การค้นหาโปรตีนที่ผิวของเซลล์เจ้าบ้านที่มีปฏิสัมพันธ์กับโปรตีน LipL 32 ของเชื้อเลปโตสไปราสายพันธุ์ก่อโรค

นางสาวมาริยา เสวกะ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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IDENTIFICATION OF HOST SURFACE PROTEINS THAT INTERACT WITH LIPL32, THE MAJOR OUTER MEMBRANE PROTEIN OF PATHOGENIC *LEPTOSPIRA*

Miss Mariya Sewaka

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Medical Microbiology
(Interdisciplinary Program)
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มาริยา เสวกะ : การค้นหาโปรตีนที่ผิวของเซลล์เจ้าบ้านที่มีปฏิสัมพันธ์กับโปรตีน LipL 32 ของเชื้อเลปโตสไปราสายพันธุ์ก่อโรค. (IDENTIFICATION OF HOST SURFACE PROTEINS THAT INTERACT WITH LIPL32, THE MAJOR OUTER MEMBRANE PROTEIN OF PATHOGENIC *LEPTOSPIRA*) อ. ที่ปรึกษาวิทยานิพนธ์หลัก : ผศ.พญ.ดร.กนิษฐา ภัทรกุล 128 หน้า.

โรคเลปโตสไปโรซิสเป็นโรคติดเชื้อจากสัตว์สู่คน ซึ่งโรคนี้เกิดจากเชื้อ *Leptospira interrogans* อย่างไรก็ตามพยาธิกำเนิดของโรคยังไม่เป็นที่ทราบแน่ชัด โดยในขั้นตอนแรกของการติดเชื้ออาจเกิดจากปฏิสัมพันธ์ระหว่างผนังชั้นนอกของเชื้อก่อโรคกับเจ้าบ้าน ดังนั้นปฏิสัมพันธ์ระหว่างผนังชั้นนอกของเชื้อก่อโรคกับเจ้าบ้านอาจเป็นปัจจัยสำคัญในการก่อพยาธิกำเนิดของโรคเลปโตสไปโรซิส โปรตีน LipL32 เป็นโปรตีนที่พบมากที่สุดในผนังชั้นนอกของเชื้อ *Leptospira interrogans* โดยพบในเชื้อเลปโตสไปราสายพันธุ์ก่อโรคเท่านั้น นอกจากนั้นยังพบว่า โปรตีน LipL32 มีการแสดงออกในระดับสูง ในหนูทดลองที่ติดเชื้อ ทั้งที่ติดเชื้อเฉียบพลันและติดเชื้อเรื้อรัง และ โปรตีน LipL32 มีคุณสมบัติในการกระตุ้นภูมิคุ้มกันในเจ้าบ้าน ดังนั้นในการศึกษานี้ ได้ค้นหาโปรตีนของเจ้าบ้านที่มีปฏิสัมพันธ์กับโปรตีน LipL32 โดย วิธี far western blot ซึ่งใช้โปรตีนที่สกัดได้จากเซลล์เจ้าบ้านมาจับกับ recombinant LipL32 หลังจากได้โปรตีนเจ้าบ้านที่จับกับโปรตีนLipL32แล้ว จากนั้นใช้วิธี liquid chromatography–mass spectrometry เพื่อวิเคราะห์ประจุของโปรตีน และหาชนิดของโปรตีนที่สอดคล้องกับประจุ โดยใช้ Mascot program ซึ่งพบโปรตีนของเจ้าบ้าน คือ peroxiredoxin นอกจากนี้ ในการศึกษานี้ได้นำเทคโนโลยีการแสดงโปรตีนบนผิวเฟจมาค้นหาโปรตีนของเจ้าบ้านที่สามารถจับกับโปรตีน LipL32 โดยใช้ T7 select® cDNA liver phage display library มาจับกับ recombinant LipL32 หลังจากขั้นตอน bio-panning ได้ค้นหาชนิดของโปรตีนเจ้าบ้านที่สอดคล้องกับลำดับอะมิโนที่วิเคราะห์ได้ โดยพบโปรตีนของเจ้าบ้าน คือ ATPsynthase ดังนั้นจากการทดลองดังที่ได้กล่าวมาข้างต้นพบว่า โปรตีนของเจ้าบ้านที่อาจเกี่ยวข้องกับพยาธิกำเนิดของโรคเลปโตสไปโรซิส ได้แก่ peroxiredoxin และ ATPsynthase อย่างไรก็ตาม จะต้องมีการทดสอบการจับกันจริงของ โปรตีนเจ้าบ้านที่อาจเกี่ยวข้องกับพยาธิกำเนิดของโรคเลปโตสไปโรซิส กับ โปรตีนของ LipL32 ในอนาคตต่อไป

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MARIYA SEWAKA : IDENTIFICATION OF HOST SURFACE PROTEINS THAT INTERACT WITH LIPL32, THE MAJOR OUTER MEMBRANE PROTEIN OF PATHOGENIC *LEPTOSPIRA*.

ADVISOR : ASST. PROF. KANITHA PATARAKUL, M.D., Ph.D., 128 pp.

Leptospirosis is a zoonotic disease that causes public health problems worldwide. Pathogenic *Leptospira interrogans* is a causative agent of leptospirosis. However, pathogenesis of leptospirosis remains unclear. Surface-exposed outer membrane proteins (OMPs) are important for initial step of interactions between pathogenic *Leptospira* and host cells. LipL32 is the most abundant surface-exposed protein of pathogenic *Leptospira*, is highly conserved in all pathogenic leptospires but is absent in non-pathogenic *Leptospira*. It is expressed at high level in leptospires during acute lethal infection and is highly immunogenic.

This study aimed to identify host proteins that interact with LipL32. Using far western blot, peroxiredoxin was identified by liquid chromatography–mass spectrometry as a putative protein that interacts with LipL32. In addition, phage display screening was performed by using recombinant LipL32 as a target molecule for biopanning with T7 select® cDNA liver phage display library. Phages with the hightest affinity to LipL32 displayed protein sequence that matched ATPsynthase. However, bacterial pull-down assay was unsuccessful to identify specific host proteins that bound to wild-type *Leptospira* but not to *lipL32*$^{-}$ mutants. Therefore, binding of LipL32 to peroxiredoxin and ATPsynthase may play a role in pathogenesis of leptospirosis. However, further studies are required to confirm true interactions between these proteins and LipL32.

Department : Medical Microbiology Student's Signature ....

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

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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
PCR	Polymerase chain reaction
OMP	Outer membrane protein
ECM	Extra cellular matrix

LPS	Lipopolysaccharide
Lig	*Leptospira* Immunoglobulin-like
NF-kB	Nuclear factor kappa B
TLR	Toll-like receptor
ROS	Reactive oxygen species
RANTES	Regulated on activation normal T cell expression
iNOS	Inducible nitric oxide synthase
TNF-α	Tumor necrotic alpha
MAT	Microscopic agglutination test
MDCK	Madin-Darby canine kidney cells
PK-15	Porcine kidney epithelial cells
EDTA	Ethylenediamine tetraacetic acid
HUVE	Human umbilical vein endothelial cells
HEp-2	Human epithelial cell lines cells
FBS	Fetal bovine serum

CHAPTER I
INTRODUCTION

Leptospirosis is a zoonotic disease that causes public health problems worldwide especially in tropical countries. Transmission of leptospirosis occurs in both industrialised and developing countries [1]. In tropical countries, farming activities, contact with animals (rats, other rodents, and livestock), poor waste disposal, rainfall, and floods are mostly related to infection [2-4]. Previous studies report increasing outbreaks of leptospirosis after severe flooding [2, 5-7]. Furthermore, risk factors for infection of leptospirosis include walking barefoot, immersion in water, contact with floodwater, drinking river water, and having skin wounds [2, 8].

Clinical manifestations of leptospirosis are extremely broad spectrum of symptoms such as non-specific including fever, chills, headache, severe myalgia, conjunctival suffusion, anorexia, nausea, vomiting and prostration [9, 10]. Severe leptospirosis may manifest as jaundice, acute renal failure, pulmonary haemorrhage syndrome, myocarditis, or meningitis with mortality rate up to 50% [9, 11, 12]. Anicteric leptospirosis and icterohaemorrhagic leptospirosis are two forms of leptospirosis [13]

Host-pathogen interactions involve in bacterial adhesion and colonization, replication and dissemination of pathogens in the host. In addition, the interaction may be importance of the host immune system. The initial step of host interactions a surface layer or adhesions were produced by many pathogenic bacteria including pathogenic spirochetes [14-16]. The outer membrane proteins OMPs are considerable for interaction between pathogenic *Leptospira* and host cells [17-21]. In contrast to non-pathogenic leptospires, pathogenic *Leptospira* are able to adhere to and colonize host cells after haematogenous dissemination before causing organ damage [22, 23].

LipL32 is 32 kDa lipoprotein, the outer membrane lipoprotein of approximately major outer membrane protein in pathogenic *Leptospira* and description up to 75% of total outer membrane protein [17, 24]. This protein is highly conserved among pathogenic leptospires but is not found in non-pathogenic *Leptospira* [25, 26]. Several studies showed that LipL32 was expressed during both chronic and acute infection [27]. LipL32 was shown expressed on the surface of *Leptospira* in the proximal tubule and the interstitium of hamster kidneys [28]. In addition, LipL32 was found to be the dominant immunoreactive protein [28, 29]. Previous studies have been shown LipL32 was bind to bind host extracellular matrix (ECM) including laminin, collagen type I, collagen type V and fibronectins [30, 31]. Moreover, the characteristic of ECM-binding domain of LipL32 was found in the C-terminal region. ECM-binding protein homologs to LipL32 were identified in *Pseudoalteromonas tunicata* of which the protein homologs are immunologically cross-reactive [30, 31]. Although, previous study demonstrated that virulence of *lipL32* mutant in the hamster model of acute infection and rat model of chronic infection showed the same as that of the wild-type strain [32]. Nevertheless, through LipL32 is the most abundant surface exposed protein, highly expression in acute infection and conservation among pathogenic leptospires. Therefore, interaction between LipL32 and host proteins cannot be declined and may be a crucial in pathogenesis of leptospirosis.

Far western blot is a high-throughput method for screening protein-protein interaction including interacting partners in a library and receptor–ligand interactions [33]. The advantage of this method is to allow a prey protein to be endogenously expressed in cells, to determine direct binding of two proteins, or to identify a third protein mediating the physical complex between them. Far western blot has been used in many applications such as identifying binding partners of a bait protein [34-38].

Phage display has been used to study protein–protein interactions [39]. The foreign genes are inserted into the phage genome and fused to the gene encoding phage capsid protein. Then, the recombinant peptides are expressed on the surface of phage particles. Phage display provides a physical linkage between a displayed peptides and its DNA encoding sequence. After bio-panning step, the inserted sequence of obtained clones corresponds to peptides responsible for protein-protein interactions [40]. Phage display library can be divided into two types including random peptide libraries (RPLs) and natural peptide libraries (NPLs). RPLs are created by random fragments of DNA from synthetic random oligonucleotides. On the other hand, the NPLs are created by random fragments of genomic DNA or cDNA from a target organism. Therefore, NPLs could display fragments of natural proteins. Moreover, phage display technology has been used in many applications such as identification of receptor–ligand interactions [41-44], affinity selection of wide varieties of target receptors [40, 45, 46], new drug discovery, isolation of recombinant antibodies [46-50], epitope mapping [51-54], and vaccine development [55]. Using phage display method, identification of protein-protein interactions has been accomplished in many bacteria [56, 57]

In this study, we planned to identify host surface proteins that interacted with LipL32. Knowledge obtained from this study may be useful for better understanding of function of LipL32 and its role in pathogenesis of leptospirosis leading to vaccine development and treatment of leptospirosis in the future.

CHAPTER II
OBJECTIVE

Hypothesis

Surface proteins of host cells that interact with wild-type, pathogenic *Leptospira* are different from those interact with the *lipL32*$^{-}$ mutant.

Objective

To identify host surface proteins that interact with LipL32, the major outer membrane protein of pathogenic *Leptospira*.

CHAPTER III

REVIEW OF RELATED LITERATURES

Leptospira spp. and leptospirosis

Taxonomy and classification

Genus *Leptospira* is a member of phylum Spirochaetes, class Spirochaetes, order Spirochaetales, family *Leptospiraceae* [58]. Serovar and genospecies are classified based on serological typing and genotyping, respectively.

Serological classification

Pathogenic leptospires are grouped within the “interrogans” complex (later, *Leptospira interrogans* sensu lato) and non-pathogenic leptospires are grouped within the “biflexa” complex (later, *L. biflexa* sensu lato). Both complexes (*L. interrogans* and *L. biflexa*) have been serologically divided into serovars by the cross-agglutination adsorption test (CAAT) using antibodies directed mainly against its lipopolysaccharide (LPS) O antigen [59, 60]. Antigenically related serovars with common O antigen are arranged into serogroups. *L. interrogans* sensu lato have been classified into more than 200 serovars while *L. biflexa* sensu lato have been classified into more than 40 serovars (Figure1) [9, 61].

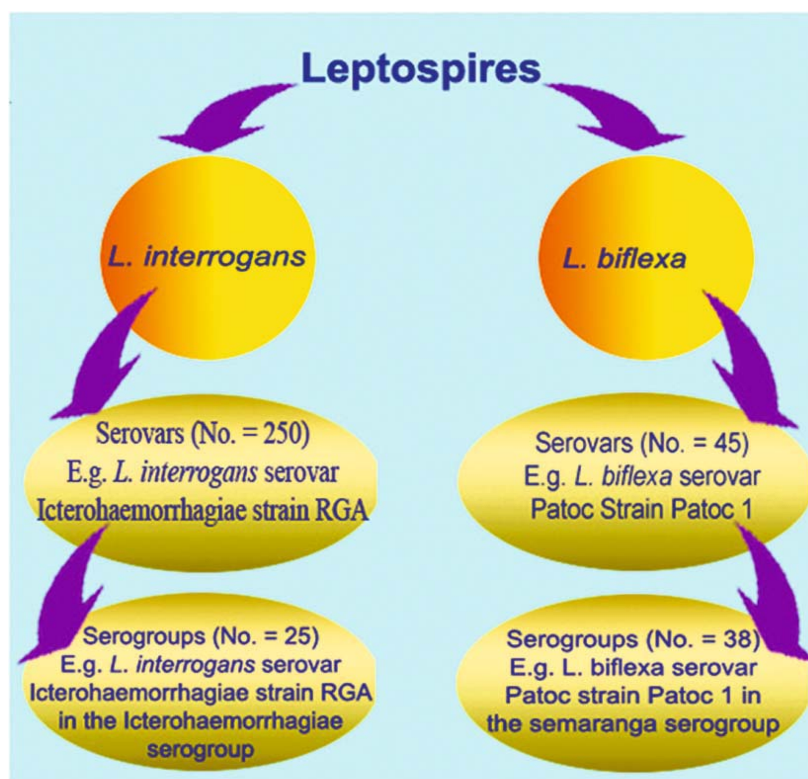

Figure 1. Serological classification of *Leptospira* spp. [61].

Genotypic classification

Genotyping is used to classify *Leptospira* into the same genospecies based on at least 70% DNA-relatedness, DNA sequence containing at most 5% unpaired bases, multilocus enzyme electrophoresis data, and 16sRNA sequences. Pathogenic *Leptospira* genospecies is currently comprised of 21 species based on 16sRNA sequences (Figure 2) [62-64]. However, genotyping classification of *Leptospira* does not correspond to serological classification. Different serovars are classified within the same genospecies and vice versa [62, 65].

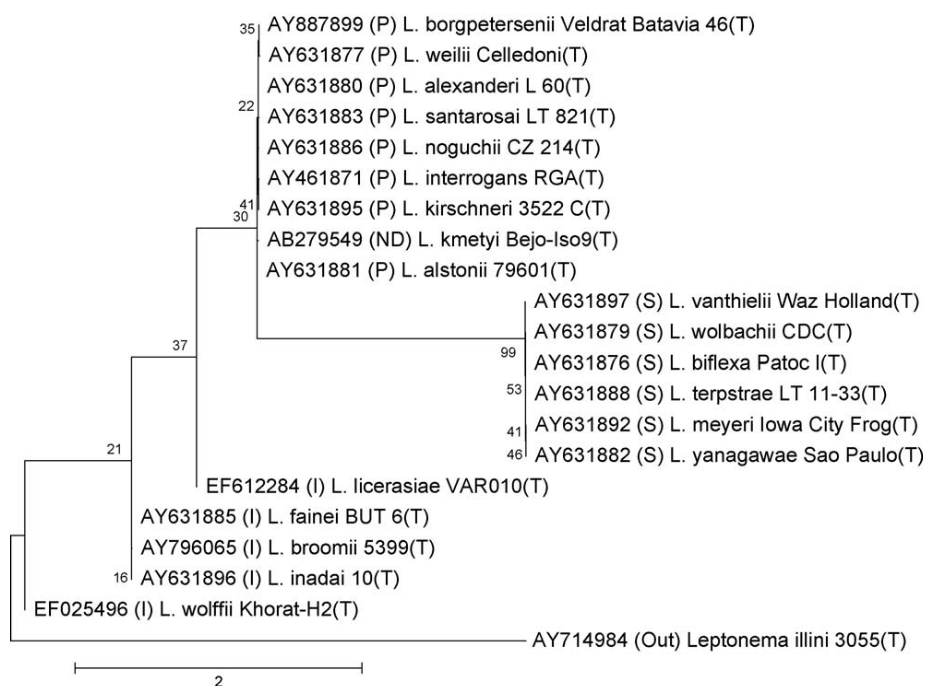

Figure 2. Phylogenetic tree based on the 16S rRNA gene sequences from *Leptospira* spp. type strains [64].

Microbiology

Leptospires are Gram negative, thin helical bacteria with a diameter of 0.2 μm, the length ranging from 10 to 20 μm and the wavelength of approximately 0.5 mm. Leptospires have characteristic hooked ends (Figure 3) [66]. The organism is highly motile and obligately aerobic bacterium [67, 68]. Two periplasmic flagella with polar insertions are responsible for motility [69].

Its double membrane structure contains the outer (OM) and inner (IM) [70]. Spirochetal outer membrane contains at least three types of proteins including lipoproteins, transmembrane proteins spanning the membrane and peripheral outer membrane proteins (OMPs) associated with the outer membrane [18]. Pathogenic *Leptospira* spp. have many outer membrane proteins of known and unknown functions (Figure 4) [17-21]. The peptidoglycan cell wall is associated with the IM [70]. The leptospiral OM contains lipopolysaccharide (LPS), the transmembrane porin outer membrane protein L1 (OmpL1) and lipoproteins such as LipL32, LipL36 (on the inner surface of the OM), LipL41 and LigB. Several TonB-dependent receptors (TBDRs) are involved in the transport of iron citrate, the siderophore desferrioxamine and hemin [19, 20]. TonB–ExbB–ExbD complex in the inner membrane are involved in the transport of energy. *Leptospira* possesses orthologues of the *E. coli* export systems that transport OMPs and lipoproteins [21], including the IM lipoprotein signal peptidase I (SPase I) and SPase II. Lipoproteins are first transported through the Sec system and then bind to the ABC transporter formed by LolC, LolD and LolE. OMPs are transported through the Sec translocon, bound to the periplasmic chaperone Skp and Omp85 before being integrated into the lipid bilayer. An incomplete set of type II secretion-like genes is also present in the *Leptospira* spp. genomes. Several cytoplasmic membrane ABC transporters are found in leptospires, including a metallic cation uptake family ABC transporter [19]. The surface-exposed Loa22, leptospiral endostatin-like protein A (LenA), LenD, LigA and LigC proteins are also known to be present at the surface of leptospires [71].

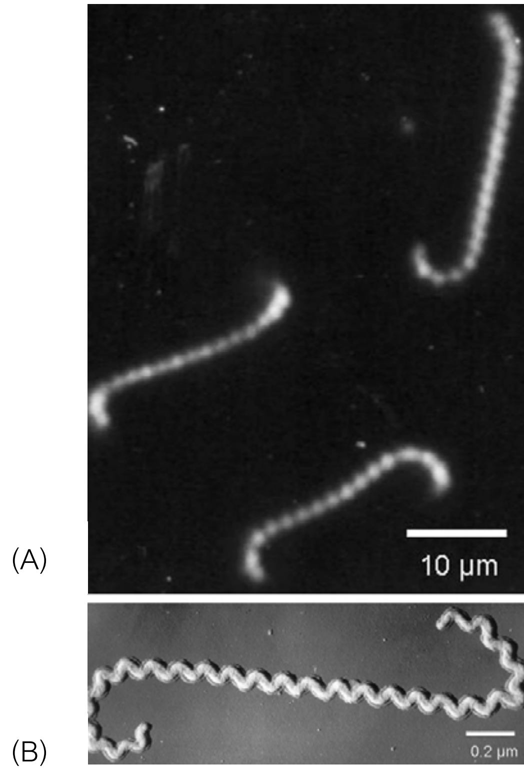

Figure 3. Visualization of *Leptospira interrogans* under dark-field microscope (A) and electron microscope (B) [66].

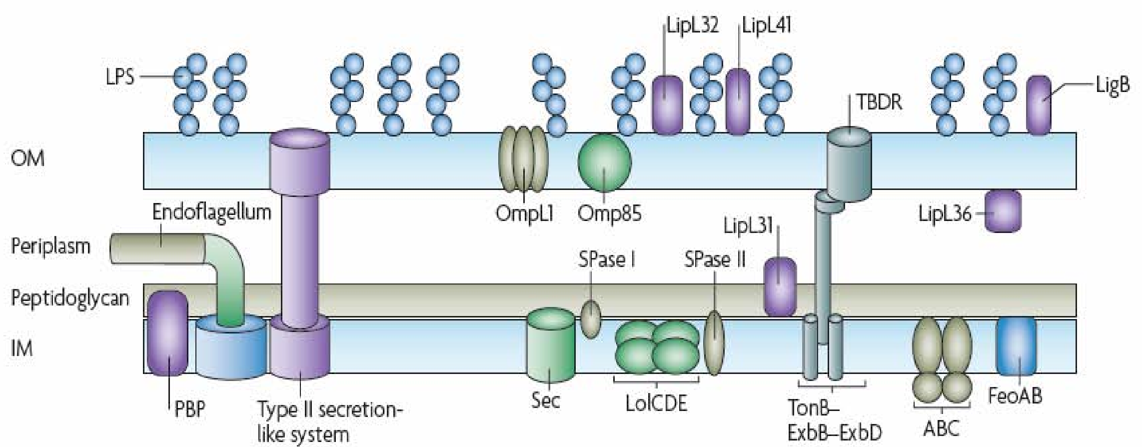

Figure 4. Schematic depiction of cell wall of pathogenic leptospires [23] .

Culture method

Leptospires are obligate aerobes. Optimal growth condition of leptospires is at pH 6.8-7.4 and at temperature of 28-30 °C [72-75]. Minimal growth temperature for saprophyte leptospires is about 5-10 °C, whereas minimal growth temperature for pathogenic leptospires is about 13-15 °C. Growth factors of leptospires are vitamin B1 and B12, ammonium salts and long-chain fatty acids. Long-chain fatty acids obtained from polysorbate (Tween) are carbon and energy sources [76, 77]. Ellinghausen-McCullough-Johnson-Harris (EMJH) medium is commonly used medium for leptospires. EMJH medium contains serum or albumin, vitamin B12, long chain fatty acids, ammonium salt and sodium pyruvate [78].

In liquid media leptospires grow about 10 to 14 days to reach log phase. Leptospires are maintained in semisolid agar. Leptospires grow near the surface of semisolid media (0.1–0.2% of agar) involving in the optimum oxygen tension and is known as a Dinger's ring or disk [9]. Leptospires are stored in liquid nitrogen for long-term preservation to maintain its virulence and give good yields [79].

Leptospires grow on solid agar, of which colony morphology depends on agar concentration and its serovar [80, 81]. Isolation of leptospires by solid media is used for separation of mixed cultures of leptospires and detection of hemolysin production[82].

Leptospira can survive longer in alkaline urine than in acid urine [66]. Pathogenic *Leptospira* may survive in the environment for a certain period of time contributed partly by biofilm formation [83]

Molecular biology

The size of leptospiral genome is approximately 5,000 kb [84, 85]. The genome is comprised of two circular chromosomes, a larger 4,400-kb chromosome and a smaller 350-kb chromosome [85]. Leptospires contain two sets of 16S and 23S rRNA genes [86]. The chromosome of *Leptospira* comprises a G+C content of 35-41 %. Previous studies comparing whole genome sequences of two pathogenic strains, *L. interrogans*, *L. borgpetersenii*, with 1 saprophytic strain, *L. biflexa* revealed that 2,052 genes (61% of whole genome) are similar to all. These findings support the idea of a common origin for leptospiral saprophytic strain and pathogenic strains. The genes common to the two pathogenic strains but absent in saprophytic strains may be responsible for pathogenesis of leptospirosis [87]. The genome of *Leptospira* spp. contains a number of genes involving in survival in the environment including genes used for production of exopolysaccharides such as genes encoding glycosyltransferases, alginate biosynthesis and lipopolysaccharide transport systems.

Until recently, the lack of genetic systems has impeded molecular analyses of pathogenic leptospires. The role of *L. interrogans* genes in virulence is currently possible to be identified by homologous recombination [88] and transposon mutagenesis [89].

Epidemiology

Leptospirosis is the most common zoonotic diseases and widespread zoonosis in the world [1], particularly, in developing countries, farming activities ,resource-poor, suburb slum poor waste disposal, rainfall [2-4] and floods are mostly related to infection [2, 5-7]. In addition, walking barefoot, drinking river water, contact with floodwater and having skin wounds are risk factors for infection of leptospirosis [2, 8].

Pathogenic *Leptospira* is a causative agent of leptospirosis. Animals concern in leptospirosis can be divided into reservoir hosts (maintenance hosts) and accidental hosts. The disease is maintained in nature by asymptomatic reservoir hosts [22]. Reservoir hosts can be domestic and wild animals such as rodents, small marsupials, cattle, pigs and dogs [9, 90]. Some serovars of pathogenic leptospires are commonly associated with certain animal reservoirs (Table 1) [60]. Proximal renal tubules are crucial for the persistence of *Leptospira* in the reservoir hosts. Pathogenic *Leptospira* spp. are shed into urine of reservoir hosts for a prolonged period of time [66] contaminating environment such as water and soil. Infection in human most commonly occurs from direct contact with infected urine or indirect contact with contaminated soil or water. Route of transmission to human is through mucous membrane and skin cuts or abrasions (Figure 5) [9, 91].

Table 1. Typical reservoir hosts of common leptospiral serovars

Reservoir host	Serovar (s)
Pigs	Pomona, Tarassovi
Cattle	Hardjo, Pomona
Horses	Bratislava
Dogs	Canicola
Sheep	Hardjo
Racoon	Grippotyphosa
Rats	Icterohaemorrhagiae, Copenhageni
Mice	Ballum, Arborea, Bim
Marsupials	Grippotyphosa
Bats	Cynopteri, Wolffi

Adapted from [22]

Leptospirosis is an emerging infectious disease in Thailand [7, 92, 93]. Before 1996, Department of Disease Control (DDC) reported approximately 200 cases per year. Most cases of leptospirosis were acquired in the central and southern regions of Thailand. In year 1996 the number of cases rised to 398 cases. The number of cases increased to 14,285 cases in year 2000 and 2,868 cases during year 2005 [94] . Paddy farmer, rat hunter and canal dredger are risk groups for leptospirosis in Thailand. Major affected areas are in the North-eastern region such as Buriram, Kalasin, Loei, Srisaket, Surin, Nakhon Sri Thammarat and Udonthani. The main outbreak season of leptospirosis in Thailand is rainfall during July to October. The Major serovars in Thailand are Icterohaemorrhagiae, Grippotyphosa, Autumnalis, Hebdomatis, Ranarum, Pyrogenes, Australis, Javanica, Sejroe, Bratislava, Pomona and Bangkok [95].

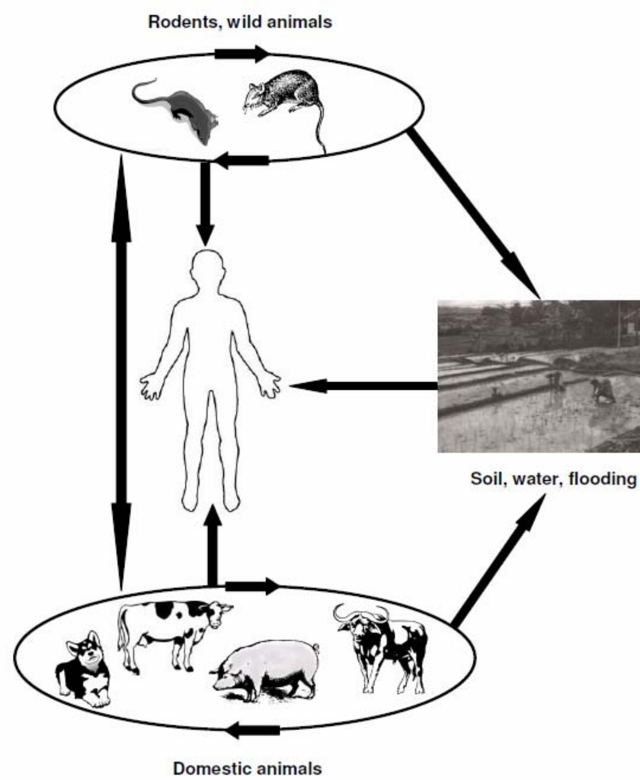

Figure 5. Epidemiology of leptospirosis. Pathogenic *Leptospira* are shed into urine of reservoir hosts. Infection in human most commonly occurs from direct contact with infected urine or indirect contact with contaminated soil or water [96].

