Dissolve in distilled water and adjust volume to 1 liter. Sterilize the solution by autoclaving at 121°C for 15 minutes.

Reagent for Protein Purification

1. **20 mM Imidazole Binding Buffer (50 ml)**

8x Phosphate buffer stock solution pH 7.4	6.25	ml
2 M Imidazole stock solution pH 7.4	0.5	ml

Dissolve in distilled water and adjust pH to 7.4 with HCl (conc.). Adjust volume with distilled water to make 50 ml volume.

2. **500 mM Elution Buffer (10 ml)**

8x Phosphate buffer stock solution pH 7.4 (GE health care)	1.25	ml
2 M Imidazole stock solution pH 7.4 (GE health care)	2.5	ml

Dissolve in distilled water and adjust pH to 7.4 with HCl (conc). Adjust volume with distilled water to make 10 ml volume.

3. **20% Ethanol (Metal-Affinity Chromatography)**

Absolute Ethanol	100	ml

Dissolve in distilled water and adjust volume to 500 ml with distilled water.

4. **20% Ethanol (Gel filtration chromatography)**

   Absolute Ethanol 200 ml

   Dissolve in Milli Q water and adjust volume to 500 ml 1 L with Milli Q water.

5. **1x Phosphate buffered saline (PBS)**

   Na_2HPO_4 4.88 g

   $NaH_2PO_4.H_2O$ 1.54 g

   NaCl 3.04 g

   Dissolve in Milli Q water and adjust pH to 7.4 with HCl (conc). Adjust volume with Milli Q water to make 10 liter volume.

Reagents for phage peptide library screening

1. **LB Medium (1Litter)**

   Bacto-Tryptone 10 g

   Yeast Extracted 5 g

   NaCl 5 g

   Dissolve in distilled water and adjust volume to 1 liter. Sterilize the solution by autoclaving at 121°C for 15 minutes. Sterilize the solution by autoclaving at 121°C for 15 minutes.

2. **LB-Tet Plates**

   Bacto-Tryptone 10 g

   Yeast Extracted 5 g

   NaCl 5 g

   Agar 15 g

   Dissolve in distilled water and adjust volume to 1 liter. Sterilize the solution by autoclaving at 121°C for 15 minutes. After autoclaved, allowed media cool down, added 1 ml Tetracycline stock, poured and stored plates at 4°C in the dark

3. **LB/IPTG/Xgal Plates(1Litter)**

Bacto-Tryptone	10	g
Yeast extract	5	g
NaCl	5	g
Agar	15	g

Dissolve in distilled water and adjust volume to 1 liter. Sterilize the solution by autoclaving at 121°C for 15 minutes. After autoclaved, allowed media cool down, added 1 ml IPTG/Xgal stock, poured and stored plates at 4°C in the dark.

4. **Agarose Top (1 Litter)**

Bacto-Tryptone	10	g
Yeast extract	5	g
NaCl	5	g
Agar	15	g
$MgCl_2 \cdot 6H_2O$	1	g
Agarose	7	g

Dissolve in distilled water and adjust volume to 1 liter. Sterilize the solution by autoclaving at 121°C for 15 minutes. After autoclaved, allowed media cool down, dispense into 50 ml aliquots. Store solid at room temperature, melt in microwave before use.

5. **Tetracycline Stock**

Tetracycline	20	mg
Ethanol	10	ml

Mix the solution and store in the dark at 4°C.Vortex before using.

6. **1 M $NaHCO_3$ pH 8.6 (20 ml)**

$NaHCO_3$	0.84	g

Dissolve in 10 ml of distilled water. Sterilize the solution by autoclaving at 121°C for 15 minutes.

7. **Blocking buffer (10 ml)**

1 M $NaHCO_3$ (pH 8.6)	1	ml
Skim milk	25	mg

Dissolve in 10 ml of distilled water. Sterilize the solution by filtration. Store at 4°C.

8. **TBS-Tween (100 ml)**

TBS	100	ml
Tween 20	100	µl (for 0.1% (v/v), 500 µl for 0.5 % (v/v))

Mix the solution and store at room temperature.