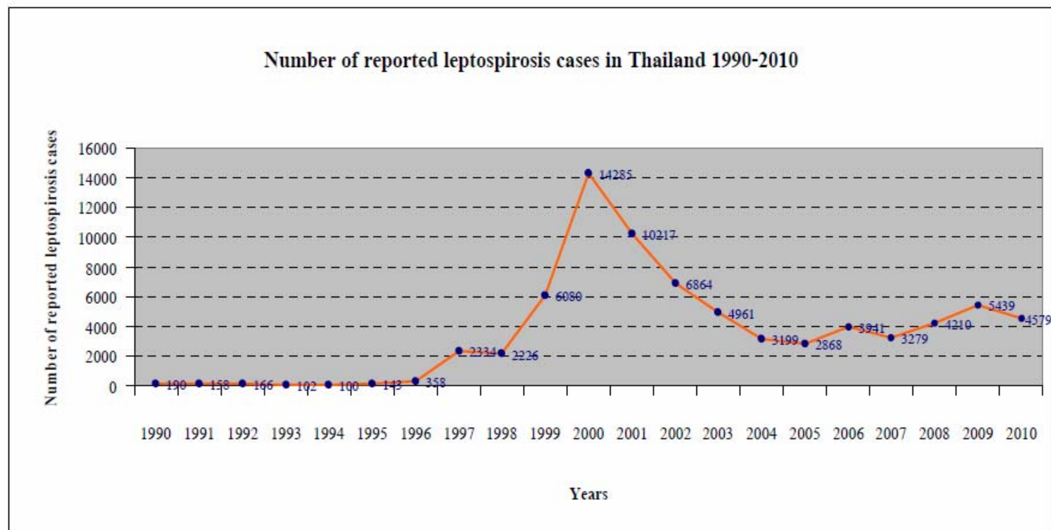

Figure 6. Reported cases of leptospirosis in Thailand during 1990-2010 [94].

Top Ten Leading Rate

1. Buri Ram	755
2. Si Sa Ket	481
3. Surin	466
4. Khon Kaen	366
5. Songkhla	288
6. Kalasin	225
7. Nakhon Ratchasima	146
8. Ubon Ratchathani	141
9. Roi Et	112
10. Nakhon Si Thammarat	110

Figure 7. Reported cases of leptospirosis by province in Thailand, 2010 [94].

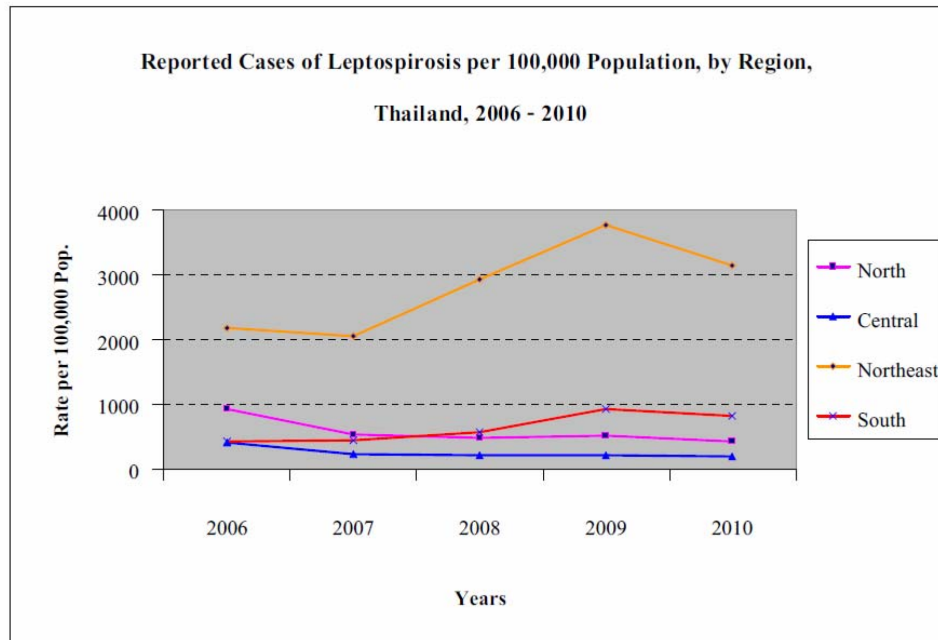

Figure 8. Reported cases of leptospirosis per 100,000 population by region in Thailand during 2006-2010 [94].

Clinical features of leptospirosis

Clinical features of leptospirosis are several ranging broad spectrum of symptoms such as fever, chills, headache, severe myalgia, conjunctival suffusion, nausea, and vomiting [9, 10]. Characteristics of severe leptospirosis include jaundice, acute renal failure, pulmonary haemorrhage syndrome, bleeding, meningitis, with case fatality rates exceeding 50% [9, 11, 12]. Clinical features of leptospirosis were devided into two forms including anicteric leptospirosis and icterohaemorrhagic leptospirosis or Weil’s disease [13].

Anicteric Leptospirosis

Major form of leptospirosis is anicteric leptospirosis. Anicteric leptospirosis manifests as subclinical or very mild symptoms such as chills, headache, myalgia, abdominal pain, and conjunctival suffusion. Anicteric leptospirosis mostly results in no mortality [97]. However, mortality occurred in 2.4% of the anicteric patients from massive pulmonary hemorrhage in a Chinese outbreak [98]. In most cases of anicteric leptospirosis the differential diagnosis must include influenza viral infection, HIV seroconversion and dengue [99, 100].

Icterohaemorrhagic Leptospirosis

Icterohaemorrhagic leptospirosis or Weil’s disease manifests severe symptoms and rapid progression, such as jaundice, renal failure and haemorrhage of target organs. Icterohaemorrhagic cases occur in about 5 and 10% of all patients with leptospirosis. Icterohaemorrhagic leptospirosis leads to high mortality rate. The mortality rate of icterohaemorrhagic leptospirosis is from 5 to 15% [9, 22]. The acute renal failure (ARF) is commonly found in 16 to 40% of leptospirosis cases [101, 102]. Pulmonary symptoms in cases of leptospirosis have been described which present with a ranging of symptoms, such as cough, dyspnea, and hemoptysis [103-109]. The death of patients in leptospirosis may be caused by pulmonary hemorrhage [12, 110-112]. Cardiac involvement in leptospirosis may be underestimated. Common cardiac

involvement in leptospirosis is myocarditis [113] resulting in about 54% mortality rate [114].

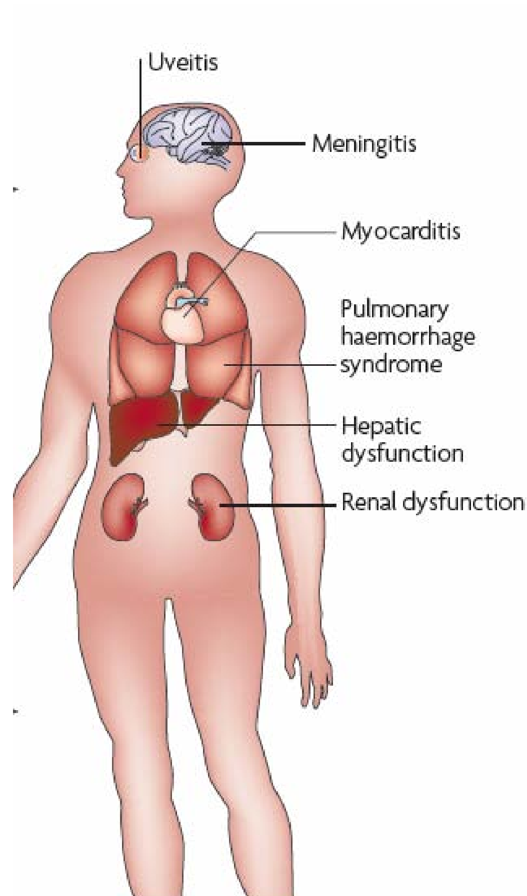

Figure 9. The symptoms of leptospirosis are multisystemic involvement such as uveitis hepatic dysfunction, acute renal failure, pulmonary haemorrhage syndrome, myocarditis and meningoencephalitis [23].

Laboratory diagnosis

Leptospires can be detected in urine, blood, tissues and CSF of patients. Diagnosis of leptospirosis is difficult because of the wide diversity of clinical manifestations and depends on laboratory assays. Therefore, confirmation tests are necessary for diagnosis of leptospirosis.

Microscopic visualization

Leptospires can be visualized by darkfield microscopy, immunofluorescence or light microscopy after appropriate staining such as silver staining. The body fluids such as blood, urine, CSF, and dialysate fluid are clinical samples for microscopic visualization. Approximately 10^4 leptospires/ml are minimal amount of leptospires required for observation by dark-field microscopy [115]. In addition, leptospires in tissues were detected by histopathological staining [116].

Cultivation

Samples of patients such as blood, CSF and dialysate fluid may be cultured during the first week of the acute illness. Urine can be cultured during the second week of symptomatic illness. However, duration of urinary excretion may vary [9, 117, 118]. The sample of patients can be cultured in semisolid medium then incubated the sample at 28 to 30°C and examined weekly by dark-field microscopy for up to 13 weeks [115, 119, 120]. Incubation periods of leptospires are long and growth rate of leptospires is slow. Therefore, culture is not used as a routine test for diagnosis [121, 122].

Serological diagnosis

Serological methods are commonly used for diagnosis of leptospirosis. Antibodies can be detected in the blood approximately 5 to 7 days after the beginning of symptoms. Microscopic agglutination test (MAT) is the gold standard for serological diagnosis. The MAT is performed by mixing patient serum with live antigen suspensions

of *Leptospira*. After incubation, agglutination of serum-antigen mixtures is detected under dark-field microscope. The positive result of MAT is determined by four-fold rising in titer between paired sera. The advantage of MAT is specificity for serovars, or at least serogroups and high sensitivity [123-127]. Many other methods have been applied to serological diagnosis, for examples, ELISA [128-133] , complement fixation test [123] , macroscopic slide agglutination [134-136], latex agglutination [137-140], dipstick ELISA [141-143] and immunofluorescence [144, 145].

Molecular diagnosis

Polymerase chain reaction (PCR) can detect DNA of leptospires in human samples. Various primer pairs for PCR detection of leptospires have been described such as some based on specific gene targets [146], 16S or 23S rRNA genes [147-151] and repetitive elements [152-156]. Recently, real-time PCR assay was reported for the rapid detection of pathogenic leptospires [151]. Moreover, lipL32 gene of pathogenic leptospires can be detected by the real-time PCR [25] .

Pathogenesis

Pathogenesis of leptospirosis remains unclear. Several virulence factors of pathogenic leptospiral were reported but the functions of virulence factors remains mostly unknown. Pathogenesis of leptospirosis may be caused by direct effect from *Leptospira* and indirect effect from host immune response to infection.

Previous studies found that many conserved OMPs of pathogenic *Leptospira* such as Loa22 [157], LipL32 [31], Lsa21 [158], LigA and LigB [159, 160] can bind to extracellular matrix proteins of host cells such as fibronectin, fibrinogen, collagen and laminin. Recent development of mutagenesis system has been used to identify virulence factors involved in pathogenesis of leptospirosis [32, 161] .

Motility may facilitate invasion of pathogenic *Leptospira* into host tissues [162, 163]. Approximately 50 hypothetical genes that involve in motility and chemotaxis were shown in whole genome sequence of pathogenic *Leptospira* [164-166].

Leptospiral lipopolysaccharide (LPS) was shown to be endotoxin with endotoxic activity less than that of other Gram-negative bacteria [167-170]. In addition, the isolation of chronically infected rat kidneys showed that the expression of O antigen of leptospiral LPS significantly higher than that isolated from the livers of guinea pigs with acute infection. Therefore, the expression of O antigen of leptospiral LPS may determine acute or chronic infection of infected hosts [171].

Analysis of genome of *L. interrogans* revealed nine genes encoding haemolysins which are not found in the saprophytic *L. biflexa* [87, 172]. Several hemolysins have been described in pathogenic leptospires [163, 172-177] such as sphingomyelinase C [177], sphingomyelinase H [172] and haemolysins-associated protein-1 (Hap-1, or LipL32). Sphingomyelinase H acted as a cytotoxic pore-forming protein on several mammalian cells. However, hemolytic activity has not been proved in *vivo* [172]. In addition, *L. interrogans* contains a microbial collagenase that may be involved in the destruction of host tissue.

Previous studies showed that adherence of pathogenic leptospires to cell monolayer of Madin-Darby canine kidney cells (MDCK), porcine kidney epithelial cells

(PK-15), and human umbilical vein endothelial cells (HUVE) was decreased after treatment with proteases [178] but there was no statistically significant change in adherence when cells were pretreated with neuraminidase, sodium metaperiodate, or lipase [178]. Therefore, host surface receptors for pathogenic *Leptospira* are proteinaceous in nature. In addition, the binding level of pathogenic *Leptospira* to human epithelial cell lines cells (HEp-2) lifted with trypsin-EDTA was less than that of cells treated with EDTA alone, a method to remove extracellular matrix proteins [179], suggesting that *L. interrogans* binds to surface proteins of host cells other than extracellular matrices. Therefore, protein-protein interactions between pathogenic *Leptospira* and proteins on mammalian cell surface may be important in pathogenesis of leptospirosis. However, these surface proteins of host cells have not been characterized [178, 179].

Immune-mediated pathogenesis of leptospirosis has been reported. Leptospires and their lipoprotein extracts stimulated monocytes leading to intracellular signaling such as p38 phosphorilation , NF-kB activation, and release of cytokines and nitric oxide [180, 181]. In addition, *Leptospira* components such as peptidoglycan and LPS are able to induce tumor necrosis factor (TNF) [182]. LPS of leptospires stimulated mouse macrophages by Toll-like receptor (TLR)2 and TLR4 [183] but in human macrophages, LPS of leptospires stimulated by TLR2 instead of TLR4 [184].

Host immunity against pathogenic leptospires remains unclear. However, the humoral immunity is the primary protective immune response against *Leptospira* [185]. LPS extract lacking endotoxic activity stimulated innate immunity, induced a serovar-specific antibody and induced protective immunity in rabbits, guinea pigs, mice, hamsters and dogs [186-190]. Several pathogenic leptospiral OMPs such a OmpL1 [191], LipL41 [191, 192], LipL32 [193, 194] ,LipL21 and Lig protein can generate broadly cross-protective immunity, which induced synergistic protection in animal models [191]. Cell-mediated immune responses to leptospirosis have been described in cattles and some human cases [195, 196]. In some cases of uveitis patients appears to involve the production of antibodies against a leptospiral antigen, which cross-react with ocular tissues [197, 198]. In addition, in some cases of leptospirosis, such antibodies

are directed against cryptantigens on damaged platelets possibly resulting in thrombocytopenia [199].

OMPs of *Leptospira*

Outer membrane of pathogenic *Leptospira* comprises of phospholipids, outer membrane protein (OMPs) and lipopolysaccharide (LPS). Previous studies reported that surface adhesins were produced by several pathogenic bacteria including pathogenic spirochetes for initial host interactions [14-16]. Surface-exposed OMPs are crucial for initial step of interaction between pathogenic *Leptospira* and host cells [17-21]. In contrast to non-pathogenic leptospires, pathogenic *Leptospira* are able to adhere to, colonize and invade host tissues. Therefore, OMPs may be crucial for pathogenesis of leptospirosis [22, 23].

Three classes of OMPs of *Leptispira* were determined based on their location and fractionation in detergents; (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 but functions of OMPs in pathogenic *Leptospira* are not well understood [200].

Previous studies found that many conserved OMPs of pathogenic *Leptospira* such as, LipL32 [31], Loa22 [157], Lsa21 [158], LigA and LigB [159, 160] can bind to extracellular matrix proteins of host cells such as fibronectin, fibrinogen, collagen and laminin.

The properties and functions of some OMPs have been described. LipL32 is the major component of the outer membrane proteome, highly conserved among pathogenic leptospires, and was shown to express during acute lethal infection [27]. However, *lipL32* deficient mutant still retained its virulence in experimental animals [32]. Loa22 is up-regulated during acute infection. Loa22 was recognized by sera of patients with leptospirosis [27]. Recent development of mutagenesis systems has been used to identify virulence factors involved in pathogenesis of leptospirosis. A surface-exposed protein Loa22 is the first virulence factor following Koch's molecular postulates. A *loa22*$^{-}$

mutant strain was shown to reduce virulence in guinea pig and hamster models compared to its corresponding wild type [161].

LipL21 is the second most abundant OMP and conserve among pathogenic strains. LipL21 was recognized by infected hamster sera [201, 202].

Endostatin-like protein A (Len A) was shown to bind human plasminogen. LenA-bound plasminogen could be converted to plasmin, resulting in degradation of fibrinogen [203]. Therefore, LenA may enhance *Leptospira* dissemination through host tissues [203].

LigA, LigB and LigC are immunoglobulin-like, surface-exposed proteins that are conserved among pathogenic leptospires [204, 205]. However, virulence of a LigB mutant strain was not reduced in animal models [88].

Osmolarity and temperature are shown to be crucial signals for regulating the expression of leptospiral OMPs that involve in infection of mammalian hosts [206, 207]. For example, *lig*A, *lig*B, *sph2, betA* were reported to be up-regulated under physiologic osmolarity [206] and *lig*B, *lig*C, *sph2* genes were up-regulated upon physiologic temperature shift [206, 207].

LipL32

LipL32 is the most abundant surface-exposed protein of pathogenic *Leptospira*, accounting for 75% of total OMPs [17, 24]. This protein is highly conserved in all pathogenic leptospires but is absent in non-pathogenic *Leptospira*. Therefore, it has been used as one of targets for diagnosis of leptospirosis such as in polymerase chain reaction and enzyme-linked immunosorbent assays [25, 26]. LipL32 was demonstrated to express at high level in leptospires during acute lethal infection [27]. It is highly immunogenic [28, 29]. Immunohistochemical analysis has shown that LipL32 was expressed on the surface of *Leptospira* in the proximal tubule and the interstitium of hamster kidneys, suggesting its function in tubular colonization [28]. Purified LipL32 induced the expression of Toll-like receptor 2 (TLR2) and stimulated the release of the monocyte chemoattractant protein–1 (MCP-1), RANTES, nitric oxide synthase (iNOS) and tumor necrosis factor–α (TNF-α) in mouse proximal renal tubule cells [208, 209]. In addition, LipL32 has been shown to act as an adhesin for extracellular matrices such as collagen, laminin and fibronectin [30, 31]. LipL32 at amino acid residues 21–272 showed $Ca^{2}+$ binding activity [210]. Surprisingly, virulence of *lipL32*$^{-}$ mutant constructed by transposon mutagenesis was not attenuated in hamsters and rats used as a model of acute and chronic infection, respectively. It is possible that function of LipL32 may be redundant and other OMPs may compensate its absence in the *lipL32* $^{-}$ mutant [32]. Similarly, potential virulence factors such as *ligB* [88], *ligC*, *lenB* and *lenE* were not essential for virulence in animal models [89]. These studies supported that *L. interrogans* has a high degree of redundancy in virulence mechanisms. However, due to its high expression in acute infection and conservation among pathogenic leptospires, interaction of LipL32 to host proteins as a part of host-microbe interaction cannot be excluded and may play a role in pathogenesis of leptospirosis.

Bacterial Pull-down assays

The pull-down assay is an *in vitro* method used to determine protein-protein interactions. Pull-down assays are useful for either confirming protein-protein interactions or initial screening for new proteins that interact with a bait protein. In a bacterial pull-down assay, surface membrane proteins on whole cell bacteria are used as bait proteins. Then, bait proteins are incubated with "prey" proteins, such as a host cell lysate and biotinylated cell lysate.

The advantages of bacterial pull-down assays are that (i) several proteins can be screened simultaneously (ii) proteins used in the assay are in their native forms due to using living cells (iii) it allows prey proteins to be expressed endogenously in cells especially if they are difficult to be purified (iv) it enables to determine direct binding of two proteins or identify a third protein mediating the physical complex between them. In addition, the method is rather simple, rapid to set up and inexpensive. However, disadvantage of this method is related to non-specific binding due to using whole cells in the assay [211].

Bacterial pull-down assays have been used to investigate protein-protein interactions in many bacteria such as *Rickettsia conorii* [212] and *Neisseria gonorrhoeae* [213]. Host surface proteins were labeled with biotin and solubilized with detergent. Cell lysate (prey proteins) was then incubated with bacteria (bait proteins) at optimized conditions. After non-specific binding proteins were removed by washing, bacteria binding proteins were subjected to SDS-PAGE and immunoblotting followed by analysis with mass spectrometry (Figure 10) [212, 213] .

In this study, bacterial pull-down assay was used to screen for host proteins that interact with LipL32.

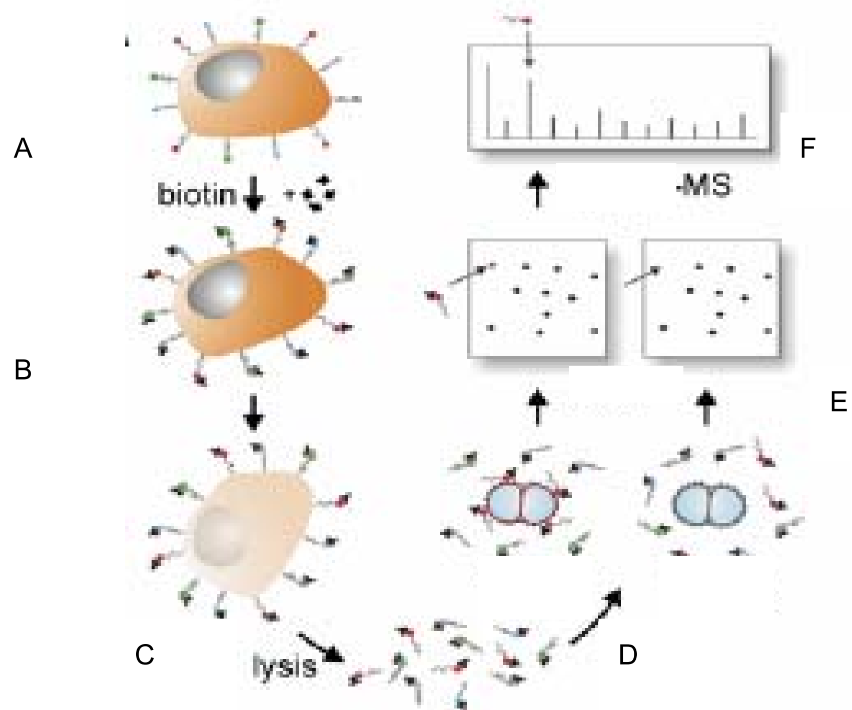

Figure 10. The bacterial pull-down assay [213].

A. The surface proteins of cells are labeled with biotin.

B. Cells were lysed with detergent.

C. Cell lysate was incubated with bacteria at optimum conditions.

D. Non-specific binding proteins were washed.

E. Bacteria binding proteins were subjected to SDS-PAGE and immunoblotting.

F. Proteins of interest were selected and analyzed by MS.

Far western blot

Far western blot has been used to study protein–protein interactions, for example, screening for interacting partners in a library and identifying receptor–ligand interactions [33]. The advantages of far western blot are that (i) it allows a prey protein to be endogenously expressed in cells, especially when prey proteins are difficult to purify (ii) it enables to determine two proteins directly binding or a third protein mediates the physical complexing between them. However, disadvantages of this method are that at least one protein should be purified to certain amounts, some protein are not native forms such as denatured proteins on SDS-PAGE and binding conditions need to be optimized to minimize non-specific binding.

Far western blot has been used in investigation of identifying binding partners of a bait protein [36, 38, 214] such as interaction between surface outer membrane proteins of *Francisella tularensis* and plasminogen [215], The sending out substrate of the *Streptococcus gordonii* accessory sec system interact with accessory Sec proteins (Asp)2 and accessory Sec proteins (Asp)2 [216] and confirming protein–protein interactions [37, 217]. Moreover, it can also be used to study the effect of post-translational modifications on protein–protein interactions. Far western blot can be performed in any laboratories where facilities for protein purification and standard western blot are available.

Far western blot detects the bait protein immobilized by its binding partner (prey protein) on the membrane at the position of the prey protein to which the bait protein binds (Figure 11). Since protein–protein interactions may depend on protein secondary and tertiary structures, protein denaturation occurring at any steps of purification or separation during SDS-PAGE may interfere with the binding result. Therefore, proteins may be denatured with guanidine or urea and then renatured by incubating with gradient-reducing concentration of guanidine or urea, which allows proteins to refold into their secondary and tertiary structures [35, 218-220]. However, refolding process of proteins to their native conformation may not be fully complete.

In this study, Far western blot was used for screening host proteins that interact with rLipL32.

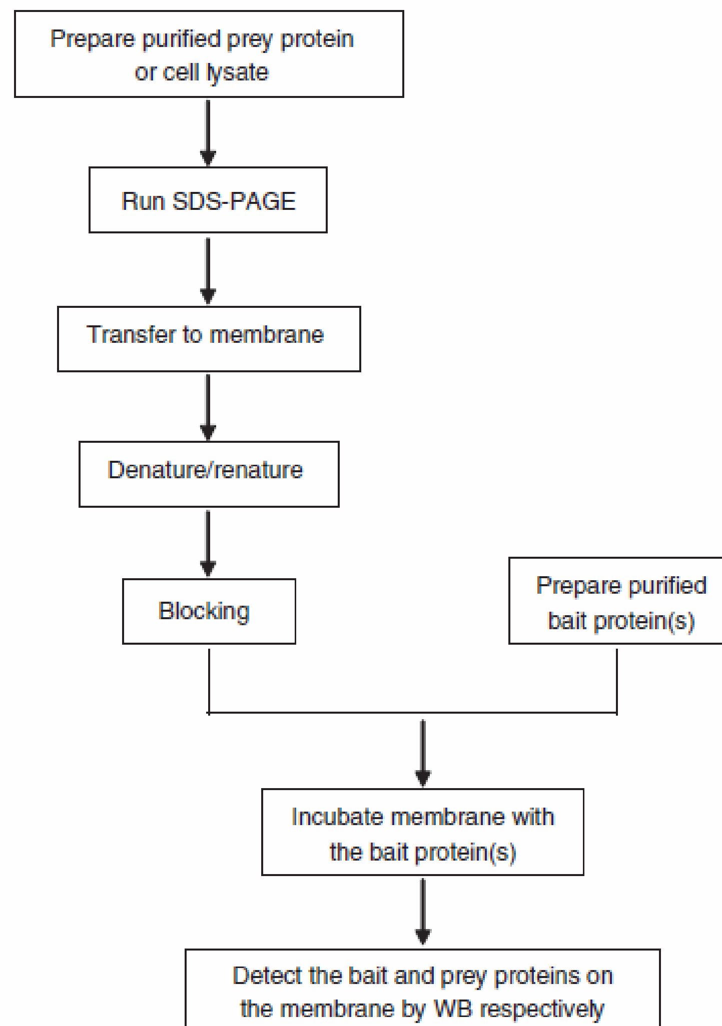

Figure 11. Flowchart of Far western blotting [218].

Phage display screening

Phage display is high-throughput screening technique to study protein–protein interactions [39]. Phage display library was constructed by based on the molecular technique. The phage genome was inserted by foreign genes. Then, the fusion of phage capsid proteins and foreign peptides was expressed on the surface of phage particles.
In phage display has been allowed a physical linkage between genotype and phenotype of phages. Phages with selection to the target protein were enriched by bio-panning and washing to take off non-specific binding phages then bound phages were amplified. At the final round of bio-panning, DNA of the bound clones was amplified by polymerase chain reaction then it was sequenced. DNA sequences correspond to the peptide sequences of displayed polypeptides on the selected phages [40, 221, 222].

The advantages of phage display technique are that (i) several proteins can be screened simultaneously; (ii) affinity selection is performed to obtain high selectivity and specificity; (iii) direct linkage between genotype and phenotype provides rapid identification of amino acid sequences; (iv) the stringency of washing during bio-panning can be adjusted to retrieve specific bound phages. In addition, the method is simple, rapid to set up and inexpensive. However, disadvantages of this method is that using host bacterial strain may result in wrong folding and modification of peptides or proteins [40].

T7 bacteriophage

T7 bacteriophage is a type of *Escherichia coli* bacteriophages. Currently, bacteriophage T7 has been used in peptide phage-display systems. The advantages of bacteriophage T7 are that (i) the system is easy to use since it is easy to grow and it replicates faster than filamentous phages resulting in decreasing the time required to perform bio-panning. Plaques of bacteriphage T7 may form within 3 hours at 37°C and cause culture lysis 1–2 hours after infection whereas lysis by filamentous phages may require 4.5 hours after infection; (ii) bacteriophage T7 can display peptides up to about 50 amino acids in size in high copy number (415 per phage), and peptides up to about 1,200 amino acids in low copy number (0.1-1 per phage); (iii) T7 phage particles are stable in severe conditions including up to 5 M sodium chloride, 4 M urea, 2 M guanidine-HCL, reducing condition with DTT at up to 100 mM and alkali up to pH 10 which not tolerated by other phages; (iv) T7 bacteriophage system is an efficient technique for investigation of protein-protein interactions.

Structure of T7 bacteriophage consists of a head encapsulating linear double-stranded DNA, a tail, and six tail fibers (Figure 12). In phage display system, T7 capsid protein display peptides or proteins on the surface of the phage. The capsid protein is divided in two forms, 10A (344 aa) and 10B (397 aa). A translational frameshift at amino acid 341 of 10A gives rise to 10B. Either the proteins 10A and 10B are responsible for functional capsids. This suggests that the T7 capsid shell may be various. Moreover, the 10B form is unique form of capsid protein then it coulde be used for phage display [223] .

Types of T7Select phage display vectors are the T7select415 vector for high-copy number peptides displaying, the T7Select10 vector for mid-copy number display of peptides or larger proteins and the T7select1 vectors for low-copy number with large proteins displaying (Table 2). In totality of the vectors, The foreign DNA are cloned into multiple cloning sites of aa 348 of the 10B protein in vectors. After that, the natural translation frameshift site in the capsid gene has been removed. Then, these vectors can be constructed only a single form of capsid protein [71].

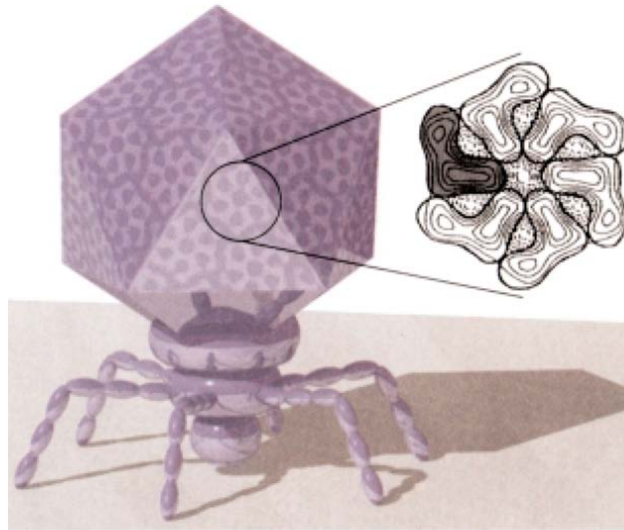

Figure 12. The T7 bacteriophage structure was shown as the capsid shell assembled of the head, head-tail connector, tail and six tail fibers [224].

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The first step of lytic phages infection occurs through an interaction between their tail fibers and lipopolysaccharide on the *E. coli* cell surface. After that, the phage genome and several proteins enter into the host cell via tail of phage [71]. Then, phage assembly takes place in the *E. coli* cytoplasm. After that, cells are lysed and mature phage virions are released (Figure 13).

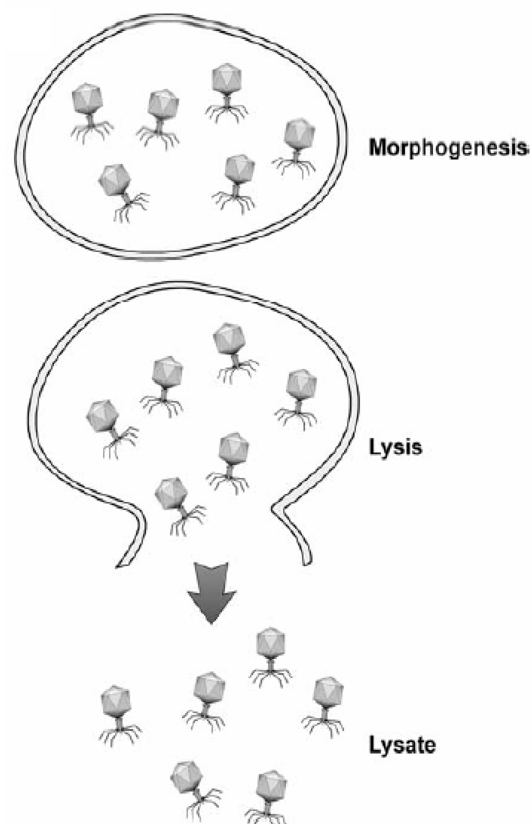


Figure 13. The life cylcle of T7 bacteriophage in host cell such as *Escherichai coli* [225].

Table 2. Phage display vector features

vector	use	display number	display limit (amino acid)
T7Select 415-1	peptides	415	40-50 aa
T7Select 1-1	peptides or proteins	≤ 1	900 aa
T7Select 1-2	Peptides or proteins	≤ 1	1200 aa

Adapted from [224]

Screening phage display libraries

Phage display selection can be executed both *in vivo* and *in vitro* [40, 57]. *In vivo* screening is performed to identify organ-specific molecules such as ligands of the brain vascular receptor [226, 227], vascular endothelium cells [228, 229] and mosquito organs [230]. The targets for *in vitro* screening may be biological or inorganic targets [231]. For inorganic targets, the solid supports such as plastic beads, nitrocellulose membrane, agarose beads, polystyrene plate and magnetic particles were immobilized on target molecules [40].

Bio-panning step begins with incubating of phage display library which displays a different pattern of peptides with the target molecules. After phage binding to the target, non-specific binding phages are removed by washing. Bound phages are eluted from target molecules. After phage amplification, bio-panning is repeated. At the final round of selection, plaques from the final eluate are isolated and sequenced. After several rounds of selection, enrichment of target-binding phages is determined by phage titering.

The washing step is necessary to remove unbound phages and to select bound phages. The high affinity and specificity of bound phages depends on stringency in the washing step such as washing time and detergent concentration. In addition, the elution is performed to release bound phages such as by competitive elution, extremes pH, ionic strength and enzymatic cleavage (Figure 14).

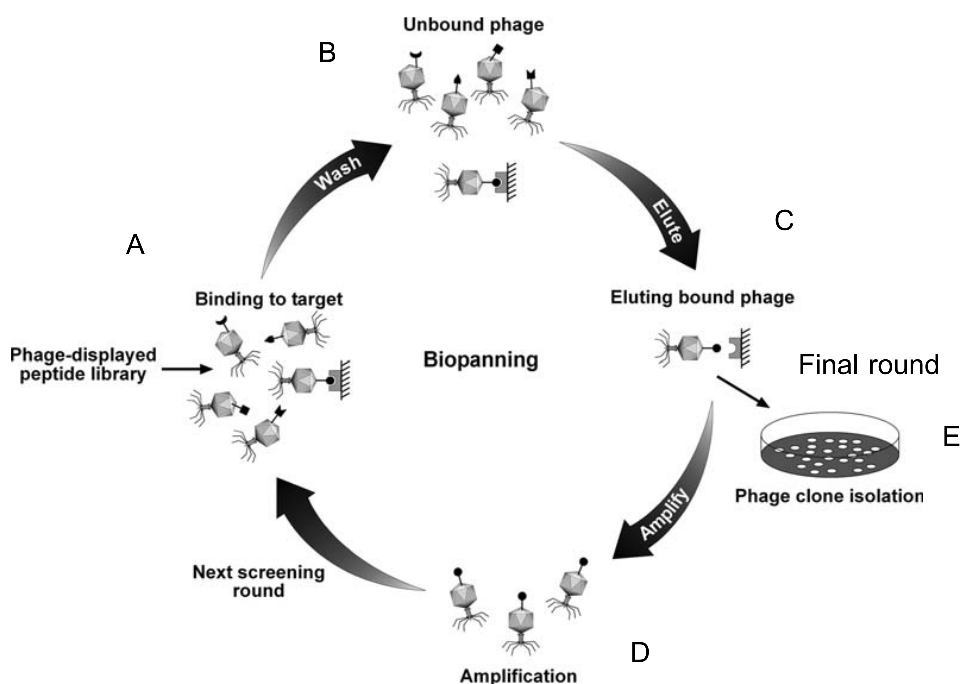


Figure 14. The phage affinity selection (bio-panning)[225] .

A. The phage display library which displays a different pattern of peptides is incubated with the target molecules.

B. Washing step to remove non-specific binding or unbound phages

C. Bound phages are eluted

D. The eluate is amplified and the bio-panning is repeated

E. At the final round, each phage clone is isolated and sequenced.

Application of phage display

Phage display technology is a used tool for study of Identification of protein-protein interactions [41-44] Identification of protein-protein interaction has been achieved by phage display method in many bacteria [56, 57, 232] such as interaction between LipL32 the major outer membrane of pathogenic *Leptospira* and candidate host proteins were identified by random phage display peptide library [232], binding of fibronectin to Group B Streptococci [233], insulin-like growth factor II receptor (IGFIIR) as a novel receptor of *Listeria monocytogenes* [234], binding of fibronectin binding protein of *Staphylococcus aureus* to platelet [235] and mapping of the laminin binding site of *Yersinia pestis* plasminogen activator [236], Moerover, selection of wide type of target receptors [40, 45, 46], separation of recombinant antibodies, novel drug discovery [46-50], epitope mapping [51-54], and vaccine advancement [55] were investigated by using phage display technology.

In this study, cDNA T7 phage display library was used for screening of host proteins that interact with LipL32.

CHAPTER IV
MATERIALS AND METHODS

Bacteria strains and growth conditions

***Leptospira* sp.**

Pathogenic wild-type *Leptospira (L. interrogans* serovar Manila*)* and *lipL32* $^{-}$ mutant of *L. interrogans* serovar Manila were cultured at 28°C in Ellinghausen-McCullough-Johnson-Harris (EMJH) broth until the density of approximately 2-5x10^{8} cells/ml(mid-log phase) was reached.

Escherichia coli

E.coli strain BL21 containing *lipL32* gene in pRSET C vector was cultivated in Luria-Bertani (LB) both or LB agar with containing 100 µg/ml ampicillin and 35 µg/ml chloramphenicol, and incubated at 37°C overnight.

Cell culture

Vero cells (African green monkey kidney fibroblasts) were maintained in Dulbecco's modified Eagle's medium (MEM, GIBCO BRL, U.S.A.) supplemented with 10% heat-inactivated fetal bovine serum (FBS, GIBCO BRL, U.S.A.), 5 mM L-glutamine , 100 unit per ml of ampicillin and 100 µg per ml of streptomycin. Vero cells were cultured in T75 flasks in a 37°C incubator containing 5% CO_2 until became confluence (Nunclon, Denmark) and the density of approximately 5x10^{6} cells was reached.

Attachment procedure

Vero cells were harvested with trypsin-PBS (see APPENDIX A). Vero cells were maintained in 2% MEM without antibiotics. About 5x10^{4} Vero cells/cm^{3} were seeded on sterile coverslips in 24-well tissue culture plate (Nunclon, Denmark). After incubating for 24 hours, the plate was washed twice with 2% MEM. Pathogenic wild-type *Leptospira (L. interrogans* serovar Manila*)* and its *lipL32* $^{-}$ mutant were harvested by centrifugation at 8,000 x *g* for 10 min, washed three times with PBS, and then suspended in warm (37°C) 2% MEM. About 5x10^{6} *Leptospiral* cells were then added to each well of the tissue culture plate (*Leptospira* : Vero cell ratio of 100:1) followed by incubation at 37°C

for 2 hours. Coverslips were washed three times with ice-cold PBS (phosphate buffer saline) to remove unbound bacteria. Experiments were performed in triplicate. Attachment of *Leptospira* spp. to Vero cells were detected by immunofluorescence staining.

Immunofluorescence staining

After removal of non-attached bacteria, Vero cells on the coverslips were fixed with acetone at room temperature for 5 minutes before washing three times in 2xPBS and air dried. Rabbit anti-leptospiral antiserum (National Animal Health and Production Institute, Bangkok, Thailand) diluted 1 to 100 in PBS was added and then incubated at 30°C in a moist chamber for 1 hour. Next, cells were washed by 2xPBS-0.1% Tween20 three times before incubating with polyclonal swine anti-rabbit immunoglobulins-FITC (Fluorescein isothiocyanate) conjugated antibody (Dako, Denmark) (diluted 1 to 20 in PBS) at 30°C in a moist chamber for 1 hour. After washing by 2xPBS-0.1% Tween20 three times, mounting medium was added. Finally, immunofluorescence staining was observed under the fluorescence microscope (Olympus). The attachment ratio was calculated as following: (average number of cells attached with leptospires /one hundred observed cells) x 100% [237]. One hundred Vero cells were observed and cells attached with leptospires were counted in triplicate (three different areas on the slide). Average numbers of 3 counts were used for calculation. Three independent experiments were performed. The statistic significance was determined by non-parametric test.

Recombinant LipL32 (rLipL32) induction and expression

E.coli strain BL21 containing *lipL32* gene in pRSET C vector was cultivated in LB broth with 35 µg/ml chloramphenicol and 100 µg/ml ampicillin with shaking at 250 rpm at 37°C. The overnight culture was added to fresh medium until OD_{600} reached 0.4. After that, isopropyl-ß-D thiogalactopyranoside (IPTG, Fermentas, U.S.A.) was added to the

culture at a final concentration of 0.5 mM. The induced culture was incubated with shaking at 200 rpm at 30°C for 3 hours before harvested by centrifugation at 8,000Xg for 15 minutes.

Recombinant LipL32 extraction and purification

The cells were centrifuged at 8,000Xg for 15 minutes and the pellet was resuspended in cold PBS. After that, the cell suspension was lysed by sonication using High intensity ultrasonic processor VC/VCX 750 sonicator with 40% amplitude for 2 minutes on ice. Then, soluble proteins in the supernatant were separated from pellet containing insoluble proteins by centrifugation at 16,000Xg for 20 minutes at 4°C. Supernatant was transferred to a new tube. Then, soluble proteins and insoluble proteins were analyzed using 15% SDS – PAGE and Western blot.

Binding buffer (see APPENDIX A) was applied to the Nickel–sepharose column (Ge Healthcare, UK.) followed by loading the soluble fraction containing rLipL32. Next step, the elution buffer (formula with 40, 60, 100, 250, or 500 mM imidazole) was added to the column. The eluate was collected and analyzed by 15% SDS-PAGE. To remove imidazole from the eluate, dialysis was performed using 10 kDa-molecular weight cut off dialysis membrane. After incubation in 2 liters of PBS at 4 °C for 16 hours, the dialysate was analyzed by 15% SDS-PAGE.

Western blot

Proteins on polyacrylamide gels were transferred to PVDF membrane by SemiDry (Ge Healthcare, UK.) at condition: 15 volt for 30 minutes. The membrane was blocked with 10% skimmed milk in the TBS-Tween (see APPENDIX A) for 1 hour at room temperature. Mouse anti-6His monoclonal antibody (1:3,000 final dilution in 5% skimmed milk in TBST; KPL, U.S.A.) was added and incubated for 1 hour at room temperature with gentle rocking. The membrane was washed with TBST buffer three times for 10 min each. The membrane was then incubated in mouse anti-His antibody

(diluted 1:3,000 in 5% milk in the TBST) (KPL, U.S.A.) with rocking for 1 hr at room temperature. After that, the membrane was washed with TBST buffer three times, each for 10 min . The blot was detected by adding BCIP (5-bromo-4-chloro-3-indolyl-phosphate) is used in conjunction with NBT (nitro blue tetrazolium) (BCIP/NBT) Phosphatase substrate (KPL, U.S.A.)

Membrane protein fractionation

Vero cells in T75 flasks were washed tree times in PBS. After that, cells were lysed with 1% Triton X-100 (pH 7.0) at room temperature. Then, cell lysate was sonicated with high intensity ultrasonic processor VC/VCX 750 sonicator with 40% amplitude for 30 minutes on ice before centrifugation at 5,000 rpm for 10 minutes at 4°C. The supernatant containing membrane proteins was separated by ultracentrifugation at 200,000 g at 4°C for 30 minutes and the supernatant was discarded. The pellet was resuspended in 1% Triton X-100 (pH 7.0), vortexed and subjected to a second round of ultracentrifugation for 30 minutes at 200,000xg at 4°C. Then, the pellet was resuspended in 1x SDS-PAGE sample buffer (see APPENDIX A). After that, protein concentration of both soluble and insoluble parts was measured by *RC DC* protein assay(Bio-Rad, U.S.A.).

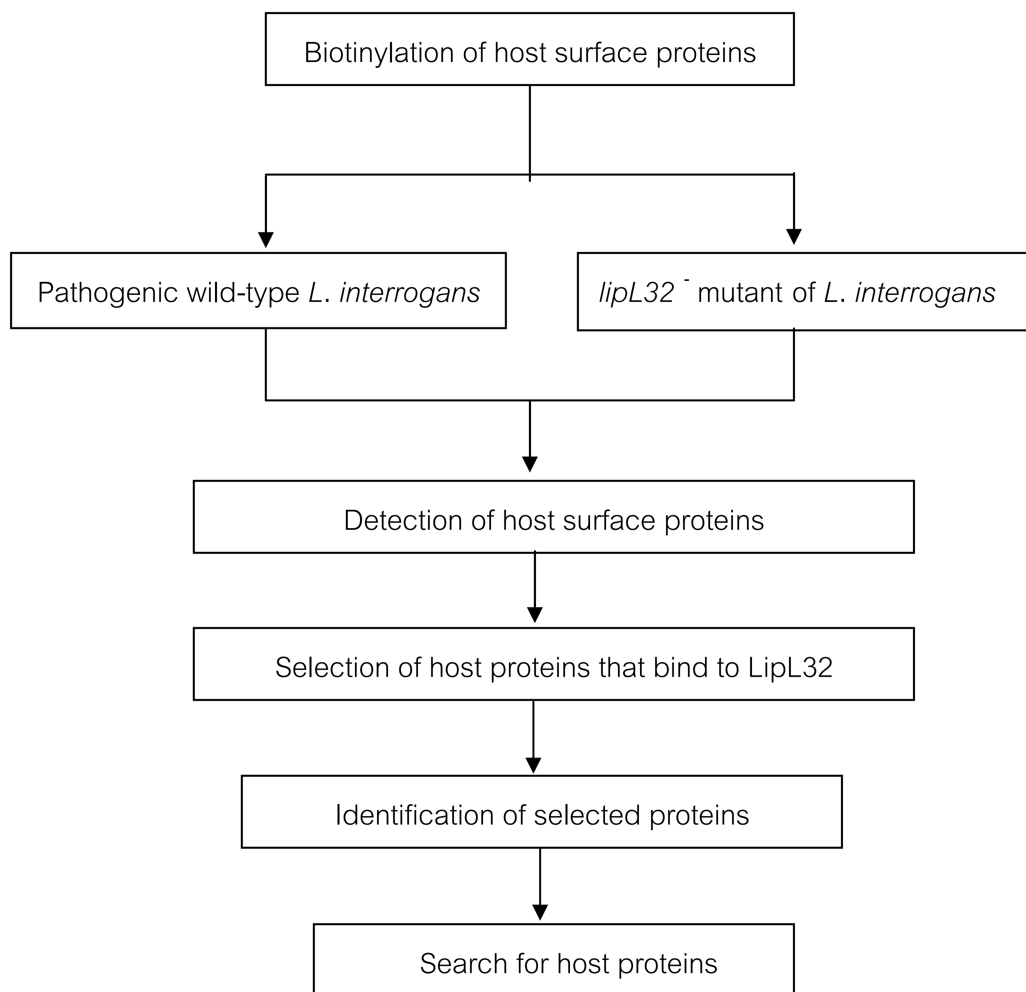
Protein assay

Protein concentration was measured by Lowry method (*RC DC* protein assay, Bio-Rad, U.S.A.) according to manufacturer's protocol as followed; Prepared reagent A by add 5 µl of *DC* reagent S to each 250 µl of *DC* reagent A. Add 127 µl of reagent A to each standard or sample. Then, 5 dilutions of BSA were prepared; 0.2, 0.5, 0.75, 1.0 and 1.5 mg/ml. About 25 µl of standards or samples was added into microcentrifuge tubes. Then, about 125 µl *RC* reagent I was added into each tube, vortex. The tube was incubate for 1 minute at room temperature. Then, about 125 µl of *RC* reagent II was added into each tube, vortex. The tube was centrifuge at 15,000xg for 5 minutes. After centrifugation, the supernatant was discarded. About 127 µl reagent A was added to each tube, vortexed, and incubated at room temperature for 5 minutes. About 1 ml of

DC reagent B was added to each tube, incubated at room temperature for 15 minutes and measured the absorbance using spectrophotometer at the wavelength of 750 nm. About 127 µl reagent A mixed with 1 ml of *DC* reagent B were used as a blank control.

Identification of host surface proteins that interact with LipL32

Bacterial Pull down assay



The experiment was performed as previously described [213]. Briefly, surface proteins of Vero cells were labeled with surface-biotinylation reagent, sulfosuccinimidyl-2-(biotinamido)-ethyl-1,3'-dithiopropionate (NHS-SS)-Biotin (Pierce, U.S.A.), for 30 min at room temperature. Cells were washed tree times in PBS and then lysed with 1% Triton X-100 (pH 7.0) at room temperature then cell lysate was separately incubated with wild-type strain of the *L. interrogans* serovar Manila and its isotype strain of *lipL32*$^{-}$ mutant for 16 hr at 4°C with constant shaking at 20 rpm. After that, *Leptospira* were centrifuged at 8000 g,10 minustes. *Leptospira* were washed with washing buffer (see APPENDIX A) to remove unbound proteins and were eluted with elution buffer (see APPENDIX A) before subjected part of crude eluate and pellet to SDS-PAGE and immunoblotting. Biotinylated proteins were detected by incubating membranes with streptavidin-horseradish peroxidase (HRP) conjugate (Fermentas, U.S.A.). Spots were detected in the wild-type but not detected in the *lipL32*$^{-}$ mutant of the *L. interrogans* were selected to be analyzed by Liquid chromatography–mass spectrometry (LC-MS).

Competitive inhibition assay to determine specific binding of host proteins to *Leptospira*

Specific host proteins that interacted with *Leptospira* were identified by competitive inhibition assay i.e., incubating leptospires with biotinylated and non-biotinylated proteins of Vero cells simultaneously. The proportion between the amount of biotinylated and non- biotinylated proteins of Vero cells was 1:10. After that, biotinylated and non-biotinylated proteins of Vero cells were separately incubated with wild-type strain of the *L. interrogans* serovar Manila and its isotype strain of *lipL32*$^{-}$ mutant followed by bacterial pull down assay, as mentioned above.

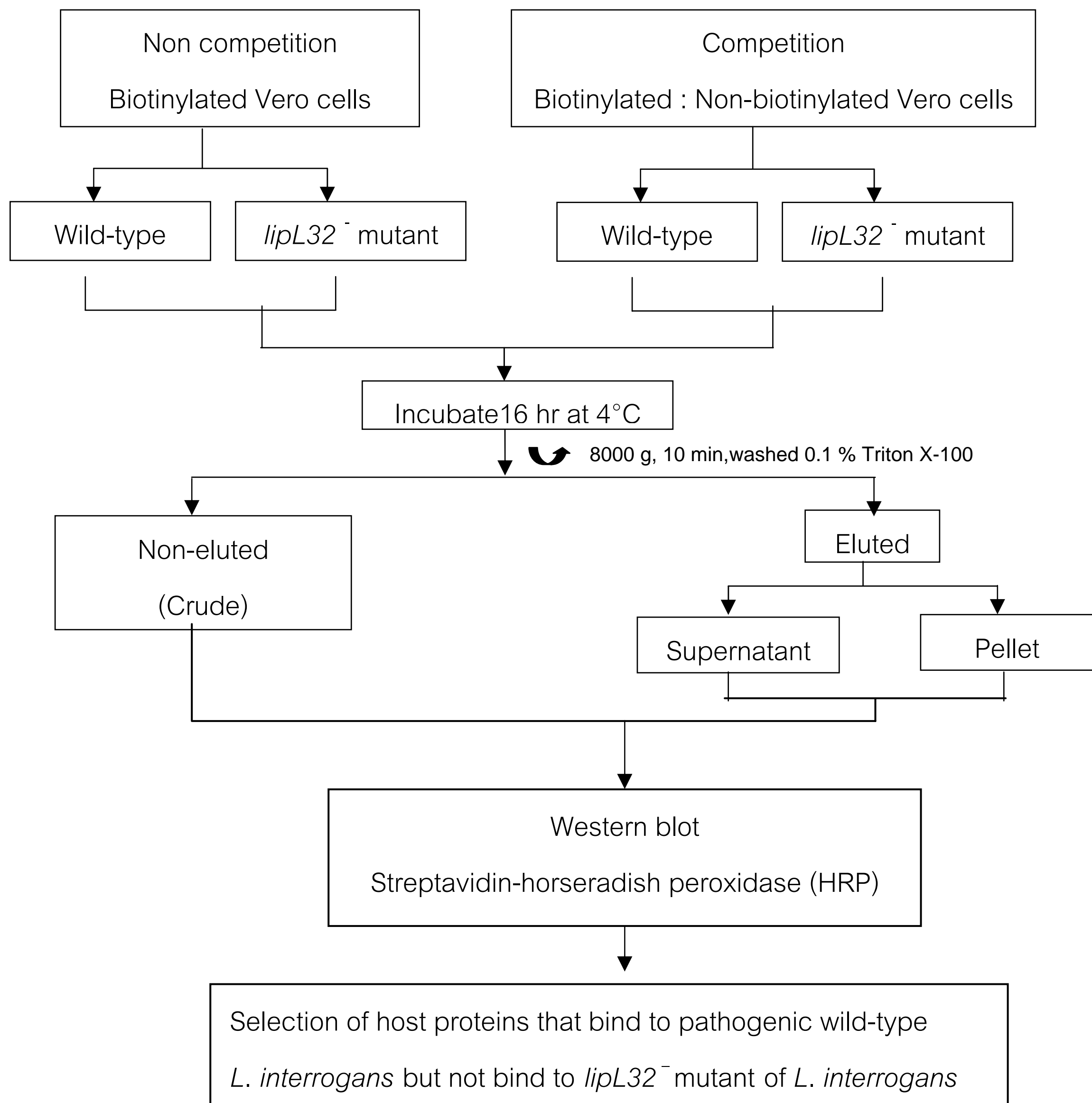
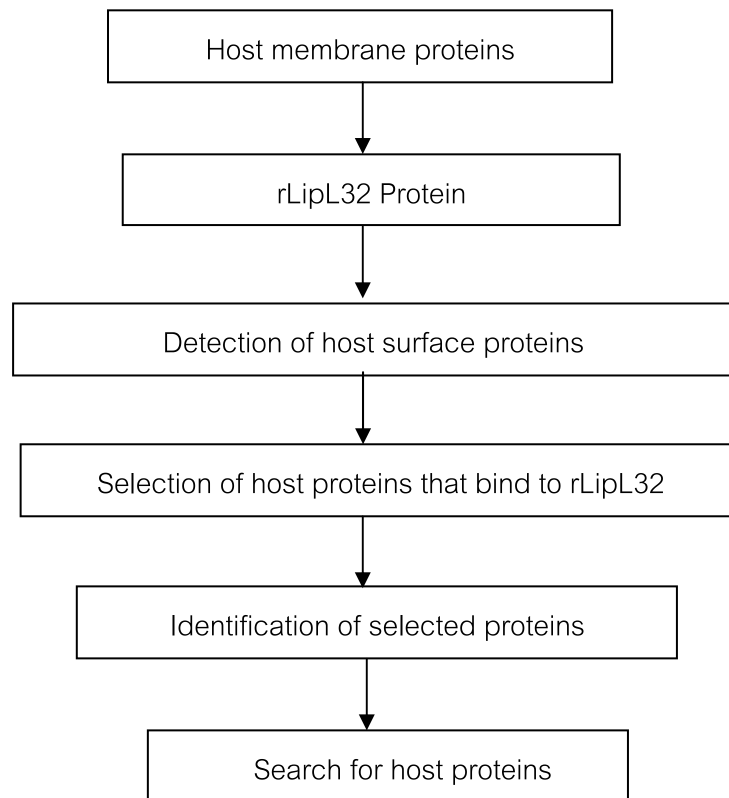


Figure 15. Detection of surface biotinylated proteins of Vero cells bound to wild-type *Leptospira* and *lipL32* $^{-}$ mutant.

Far western blot



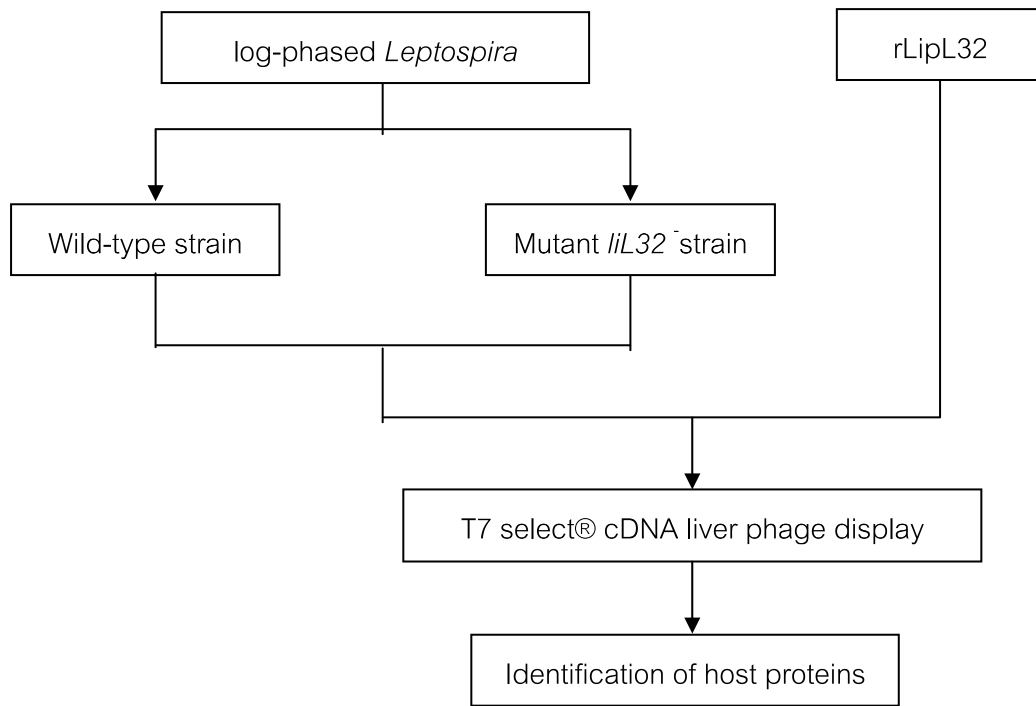
Approximately 100 µg of membrane proteins of Vero cells obtained from membrane protein fraction by ultracentrifugation were boiled in SDS-PAGE sample buffer (see APPENDIX A). The samples were subjected to 12% SDS-PAGE and transferred to a PVDF membrane(Ge Healthcare, UK.). The proteins on the membrane were renatured by incubating the membrane in the denaturing and renaturing buffer (see APPENDIX A) containing decreasing concentration of guanidine HCl. The membrane was washed with the AC buffer containing 6 M and 3 M guanidine–HCl each round for 30 min at room temperature, then washed with AC buffer containing 0.1 M at 4 °C for 30 min before incubating with denaturing and renaturing buffer containing no guanidine–HCl at 4 °C for 16 hours. After that, the PVDF membrane was blocked with 5% skimmed milk in the PBS-Tween buffer (see APPENDIX A) for 1 hour at

room temperature. The membrane was incubated with a total 5 ug of LipL32 (1 ug/mL) in 3% skimmed milk in PBST for 16 hours with rocking at 4°C. Unbound LipL32 was washed by PBST buffer three times, each for 10 minutes. After that, mouse anti-LipL32 antibody (diluted 1:5,000 in 3% skimmed milk in PBST) was added and incubated for 1 hour at room temperature. The membrane was washed by PBST buffer three times, each for 10 minutes. The membrane was then incubated in anti-mouse-IgG antibody horseradish peroxidase (HRP) conjugate (diluted 1:5,000 in 3% skimmed milk in the PBST) for 1 hr at room temperature. After that, the membrane was washed by PBST buffer three times, each for 10 minutes. The blot was developed using chemiluminescence reagents (GE) and imaged using a Bio-Rad ChemiDoc XRS system. The band of interest on polyacrylamide gel was further analyzed by liquid chromatography–mass spectrometer.