9. **PEG/NaCl (100 ml)**

Polyethylene glycol–800	20	g
NaCl	14.61	g

Dissolve in distilled water and adjust volume to 500 ml with distilled water. Sterilize the solution by autoclaving at 121°C for 15 minutes.

10. **IPTG/Xgal**

IPTG (isopropyl β-D-thiogalactoside)	1.5	g
Xgal (5-Bromo-4-chloro-3-indolyl- β -D-galactoside)	1.0	g
Dimethyl formamide	25	ml

Mix the solution and store at -20°C in the dark.

11. 2 M Glycine-HCl pH 2.2 (10 ml)

Glycine	1.5	g

Dissolve in distilled water and adjust pH to 2.2 with HCl (conc). Adjust volume with distilled water to make 10 ml volume. Sterilize the solution by autoclaving at 121°C for 15 minutes.

12. Elution buffer (10 ml)

2 M Glycine-HCl pH 2.2	1	ml
Skim milk	10	mg

Dissolve in distilled water and adjust volume to 10 ml with distilled water.

13. 1 M Tris-HCl pH 9.1 (10 ml)

Tris base	12.11	g

Dissolve in distilled water and adjust pH to 9.1 with HCl (conc). Adjust volume with distilled water to make 100 ml. Sterilize the solution by autoclaving at 121°C for 15 minutes.

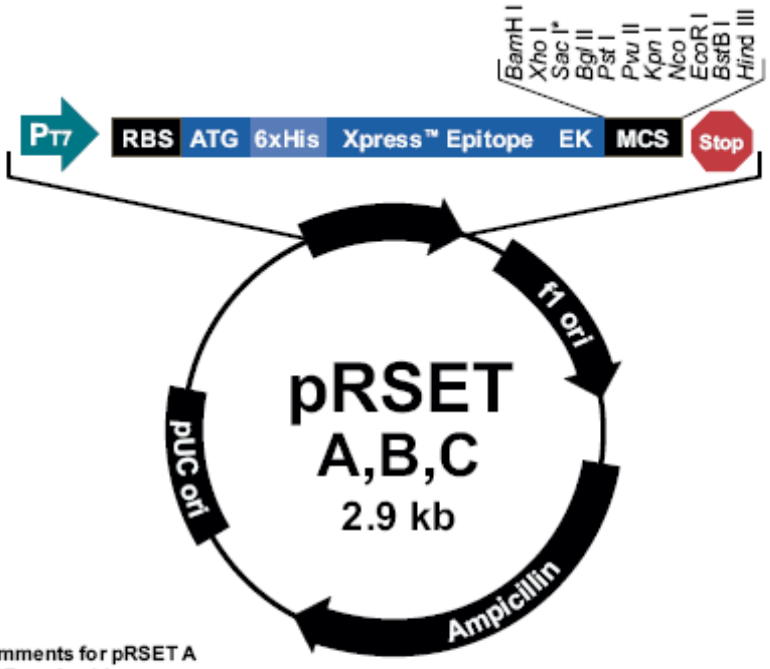
APPENDIX B

1. Complete sequence of *lipL32* gene

>w_lipl32_Pomona_/1/1692582/1693400

ATGAAAAAACTTTCGATTTTGGCTATCTCCGTTGCACTCTTTGCAAGCATTACCGCTTGTGGTGCTTT
CGGTGGTCTGCCAAGCCTAAAAAGCTCTTTTGTTCTGAGCGAGGACACAATCCCAGGGACAAACG
AAACCGTAAAAACGTTACTTCCCTACGGATCTGTGATCAACTATTACGGATACGTAAAGCCAGGACA
AGCGCCGGACGGTTTAGTCGATGGAAACAAAAAAGCATACTATCTCTATGTTTGGATTCCTGCCGTA
ATCGCTGAAATGGGAGTTCGTATGATTTCCCCAACAGGCGAAATCGGTGAACCAGGCGACGGAGA
CTTAGTAAGCGACGCTTTCAAAGCGGCTACCCCAGAAGAAAAATCAATGCCACATTGGTTTGATACT
TGGATCCGTGTAGAAAGAATGTCGGCGATTATGCCTGACCAAATCGTCAAAGCTGCGAAAGCAAAA
CCAGTTCAAAAATTGGACGATGATGATGATGGTGACGATACTTATAAAGAAGAGAGACACAACAAG
TACAACTCTCTTACTAGAATCAAGATCCCTAATCCTCCAAAATCTTTTGACGATCTGAAAAACATCGA
CACTAAAAAACTTTTAGTAAGAGGTCTTTACAGAATTTCTTTCACTACCTACAAACCAGGTGAAGTGA
AAGGATCTTTCGTTGCATCTGTTGGTCTGCTTTTCCCACCAGGTATTCCAGGTGTGAGCCCGCTGAT
CCACTCAAATCCTGAAGAATTGCAAAAACAAGCTATCGCTGCTGAAGAGTCTTTGAAAAAAGCTGCT
TCTGACGCGACTAAGTAA