Liquid chromatography–mass spectrometry

The band of interest on Coomassie-stained SDS-PAGE gels corresponding to the detected band on the membrane was cut into small pieces. Then, gel slice was washed by adding 200µl of MilliQ water with gentle rocking for 5 minutes followed by incubating in 200µl of 50% acetonitrile/100 mM ammonium bicarbonate until colorless, and then washing with 200µl of MilliQ water for 5 minutes. Then, the gel slice was dehydrated by incubating with 200µl of 100 % acetonitrile for 5 minutes at room temperature, and allowed gel to dry at room temperature for 5 minutes. The protein was digested by adding 20 µl of 20 ng/ µl trypsin solution and incubated at room temperature for 3 hours. Peptides were extracted from the gel slice via sonication in 30 µl 50% acetonitrile/1% trifluoroacetic acid (TFA) and incubated 40°C for 4 hours. The peptides were analysed on an UltiMate™ NanoLC system (Dionex-LC Packings) coupled to a Bruker micrOTOF-Q II™, equipped with an online nanoESI source. The complete LC-MS setup was controlled by HyStar™software. The Mascot search engine (http://www.matrixscience.com/) was used to search the obtained amino acid sequences for matched proteins. Significant matching required probability-based Mowse score of > 43 ($p<0.05$)

T7 select® cDNA liver phage display screening



Phage titering

Single colony of BLT 5403 was inoculated in LB broth 5-10 ml and incubated at 37 ^{o} C under shaking condition until OD_{600} get to 1.0. Pending cells were growth, agarose top was melted in microwave then, approximately 3 ml of agarose top was poured into sterile tubes, one tube per one T7phage dilution, and kept at 45°C before use. T7phage were diluted in LB broth to prepare 10-fold serial dilution of T7phage. Approximately 10^{-8}-10^{-11} was the range of phage dilution for amplified phage culture supernatants and approximately 10^{-2}-10^{-4} was the dilution range of phage dilution for unamplified panning elute. After *E. coli* strain BLT 5403 has reached to 1.0 of OD_{600}250 μl of cells were aliquoted to sterile microcentrifuge tubes, one for each phage dilution. About 100 μl of each phage dilution were added to tubes that contained 250 μl of BLT 5403 culture. the tube was vortexed immediately and the infected cells were added to culture tubes containing melted agarose top, vortexed and quickly poured to LB plate containing 5 μl/ml carbenicillin. Agarose top was spreaded, allowed plate to cool

5 minutes and incubated the plate overnight at room temperature. Then, clear areas (plaques) that appeared on the plate were counted. Afterwards, phage titers were calculated by counting number of plaques on the plate. Phage titer (plaque forming units (pfu) per ml) was calculated by using the following formula.

Pfu/ml = Plaques/(D x V)

Where D = Dilution factor

V = Volume of diluted virus added to plate

Panning Procedure of T7 select® cDNA liver phage display library incubated with pathogenic wild-type *Leptospira* and subtracted with *lipL32*$^-$ mutant of *L. interrogans*

Fifth rounds of bio-panning were undertaken according to the protocol of The T7 select® cDNA liver phage display library kit (Novagen, U.S.A.). First step, number of phage titer before incubating with target were determined by amplification once prior to bio-panning. About 5 µl of T7 select® cDNA liver phage display library was amplified by infecting 50 ml of mid-log phase of BLT 5403 and incubated at 37°C for 1.5 hours at shaking condition. After that, amplified lysate was centrifugated at 12,000 rpm at 4°C for 15 minutes and the upper 80% of supernatant was transferred to fresh tube. Then, phage were precipitated by adding 1/6 volume of PEG/NaCl and incubated at 4°C overnight. After precipitation, phage were collected by centrifugation at 10,000 rpm 4°C for 15 minutes, discarded supernatant and respun. Afterward, pellet was suspended by adding 1 ml TBS and transferred to sterile microcentrifuge tube. Then, phages were collected by centrifugation at 10,000 rpm 4°C for 5 minutes. Next step, phages were re-precipitated with 1/6 volume of PEG/NaCl and incubated on ice for 1 hour. After incubation, centrifugation was followed at 10,000 rpm 4°C for 10 minutes, discarded supernatant, re-spun and discarded supernatant. Then, pellet was suspended in 200 µl of TBS+0.02% NaN_3 , incubated on ice for 1 hour, centrifuged at 12,000 rpm 4°C for 2 minutes and transferred supernatant to fresh microcentrifuge tube. The amplified phage was taken for phage titering.

Pathogenic wild-type *Leptospira (L. interrogans* serovar Manila*) lipL32* $^-$ mutant of *L. interrogans* serovar Manila were grown until amount get to $1x10^9$ cell/ml, then, incubated at 37°C for 16 hours. After that, about 1.5 ml of blocking buffer was added to sterile microcentrifuge tube for binding non-specific residues at 4°C for 16 hours. Then, cells were centrifuged at 8,000X g 4°C for 10 minutes. Pellet was collected and resuspended in 500 µl TBS. Then, the suspension was centrifuged at 8,000xg at 4°C for 10 minutes and discarded supernatant. The pellet was washed with 0.1% TBST for 10 times. In first round of panning, added 100 µl of T7 select® cDNA liver phage display library to each blocked tube that contained pathogenic *Leptospira interrogans* serovar Manila and gently rocked at room temperature for 1hour. Then, centrifugation was performed at 8,000 g at 4°C for 10 minutes , Unboud phages which contain in supernate were discarded and leptospires pellet was washed pellet with 0.1% TBST for 10 times.

Next step, bound phage were eluted from *Leptospira* cells with 200 µl of elution buffer (1% SDS; Appendix A) and gently rocked for 17 minutes. Then, transferred the eluate into a fresh microcentrifuge tube. About 10 µl of eluate was amplified by infecting 50 ml of mid-log phase of BLT 5403 which had OD_{600} reached to 0.5 and incubate at 37°C for 1.5 hours at 250 rpm. After that, amplified lysate was centrifugated at 12,000 rpm at 4°C for 15 minutes and transferred the upper 80% of supernatant to fresh tube. Then, phage were precipitated by adding 1/6 volume of PEG/NaCl and incubated at 4°C overnight. After precipitation, phages were collected by centrifugation at 10,000 rpm 4°C for 15 minutes, supernatant was discarded supernatant and respun. Afterward, pellet was suspended by adding 1 ml TBS and transferred to sterile microcentrifuge tube. Then, phages were collected by centrifugation at 10,000 rpm 4°C for 5 minutes. Next step, phage were re-precipitated with 1/6 volume of PEG/NaCl and incubated on ice for 1 hour. After incubation, centrifugation was followed at 10,000 rpm 4°C for 10 minutes, discarded supernatant, re-spun and discarded supernatant. Then, pellet was suspended by adding 200 µl of TBS+0.02% NaN_3 , incubated on ice for 1 hour, centrifuged at 12,000 rpm 4°C for 2 minutes and transferred supernatant to fresh

microcentrifuge tube. The amplified phage was taken for phage titering. After that, bound phages on wild-type *Leptospira interrogans* serovar Manila was incubated with *lipL32*$^-$ mutant and gently rocked at room temperature for 30 minutes. After that, the solution was centrifuged and phages which unbound with mutant leptospires were collected. Then, centrifugation was performed at 8,000 g at 4°C for 10 minutes , Unboud phages in the supernatant were discarded and leptospire pellet was washed with 0.1% TBST for 10 times. In the second to fifth rounds of bio-panning ,the supernatant containing unbound phage with mutant *lipL32*$^-$ *Leptospira* was taken to incubate with wild type *Leptospira interrogans* serovar Manila for 1 hour at room temperature.

In the second to fifth rounds of bio-panning were the same as in the first round of panning, other than, the washing step were used with 0.5% TBST. Moreover, unamplified phages in the fifth round of bio-panning were selected for phage titering and clear plaques from this titering were used for sequencing.

Panning Procedure of T7 select® cDNA liver phage display library incubated with LipL32 purified protein

The procedure of bio-panning with rLipL32 were the same as above, except this experiment was perform in microtiter plate which described as; A 96 well microtiter plate (Greiner bio-one, Germany) was coated about 100 µl purified rLipL32 (100 µg/ml in 0.1 M $NaHCO_3$ pH 8.6) Then, incubation were followed at 4°C for 16 hours in a humidified package. Next step, coated wells were washed with 0.1% TBST for 10 times , added blocking buffer in the wells , incubated at 4°C for 2 hours, discarded the blocking solution and washed each well with 0.1%TBST for 10 times.

In first round of panning, added 100 µl of T7 select® cDNA liver phage display library onto coated plate and gently rocked at room temperature for 1 hour. After that, non-binding phage were discarded by poured off and slapped plate onto a clean paper towel and washed with 0.1% TBS for 10 times. Then, bound phage were eluted from the wells with 200 µl elution buffer (1%SDS, see APPENDIX A), gently rocked for 20 minutes. Next step, the eluate was removed to microcentrifuge tube. About 10 µl of

eluate was amplified and precipitated. Moreover, unamplified phage in the sixth round of bio-panning was selected for phage titering and clear plaques from this titering were used for sequencing.

Plaque amplification

Overnight culture of BLT 5403 was diluted 1:50 in LB and incubated at 37 °C under shaking condition until OD_{600} get to 0.5. The cultured was aliquoted about 1ml per tube to sterile microcentirifuge tube. After that, picked up clear plaques from plate to each tube which contained culture. The culture was incubated at 37°C for 1.5 hours at shaking condition. Next step, the culture were centrifuged at 1,200 rpm 4°C for 10 minutes. After centrifugation, 80% upper supernatant was transferred to sterile tube then, diluted 1:1 with steriled glycerol and store at -20°C for long-term storage.

DNA Sequencing

Generation of sequencing template by polymerase chain reaction (PCR)

The insert cDNA template was generated in a total volume about 50 µl of PCR mixture : 1x PCR Polymerase *Taq*, 2 mM $MgCl_2$, 0.2 dNTP mix, 0.2 pmol forward primer, 0.2 pmol reverse primer, 1.25 units Taq polymerase and 4 µl of an amplified phage which preparation of individual plaque. The PCR amplification was followed using the condition; Primary denaturation at 94°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 1 minutes, annealing at 50°C for 1 minutes, extension at 72°C for 1 minute and final extension at 72°C for 10 minutes. Then, PCR products were analyzed which using 2.5% agarose gel electrophoresis at 100 volt. Next step, PCR product was purified by QIAquick® PCR Purification Kit (QIAGEN, U.S.A.), Then, purified PCR product sequences were determined by T7 promoter and T7 terminator primers (First BASE Laboratories, Malaysia). Finally, DNA sequencing results were compared to human sequence in GenBank database.

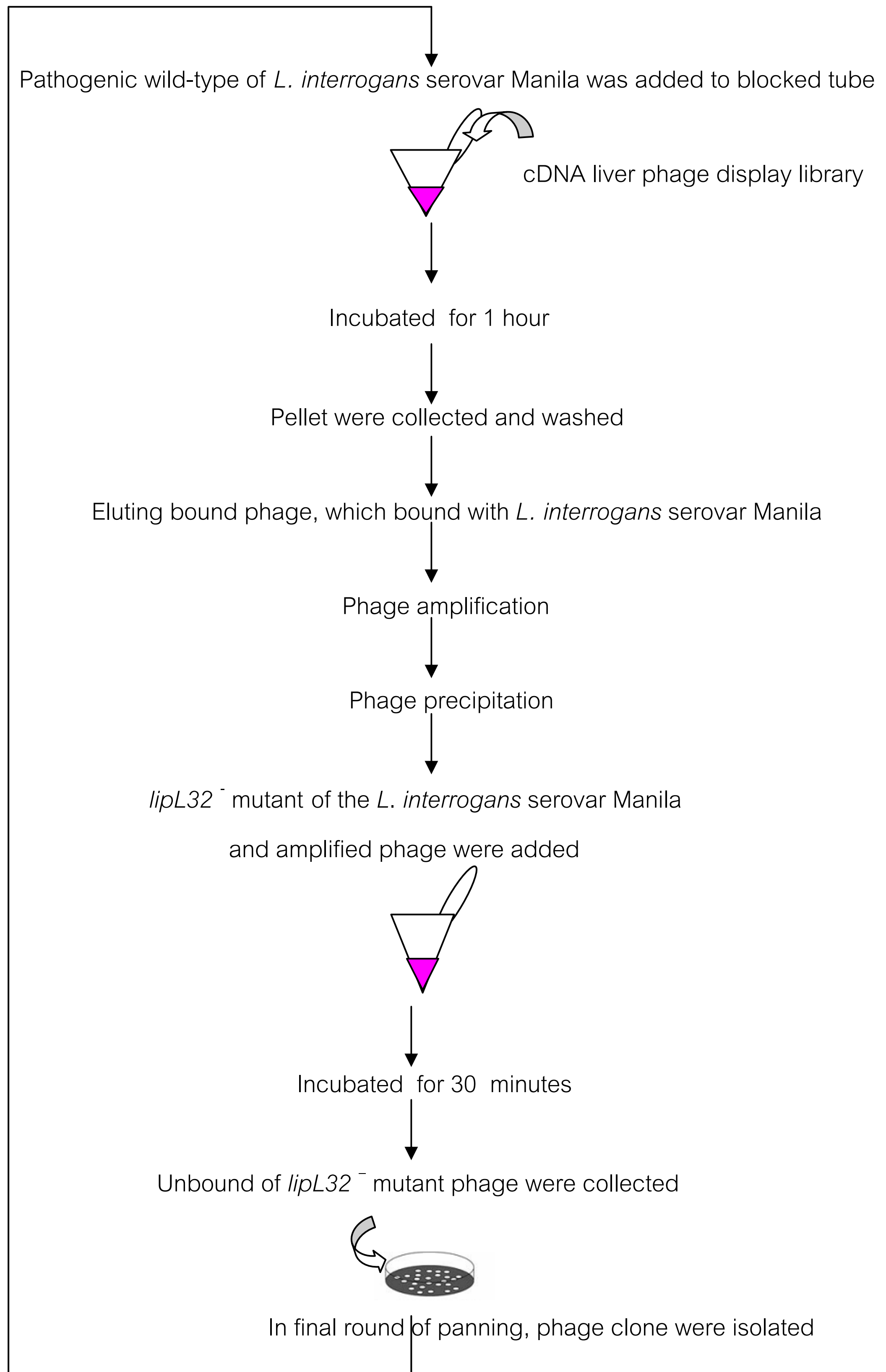


Figure 16. The phage affinity selection (bio-panning and subtraction).

CHAPTER V

RESULTS

Attachment of *Leptospira* to Vero cells

Leptospira were incubated with Vero cells (*Leptospira*:Vero cell ratio of 100:1) at 37°C for 2 h. Attachment of *Leptospira* with Vero cells was detected by immunofluorescence staining (Figure 17). The results showed that the wild-type strain of the *L. interrogans* serovar Manila attached to 71% of Vero cells whereas *lipL32* $^-$ mutant strain attached to 50% of Vero cells and saprophytic *L. biflexa* attached to 37% of Vero cells. The results suggested that attachment of pathogenic *Leptospira* with *in vitro* cultured mammalian cells may be related to virulence. The next step we plan to find host surface proteins that interact with LipL32 by identifying host proteins that bind exclusively to wild-type *L. interrogans* but not to *lipL32*$^-$ mutant and non-pathogenic *Leptospira*.

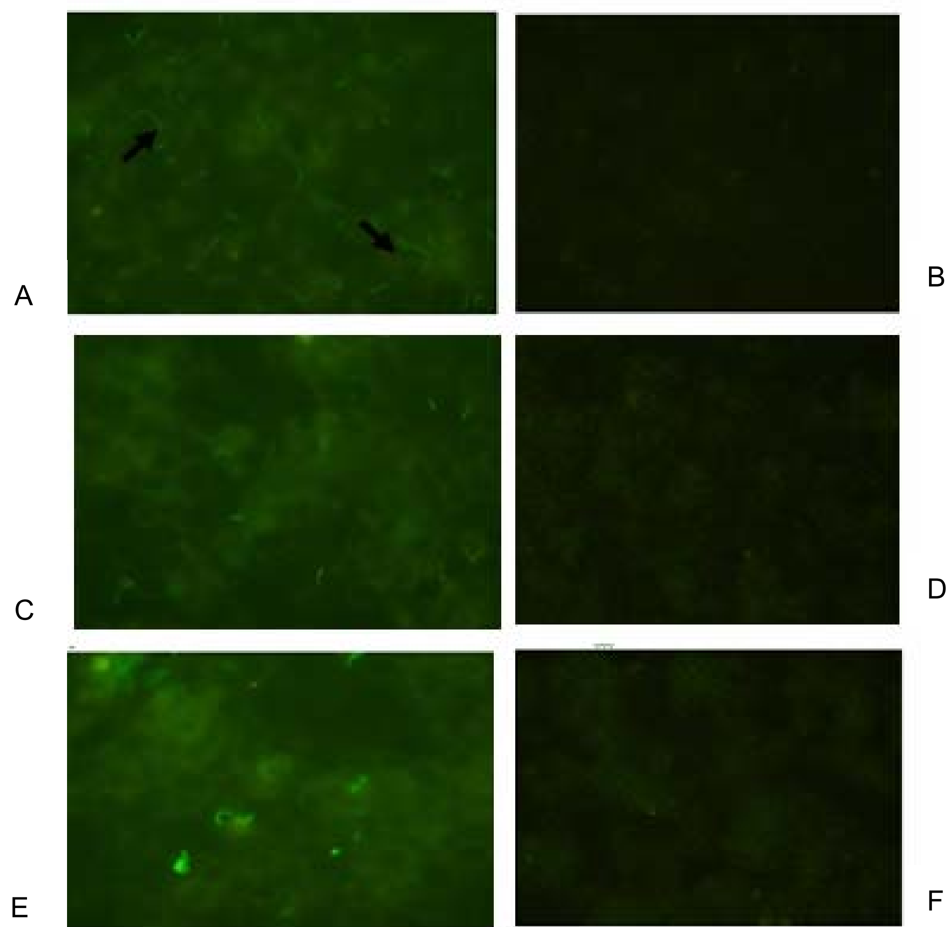


Figure 17. Immunofluorescence staining of *Leptospira* incubated with Vero cells. Cells were detected with (A) anti-leptospire antibody followed by FITC-conjugated rabbit anti-Ig (arrows indicate positively stained leptospires). (B) negative control using FITC-conjugated rabbit anti-Ig only. (C) *lipL32*$^{-}$ mutant strain of the *L. interrogans* serovar Manila strain incubated with Vero cells. (D) *lipL32*$^{-}$ mutant strain of the *L. interrogans* serovar Manila strain incubated with Vero cells staining with only secondary antibody. (E) Non-pathogenic *Leptospira* (*L. biflexa* serovar Patoc) incubated with Vero cells. (F) Non-pathogenic *Leptospira* (*L. biflexa* serovar Patoc) incubated with Vero cells staining with only secondary antibody.

Bacterial Pull down assay

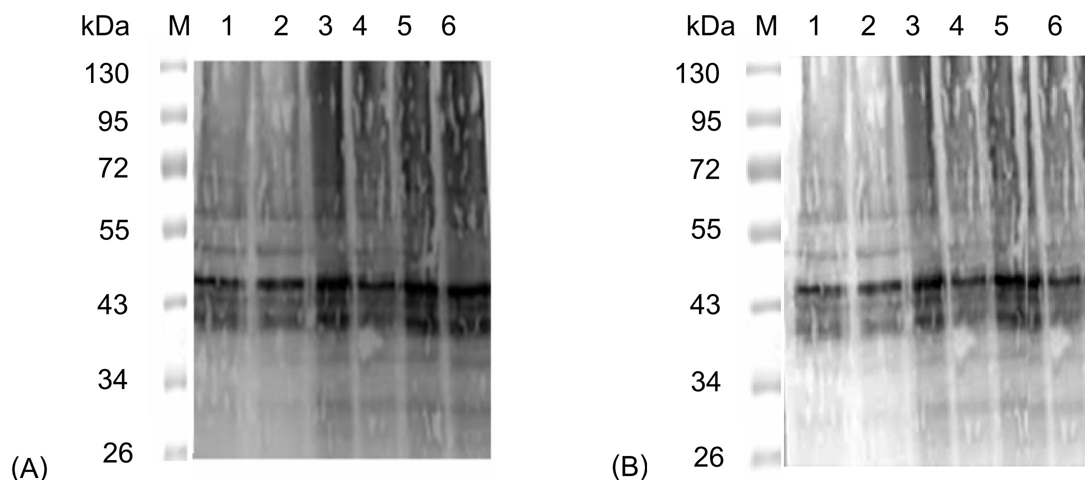


Figure 18. Detection of surface biotinylated proteins of Vero cells bound to wild-type *Leptospira* (A) and *lipL32* $^{-}$ mutant (B). After *Leptospira* were incubated with biotinylated surface proteins from Vero cells. Bacterial cells were harvested and eluted with 0.2% triton X-100 and then separated into the supernatant (eluted bound proteins) (lane 1 and lane 2) ; pellet (lane 3 and lane 4) . Crude proteins (uneluted cells) were shown in lane 5 and 6. To determine binding specificity, *Leptospira* were incubated with surface biotinylated proteins of Vero cells (lane 1, lane 3 and lane 5) or biotinylated proteins combined with non-biotinylated proteins of Vero cell lysate at the ratio of 1:10 (lane 2, lane 4 and lane 6); M, protein MW markers.

Surface proteins of Vero cells were labeled with NHS-SS-biotin which is used for specific labeling of cell surface proteins due to its membrane impermeability. Proteins of Vero cell lysate which interacted with wild-type pathogenic *L. interrogans* but not interacted with *lipL32* $^{-}$ mutant of *L. interrogans* were not observed indicating that interaction between whole cell of *L. interrogans* and Vero cell lyaste can not be identified by bacterial pull down assay. However, the competitive inhibition by adding non-biotinylated proteins did not decrease the intensity of any biotinylated proteins on

the membrane. Therefore, no additional data on specific binding were derived from the competitive inhibition assay at the conditions used in our study.

Protein Extraction and Purification

Expression of LipL32 in *E.coli* strain BL21(LE3)pLysS was induced by IPTG. Recombinant LipL32 with its correct size of 32.2 kDa was detected in the soluble and insoluble parts as shown on SDS-PAGE and Western blot (Figure 19).

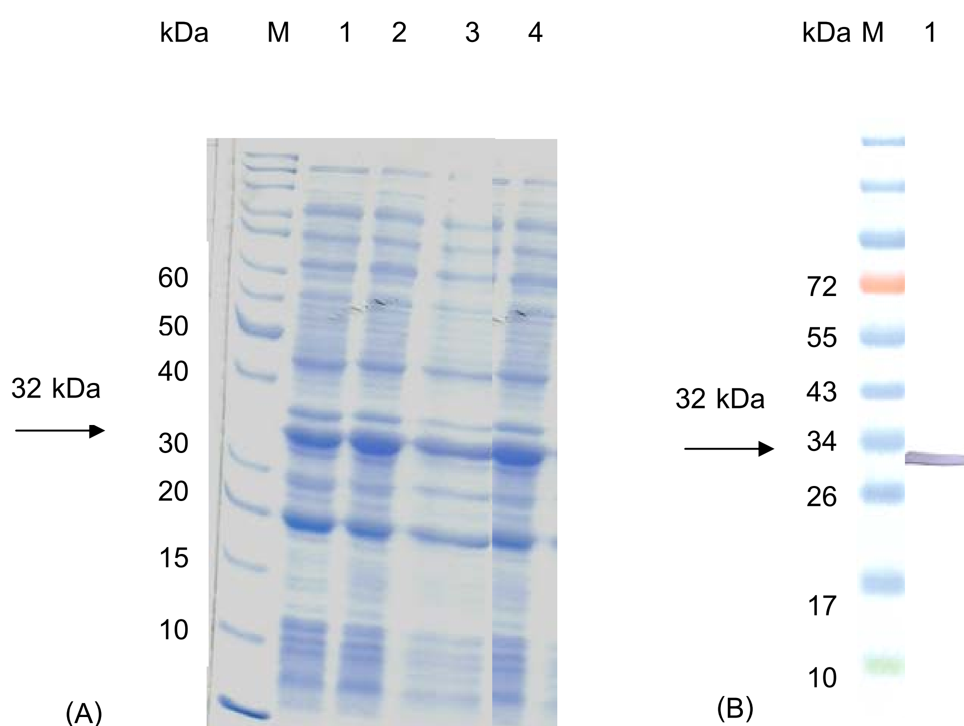


Figure 19. Detection of rLipL32 by SDS-PAGE and Western blot. The rLipL32 was detected by Coomassie blue staining on SDS-PAGE gel and by Western blot using anti-His antibody. (A) Crude proteins from *E. coli* extraction; M, protein MW ladder; lane 1, Curde proteins un-induce; lane 2, Curde proteins un-induce; lane3, Proteins in soluble part; lane4, Proteins in insoluble part. (B) Soluble part of LipL32 expression in *E. coli* was detected by Western blot; M, pre-stained protein ladder.

The soluble part of LipL32 was purified by metal-affinity chromatography. The purified rLipL32 were dominant on the SDS-PAGE gel (Figure 20). However, the contaminated proteins were found in some fractions of the eluate. Therefore, we selected the fraction of purified rLipL32 protein with the least contaminated proteins for further investigation.

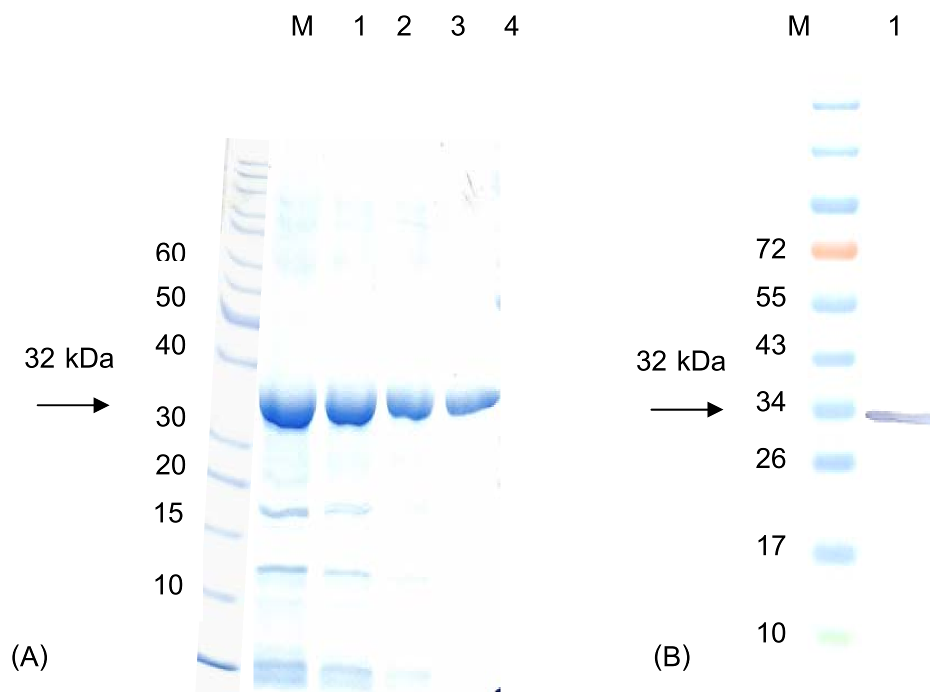


Figure 20. Detection of purified rLipL32 from metal-affinity chromatography. The purified rLipL32 was detected by Coomassie blue staining on SDS-PAGE gel and by Western blot using anti-His antibody. (A) M, unstained protein MW ladder; lanes 1-4 rLipL32 in fractions eluted with 100 mM imidazole. (B) M, pre-stained protein MW ladder. lane1, purified rLipL32 in whole cell lysate of *E. coli* was detected by Western blot

Imidazole was removed from rLipL32 due to its possible interference during the protein-protein interaction study. The purified rLipL32 fraction was dialyzed and detected by SDS-PAGE after dialysis was complete (Figure 21).

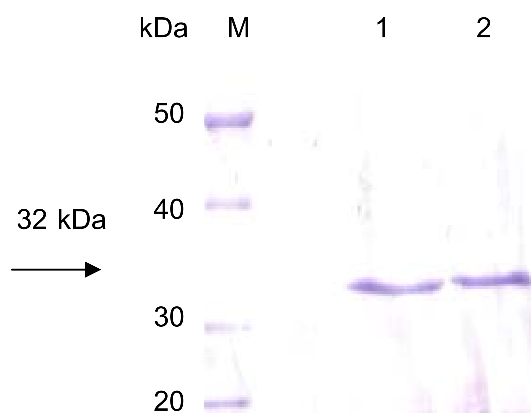


Figure 21. Detection of purified rLipL32 after dialysis. M, unstained protein MW ladder; lanes 1, purified rLipL32 before dialysis; lane 2, purified rLipL32 after dialysis.

Far western blot

We identified host membrane proteins that interact with rLipL32 by Far western blot. Membrane protein fraction of Vero cells was subjected to SDS-PAGE and transferred to the membrane. The proteins on the membrane were renatured by incubating the membrane in the denaturing and renaturing buffer. After that, membrane proteins of Vero cells on the membrane were incubated with rLipL32, washed non-specific binding and detected by using anti-LipL32 and therefore membrane protein of Vero cells bound to rLipL32 was detected.

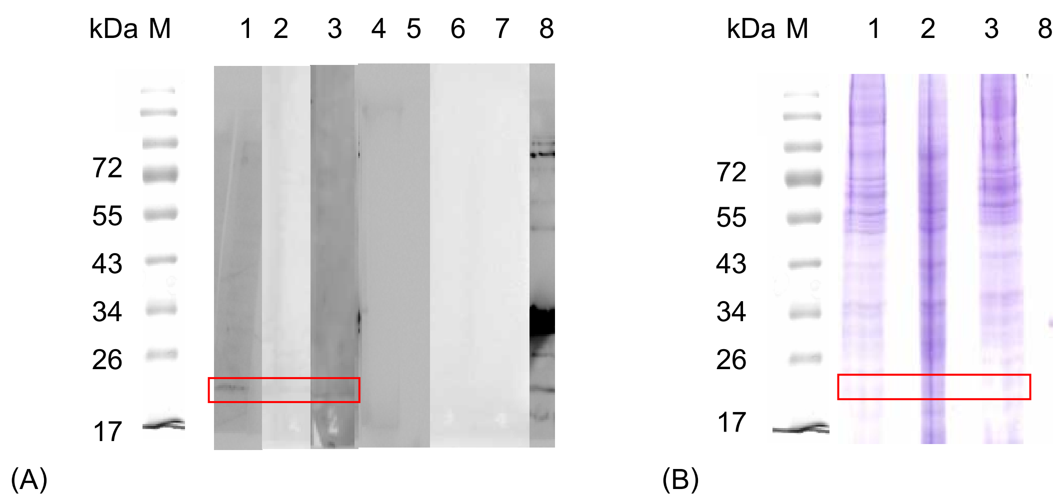


Figure 22. Detection of membrane proteins of Vero cells bound to rLipL32 by Far western blot (A) and corresponding band was detected on SDS-PAGE gel with Coomassie blue staining (B). After incubated with rLipL32, membrane proteins of Vero cells on the PVDF membrane that bound to rLipL32 were detected by anti-LipL32. ; lane 1, crude membrane proteins; lane 2, soluble membrane proteins; lane 3, insoluble membrane proteins; lane 4, rLipL32 incubating with only secondary antibody ; lane 5, BSA (Negative control) ; lane 6, crude membrane proteins that were not incubated with rLipL32 and detected with anti-LipL32 ; lane 7, crude membrane proteins that were not incubated with rLipL32 and incubated with only secondary antibody; lane 8, rLipL32 detected with anti-LipL32;. M, pre-stained protein MW ladders.

Proteins matching were searched by the Mascot search engine

1. gi|4505591 Mass: 22324 Score: 117 Matches: 6(3) Sequences: 6(3) emPAI: 0.88
peroxiredoxin-1 [Homo sapiens]
Check to include this hit in error tolerant search

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Unique	Peptide
56	819.4311	818.4238	818.4134	0.0104	0	17	27	1		R.SVDETLR.L
131	554.3145	1106.6144	1106.5972	0.0172	0	58	0.002	1	U	R.TIAQDYGVLK.A
138	563.3008	1124.5871	1124.5713	0.0158	1	18	15	1	U	K.DISLSDYKGK.Y
153	598.8300	1195.6454	1195.6237	0.0217	0	37	0.2	1		R.LVQAFQFTDK.H
156	606.3513	1210.6881	1210.6670	0.0211	0	66	0.00026	1		R.QITVNDLPVGR.S
185	680.4143	1358.8140	1358.7922	0.0218	1	44	0.042	1		R.GLFIIDDKGILR.Q

Proteins matching the same set of peptides:
gi|55959887 Mass: 19135 Score: 117 Matches: 6(3) Sequences: 6(3)
peroxiredoxin 1 [Homo sapiens]
gi|119627382 Mass: 20943 Score: 117 Matches: 6(3) Sequences: 6(3)
peroxiredoxin 1, isoform CRA_b [Homo sapiens]
gi|164519504 Mass: 22147 Score: 117 Matches: 6(3) Sequences: 6(3)
Chain A, Crystal Structure Of Human Peroxiredoxin I In Complex With Sulfiredoxin
gi|260656338 Mass: 23072 Score: 117 Matches: 6(3) Sequences: 6(3)
Chain A, Crystal Structure Of Sulfiredoxin In Complex With Peroxiredoxin I And Atp:mg2+

Figure 23. The protein derived from Far Western blot using rLipL32 binding to the crude membrane proteins was identified by LC-MS and was shown to be peroxiredoxin 1.

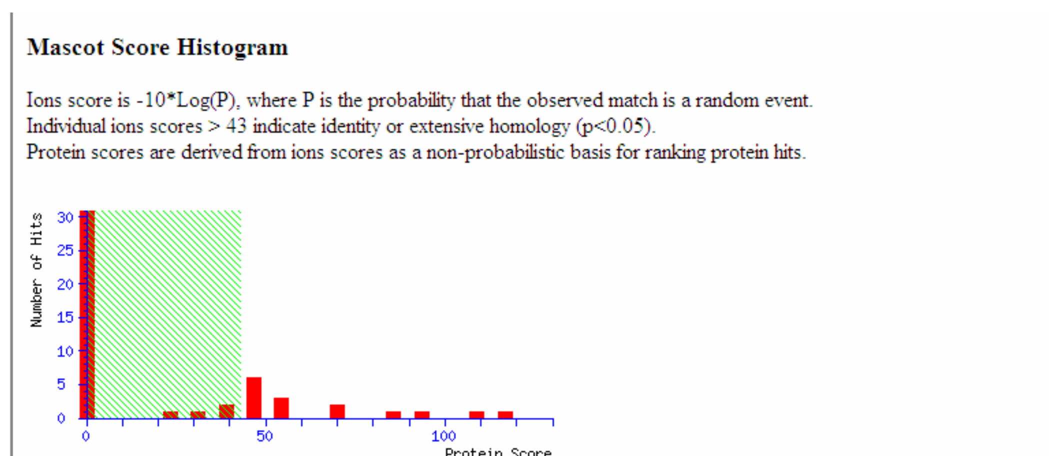


Figure 24. Mascot score histogram of matched protein derived from Far Western blot using rLipL32 binding to the crude membrane proteins.

1. gi|4505591 Mass: 22324 Score: 361 Matches: 10(0) Sequences: 8(0) emPAI: 0.61
peroxiredoxin-1 [Homo sapiens]
☐ Check to include this hit in error tolerant search

	Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Unique	Peptide
☑	36	410.2198	818.4251	818.4134	0.0117	0	41	7.4	1		R.SVDETLR.L
☑	37	819.4324	818.4251	818.4134	0.0117	0	(18)	1.5e+03	1		R.SVDETLR.L
☑	76	940.4798	939.4726	939.4549	0.0177	0	40	7.2	1	U	K.DISLSDYK.G
☑	122	1107.6276	1106.6203	1106.5972	0.0232	0	58	0.1	1		R.TIAQDYGVLK.A
☑	123	554.3175	1106.6203	1106.5972	0.0232	0	(46)	2	1		R.TIAQDYGVLK.A
	126	563.3043	1124.5940	1124.5713	0.0227	1	35	20	2	U	K.DISLSDYKGK.Y
☑	141	598.8316	1195.6486	1195.6237	0.0249	0	55	0.21	1		R.LVQAFQFTDK.H
☑	145	606.3543	1210.6941	1210.6670	0.0271	0	57	0.12	1		R.QITVNDLPVGR.S
☑	173	680.4178	1358.8211	1358.7922	0.0290	1	54	0.27	1		R.GLFIIDDKGILR.Q
	221	661.6879	1982.0418	1982.0109	0.0309	1	21	4e+02	3		R.TIAQDYGVLKADEGISFR.G

Proteins matching the same set of peptides:
gi|55824562 Mass: 21081 Score: 361 Matches: 10(0) Sequences: 8(0)
peroxiredoxin 1 [Macaca fascicularis]
gi|55959887 Mass: 19135 Score: 361 Matches: 10(0) Sequences: 8(0)
peroxiredoxin 1 [Homo sapiens]
gi|60654321 Mass: 22399 Score: 361 Matches: 10(0) Sequences: 8(0)
peroxiredoxin 1 [synthetic construct]
gi|75043305 Mass: 22338 Score: 361 Matches: 10(0) Sequences: 8(0)
RecName: Full=Peroxiredoxin-1
gi|90075488 Mass: 22004 Score: 361 Matches: 10(0) Sequences: 8(0)

Figure 25. The protein derived from Far Western blot using rLipL32 binding to the soluble part of membrane proteins was identified by LC-MS and was shown to be peroxiredoxin 1.

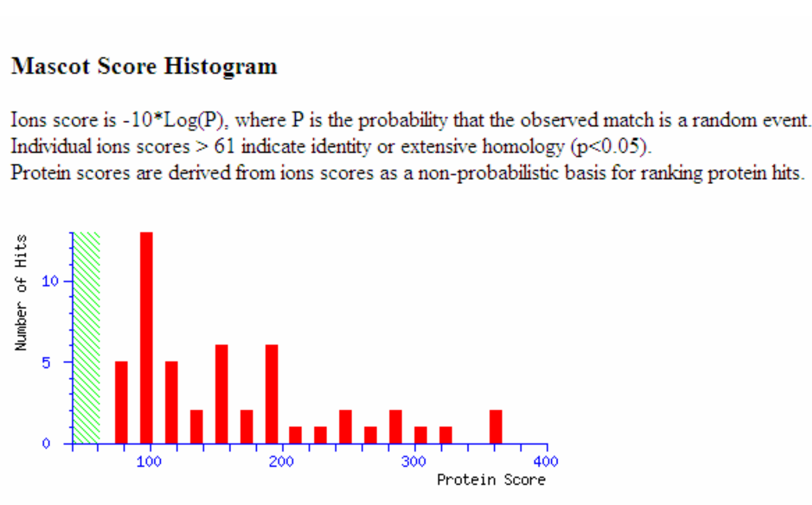


Figure 26. Mascot score histogram of matched protein derived from Far Western blot using rLipL32 binding to the soluble part of membrane proteins.

1. gi|4505591 Mass: 22324 Score: 145 Matches: 11(4) Sequences: 9(4) emPAI: 2.03
peroxiredoxin-1 [Homo sapiens]
Check to include this hit in error tolerant search

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Unique	Peptide
23	819.4360	818.4287	818.4134	0.0153	0	(20)	17	2	U	R.SVDETLR.L
24	410.2220	818.4295	818.4134	0.0161	0	41	0.12	1	U	R.SVDETLR.L
53	940.4801	939.4729	939.4549	0.0180	0	24	3.9	1	U	K.DISLSDYK.G
88	554.3177	1106.6208	1106.5972	0.0237	0	46	0.031	1	U	R.TIAQDYGVLK.A
91	563.3046	1124.5947	1124.5713	0.0234	1	36	0.27	1	U	K.DISLSDYKGK.Y
106	598.8340	1195.6534	1195.6237	0.0297	0	56	0.0028	1	U	R.LVQAFQFTDK.H
110	606.3555	1210.6965	1210.6670	0.0295	0	58	0.0016	1	U	R.QITVNDLPVGR.S
146	680.4164	1358.8182	1358.7922	0.0260	1	(36)	0.27	1	U	R.GLFIIDDKGILR.Q
147	680.4198	1358.8250	1358.7922	0.0328	1	60	0.0011	1	U	R.GLFIIDDKGILR.Q
186	819.9411	1637.8677	1637.8447	0.0230	0	37	0.18	1	U	K.QGGLGPMNIPLVSDPK.R + Oxidation (M)
220	992.0314	1982.0483	1982.0109	0.0374	1	7	1.6e+02	1	U	R.TIAQDYGVLKADEGISFR.G

Proteins matching the same set of peptides:
gi|55959887 Mass: 19135 Score: 145 Matches: 11(4) Sequences: 9(4)
peroxiredoxin 1 [Homo sapiens]
gi|119627382 Mass: 20943 Score: 145 Matches: 11(4) Sequences: 9(4)
peroxiredoxin 1, isoform CRA_b [Homo sapiens]
gi|164519504 Mass: 22147 Score: 145 Matches: 11(4) Sequences: 9(4)
Chain A, Crystal Structure Of Human Peroxiredoxin I In Complex With Sulfiredoxin
gi|260656338 Mass: 23072 Score: 145 Matches: 11(4) Sequences: 9(4)
Chain A, Crystal Structure Of Sulfiredoxin In Complex With Peroxiredoxin I And Atp:mg2+

Figure 27. The protein derived from Far Western blot using rLipL32 binding to the insoluble part of membrane proteins was identified by LC-MS and was shown to be peroxiredoxin 1.

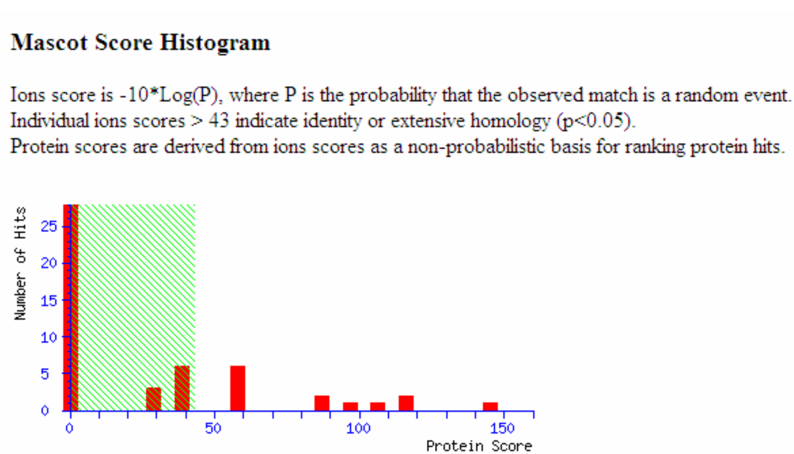


Figure 28. Mascot score histogram of matched protein derived from Far Western blot using rLipL32 binding to the insoluble part of membrane proteins.

Protein analysis

The rLipL32 was shown to bind to proteins with predicted size of approximately 22 kDa in both soluble and insoluble parts of membrane proteins. The protein of interest was analysed by liquid chromatography–mass spectrometer. After that, the obtained amino acid sequences were searched by the Mascot search engine and was shown to match peroxiredoxin. Peroxiredoxin was present in both soluble and insoluble parts of membrane proteins (Figure 23, 25 and 27).

Phage display screening

T7 select® cDNA liver phage display library was used to screen for proteins that interact with whole cells of wild-type *Leptospira* and subtracted with *lipL32*$^-$ mutant strain of *L. interrogans*. The results may reveal host proteins that interact with pathogenic *Leptospira* which may be important for the initial step of host-protein interaction. Two independent experiments were performed. The results obtained from the experiment yielding higher enrichment were reported.

The phage titer of bound phages from each round of bio-panning was counted as plaque forming unit (pfu). The recovery rate was calculated by the proportion of output to input phage titers.

After four rounds of bio-panning only 10-fold enrichment was obtained. However, the fifth round of bio-panning was able to increase 317-fold of specific clones in comparison to the first round of bio-panning. This finding is similar to the results obtained from previous study using T7 cDNA phage display library [238].

Table 3. Titers of input and output phages bound to pathogenic *Leptospira interrogans* serovar Manila (wild type LipL32) after each round of bio-panning

Round	% Tween 20	Input phage titer (pfu)	Output phage titer (pfu)	Recovery Rate*
1	0.1	$1x10^{12}$	$3 x10^{9}$	$3 x10^{-3}$
2	0.5	$1.1x10^{9}$	$1x10^{8}$	$9x10^{-2}$
3	0.5	$1.x10^{9}$	$8x10^{7}$	$8 x10^{-2}$
4	0.5	$1.3x10^{10}$	$4x10^{8}$	$3x10^{-2}$
5	0.5	$2.1x10^{10}$	$2x10^{8}$	$9.52x10^{-1}$

Recovery rate is the proportion of output to input phage titer

Table 4. Titers of input and output phages subtracted with mutant *lipl32*$^-$ strain of *Leptospira interrogans* serovar Manila after each round of panning

Round	% Tween 20	Input phage titer (pfu)	Output phage titer (pfu)	Recovery Rate*
1	0.1	$1x10^{10}$	$1.1x10^{9}$	$1.1x10^{-1}$
2	0.5	$8x10^{9}$	$1x10^{9}$	$1.2x10^{-1}$
3	0.5	$1.2x10^{11}$	$1.3x10^{10}$	$1.08x10^{-1}$
4	0.5	$1.2.x10^{11}$	$2.1x10^{10}$	$1.7x10^{-1}$
5	0.5	$4.8x10^{11}$	$6.7x10^{10}$	$1.3x10^{-1}$

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

Seventeen plaques were randomly selected from the fifth round of bio-panning and were subjected to sequencing. Approximately 317 folds increase of recovery rate after fifth round of bio-panning.

Sequencing

After plaque amplification, the purified PCR product of 16 plaques were analysed by gel electrophoresis (Figure 29). PCR products of various sizes were subjected to sequencing.

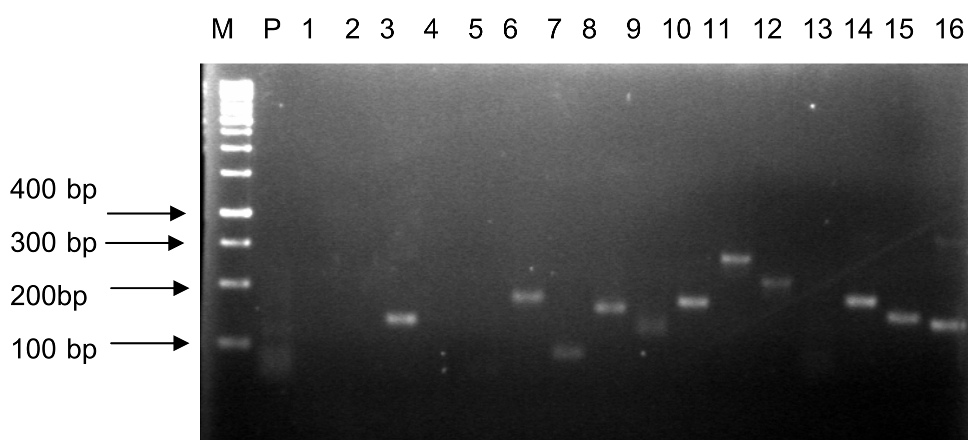


Figure 29. PCR products of individual plaque amplification on agarose gel. M, 1 kb DNA Ladder; P, positive control is DNA of T7 phage; lanes 1-16, PCR products of different clones of plaques obtained from phage display sceening.

DNA sequencing results of selected plaques were searched for matched human proteins in GenBank database as shown in Table 5. Several different proteins were retrieved from phage display screening using whole-cell *Leptospira*. Serpin peptidase inhibitor, clade A is the most common proteins identified (Figure 30), 12.5 % of total sequences (2 clones out of total 16 clones). Serine/threonine kinase 35, angiopoietin 2, and albumin were each found in 6.25 % of total sequences (1 clone out of total 16 clones)

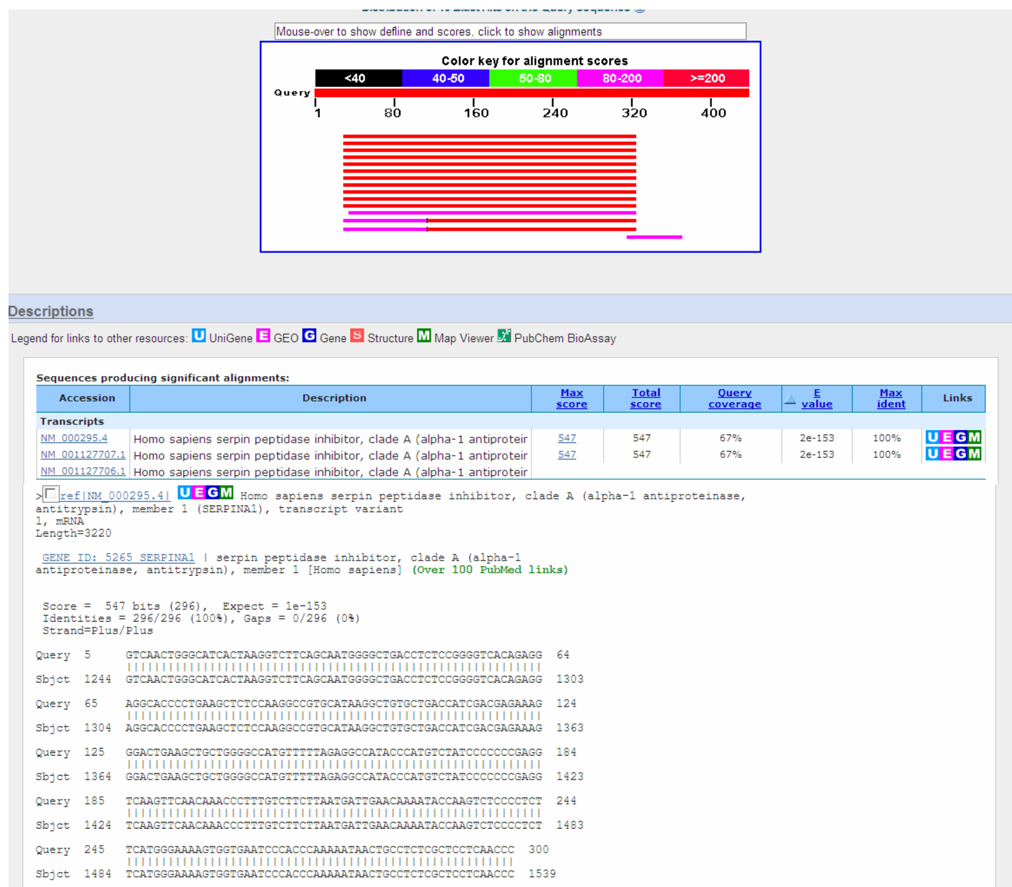


Figure 30. Sequence alignment of peptide sequence 1 to serpin peptidase inhibitor, clade A

Table5. Putative proteins from BLAST analysis and their functions

Sequences	Matched proteins	Reported function	Expression cell	References
Sequence 1 12.5% of total sequences (2 clones out of total 16 clones)	Serpin peptidase inhibitor, clade A	Inhibitor of serine proteases, Involve in the protection of the lower respiratory tract against proteolytic destruction by human leukocyte elastase.	Expressed in extracellular space, Platelet alpha granule lumen, proteinaceous extracellular matrix	[239-244]
Sequence 2 6.25% of total sequences (1 clones out of total 16 clones)	Serine/threonine kinase 35	Kinase protein, Interacts with PDLIM1/CLP-36	located in cytoplasm and nucleus and nucleolus of endothelial cells. , localized to actin stress fibers	[245-247]
Sequence 3 6.25% of total sequences (1 clones out of total 16clones)	Angiopoietin	Induce tyrosine phosphorylation of TIE2, In the absence it, inducers may induce endothelial cell apoptosis.	Expressed in extracellular space	[248]

Table5 (Continue)

Sequences	Matched proteins	Reported function	Expression cell	References
Sequence 4 6.25% of total sequences (1 clones out of total 16 clones)	Albumin	Regulate of the colloidal osmotic pressure of blood, transport zinc in plasma	Expressed in plasma, extracellular space, Platelet alpha granule lumen	[249-253]

In the phage display screening using whole-cell *Leptospira,* various proteins were identified. It is possible that several OMPs on the surface can bind to different target proteins on *Leptospira* and leptospiral cell surface may be tenacious. Therefore, single protein, rLipL32, was subsequently used as a target for T7 select® cDNA liver phage display library to get higher specificity of protein binding than the results obtained from using whole-cell *Leptospira* as a target. The recovery rate of bound phage from each round of bio-panning was calculated as the proportion of output to input phage titer.

Table 6. Recovery phage of T7 select® cDNA liver phage display library incubated with LipL32 purified protein

Round	% Tween 20	Input phage titer (pfu)	Output phage titer (pfu)	Recovery rate*
1	0.1	$8x10^{10}$	$7.4x10^{4}$	$9.25x10^{-7}$
2	0.5	$1.84x10^{10}$	$1x10^{5}$	$5.43x10^{-6}$
3	0.5	$1.6x10^{10}$	$1x10^{6}$	$6.25x10^{-5}$
4	0.5	$2.44x10^{8}$	$8x10^{4}$	$3.27x10^{-4}$
5	0.5	$8x10^{10}$	$3x10^{8}$	$3.7x10^{-2}$
6	0.5	$1.2x10^{11}$	$4x10^{9}$	$3.3x10^{-2}$

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

Approximately 3×10^{4} fold-increase of recovery rate was obtained after the sixth round of bio-panning. Twenty-eight plaques were randomly selected from the sixth round output phages and were sequenced.

After plaque amplification, PCR product from 1-14 plaques (Figure 31) and 15-28 plaques (Figure 32) were analysed by gel electrophoresis and sequenced. We found that the dominant size of PCR products obtained from 19 plaques out of total 28 plaques was approximately 600 bp, whereas the phage display screening using whole-cell *Leptospira* resulted in distinct sizes of PCR products.

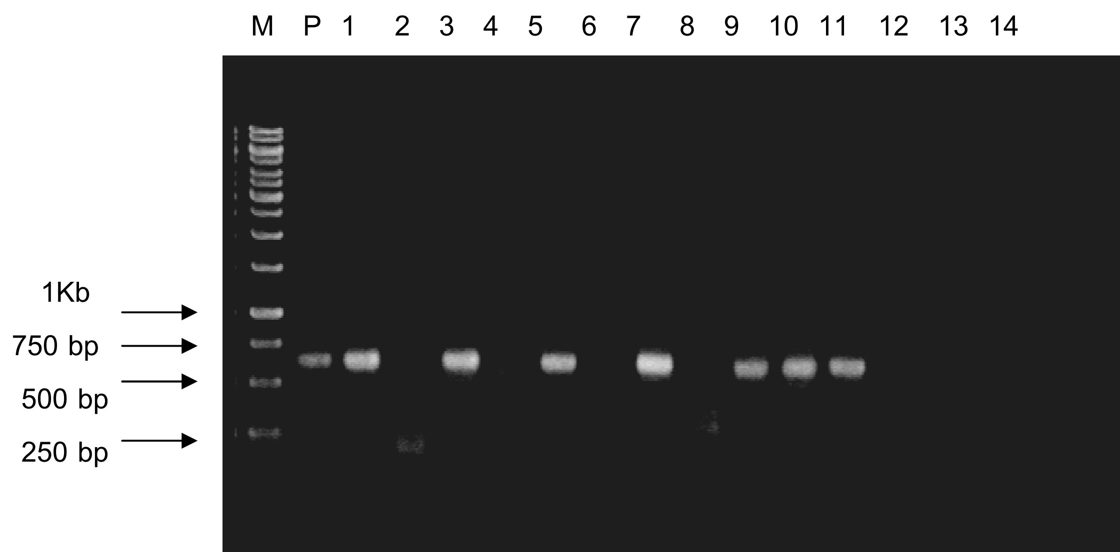


Figure 31. PCR products of individual plaque amplification on agarose gel. M, 1 kb DNA Ladder;P. positive control is DNA of T7 phage; lanes 1-14, PCR products of different clones of plaques obtained from phage display screening.

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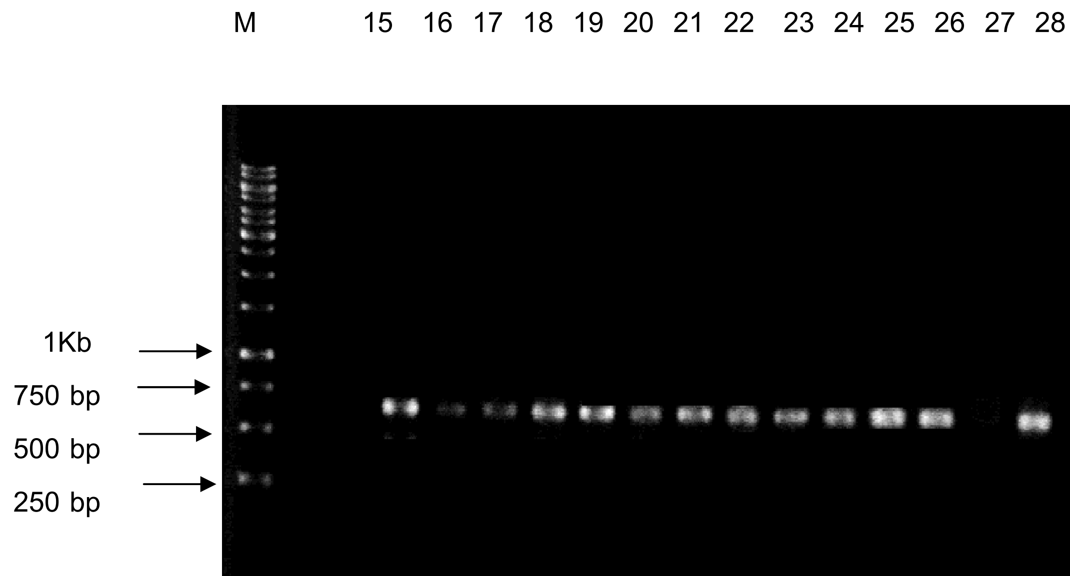

Figure 32. PCR products analyzed of individual plaques amplification on agarose gel. The gel shows the PCR product from individual plaques. ; M, 1 kbp DNA Ladder; lanes 15-28 show PCR product.

After sequencing of selected plaques, DNA sequencing results were compared to human sequence in GenBank database as shown in Table7. ATP synthase is the major proteins identified (Figure 33) from 68% of total sequences (19 clones out of total 28 clones).

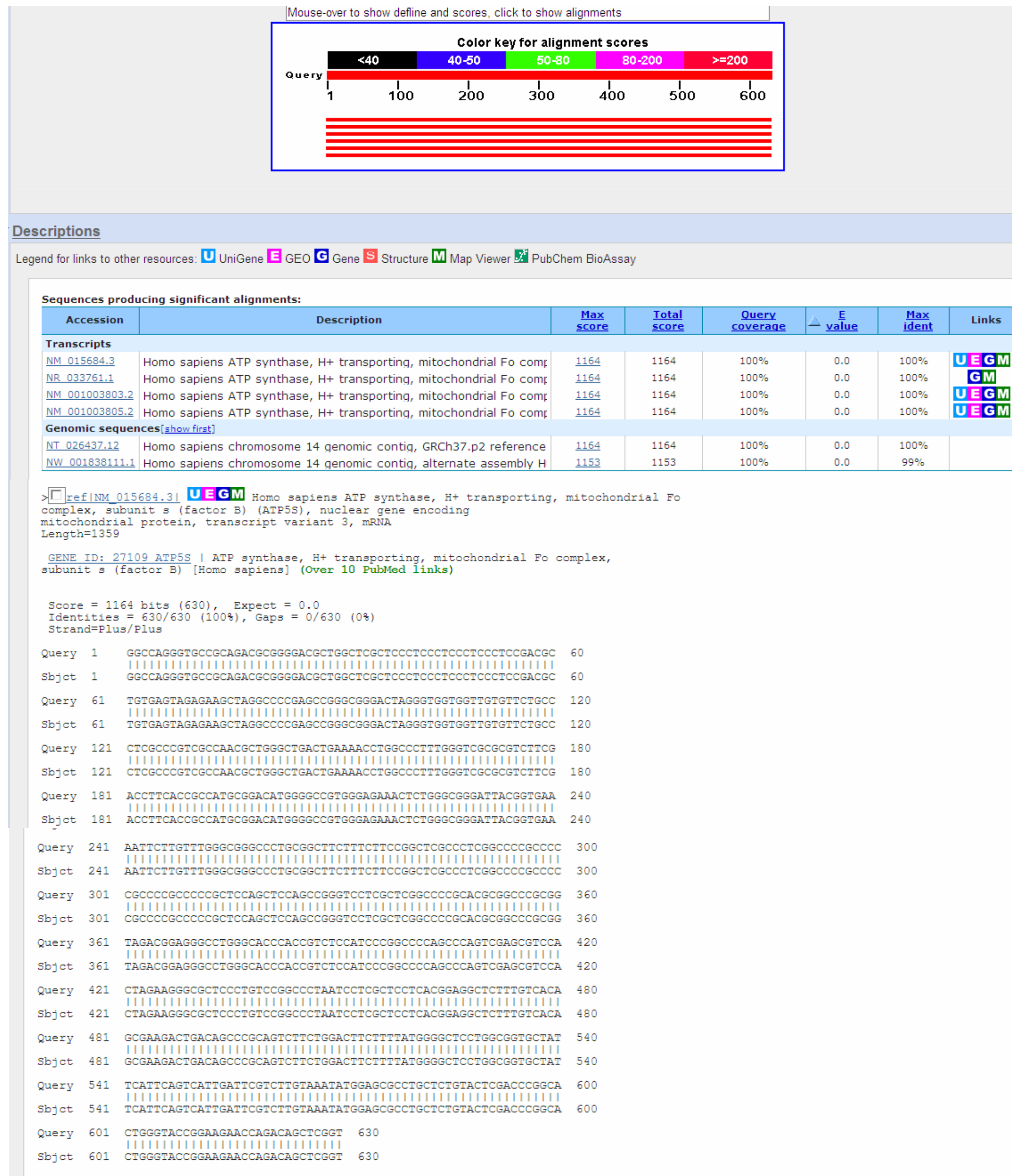

Figure 33. Sequence alignment of peptide sequence 1 to ATP synthase.