2. Map of pRSET vector

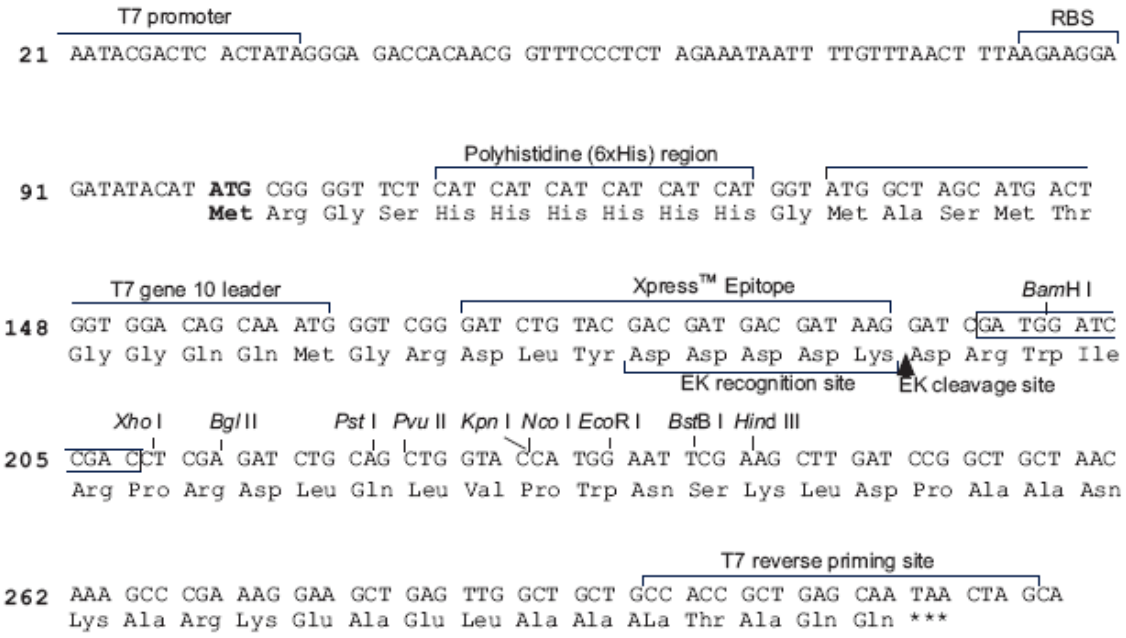


Comments for pRSET A
2897 nucleotides

*Version C does not contain *Sac* I

T7 promoter: bases 20-39
6xHis tag: bases 112-129
T7 gene 10 leader: bases 133-162
Xpress™ epitope: bases 169-192
Multiple cloning site: bases 202-248
T7 reverse priming site: bases 295-314
T7 transcription terminator: bases 256-385
f1 origin: bases 456-911
bla promoter: bases 943-1047
Ampicillin (*bla*) resistance gene (ORF): bases 1042-1902
pUC origin: bases 916-2852 (C)

3. Multi Cloning site of pRSET C



BIOGRAPHY

NAME	Miss Suwittra Chaemchuen
DATE OF BIRTH	17th May 1984
PLACE OF BIRTH	Surin, Thailand
INSTITUTION ATTENDED	King Mongkut's Institute of Technology Ladkrabang, 2002-2005
	Bachelor of Sciences (Biotechnology)