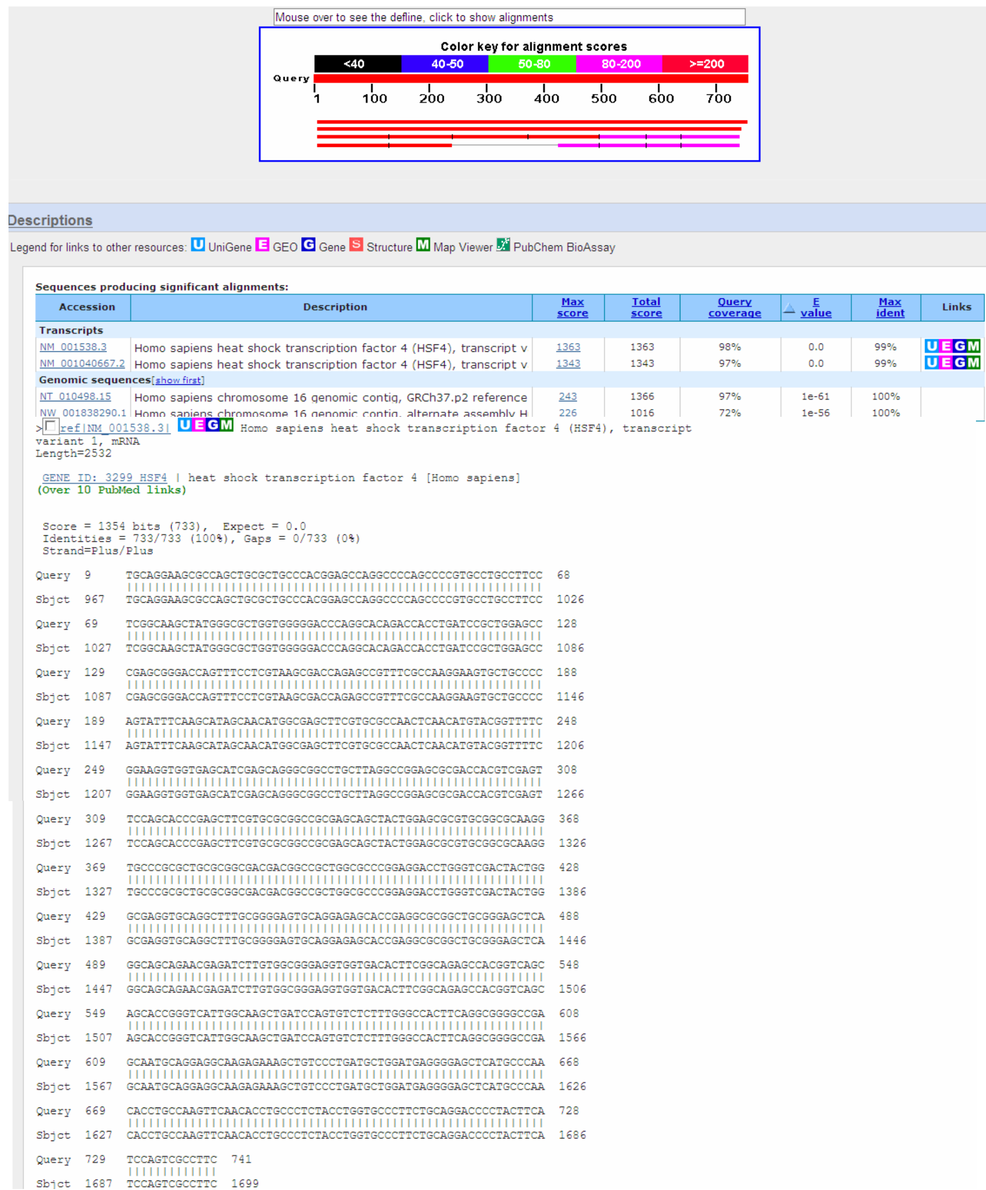

Figure 34. Sequence alignment of peptide sequence 2 to heat shock transcription factor 4.

Table 7. Putative proteins from BLAST analysis and their functions

Sequcenes	Matched proteins	Reported function	Expression cell	References
Sequence 1 68% of total sequences (19 clones out of total 28 clones)	ATP synthase	Located in microchondrion inner membrane and peripheral membrane protein, produced ATP which is energy for the cell, ATP systhase on the surface of human cells able to interacted with many proteins such as plasminogen and histidine-rich glycoprotein	Expressed in fetal lung, heart, liver, gut and kidney. Expressed at higher levels in the fetal brain, retina and spinal cord.	[254-257]
Sequence 2 7% of total sequences (2 clones out of total 28 clones)	Heat shock transcription factor 4	DNA-binding protein that regulate the expression of the heat shock proteins, the expression of heat shock proteins is increased when cells were induced by higher temperatures or other stress.	Expressed in heart, skeletal muscle, eye , brain, and at much lower levels in some other tissues.	[258-261]

CHAPTER VI
DISCUSSION

Host-microbe interactions generally involve in bacterial adhesion and colonization, invasion, immune cell activation, and phagocytosis. Several pathogenic bacteria including pathogenic spirochetes produce a surface layer or adhesins for initial host interactions [14-16]. Surface-exposed outer membrane proteins (OMPs) are important for interaction between pathogenic *Leptospira* and host cells [17-21]. In contrast to non-pathogenic leptospires, pathogenic *Leptospira* are able to adhere to and colonize host cells after haematogenous dissemination before causing organ damage [22, 23]. However, the function of several OMPs of pathogenic *Leptospira* remains unclear.

LipL32 is the most abundant surface-exposed protein of pathogenic *Leptospira*, . This protein is highly conserved among pathogenic leptospires and not found in non-pathogenic *Leptospira* [17, 24]. LipL32 was expressed in leptospires during acute lethal infection [27] and highly immunogenic [28, 29]. Several studies reported the immuogenicity of LipL32. Antibody against LipL32 was generated while infection both in patients and animal model [209, 262, 263] and was shown to help protection in animal model [193, 264]. In addition, the C-terminus of LipL32 was found to bind to ECM proteins [30, 31]. Surprisingly, *lipL32*$^{-}$ mutant constructed by transposon mutagenesis was not attenuated both in animal models of acute and chronic infection [32]. It is possible that other OMPs may compensate for its function for its absence in the *lipL32*$^{-}$ mutant. Since LipL32 is highly conserved in pathogenic *Leptospira* and is expressed during *Leptospira* infection in animals and patients, LipL32 must play a role in pathogenesis of leptospirosis. Therefore, the interaction of LipL32 to host proteins should be investigated.

In this study, we used far western blot as an approach to identify host proteins that interact with LipL32. The recombinant LipL32 was utilized as a target protein. After duplicate experiments, the same host protein bound to rLipL32, peroxiredoxin, was identified. Surprisingly, the result showed that several size of LipL32 was detected by anti-LipL32 (lane 11, figure 22). It is possible that (i) several isoforms of LipL32 was separated on SDS-PAGE [210]; (ii) LipL32 was detected by polyclonal anti-LipL32. Therefore, the experiment should be performed again using monoclonal antibody against LipL32. The interaction between peroxiredoxin and LipL32 should be further investigated.

Peroxiredoxins (Prx) are in the family of peroxidases with antioxidant properties. Prx catalyzes the reduction of hydrogen peroxide, alkyl hydroperoxides, and peroxinitrite [265-267]. During infectious process, reactive oxygen species (ROS) released by several inflammatory cells can cause damage of cellular macromolecules such as DNAs, lipids and proteins leading to injury of cells and tissues. Therefore, cells must produce antioxidant enzymes against oxidative stress. Previous studies showed that oxidant/antioxidant imbalance may be contributed to pathogenesis of the processes of inflammation and fibrosis [268, 269].

Mammalian peroxiredoxins are classified into six types based on the presence of a conserved amino acid sequence and catalysis mechanism. Peroxiredoxins are present in diversed tissues [270, 271]. All six types of peroxiredoxins are expressed in the lung such as bronchial and alveolar epithelial cells [272-274]. Moreover, Prx I and Prx II have a molecular mass of 23 and 21.8 kDa [270] , respectively which a molecular mass of their is similar to rLipL32 binding protein in our study (Figure 22). Although peroxiredoxins are generally located in the cytoplasm and mitochondria, the current reports suggested that Prx was secreted from cells and function in the extracellular space [275, 276]. Prx II can be linked to the erythrocyte membrane [277-283] and invole in mechanisms of defense against lipid peroxidation [278]. In addition, Prx I is expressed in alveolar macrophages [284]. Secreted Prxs has been shown to function as

a cytokine, can activate NFk-B [285]. In addition, Th2 responses were activated by Prxs in helminths parasites infection. Recently, secreted Prxs is classified as one of damage-associated molecular patterns (DAMPs) which are host derived molecules with the capability to induce immune responses and activate immune cells leading to inflammation [286-288].

Previous studies reported that LipL32 preparation from *L. shermani* caused dose-dependent expression of monocyte chemoattractant protein (MCP)-1, regulated on activation normal T cell expression (RANTES), iNOS, TNF-α, NFk-B and AP1 transcription factors in proximal tubule cells [209]. Therefore, interaction between secreted peroxiredoxin and LipL32 of pathogenic *Leptospira* may cause immune modulation in the host.

Furthermore, previous studies reported that Prx I-deficient (Prx $I^{-/-}$) mice showed a higher sensitivity to ferric-nitrilotriacetate-induced oxidative tissue damage in the liver and kidney [289]. The Prx $I^{-/-}$ mice showed more severe gastritis induced by *Helicobacter pylori* [290]. These findings suggest that Prx I plays a critical role in protection against tissue inflammation and fibrosis [291] [292, 293]. In addition, the interaction of Prx1 with a novel interaction partner p66CH2CB was reported to reduce its ability to induce mitochondrial rupture [294]. Binding to LipL32 may impede antioxidant function of peroxiredoxins leading to inflammation in leptospirosis [268, 269]. However, binding of LipL32 to Prx and its role in pathogenesis of leptospirosis needs to be verified.

Moreover, we used phage display technique for screening surface proteins that interacted with recombinant protein LipL32. The target proteins for bio-panning with cDNA liver phage library are recombinant LipL32. Approximately 3 x 10^4 folds increase of recovery rate after sixth round of bio-panning as indicating that its is high enrichment .The result showed that ATP synthase was identified in 68% out of total sequences which is high affinity selection. Approximately sixth folds of recovery rate increase

indicating binding between ATP synthase and LipL32 is specific. While the target proteins for bio-panning with cDNA liver phage library are whole cell *Leptospira* and subtracted with *lipL32*$^{-}$ mutant strain of *L*. *interrogans*. Approximately 317 folds increase of recovery rate after fifth round of bio-panning as indicating that its is low enrichment .Then, the phage display screening using whole cell *Leptospira* various proteins were identified.

ATP synthase is an enzyme responsible for ATP synthesis. Although ATP synthase is mainly found in the inner membrane of mitochondria [295], the recent reports demonstrated that the ATP synthase components were present on the outer face of the plasma membrane of human, mouse and rat cell types. In addition, ATP synthase functions as receptors for multiple ligands on plasma membrane [255, 296] . ATP synthase is responsible for various processes such as regulation of modulates angiogenesis, cellular immunity , regulates intracellular pH , lipid metabolism, cholesterol homeostasis [297, 298], control of proliferation, differentiation of endothelial cells [299] and immune recognition of tumors [300] or human innate immunity [295]. Previous studies showed that *Candidatus Phytoplasma asteris*, wall-less plant pathogenic bacteria interacts with ATP Synthase [38] . In several biological systems, cell membrane ATP synthase is related to the production of extracellular ATP [296, 301-304]. ATP binding cassette transporter in *Spiroplasma citri* is involved in salivary gland colonization of the vector *Circulifer haematoceps* [305]. On the other hand, previous study suggested that ATP synthase may be crucial for NK-mediated tumor cell cytotoxicity [306]. LipL32 may interact with ATP synthase and interfere function of host cells.

In the phage display screening using whole cell *Leptospira*, various proteins were identified. Therefore, we will only discuss about proteins that may have a potential role in pathogenesis of leptospirosis. First, serpin peptidase inhibitor, clade A is secreted and located in extracellular matrix [241]. It was shown to be responsible for inhibition of proteolytic destruction of the lower respiratory tract by human leukocyte

elastase [241]. Interaction between LipL32 and serpin peptidase inhibitor may interfere function of serpin peptidase inhibitor resulting in tissue damage in leptospirosis.

Identification of host proteins by bacterial pull down assay using whole cell *Leptospira*, there was no difference of surface biotinylated proteins of Vero cell lysate that interacted with wild-type pathogenic *L*. *interrogans* and *lipL32*$^{-}$ mutant. Moreover, non-biotinylated proteins of Vero cells were unable to competitively inhibit binding of biotinylated membrane proteins of Vero cells to whole cell *Leptospira*. The result indicated that specific host proteins binding to LipL32 can not be achieved by bacterial pull down assay. In addition, identification of host proteins by phage display screening or bacterial pull down assay using whole cell *Leptospira* showed that various proteins were identified. This finding may be because (i) there are several OMPs on the surface of *Leptospira* [31], Loa22 [157], Lsa21 [158], LigA and LigB [159, 160] which can bind to host proteins (ii) leptospiral cell may be tenacious resulting in non-specific binding of whole cell *L*. *interrogans* to host proteins.

In this study, the hypothesis was unable to achieve by bacterial pull-down assay or phage display screening using whole cell *Leptospira*. However, we can identify host proteins that interact with LipL32 by Far western blot and phage display screening using purified rLipL32.

CHAPTER VII
SUMMARY

The objective of this study is to identify host proteins that interact with LipL32 by utilization of bacterial pull-down assay, far western blot, and phage display technology. In far western blot method, we obtained a single band of host protein that interacted with LipL32. Peroxiredoxin was then identified by liquid chromatography–mass spectrometry followed by searching for matched protein with the Mascot program. Using T7 cDNA liver phage display library, enrichment of bound phages against recombinant LipL32 obtained high-affinity selected phages displaying peptide sequence that matched to ATPsynthase. However, surface host proteins that interacted with LipL32; i,e. bind to wild-type *Leptospira* but not to *lipL32*$^-$ mutant, could not be identified by bacterial pull down assay.

Host proteins such as peroxiredoxin and ATPsynthase have possible roles in pathogenesis of leptospirosis. However, Further investigations to confirm protein-protein interactions between these proteins and LipL32 *in vitro* and *in vivo* are required.

The knowledge acquired from this study may be useful for better understanding of the function of LipL32 and pathogenesis of leptospirosis.

REFERENCES

[1] Bovet, P., Yersin, C., Merien, F., Davis, C.E., and Perolat, P. Factors associated with clinical leptospirosis: a population-based case-control study in the Seychelles (Indian Ocean). Int J Epidemiol 28 (June 1999) : 583-590.

[2] Bhardwaj, P., Kosambiya, J.K., and Desai, V.K. A case control study to explore the risk factors for acquisition of leptospirosis in Surat city, after flood. Indian J Med Sci 62 (November 2008) : 431-438.

[3] Kawaguchi, L.,,et al. Seroprevalence of leptospirosis and risk factor analysis in flood-prone rural areas in Lao PDR. Am J Trop Med Hyg 78 (June 2008) : 957-961.

[4] Lau, C., Smythe, L., and Weinstein, P. Leptospirosis: an emerging disease in travellers. Travel Med Infect Dis 8 (January) : 33-39.

[5] Maskey, M., Shastri, J.S., Saraswathi, K., Surpam, R., and Vaidya, N. Leptospirosis in Mumbai: post-deluge outbreak 2005. Indian J Med Microbiol 24 (October 2006) : 337-338.

[6] Sehgal, S.C., Sugunan, A.P., and Vijayachari, P. Outbreak of leptospirosis after the cyclone in Orissa. Natl Med J India 15 (January 2002) : 22-23.

[7] Niwetpathomwat, A., Niwatayakul, K., and Doungchawee, G. Surveillance of leptospirosis after flooding at Loei Province, Thailand by year 2002. Southeast Asian J Trop Med Public Health 36 (2005) : 202-205.

[8] Yersin, C., Bovet, P., Merien, F., Wong, T., Panowsky, J., and Perolat, P. Human leptospirosis in the Seychelles (Indian Ocean): a population-based study. Am J Trop Med Hyg 59 (December 1998) : 933-940.

[9] Levett, P.N. Leptospirosis. Clin Microbiol Rev 14 (April 2001) : 296-326.

[10] Nery, L.E., de Paula, A.B., Nakatani, J., dos Santos, M.L., and Ratto, O.R. Clinical, radiological and functional pulmonary manifestations in patients with leptospirosis. Rev Inst Med Trop Sao Paulo 19 (November 1977) : 366-373.

[11] Silverstein, C.M. Pulmonary manifestations of leptospirosis. Radiology 61 (September 1953) : 327-334.

[12] Yersin, C., et al. Pulmonary haemorrhage as a predominant cause of death in leptospirosis in Seychelles. Trans R Soc Trop Med Hyg 94 (January 2000) : 71-76.

[13] Vinetz, J.M., Glass, G.E., Flexner, C.E., Mueller, P., and Kaslow, D.C. Sporadic urban leptospirosis. Ann Intern Med 15 (November 1996) : 794-798.

[14] Kline, K.A., Falker, S., Dahlberg, S., Normark, S., and Henriques, B. Bacterial adhesins in host-microbe interactions. Cell Host Microbe 18 (June 2009) : 580-592.

[15] Kronvall, G., and Jonsson, K. Receptins: a novel term for an expanding spectrum of natural and engineered microbial proteins with binding properties for mammalian proteins. J Mol Recognit 12 (January 1999) : 38-44.

[16] Dehio, C., Gray, S.D., and Meyer, T.F. The role of neisserial Opa proteins in interactions with host cells. Trends Microbiol 6 (December 1998) : 489-495.

[17] Cullen, P.A., et al. Surfaceome of Leptospira spp. Infect Immun 73 (August 2005) : 853-4863.

[18] Schulz, G.E. The structure of bacterial outer membrane proteins. Biochim Biophys Acta 1565 (October 2002) : 308-317.

[19] Louvel, H., et al. Comparative and functional genomic analyses of iron transport and regulation in *Leptospira* spp. J Bacteriol 188 (November 2006) : 7893-7904.

[20] Asuthkar, S., Velineni, S., Stadlmann, J., Altmann, F., and Sritharan, M. Expression and characterization of an iron-regulated hemin-binding protein, HbpA, from *Leptospira interrogans* serovar Lai. Infect Immun 75 (September 2007) : 4582-4591.

[21] Bos, M.P., Robert, V., and Tommassen, J. Biogenesis of the gram-negative bacterial outer membrane. Annu Rev Microbiol 61 (2007) : 191-214.

[22] Bharti, A.R., et al. Leptospirosis: a zoonotic disease of global importance. Lancet Infect Dis 3 (December 2003) : 757-771.

[23] Ko, A.I., Goarant, C., and Picardeau, M. *Leptospira*: the dawn of the molecular genetics era for an emerging zoonotic pathogen. Nat Rev Microbiol 7 (October 2009) : 736-747.

[24] Cullen, P.A., Cordwell, S.J., Bulach, D.M., Haake, D.A., and Adler, B. Global analysis of outer membrane proteins from *Leptospira interrogans* serovar Lai. Infect Immun 70 (May 2002) : 2311-2318.

[25] Levett, P.N., Morey, R.E., Galloway, R.L., Turner, D.E., Steigerwalt, A.G., and Mayer, L.W. Detection of pathogenic leptospires by real-time quantitative PCR. J Med Microbiol 54 (January 2005) : 45-49.

[26] Flannery, B., et al. Evaluation of recombinant *Leptospira* antigen-based enzyme-linked immunosorbent assays for the serodiagnosis of leptospirosis. J Clin Microbiol 39 (September 2001) : 3303-3310.

[27] Nally, J.E., Whitelegge, J.P., Bassilian, S., Blanco, D.R., and Lovett, M.A. Characterization of the outer membrane proteome of *Leptospira interrogans* expressed during acute lethal infection. Infect Immun 75 (February 2007) : 766-773.

[28] Haake, D.A., et al. The leptospiral major outer membrane protein LipL32 is a lipoprotein expressed during mammalian infection. Infect Immun 68 (April 2000) : 2276-2285.

[29] Haake, D.A., Suchard, M.A., Kelley, M.M., Dundoo, M., Alt, D.P., and Zuerner, R.L. Molecular evolution and mosaicism of leptospiral outer membrane proteins involves horizontal DNA transfer. J Bacteriol 186 (May 2004) : 2818-2828.

[30] Hoke, D.E., Egan, S., Cullen, P.A., and Adler, B. LipL32 is an extracellular matrix-interacting protein of *Leptospira* spp. and *Pseudoalteromonas tunicata*. Infect Immun 76 (May 2008) : 2063-2069.

[31] Hauk, P., et al. 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 (June 2008) : 2642-2650.

[32] Murray, G.L., et al. Major surface protein LipL32 is not required for either acute or chronic infection with *Leptospira interrogans*. Infect Immun 77 (March 2009) : 952-958.

[33] Kaido, M., et al. Downregulation of the NbNACa1 gene encoding a movement-protein-interacting protein reduces cell-to-cell movement of Brome mosaic virus in *Nicotiana benthamiana*. Mol Plant Microbe Interact 20 (June 2007) : 671-681.

[34] Vasavada, H.A., Ganguly, S., Germino, F.J., Wang, Z.X., and Weissman, S.M. A contingent replication assay for the detection of protein-protein interactions in animal cells. Proc Natl Acad Sci U S A 88 (December 1991) : 10686-10690.

[35] Hall, R.A. Studying protein-protein interactions via blot overlay or Far Western blot. Methods Mol Biol 261 (2004) : 167-174.

[36] Gupta, R., et al. FANCJ (BACH1) helicase forms DNA damage inducible foci with replication protein A and interacts physically and functionally with the single-stranded DNA-binding protein. Blood 110 (October 2007) : 2390-2398.

[37] Feldman, M., Zusman, T., Hagag, S., and Segal G. Coevolution between nonhomologous but functionally similar proteins and their conserved partners in the *Legionella* pathogenesis system. Proc Natl Acad Sci U S A 102 (August 2005) : 12206-12211.

[38] Galetto, L., Bosco, D., Balestrini, R., Genre, A., Fletcher, J., and Marzachi, C. The Major Antigenic Membrane Protein of *Candidatus Phytoplasma asteris* Selectively Interacts with ATP Synthase and Actin of Leafhopper Vectors. PLoS One 6 (July 2011) : 1-12.

[39] Smith, G.P. Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surface. Science 228 (June 1985) : 1315-1317.

[40] Smith, G.P., and Petrenko, V.A. Phage Display. Chem Rev 97 (April 1997) : 391-410.

[41] Mongiovi, A.M., et al. A novel peptide-SH3 interaction. EMBO J 18 (October 1999) : 5300-5309.

[42] Pillutla, R.C., et al. Peptides identify the critical hotspots involved in the biological activation of the insulin receptor. J Biol Chem 277 (June 2002) : 22590-22594.

[43] Binetruy-Tournaire, R., et al. Identification of a peptide blocking vascular endothelial growth factor (VEGF)-mediated angiogenesis. EMBO J 19 (April 2000) : 1525-1533.

[44] Sidhu, S.S., and Koide, S. Phage display for engineering and analyzing protein interaction interfaces. Curr Opin Struct Biol 17 (August 2007) : 481-487.

[45] Koivunen, E., Arap, W., Rajotte, D., Lahdenranta, J., and Pasqualini, R. Identification of receptor ligands with phage display peptide libraries. J Nucl Med 40 (May1999) : 883-888.

[46] Lowman, H.B. Bacteriophage display and discovery of peptide leads for drug development. Annu Rev Biophys Biomol Struct 26 (1997) : 401-424.

[47] Hyde-DeRuyscher, R., et al. Detection of small-molecule enzyme inhibitors with peptides isolated from phage-displayed combinatorial peptide libraries. Chem Biol 7 (January 2000) : 17-25.

[48] Stockwin, L.H., and Holmes, S. The role of therapeutic antibodies in drug discovery. Biochem Soc Trans 31 (April 2003) : 433-436.

[49] Schmitz, U., Versmold, A., Kaufmann, P., and Frank, H.G. Phage display: a molecular tool for the generation of antibodies--a review. Placenta 21 (March 2000) : 106-112.

[50] Sergeeva, A., Kolonin, M.G., Molldrem, J.J., Pasqualini, R., and Arap, W. Display technologies: application for the discovery of drug and gene delivery agents. Adv Drug Deliv Rev 58 (December 2006) : 1622-1654.

[51] Kola, A., et al. Epitope mapping of a C5a neutralizing mAb using a combined approach of phage display, synthetic peptides and site-directed mutagenesis. Immunotechnology 2 (June 1996) : 115-126.

[52] Ramasoota, P., Tungtrakanpoung, R., Pitaksajjakul, P., Ekpo, P., Froman, G., and Chaicumpa, W. Epitope mapping of monoclonal antibodies specific to serovar of *Leptospira*, using phage display technique. Southeast Asian J Trop Med Public Health 36 (2005) : 206-212.

[53] Oleksiewicz, M.B., Botner, A., Toft, P., Normann, P., and Storgaard, T. 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 (April 2001) : 3277-3290.

[54] Mullaney, B.P., Pallavicini, M.G., and Marks, J.D. Epitope mapping of neutralizing botulinum neurotoxin A antibodies by phage display. Infect Immun 69 (October 2001) : 6511-6514.

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

[56] Mullen, L.M., Nair, S.P., Ward, J.M., Rycroft, A.N., Williams, R.J., and Henderson, B. Comparative functional genomic analysis of *Pasteurellaceae* adhesins using phage display. Vet Microbiol 122 (May 2007) : 123-134.

[57] Antonara, S., Chafel, R.M., LaFrance, M., and Coburn, J. *Borrelia burgdorferi* adhesins identified using in vivo phage display. Mol Microbiol 66 (October 2007) : 262-276.

[58] Paster, B.J., et al. Phylogenetic analysis of the spirochetes. J Bacteriol 173 (October 1991) : 6101-6109.

[59] Hyde, F.W., and Johnson, R.C. Genetic relationship of lyme disease spirochetes to *Borrelia*, *Treponema*, and *Leptospira* spp. J Clin Microbiol 20 (August 1984) : 151-154.

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

[61] Vijayachari, P., Sugunan, A.P., and Shriram, A.N. Leptospirosis: an emerging global public health problem. J Biosci 33 (November 2008) : 557-569.

[62] Brenner, D.J., Kaufmann, A.F., Sulzer, K.R., Steigerwalt, A.G., Rogers, F.C., and Weyant, R.S. 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 (April 1999) : 839-858.

[63] Sasaki, F., Kano, R., Nakamura, Y., Tsujimoto, H., Yamamoto, S., and Hasegawa, A. Phylogenetic analysis of *Leptospira* strains of pathogenic serovars using 23S rDNA gene sequences. Microbiol Res 154 (September 1999) : 167-172.

[64] Cerqueira, G.M., and Picardeau, M. A century of *Leptospira* strain typing. Infect Genet Evol 9 (September 2009) : 760-768.

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

[66] Adler, B., and de la Pena Moctezuma, A. *Leptospira* and leptospirosis. Vet Microbiol (Mar 2009).

[67] Li, C., Motaleb, A., Sal, M., Goldstein, S.F., and Charon, N.W. Spirochete periplasmic flagella and motility. J Mol Microbiol Biotechnol 2 (October 2000) : 345-354.

[68] Ellis, W.A., and Michno, S.W. Bovine leptospirosis: a serological and clinical study. Vet Rec 99 (November 1976) : 387-391.

[69] Picardeau, M., Brenot, A., and Saint Girons, I. First evidence for gene replacement in *Leptospira* spp. Inactivation of *L. biflexa* flaB results in non-motile mutants deficient in endoflagella. Mol Microbiol 40 (April 2001) : 189-199.

[70] Cullen, P.A., Haake, D.A., and Adler, B. Outer membrane proteins of pathogenic spirochetes. FEMS Microbiol Rev 28 (June 2004) : 291-318.

[71] Kemp, P., Garcia, L.R., and Molineux, I.J. Changes in bacteriophage T7 virion structure at the initiation of infection. Virology 340 (September 2005) : 307-317.

[72] Ellinghausen, H.C., Jr., and McCullough, W.G. Nutrition of *Leptospira* Pomona and Growth of 13 Other Serotypes: Fractionation of Oleic Albumin Complex and a Medium of Bovine Albumin and Polysorbate 80. Am J Vet Res 26 (January 1965) : 45-51.

[73] Ellinghausen, H.C, Jr., and McCullough, W.G. Nutrition of *Leptospira* Pomona and Growth of 13 Other Serotypes: A Serum-Free Medium Employing Oleic Albumin Complex. Am J Vet Res 26 (January 1965) : 39-44.

[74] Johnson, R.C., and Rogers, P. Metabolism of leptospires. II. The action of 8-azaguanine. Can J Microbiol 13 (December 1967) : 1621-1629.

[75] Smith, C.E., and Turner, L.H. The effect of pH on the survival of leptospires in water. Bull World Health Organ 24 (1961) : 35-43.

[76] Henneberry, R.C., and Cox, C.D. Beta-oxidation of fatty acids by *Leptospira*. Can J Microbiol 16 (January 1970) : 41-45.

[77] Johnson, R.C., Walby, J., Henry, R.A., and Auran, N.E. Cultivation of parasitic leptospires: effect of pyruvate. Appl Microbiol 26 (July 1973) : 118-119.

[78] Menges, R.W., and Galton, M.M. Direct cultural methods for the isolation of leptospires from experimentally infected guinea pigs. Am J Vet Res 22 (November 1961) : 1085-1092.

[79] Adler, B., and de la Pena Moctezuma, A. *Leptospira* and leptospirosis. Vet Microbiol 140 (January 2009) : 287-296.

[80] Roth, E.E., Linder, D., and Adams, W.V. The use of agar plates as an aid for the isolation of leptospires. Am J Vet Res 22 (March 1961) : 308-312.

[81] Tripathy, D.N., Hanson, L.E., and Jones, F.C. Growth of hebdomadis group of leptospires in solid medium. Am J Vet Res 41 (July 1980) : 1153-1154.

[82] Thiermann, A.B. Use of solid medium for isolation of leptospires of the Hebdomadis serogroup from bovine milk and urine. Am J Vet Res 42 (December 1981) : 2143-5.

[83] Ristow, P., et al. Biofilm formation by saprophytic and pathogenic leptospires. Microbiology 154 (May 2008) : 1309-1317.

[84] Baril, C., and Saint Girons, I. Sizing of the *Leptospira* genome by pulsed-field agarose gel electrophoresis. FEMS Microbiol Lett 59 (September 1990) : 95-99.

[85] Zuerner, R.L. Physical map of chromosomal and plasmid DNA comprising the genome of *Leptospira interrogans*. Nucleic Acids Res 19 (September 1991) : 4857-4860.

[86] Fukunaga, M., and Mifuchi, I. The number of large ribosomal RNA genes in *Leptospira interrogans* and *Leptospira biflexa*. Microbiol Immunol 33 (1989) : 459-466.

[87] Picardeau, M., et al. Genome sequence of the saprophyte *Leptospira biflexa* provides insights into the evolution of *Leptospira* and the pathogenesis of leptospirosis. PLoS One 3 (February 2008) : e1607.

[88] Croda, J., et al. Targeted mutagenesis in pathogenic *Leptospira* species: disruption of the LigB gene does not affect virulence in animal models of leptospirosis. Infect Immun 76 (December 2008) : 5826-5833.

[89] Murray, G.L., et al. Genome-wide transposon mutagenesis in pathogenic *Leptospira* species. Infect Immun 77 (February 2009) : 810-816.

[90] Epstein, P.R., Calix, O., and Blanco Racedo, J. Climate and disease in Colombia. Lancet 346 (November 1995) : 1243-1244.

[91] Rathinam, S.R., Rathnam, S., Selvaraj, S., Dean, D., Nozik, R.A., and Namperumalsamy, P. Uveitis associated with an epidemic outbreak of leptospirosis. Am J Ophthalmol 124 (July 1997) : 71-79.

[92] Tangkanakul, W., Smits, H.L., Jatanasen, S., and Ashford, D.A. Leptospirosis: an emerging health problem in Thailand. Southeast Asian J Trop Med Public Health 36 (March 2005) : 281-288.

[93] Ellis, R.D., et al. Causes of fever in adults on the Thai-Myanmar border. Am J Trop Med Hyg 74 (January 2006) : 108-113.

[94] สำนักระบาดวิทยา. สรุปรายงานการเฝ้าระวังโรคเลปโตสไปโรซิส. สำนักระบาดวิทยา, กระทรวงสาธารณสุข 2553.

[95] Organization WH. Leptospirosis situation in the WHO South-East Asia Region. WHO India and Regional Medical Research Centre. WHO Collaborating Centre for Diagnosis, Research, Reference and Training in Leptospirosis 2009.

[96] Victoriano, A.F., et al. Leptospirosis in the Asia Pacific region. BMC Infect Dis 9 (2009) : 147.

[97] Viviani, M., Berlot, G., Poldini, F., Silvestri, L., Sabadini, D., and Dezzoni, R. Leptospirosis. Description of a clinical case and review of the literature. Minerva Anestesiol 64 (October 1998) : 465-469.

[98] Wang, C.N., John, L., Chang, T.F., Cheng, W.J., Lou, M.Y., and Hung, A.T. Studies on Anicteric Leptospirosis. I. Clinical Manifestations and Antibiotic Therapy. Chin Med J (Engl) 84 (May 1965) : 283-291.

[99] Hudson, C.P., Levett, P.N., Edwards, C.N., Moosai, R., and Roach, T.C. Severe primary HIV-1 infection among black persons in Barbados. Int J STD AIDS 8 (June 1997) : 393-397.

[100] Sanders, E.J., et al. Increase of letospirosis in dengue-negative patients after a hurricane in Puerto Rico in 1996. Am J Trop Med Hyg 61 (September 1999) : 399-404.

[101] Abdulkader, R.C. Acute renal failure in leptospirosis. Ren Fail 19 (March 1997) : 191-198.

[102] Edwards, C.N., Nicholson, G.D., Hassell, T.A., Everard, C.O., and Callender, J. Leptospirosis in Barbados. A clinical study. West Indian Med J 39 (March 1990) : 27-34.

[103] Alani, F.S., Mahoney, M.P., Ormerod, L.P., Wright, P.A., and Garrues, M. Leptospirosis presenting as atypical pneumonia, respiratory failure and pyogenic meningitis. J Infect 27 (November 1993) : 281-283.

[104] Allen, P., Raftery, S., and Phelan, D. Massive pulmonary haemorrhage due to leptospirosis. Intensive Care Med 15 (1989) : 322-324.

[105] Berendsen, H.H., Rommes, J.H., Hylkema, B.S., Meinesz, A.F., and Sluiter, H.J. Adult espiratory failure with leptospirosis. Ann Intern Med 101 (September 1984) : 402.

[106] Burke, B.J., and Searle, J.F., Mattingly D. Leptospirosis presenting with profuse haemoptysis. Br Med J 2 (October 1976) : 982.

[107] Chee, H.D., Ossenkoppele, G.J., Bronsveld, W., and Thijs, L.G. Adult respiratory distress syndrome in *leptospira icterohaemorrhagiae* infection. Intensive Care Med 11 (1985) : 254-256.

[108] de Koning, J., van der Hoeven, J.G., and Meinders, A.E. Respiratory failure in leptospirosis (Weil's disease). Neth J Med 47 (November 1995) : 224-229.

[109] Dive, A.M., Bigaignon, G., and Reynaert, M. Adult respiratory distress syndrome in *leptospira icterohaemorrhagiae* infection. Intensive Care Med 13 (1987) : 214.

[110] Im, J.G., et al. Leptospirosis of the lung: radiographic findings in 58 patients. AJR Am J Roentgenol 152 (May 1989) : 955-959.

[111] Trevejo, R.T., et al. Epidemic leptospirosis associated with pulmonary hemorrhage-Nicaragua, 1995. J Infect Dis 178 (November 1998) : 1457-1463.

[112] Zaki, S.R., and Shieh, W.J. Leptospirosis associated with outbreak of acute febrile illness and pulmonary haemorrhage, Nicaragua, 1995. The Epidemic Working Group at Ministry of Health in Nicaragua. Lancet 347 (February 1996) : 535-536.

[113] Lin, C., Ma, T.L., Chen, Y.C., and Cheng, W.J. Studies on Anicteric Leptospirosis. Observations on Electrocardiograms. Chin Med J (Engl) 84 (May 1965) : 291-298.

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

[115] Turner, L.H. Leptospirosis. 3. Maintenance, isolation and demonstration of leptospires. Trans R Soc Trop Med Hyg 64 (1970) : 623-646.

[116] Vijayachari, P., Sugunan, A.P., Umapathi, T., and Sehgal, S.C. Evaluation of darkground microscopy as a rapid diagnostic procedure in leptospirosis. Indian J Med Res 114 (August 2001) : 54-58.

[117] Levett, P.N. Usefulness of serologic analysis as a predictor of the infecting serovar in patients with severe leptospirosis. Clin Infect Dis 36 (February 2003) : 447-452.

[118] Bal, A.E., Gravekamp, C., Hartskeerl, R.A., De Meza-Brewster, J., Korver, H., and Terpstra, W.J. Detection of leptospires in urine by PCR for early diagnosis of leptospirosis. J Clin Microbiol 2 (August 1994) : 1894-1898.

[119] Rittenberg, M.B., Linscott, W.D., and Ball, M.G. Simple method for separating leptospirae from contaminating microorganisms. J Bacteriol 76 (December 1958) : 669-670.

[120] Sulzer, A.J., Sulzer, K.R., Cantella, R.A., Colichon, H., Latorre, C.R., and Welch, M. Study of coinciding foci of malaria and leptospirosis in the Peruvian Amazon area. Trans R Soc Trop Med Hyg 72 (1978) : 76-83.

[121] Ellinghausen, H.C., and Jr. Growth, cultural characteristics, and antibacterial sensitivity of *Leptospira interrogans* serovar hardjo. Cornell Vet 73 (July 1983) : 225-239.

[122] Jeandel, P., Raoult, D., Rougier, Y., Auger, C., and Mailloux, M. Late positive blood cultures in leptospiroses (report of four cases). Trans R Soc Trop Med Hyg 78 (1984) : 143-145.

[123] Turner, L.H. Leptospirosis. II. Serology. Trans R Soc Trop Med Hyg 62 (1968) : 880-899.

[124] Cole, J.R., Jr., Sulzer, C.R., and Pursell, A.R. Improved microtechnique for the leptospiral microscopic agglutination test. Appl Microbiol 25 (June 1973) : 976-980.

[125] Galton, M.M., Sulzer, C.R., Santarosa, C.A., and Fields, M.J. Application of a Microtechnique to the Agglutination Test for Leptospiral Antibodies. Appl Microbiol 13 (January 1965) : 81-85.

[126] Sulzer, C.R., and Jones, W.L. A modified semi-micro method for the test for leptospirosis. Health Lab Sci 10 (January 1973) : 13-17.

[127] Cominazzini, C. Laboratory diagnosis of leptospirosis; practical notes and critical study. Minerva Med 45 (February 1954) : 340-347.

[128] Adler, B., Murphy, A.M., Locarnini, S.A., and Faine, S. Detection of specific anti-leptospiral immunoglobulins M and G in human serum by solid-phase enzyme-linked immunosorbent assay. J Clin Microbiol 11 (May 1980) : 452-457.

[129] Mailloux, M., Mazzonelli, J.G., and Dufresne, Y. Application of an immuno-enzyme technique to titration of antibodies in leptospirosis: ELISA (enzyme-linked immunosorbent assay). Zentralbl Bakteriol Mikrobiol Hyg A 257 (September 1984) : 511-513.

[130] Terpstra, W.J., Ligthart, G.S., and Schoone, G.J. ELISA for the detection of specific IgM and IgG in human leptospirosis. J Gen Microbiol 131 (February 1985) : 377-385.

[131] Surujballi, O., and Elmgren, C. 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 (January 2000) : 149-159.

[132] Sharma, R., Tuteja, U., Khushiramani, R., Shukla, J., and Batra, H.V. Application of rapid dot-ELISA for antibody detection of leptospirosis. J Med Microbiol 56 (June 2007) : 873-874.

[133] Vitale, G., et al. Evaluation of an IgM-ELISA test for the diagnosis of human leptospirosis. New Microbiol 27 (April 2004) : 149-154.

[134] Galton, M.M., Powers, D.K., Hall, A.D., and Cornell, R.G. A rapid macroscopicslide screening test for the serodiagnosis of leptospirosis. Am J Vet Res 19 (April 1958) : 505-512.

[135] Andreescu, N. 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 (July 1990) : 223-227.

[136] Lilenbaum, W., Ristow, P., Fraguas, S.A., and da Silva, E.D. Evaluation of a rapid slide agglutination test for the diagnosis of acute canine leptospirosis. Rev Latinoam Microbiol 44 (July 2002) : 124-128.

[137] Senthilkumar, T., Subathra, M., Phil, M., Ramadass, P., and Ramaswamy, V. Rapid serodiagnosis of leptospirosis by latex agglutination test and flow-through assay. Indian J Med Microbiol 26 (January 2008) : 45-49.

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

[139] Hull-Jackson, C., et al. Evaluation of a commercial latex agglutination assay for serological diagnosis of leptospirosis. J Clin Microbiol 44 (May 2006) : 1853-1855.

[140] Pradutkanchana, S., and Nakarin, J. The use of latex agglutination for the diagnosis of acute human leptospirosis. J Med Assoc Thai 88 (October 2005) : 1395-1400.

[141] Dey, S., Mohan, C.M., Ramadass, P., and Nachimuthu, K. Recombinant antigen-based dipstick ELISA for the diagnosis of leptospirosis in dogs. Vet Rec 160 (February 2007) : 186-188.

[142] Boonyod, D., Poovorawan, B., and Chirathaworn, C. LipL32, an outer membrane protein of *Leptospira*, as an antigen in a dipstick assay for diagnosis of leptospirosis. Asian Pac J Allergy Immunol 23 (June 2005) : 133-141.

[143] Smits, H.L., et al. International multicenter evaluation of the clinical utility of a dipstick assay for detection of *Leptospira*-specific immunoglobulin M antibodies in human serum specimens. J Clin Microbiol 37 (September 1999) : 2904-2909.

[144] Appassakij, H., Silpapojakul, K., Wansit, R., and Woodtayakorn, J. Evaluation of the immunofluorescent antibody test for the diagnosis of human leptospirosis. Am J Trop Med Hyg 52 (April 1995) : 340-343.

[145] Torten, M., Shenberg, E., and Van der Hoeden, J. The use of immunofluorescence in the diagnosis of human leptospirosis by a genus-specific antigen. J Infect Dis 116 (December 1966) : 537-543.

[146] Renesto, P., Lorvellec-Guillon, K., Drancourt, M., and Raoult, D. rpoB gene analysis as a novel strategy for identification of spirochetes from the genera *Borrelia*, *Treponema*, and *Leptospira*. J Clin Microbiol 38 (June 2000) : 2200-2203.

[147] Hookey, J.V. Detection of *Leptospiraceae* by amplification of 16S ribosomal DNA. FEMS Microbiol Lett 69 (January 1992) : 267-274.

[148] Merien, F., Amouriaux, P., Perolat, P., Baranton, G., and Saint, Girons I. Polymerase chain reaction for detection of *Leptospira* spp. in clinical samples. J Clin Microbiol 30 (September 1992) : 2219-2224.

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

[150] Wagenaar, J.A., Segers, R.P., and Van der Zeijst, B.A. Rapid and specific detection of pathogenic *Leptospira* species by amplification of ribosomal sequences. Mol Biotechnol 2 (August 1994) : 1-14.

[151] Woo, T.H., Patel, B.K., Smythe, L.D., Norris, M.A., Symonds, M.L., and Dohnt, M.F. Identification of pathogenic *Leptospira* by TaqMan probe in a LightCycler. Anal Biochem 256 (February 1998) : 132-134.

[152] Pacciarini, M.L., Savio, M.L., Donini, G., and Tagliabue, S. The search for improved methods for diagnosing leptospirosis: the approach of a laboratory in Brescia, Italy. Rev Sci Tech 12 (June 1993) : 647-663.

[153] Savio, M.L., Rossi, C., Fusi, P., Tagliabue, S., and Pacciarini, M.L. Detection and identification of *Leptospira interrogans* serovars by PCR coupled with restriction endonuclease analysis of amplified DNA. J Clin Microbiol 32 (April 1994) : 935-941.

[154] Woodward, M.J., Sullivan, G.J., Palmer, N.M., Woolley, J.C., and Redstone, J.S. Development of a PCR test specific for *Leptospira hardjo* genotype bovis. Vet Rec 128 (March 1991) : 282-283.

[155] Zuerner, R.L., Alt, D., and Bolin, C.A. IS1533-based PCR assay for identification of *Leptospira interrogans* sensu lato serovars. J Clin Microbiol 33 (December 1995) : 3284-3289.

[156] Zuerner, R.L., and Bolin, C.A. Differentiation of *Leptospira interrogans* isolates by IS1500 hybridization and PCR assays. J Clin Microbiol 35 (October 1997) : 2612-2617.

[157] Barbosa, A.S., et al. A newly identified leptospiral adhesin mediates attachment to laminin. Infect Immun 74 (November 2006) : 6356-6364.

[158] Atzingen, M.V., et al. Lsa21, a novel leptospiral protein binding adhesive matrix molecules and present during human infection. BMC Microbiol 8 (2008) : 70.

[159] Choy, H.A., Kelley, M.M., Chen, T.L., Moller, A.K., Matsunaga, J., and Haake, D.A. Physiological osmotic induction of *Leptospira interrogans* adhesion: LigA and LigB bind extracellular matrix proteins and fibrinogen. Infect Immun 75 (May 2007) : 2441-2450.

[160] Lin, Y.P., and Chang, Y.F. A domain of the *Leptospira* LigB contributes to high affinity binding of fibronectin. Biochem Biophys Res Commun 362 (October 2007) : 443-448.

[161] Ristow, P., et al. The OmpA-like protein Loa22 is essential for leptospiral virulence. PLoS Pathog 3 (July 2007) : e97.

[162] Bulach, D.M., et al. Genome reduction in *Leptospira borgpetersenii* reflects limited transmission potential. Proc Natl Acad Sci U S A 103 (September 2006) : 14560-14565.

[163] Bernheimer, A.W., and Bey, R.F. Copurification of *Leptospira interrogans* serovar pomona hemolysin and sphingomyelinase C. Infect Immun 54 (October 1986) : 262-264.

[164] Ren, S.X., et al. Unique physiological and pathogenic features of *Leptospira interrogans* revealed by whole-genome sequencing. Nature 422 (April 2003) : 888-893.

[165] Lux, R., Moter, A., and Shi, W. Chemotaxis in pathogenic spirochetes: directed movement toward targeting tissues. J Mol Microbiol Biotechnol 2 (October 2000) : 355-364.

[166] Yuri, K., Takamoto, Y., Okada, M., Hiramune, T., Kikuchi, N., and Yanagawa, R. Chemotaxis of leptospires to hemoglobin in relation to virulence. Infect Immun 61 (May 1993) : 2270-2272.

[167] De-Souza, L., and Koury, M.C. Chemical and biological properties of endotoxin from *Leptospira interrogans* serovars canicola and icterohaemorrhagiae. Braz J Med Biol Res 25 (1992) : 467-475.

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

[169] Masuzawa, T., Nakamura, R., Shimizu, T., and Yanagihara, Y. Biological activities and endotoxic activities of protective antigens (PAgs) of *Leptospira interrogans*. Zentralbl Bakteriol 274 (October 1990) : 109-117.

[170] Nie, D.K., et al. Studies on endotoxin of Leptospira: I. Extraction of lipopolysaccharides from *Leptospira interrogans* serovar Lai and analysis of their chemical and biological properties. Zhongguo Yi Xue Ke Xue Yuan Xue Bao 6 (October 1984) : 321-325.

[171] Nally, J.E., Chow, E., Fishbein, M.C., Blanco, D.R, and Lovett, M.A. Changes in lipopolysaccharide O antigen distinguish acute versus chronic *Leptospira interrogans* infections. Infect Immun 73 (June 2005) : 3251-3260.

[172] Lee, S.H., Kim, S., Park, S.C., and Kim, M.J. Cytotoxic activities of *Leptospira interrogans* hemolysin SphH as a pore-forming protein on mammalian cells. Infect Immun 70 (January 2002) : 315-322.

[173] Lee, S.H., Kim, K.A., Park, Y.G., Seong, I.W., Kim, M.J., and Lee, Y.J. Identification and partial characterization of a novel hemolysin from *Leptospira interrogans* serovar lai. Gene 254 (August 2000) : 19-28.

[174] del Real, G., Segers, R.P., van der Zeijst, B.A., and Gaastra, W. Cloning of a hemolysin gene from *Leptospira interrogans* serovar hardjo. Infect Immun 57 (August 1989) : 2588-2590.

[175] Zhang, Y.X., et al. Identification and classification of all potential hemolysin encoding genes and their products from *Leptospira interrogans* serogroup Icterohae-morrhagiae serovar Lai. Acta Pharmacol Sin 26 (April 2005) : 453-461.

[176] Hauk, P., Negrotto, S., et al. Expression and characterization of HlyX hemolysin from *Leptospira interrogans* serovar Copenhageni: potentiation of hemolytic activity by LipL32. Biochem Biophys Res Commun 333 (August 2005) : 1341-1347.

[177] Segers, R.P., van der Drift, A., de Nijs, A., Corcione, P., van der Zeijst, B.A., and Gaastra, W. Molecular analysis of a sphingomyelinase C gene from *Leptospira interrogans* serovar hardjo. Infect Immun 58 (July 1990) : 2177-2185.

[178] Thomas, D.D., and Higbie, L.M. In vitro association of leptospires with host cells. Infect Immun 58 (March 1990) : 581-585.

[179] Breiner, D.D., Fahey, M., Salvador, R., Novakova, J., and Coburn, J. *Leptospira interrogans* binds to human cell surface receptors including proteoglycans. Infect Immun 77 (December 2009) : 5528-5536.

[180] Cinco, M., Domenis, R., Perticarari, S., Presani, G., Marangoni, A., and Blasi, E. Interaction of leptospires with murine microglial cells. New Microbiol 29 (July 2006) : 193-199.

[181] Blasi, E., et al. NF-kB activation and p38 phosphorilation in microglial cells infected with *Leptospira* or exposed to partially purified leptospiral lipoproteins. Microb Pathog 42 (March 2007) : 80-87.

[182] Cinco, M., Vecile, E., Murgia, R., Dobrina, P., and Dobrina, A. *Leptospira interrogans* and *Leptospira* peptidoglycans induce the release of tumor necrosis factor alpha from human monocytes. FEMS Microbiol Lett 138 (May 1996) : 211-214.

[183] Werts, C., et al. Leptospiral lipopolysaccharide activates cells through a TLR2-dependent mechanism. Nat Immunol 2 (April 2001) : 346-352.

[184] Nahori, M.A., et al. Differential TLR recognition of leptospiral lipid A and lipopolysaccharide in murine and human cells. J Immunol 175 (November 2005) : 6022-6031.

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

[186] Faine, S., Adler, B., and Palit, A. Chemical, serological and biological properties of a serotype-specific polysaccharide antigen in *Leptospira*. Aust J Exp Biol Med Sci 52 (April 1974) : 311-319.

[187] Jost, B.H., Adler, B., and Faine, S. Experimental immunisation of hamsters with lipopolysaccharide antigens of *Leptospira interrogans*. J Med Microbiol 29 (June 1989) : 115-120.

[188] Jost, B.H., Adler, B., Vinh, T., and Faine, S. A monoclonal antibody reacting with a determinant on leptospiral lipopolysaccharide protects guinea pigs against leptospirosis. J Med Microbiol 22 (November 1986) : 269-275.

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

[190] Masuzawa, T., Nakamura, R., Beppu, Y., and Yanagihara, Y. Immunochemical characteristics and localization on cells of protective antigen (PAg) prepared from *Leptospira interrogans* serovar lai. Microbiol Immunol 40 (1996) : 237-241.

[191] Haake, D.A., Mazel, M.K., et al. Leptospiral outer membrane proteins OmpL1 and LipL41 exhibit synergistic immunoprotection. Infect Immun 67 (December 1999) : 6572-6582.

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

[193] Branger, C., et al. Protection against *Leptospira interrogans* sensu lato challenge by DNA immunization with the gene encoding hemolysin-associated protein 1. Infect Immun 73 (July 2005) : 4062-4069.

[194] Seixas, F.K., et al. Recombinant Mycobacterium bovis BCG expressing the LipL32 antigen of *Leptospira interrogans* protects hamsters from challenge. Vaccine 26 (December 2007) : 88-95.

[195] Ratnam, S., Sundararaj, T., Subramanian, S., Madanagopalan, N., and Jayanthi, V. Humoral and cell-mediated immune responses to leptospires in different human cases. Trans R Soc Trop Med Hyg 78 (1984) : 539-542.

[196] Klimpel, G.R., Matthias, M.A., and Vinetz, J.M. *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 (August 2003) : 1447-1455.

[197] Lucchesi, P.M., and Parma, A.E. A DNA fragment of *Leptospira interrogans* encodes a protein which shares epitopes with equine cornea. Vet Immunol Immunopathol 71 (November 1999) : 173-179.

[198] Parma, A.E., Fernandez, A.S., Santisteban, C.G., Bowden, R.A., and Cerone, S.I. Tears and aqueous humor from horses inoculated with *Leptospira* contain antibodies which bind to cornea. Vet Immunol Immunopathol 14 (February 1987) : 181-185.

[199] van der Lelie, J., van der Plas-Van Dalen, C.M., and von dem Borne, A.E. Platelet autoantibodies in septicaemia. Br J Haematol 58 (December 1984) : 755-760.

[200] Feng, C.Y., Li, Q.T., Zhang, X.Y., Dong, K., Hu, B.Y., and Guo, X.K. Immune strategies using single-component LipL32 and multi-component recombinant

LipL32-41-OmpL1 vaccines against *leptospira*. Braz J Med Biol Res 42 (September 2009) : 796-803.

[201] Cullen, P.A., Haake, D.A., Bulach, D.M., Zuerner, R.L., and Adler, B. LipL21 is a novel surface-exposed lipoprotein of pathogenic *Leptospira* species. Infect Immun 71 (May 2003) : 2414-2421.

[202] He, H.J., Wang, W.Y., Wu, Z.D., Lv, Z.Y., Li, J., and Tan, L.Z. Protection of guinea pigs against *Leptospira interrogans* serovar Lai by LipL21 DNA vaccine. Cell Mol Immunol 5 (October 2008) : 385-391.

[203] Verma, A., Brissette, C.A., Bowman, A.A., Shah, S.T., Zipfel, P.F., and Stevenson, B. Leptospiral endostatin-like protein A is a bacterial cell surface receptor for human plasminogen. Infect Immun 78 (May) : 2053-2059.

[204] Koizumi, N., and Watanabe, H. Leptospiral immunoglobulin-like proteins elicit protective immunity. Vaccine 22 (March 2004) : 1545-1552.

[205] Matsunaga, J., et al. Pathogenic *Leptospira* species express surface-exposed proteins belonging to the bacterial immunoglobulin superfamily. Mol Microbiol 49 (August 2003) : 929-945.

[206] Matsunaga, J., Lo, M., Bulach, D.M., Zuerner, R.L., Adler, B., and Haake, D.A. Response of *Leptospira interrogans* to physiologic osmolarity: relevance in signaling the environment-to-host transition. Infect Immun 75 (June 2007) : 2864-2874.

[207] Lo, M., et al. Effects of temperature on gene expression patterns in *Leptospira interrogans* serovar Lai as assessed by whole-genome microarrays. Infect Immun 74 (October 2006) : 5848-5859.

[208] Yang, C.W., et al. Toll-like receptor 2 mediates early inflammation by leptospiral outer membrane proteins in proximal tubule cells. Kidney Int 69 (March 2006) : 815-822.

[209] Yang, C.W., Wu, M.S., Pan, M.J., Hsieh, W.J., Vandewalle, A., and Huang, C.C. The *Leptospira* outer membrane protein LipL32 induces tubulointerstitial nephritis-mediated gene expression in mouse proximal tubule cells. J Am Soc Nephrol 13 (August 2002) : 2037-2045.

[210] Hauk, P., Guzzo, C.R., Roman Ramos, H., Ho, P.L., and Farah, C.S. Structure and calcium-binding activity of LipL32, the major surface antigen of pathogenic *Leptospira* sp. J Mol Biol 390 (July 2009) : 722-736.

[211] Einarson, T.R., Metge, C.J., Iskedjian, M., and Mukherjee, J. An examination of the effect of cytochrome P450 drug interactions of hydroxymethylglutaryl-coenzyme A reductase inhibitors on health care utilization: a Canadian population-based study. Clin Ther 24 (December 2002) : 2126-2136.

[212] Martinez, J.J., Seveau, S., Veiga, E., Matsuyama, S., and Cossart, P. Ku70, a component of DNA-dependent protein kinase, is a mammalian receptor for *Rickettsia conorii*. Cell 123 (December 2005) : 1013-1023.

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

[214] Cantor, S.B., et al. BACH1, a novel helicase-like protein, interacts directly with BRCA1 and contributes to its DNA repair function. Cell 105 (April 2001) : 149-160.

[215] Clinton, S.R., Bina, J.E., Hatch, T.P., Whitt, M.A., and Miller, M.A. Binding and activation of host plasminogen on the surface of *Francisella tularensis*. BMC Microbiol 10 (March 2010) : 1471-1480.

[216] Yen, Y.T., Seepersaud, R., Bensing, B.A., and Sullam, P.M. Asp2 and Asp3 interact directly with GspB, the export substrate of the *Streptococcus gordonii* accessory Sec System. J Bacteriol 93 (July 2011) : 3165-3174.

[217] Barclay, J.K., Murrant, C.L., Woodley, N.E., and Reading, S.A. Potential interactions among vascular and muscular functional compartments during active hyperemia. Can J Appl Physiol 28 (October 2003) : 737-753.

[218] Wu, Y., Li, Q., and Chen, X.Z. Detecting protein-protein interactions by Far western blotting. Nat Protoc 2 (December 2007) : 3278-284.

[219] Fuentes, M., et al. Determination of protein-protein interactions through aldehyde-dextran intermolecular cross-linking. Proteomics 4 (September 2004) : 2602-2607.

[220] Fields, S., and Song, O. A novel genetic system to detect protein-protein interactions. Nature 340 (July 1989) : 245-246.

[221] Tim, C. H.B. Phage display. New York: Oxford university press 6 (November 2004) : 12-26.

[222] Kehoe, J.W.. Filamentous phage display in the new millennium. Chem Rev 105 (November 2005) : 4056-4072.

[223] Condron, B.G., Atkins, J.F., and Gesteland, R.F. Frameshifting in gene 10 of bacteriophage T7. J Bacteriol 173 (November 1991) : 6998-7003.

[224] Rosenberg, S., et al. T7Select® Phage Display System: A powerful new protein display system based on bacteriophage T7. Advance product sandprotocols for molecularbiology research 6 (December 1996) : 1-6.

[225] Krumpe, L.R.H., and Mori, T. The Use of Phage-Displayed Peptide Libraries to Develop Tumor-Targeting Drugs. International Journal of Peptide Research and Therapeutics 12 (March 2006) : 79-91.

[226] Rahim, A.A. Pyrosequencing of phage display libraries for the identification of cell-specific targeting ligands. Methods Mol Biol 373 (2007) : 135-146.

[227] Valadon, P., Garnett, J.D., Testa, J.E., Bauerle, M., Oh, P., and Schnitzer, J.E. Screening phage display libraries for organ-specific vascular immunotargeting in vivo. Proc Natl Acad Sci U S A 103 (January 2006) : 407-412.

[228] El-Mousawi, M., et al. A vascular endothelial growth factor high affinity receptor 1-specific peptide with antiangiogenic activity identified using a phage display peptide library. J Biol Che 278 (November 2003) : 46681-46691.

[229] Hetian, L., et al. 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 (November 2002) : 43137-43142.

[230] Lauterbach, S.B., Lanzillotti, R., and Coetzer, T.L. Construction and use of *Plasmodium falciparum* phage display libraries to identify host parasite interactions. Malar J 2 (December 2003) : 47.

[231] Whaley, S.R., English, D.S., Hu, E.L., Barbara, P.F., and Belcher, A.M. Selection of peptides with semiconductor binding specificity for directed nanocrystal assembly. Nature 405 (June 2000) : 665-668.

[232] Chaemchuen, S., Rungpragayphan, S., Poovorawan, Y., and Patarakul, K. Identification of candidate host proteins that interact with LipL32, the major outer membrane protein of pathogenic Leptospira, by random phage display peptide library. Vet Microbiol 23 (April 2011) : 178-185.

[233] Beckmann, C., Waggoner, J.D., Harris, T.O., Tamura, G.S., and Rubens, C.E. Identification of novel adhesins from Group B *streptococci* by use of phage display reveals that C5a peptidase mediates fibronectin binding. Infect Immun 70 (June 2002) : 2869-2876.

[234] Gasanov, U., Koina, C., Beagley, K.W., Aitken, R.J., and Hansbro, P.M. Identification of the insulin-like growth factor II receptor as a novel receptor for binding and invasion by *Listeria monocytogenes*. Infection and immunity 74 (January 2006) : 566-577.

[235] Heilmann, C., Niemann, S., Sinha, B., Herrmann, M., Kehrel, B.E., and Peters, G. *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 (July 2004) : 321-329.

[236] Benedek, O., et al. Identification of laminin-binding motifs of *Yersinia pestis* plasminogen activator by phage display. Int J Med Microbiol 295 (June 2005) : 87-98.

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

[238] Gao, W., et al. Identification of NCAM that interacts with the PHE-CoV spike protein. Virol J 7 (September 2010) : 254-265.

[239] Mordwinkin, N.M., and Louie, S.G. Aralast: an alpha 1-protease inhibitor for the treatment of alpha-antitrypsin deficiency. Expert Opin Pharmacother 8 (October 2007) : 2609-2614.

[240] Tanaka, N., Sekiya, S., Takamizawa, H., Kato, N., Moriyama, Y., and Fujimura, S. Characterization of a 54 kDa, alpha 1-antitrypsin-like protein isolated from ascitic fluid of an endometrial cancer patient. Jpn J Cancer Res 82 (June 1991) : 693-700.

[241] Niemann, M.A., Narkates, A.J., and Miller, E.J. Isolation and serine protease inhibitory activity of the 44-residue, C-terminal fragment of alpha 1-antitrypsin from human placenta. Matrix 12 (June 1992) : 233-241.

[242] Seyama, K., Nukiwa, T., Takabe, K., Takahashi, H., Miyake, K., and Kira, S. Siiyama (serine 53 (TCC) to phenylalanine 53 (TTC)). A new alpha 1-antitrypsin-deficient variant with mutation on a predicted conserved residue of the serpin backbone. J Biol Chem 266 (July 1991) : 12627-12632.

[243] Holmes, M.D., Brantly, M.L., Fells, G.A., and Crystal, R.G. Alpha 1-antitrypsin Wbethesda: molecular basis of an unusual alpha 1-antitrypsin deficiency variant. Biochem Biophys Res Commun 170 (August 1990) : 1013-1020.

[244] Graham, A., Kalsheker, N.A., Bamforth, F.J., Newton, C.R., and Markham, A.F. Molecular characterisation of two alpha-1-antitrypsin deficiency variants: proteinase inhibitor (Pi) Null(Newport) (Gly115----Ser) and (Pi) Z Wrexham (Ser-19----Leu). Hum Genet 85 (October 1990) : 537-540.

[245] Vallenius, T., and Makela, T.P. Clik1: a novel kinase targeted to actin stress fibers by the CLP-36 PDZ-LIM protein. J Cell Sci 115 (May 2002) : 2067-2073.

[246] Goyal, P., Behring, A., Kumar, A., and Siess, W. Identifying and characterizing a novel protein kinase STK35L1 and deciphering its orthologs and close-homologs in vertebrates. PLoS One 4 (September 2009) : e6981.

[247] Goyal, P., Behring, A., Kumar, A., and Siess, W. STK35L1 associates with nuclear actin and regulates cell cycle and migration of endothelial cells. PLoS One 6 (January 2011) : e16249.

[248] Vieira, O.V., Botelho, R.J., and Grinstein, S. Phagosome maturation: aging gracefully. Biochem J 366 (September 2002) : 689-704.

[249] Bhattacharya, A.A., Curry, S., and Franks, N.P. Binding of the general anesthetics propofol and halothane to human serum albumin. High resolution crystal structures. J Biol Chem 275 (December 2000) : 38731-38738.

[250] Lu, J., Stewart, A.J., Sadler, P.J., Pinheiro, T.J., and Blindauer, C.A. Albumin as a zinc carrier: properties of its high-affinity zinc-binding site. Biochem Soc Trans 36 (December 2008) : 1317-1321.

[251] Zhou, W., et al. An initial characterization of the serum phosphoproteome. J Proteome Res 8 (December 2009) : 5523-5531.

[252] Carter, D.C., and He, X.M. Structure of human serum albumin. Science 249 (July 1990) : 302-303.

[253] Minchiotti, L., Galliano, M., Zapponi, M.C., and Tenni, R. The structural characterization and bilirubin-binding properties of albumin Herborn, a [Lys240-->Glu] albumin mutant. Eur J Biochem 214 (June 1993) : 437-444.

[254] Godbout, R., Bisgrove, D.A., Honore, L.H., and Day, R.S. Amplification of the gene encoding the alpha-subunit of the mitochondrial ATP synthase complex in a human retinoblastoma cell line. Gene 123 (January 1993) : 195-201.

[255] Moser, T.L., et al. Angiostatin binds ATP synthase on the surface of human endothelial cells. Proc Natl Acad Sci U S A 96 (March 1999) : 2811-2816.

[256] Ohta, T., Ikemoto, Y., Usami, A., Koide, T., and Wakabayashi, S. High affinity interaction between histidine-rich glycoprotein and the cell surface type ATP synthase on T-cells. Biochim Biophys Acta 1788 (May 2009) : 1099-1107.

[257] Wang, Z.G., White, P.S., and Ackerman, S.H. Atp11p and Atp12p are assembly factors for the F(1)-ATPase in human mitochondria. J Biol Chem 276 (August 2001) : 30773-30778.

[258] Bu, L., et al. Mutant DNA-binding domain of HSF4 is associated with autosomal dominant lamellar and Marner cataract. Nat Genet 31 (July 2002) : 276-278.

[259] Hu, Y., and Mivechi, N.F. Association and regulation of heat shock transcription factor 4b with both extracellular signal-regulated kinase mitogen-activated

protein kinase and dual-specificity tyrosine phosphatase DUSP26. Mol Cell Biol 26 (April 2006) : 3282-3294.

[260] Hietakangas, V., et al. PDSM, a motif for phosphorylation-dependent SUMO modification. Proc Natl Acad Sci U S A 103 (January 2006) : 45-50.

[261] Ke, T., et al. Novel HSF4 mutation causes congenital total white cataract in a Chinese family. Am J Ophthalmol 142 (August 2006) : 298-303.

[262] Gamberini, M., et al. Whole-genome analysis of *Leptospira interrogans* to identify potential vaccine candidates against leptospirosis. FEMS Microbiol Lett 244 (March 2005) : 305-313.

[263] Guerreiro, H., et al. Leptospiral proteins recognized during the humoral immune response to leptospirosis in humans. Infect Immun 69 (August 2001) : 4958-4968.

[264] Seixas, F.K., Fernandes, C.H., Hartwig, D.D., Conceicao, F.R., Aleixo, J.A, and Dellagostin, O.A. 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 (April 2007) : 472-479.

[265] Ishii, T., et al. Cloning and characterization of a 23-kDa stress-induced mouse peritoneal macrophage protein. J Biol Chem 268 (September 1993) : 18633-18636.

[266] Prosperi, M.T., Ferbus, D., Karczinski, I., and Goubin, G. A human cDNA corresponding to a gene overexpressed during cell proliferation encodes a product sharing homology with amoebic and bacterial proteins. J Biol Chem 268 (May 1993) : 11050-11056.

[267] Ishii, T., Itoh, K., et al. Transcription factor Nrf2 coordinately regulates a group of oxidative stress-inducible genes in macrophages. J Biol Chem 275 (May 2000) : 16023-16029.

[268] Chow, C.W., Herrera Abreu, M.T., Suzuki, T., and Downey, G.P. Oxidative stress and acute lung injury. Am J Respir Cell Mol Biol 29 (October 2003) : 427-431.

[269] Kinnula, V.L., Fattman, C.L., Tan, R.J., and Oury, T.D. Oxidative stress in pulmonary fibrosis: a possible role for redox modulatory therapy. Am J Respir Crit Care Med 172 (August 2005) : 417-422.

[270] Harris, J.R., et al. Comparison of the decameric structure of peroxiredoxin-II by transmission electron microscopy and X-ray crystallography. Biochim Biophys Acta 1547 (June 2001) : 221-234.

[271] Wood, Z.A., Poole, L.B., and Karplus, P.A. Peroxiredoxin evolution and the regulation of hydrogen peroxide signaling. Science 300 (April 2003) : 650-653.

[272] Lehtonen, S.T., et al. Peroxiredoxins, a novel protein family in lung cancer. Int J Cancer 111 (September 2004) : 514-521.

[273] Schremmer, B., Manevich, Y., Feinstein, S.I., and Fisher, A.B. Peroxiredoxins in the lung with emphasis on peroxiredoxin VI. Subcell Biochem 44 (September 2007) : 317-344.

[274] Kikuchi, N., et al. Nrf2 protects against pulmonary fibrosis by regulating the lung oxidant level and Th1/Th2 balance. Respir Res 11 (March 2010) : 31-43.

[275] Matsumoto, A., et al. Cloning of the peroxiredoxin gene family in rats and characterization of the fourth member. FEBS Lett 443 (January 1999) : 246-250.

[276] Iwata, Y., et al. Autoantibody against peroxiredoxin I, an antioxidant enzyme, in patients with systemic sclerosis: possible association with oxidative stress. Rheumatology (Oxford) 46 (May 2007) : 790-795.

[277] Moore, R.B., Mankad, M.V., Shriver, S.K., Mankad, V.N., and Plishker, G.A. Reconstitution of Ca(2+)-dependent K+ transport in erythrocyte membrane vesicles requires a cytoplasmic protein. J Biol Chem 266 (October 1991) : 18964-18968.

[278] Cha, M.K., Yun, C.H., and Kim, I.H. Interaction of human thiol-specific antioxidant protein 1 with erythrocyte plasma membrane. Biochemistry 39 (June 2000) : 6944-6950.

[279] Moore, R.B., Shriver, S.K., Jenkins, L.D., Mankad, V.N., Shah, A.K., Plishker, G.A. Calpromotin, a cytoplasmic protein, is associated with the formation of dense cells in sickle cell anemia. Am J Hematol 56 (October 1997) : 100-106.

[280] Murphy, S.C., et al. Erythrocyte detergent-resistant membrane proteins: their characterization and selective uptake during malarial infection. Blood 103 (March 2004) : 1920-1928.

[281] Plishker, G.A., Chevalier, D., Seinsoth, L., and Moore, R.B. Calcium-activated potassium transport and high molecular weight forms of calpromotin. J Biol Chem 267 (October 1992) : 21839-21843.

[282] Rocha, S., et al. Presence of cytosolic peroxiredoxin 2 in the erythrocyte membrane of patients with hereditary spherocytosis. Blood Cells Mol Dis 41 (Juiy 2008) : 5-9.

[283] Low, T.Y., Seow, T.K., and Chung, M.C. Separation of human erythrocyte membrane associated proteins with one-dimensional and two-dimensional gel electrophoresis followed by identification with matrix-assisted laser desorption/ionization-time of flight mass spectrometry. Proteomics 2 (September 2002) : 1229-1239.

[284] Kinnula, V.L., et al. Cell specific expression of peroxiredoxins in human lung and pulmonary sarcoidosis. Thorax 57 (February 2002) : 157-164.

[285] Haridas, V., et al. TRANK, a novel cytokine that activates NF-kappa B and c-Jun N-terminal kinase. J Immunol 161 (July 1998) : 1-6.

[286] Bianchi, M.E. DAMPs, PAMPs and alarmins: all we need to know about danger. J Leukoc Biol 81 (January 2007) : 1-5.

[287] Rubartelli, A., and Lotze, M.T. Inside, outside, upside down: damage-associated molecular-pattern molecules (DAMPs) and redox. Trends Immunol 28 (October 2007) : 429-436.

[288] Bianchi, M.E., and Manfredi, A.A. Immunology. Dangers in and out. Science 323 (March 2009) : 1683-1684.

[289] Uwayama, J., et al. Tissue Prx I in the protection against Fe-NTA and the reduction of nitroxyl radicals. Biochem Biophys Res Commun 339 (January 2006) : 226-231.

[290] Sato, D., et al. Peroxiredoxin I protects gastric mucosa from oxidative injury induced by H. pylori infection. J Gastroenterol Hepatol 23 (April 2008) : 652-659.

[291] Jung, H., Kim, T., Chae, H.Z., Kim, K.T., and Ha, H. Regulation of macrophage migration inhibitory factor and thiol-specific antioxidant protein PAG by direct interaction. J Biol Chem 276 (May 2001) : 15504-15510.

[292] Donnelly, S.C., et al. Regulatory role for macrophage migration inhibitory factor in acute respiratory distress syndrome. Nat Med 3 (March 1997) : 320-323.

[293] Kikuchi, N., et al. Aggravation of Bleomycin-Induced Pulmonary Inflammation and Fibrosis in Mice Lacking Peroxiredoxin I. Am J Respir Cell Mol Biol 14 (January 2011) : 600-609.

[294] Gertz, M., Fischer, F., Leipelt, M., Wolters, D., and Steegborn, C. Identification of Peroxiredoxin 1 as a novel interaction partner for the lifespan regulator protein p66Shc. Aging (Albany NY) 1 (February 2009) : 254-265.

[295] Champagne, E., Martinez, L.O., Collet, X., and Barbaras, R. Ecto-F1Fo ATP synthase/F1 ATPase: metabolic and immunological functions. Curr Opin Lipidol 17 (June 2006) : 279-284.

[296] Martinez, L.O., et al. Ectopic beta-chain of ATP synthase is an apolipoprotein A-I receptor in hepatic HDL endocytosis. Nature 421 (January 2003) : 75-79.

[297] Arakaki, N., Kita, T., Shibata, H., and Higuti, T. Cell-surface H+-ATP synthase as a potential molecular target for anti-obesity drugs. FEBS Lett 581 (July 2007) : 3405-3409.

[298] Lyly, A., et al. Deficiency of the INCL protein Ppt1 results in changes in ectopic F1-ATP synthase and altered cholesterol metabolism. Hum Mol Genet 17 (May 2008) : 1406-1417.

[299] Arakaki, N., et al. Possible role of cell surface H+ -ATP synthase in the extracellular ATP synthesis and proliferation of human umbilical vein endothelial cells. Mol Cancer Res 1 (November 2003) : 931-939.

[300] Scotet, E., et al. Tumor recognition following Vgamma9Vdelta2 T cell receptor interactions with a surface F1-ATPase-related structure and apolipoprotein A-I. Immunity 22 (January 2005) : 71-80.

[301] Kim, B.W., Choo, H.J., Lee, J.W., Kim, J.H., and Ko, Y.G. Extracellular ATP is generated by ATP synthase complex in adipocyte lipid rafts. Exp Mol Med 36 (October 2004) : 476-485.

[302] Mangiullo, R., Gnoni, A., Leone, A., Gnoni, G.V., Papa, S., and Zanotti, F. Structural and functional characterization of F(o)F(1)-ATP synthase on the extracellular surface of rat hepatocytes. Biochim Biophys Acta 1777 (October 2008) : 1326-1335.

[303] Moser, T.L., et al. Endothelial cell surface F1-F0 ATP synthase is active in ATP synthesis and is inhibited by angiostatin. Proc Natl Acad Sci U S A 98 (June 2001) : 6656-6661.

[304] Lin, X., Kim, Y.A., Lee, B.L., Soderhall, K., and Soderhall, I. Identification and properties of a receptor for the invertebrate cytokine astakine, involved in hematopoiesis. Exp Cell Res 315 (April 2009) : 1171-1180.

[305] Boutareaud, A., Danet, J.L., Garnier, M., and Saillard, C. Disruption of a gene predicted to encode a solute binding protein of an ABC transporter reduces transmission of *Spiroplasma citri* by the leafhopper *Circulifer haematoceps*. Appl Environ Microbiol 70 (July 2004) : 3960-3967.

[306] Das, B., Mondragon, M.O., Sadeghian, M., Hatcher, V.B., and Norin, A.J. A novel ligand in lymphocyte-mediated cytotoxicity: expression of the beta subunit of H+ transporting ATP synthase on the surface of tumor cell lines. J Exp Med 180 (July 1994) : 273-281.

APPENDICES

APPENDIX A

BUFFER AND REAGENTS

Reagents for EMJH media

1. Albumin fatty acid supplement stock solution

$CaCl_2$ + $MgCl_2$•$6H_2O$	0.076	g
$ZnSO_4$•$7H_2O$	0.04	g
$CuSO_4$•H_2O	0.03	g
Vitamin B12	0.002	g
Tween 80	1	g
Glycerol	1	g

All reagents are stored at -20°C until use.

Dissolve each reagent separately in 10 ml of distilled water.

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

BSA	5	g
$CaCl_2$ + $MgCl_2$ • $6H_2O$	750	μl
$ZnSO_4$•$7H_2O$	500	μl
$CuSO_4$•$5H_2O$	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 Cell Culture

1. **2X Medium MEM**

MEM with Earle's salts, with L-glutamine, Without $NaHCO_3$	9.9 g
Sterilized deionized distilled water	500 ml

Filtration and store at 4°C

2. **10% $NaHCO_3$**

$NaHCO_3$	10 g
Deionized distilled water	100 ml

Sterile the solution by autoclaving at 121°C 15 minutes

3. **10% MEM medium**

2X MEM with Earle's salts, with L-glutamine without $NaHCO_3$	50 ml
1M HEPES	2 ml
Fetal bovine serum	20 ml
Penicillin/Streptomycin antibiotic (100X)	2 ml
10% $NaHCO_3$ adjusted to pH 7.4	1 ml
Sterilized deionized distilled water	125 ml

4. **2% MEM medium (without antibiotic)**

2X MEM with Earle's salts, with L-glutamine without $NaHCO_3$	50 ml
1M HEPES	2 ml

Fetal bovine serum	4 ml
10% $NaHCO_3$ adjusted to pH 7.4	1 ml
Sterilized deionized distilled water	143 ml

5. 10X PBS (Phosphate-buffer saline)

NaCl	40 g
KCl	1 g
$NaHPO_4$	5.75 g
KH_2PO_4	1 g
Deionized distilled water	1000 ml

Sterilized the solution by autoclaving 121 °C 15 minutes

6. 1X PBS

10X PBS	100 ml
Sterilized deionized distilled water	900 ml

7. 10X Trypsin

Trypsin	0.5 g
EDTA	0.2 g
NaCl	9 g
Deionized distilled water	100 ml

Sterilized by filtration and kept at-20°C

8. 1X Trypsin

10X stock trypsin	10 ml
1X PBS	90 ml

Store at 4 °C

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. 10mg/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)
1xTAE	20	ml

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

Reagents 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 500 ml volume.

2. 500 mM Elution Buffer (10 ml)

8x Phosphate buffer stock solution pH 7.4	1.25	ml
2 M Imidazole stock solution pH 7.4	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. 1x Phosphate buffer saline (PBS)

Na_2HPO_4	4.88	g
$NaH_2PO_4 \cdot H_2O$	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 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

1. 1M 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. 4x Tris 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 Dithiothreitol (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. **1x sample buffer with DTT (12 ml)**

6x sample buffer with DTT	2	ml
Distilled water	10	ml

Mix the solution and store at-20°C.

7. **10% Ammonium Persulfate (APS)**

APS	1	g
Distilled water	10	ml

Mix the solution and store at-20°C.

8. **10% Sodium dodecyl sulfate (SDS)**

Sodium dodecyl sulfate (SDS)	1	g
Distilled water	10	ml

Mix the solution and store at room temperature.

9. **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 the dark at room temperature.

10. 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 Western blot

1. TBS (Tris-buffered saline)(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.

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

TBS	500	ml
Tween-20	500	μl

Mix the solution and store at room temperature.

3. Bltotting Buffer (1 liter)

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.

Reagents for far western blot

1. **PBS-0.05 % (v/v)Tween (500 ml)**

PBS 500 ml

Tween-20 250 μl

Mix the solution and store at room temperature.

2. **Denaturing and renaturing buffer**

Concentration of guanidine–HCl (M)	6	3	1	0.1	0
Glycerol (ml)	2.5	2.5	2.5	2.5	2.5
5 M NaCl (ml)	0.5	0.5	0.5	0.5	0.5
1 M Tris, pH 7.5 (ml)	0.5	0.5	0.5	0.5	0.5
0.5 M EDTA (ml)	0.05	0.05	0.05	0.05	0.05
10% Tween-20 (ml)	0.25	0.25	0.25	0.25	0.25
Guanidine–HCl (8 M) (ml)	18.75	9.30	3.13	0.31	0
Milk powder (g)	0.5	0.5	0.5	0.5	0.5
1 M DTT (μl)	25	25	25	25	25
ddH_2O (ml)	2.45	12.82	18.07	20.89	21.20
Total volume (ml)	25	25	25	25	25
Time/temperature	30 min/room temperature (RT)	30 min/RT	30 min/RT	30 min/4°C	overnight/ 4°C

Reagents for Bacterial pull down assay

1. Washing buffer (0.1% (v/v) Triton X-100, 20 mM Hepes (N-2-hydroxylethlpiperazine-N'-2-ethanesulfonic acid)) 50 ml

10% Triton X-100	0.5	ml
1M Hepes	1	ml
1X PBS	48.5	ml

2. Elution buffer (1.0 M NaCl, 0.2 % (v/v) Tritonx-100) 500 µl

2% Triton X-100	50	µl
1M Hepes	10	µl
2M Nacl	250	µl
1X PBS	190	µl

Reagents for phage peptide library screening

LB Medium (1 Liter)

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.

1. LB- Carbenicillin plate

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 and allow media to cool down before adding 1ml carbenicillin stock, pouring into plates and store at 4°C in the dark.

2. **Carbenicillin stock**

Carbenicillin	50	ml
Distilled water	1	ml

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

3. **Agarose Top (1 Liter)**

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 and allow media to cool down before dispensing into 50 ml aliquots. Store at room temperature and melt in microwave oven before use.

4. **1M $NaHCO_3$ 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.

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

1M $NaHCO_3$ pH 8.6	1	ml
BSA	25	mg

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

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

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

Mix the solution and store at room temperature.

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

Polyethylene glycon-800	20	g
NaCl	14.61	g

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

8. **Elution buffer (10 ml)**

SDS	0.1	g
TBS	10	ml

Mix the solution and store at room temperature.

APPENDIX B

1. Complete sequence of proteins that interact with whole cells of wild-type *Leptospira* and subtracted with *lipL32*$^-$ mutant strain of *L. interrogans* which were identified by T7 select® cDNA liver phage display library

Sequence 1 found 2 in 16 clones (12.5%)

CAGCGTCAACTGGGCATCACTAAGGTCTTCAGCAATGGGGCTGACCTCTCCGGGGTC
ACAGAGGAGGCACCCCTGAAGCTCTCCAAGGCCGTGCATAAGGCTGTGCTGACCAT
CGACGAGAAAGGGACTGAAGCTGCTGGGGCCATGTTTTTAGAGGCCATACCCATGTC
TATCCCCCCCGAGGTCAAGTTCAACAAACCCTTTGTCTTCTTAATGATTGAACAAAATA
CCAAGTCTCCCCTCTTCATGGGAAAAGTGGTGAATCCCACCCAAAAATAACTGCCTCT
CGCTCCTCAACCCTACTCCTAATCACATAACCTATTCCCCCGAGCAATCTCAATTACA

Sequence 2 found 1 in 16 clones (6.25%)

GGGGGGTTTGCTCGGGGGACGATTCAAGCCCTGCATCTGGTAGGGCCAGCCCACCA
AGGAGTCCAGGAGTTCCTGCATCTGGTAGGGAGGCGTGGCCCAGAGGCCTCCTTGT
GTCCTCACGCCCCGTTCCAGAAGAAACCCCCAGATTCCCTATGGGGAGGCAGGCCT
TCTCTAGGGAAGTGAGGTGTCATAAGTAGAACTGAGAAAAGTATAAAGAAGGAAGAC
AGAGGGAAAGTGAAGGACAAAACCAAAAATAGCTCCCTAACCCCTTATAAAAAGTAAA
AGTGAAATTTACACATATGCCTTGCCCACATTGAATTTCTTTAAAGAAATTGGTGCCTGT
CCACAGCATTGTGTCCTGCTCTTCTGAAGTCTCAATGGTTTAAATTTAAAAAAAGGAAA
GAGAAAAAAAAAAAAAAAAAAAAAAGGGAAAAAATAAAGCAAAACTAAAAAAAAGCGG
GAAAACCCAAACAAAACCTTGCGGCCGCACTCGAGAAACTAGTTAACCCCTTGGGGC
CTCTAAACGGGTCTGGAGGGGTTAAA

Sequence 3 found 1 in 16 clones (6.25%)

GGGGGGGTTTGGCTCGGGGGACGATTCATAAACCTTCTTAAATATAAAGTAGATACAG
TTCTAAGATAGGGAGGTTCTTAACTAGTTAAATAGTTGTTGGAAAAGTGCACCTTGGTG
GAAATAAAACAGAGCCTTGACTTTGCCAGAGTCCATCATTGACTCCAAATATGTAGCA

ACACCTGTGTGTTCTAAAACTACGTCAAGTGGTGGGGAGAAGTTGGGGTAAAATA
AATTANATTTTGAAATGGAATAAAGAAAAAATAATGGTAGAACAAAGAGAGGTGAAGAA
AAATATATAGTATATGTTATTTACAGAATGGATTA

Sequence 4 found 1 in 16 clones (6.25%)

CAGCCTGAAACATTCACCTTCCATGCAGATATATGCACACTTTCTGAGAAGGAGAGAC
AAATCAAGAAACAAACTGCACTTGTTGAGCTCGTGAAACACAAGCCCAAGGCAACAA
AAGAGCAACTGAAAGCTGTTATGGATGATTTCGCAGCTTTTGTAGAGAAGTGCTGCAA
GGCTGACGATAAGTAGACCTGCTTTGCCGAGGAGGGTAAAAAACTTGTTGCTGCAAG
TCAAGCTGCCTTAGGCTTATAACATCACATTTAAAAGCTTGCGGCCGCACTCGAGTAA
CTAGTTAACCCCTTGGGGCCTCTAAACGGGTCTTGAGGGGTTAAC

Sequence 5 found 1 in 16 clones (6.25%)

GGGGGGTTGGCTCGGGGGACGGATTCAGCCGCAACCGCAAAGGCTACCGTTCACA
ACGAGGCCACAGCCGTGGCCGCAACCAGAACTCCCGCCGGCCATCCCGCGCCACG
TGGCTGTCCTTGTTCTCCAGTGAGGAGAGCAACTTGGGAGCCAACAACTATGATGACT
ACAGGATGGACTGGCTTGTGCCTGCCACCTGTGAACCCATCCACAGTGTCCACTCGC
CGTGTGCGAAGGTTGACTCGGTAGTACTTGTCTCCAGAGAAGA

Sequence 6 found 1 in 16 clones (6.25%)

CAAGCGCCACCAGTCCTTTCTGCCGGATATTCACTATTAACAATCAGCACTCCAGCTG
TCTCATAAACAAAAAGTTGATCATCGCTGCTCAGTAAGGACTGGTGGCCATTCTCTCG
AGGAGAAAGCTCTAATAAATCTTGTATTCTATTCAAAATATCCTCAATGAAAGGATTCAT
TTGCTTATTGAGAGATTTGACAAATCTAGAAAACAGGTAAGCCGTCCTGCTCCGAACTT
TTGCACTGGA

Sequence 7 found 1 in 16 clones (6.25%)

CAAGCGCCACCAGTCCTTTCTGCCGGATATTCACTATTAACAATCAGCACTCCAGCTG
TCTCATAAACAAAAAGTTGATCATCGCTGCTCAGTAAGGACTGGTGGCCATTCTCTCG
AGGAGAAAGCTCTAATAAATCTTGTATTCTATTCAAAATATCCTCAATGAAAGGATTCAT

TTGCTTATTGAGAGATTTGACAAATCTAGAAAACAGGTAAGCCGTCCTGCTCCGAA

CTTTTGCACTGGA

Sequence 8 found 1 in 16 clones (6.25%)

TCCAGCTTGGGCAACAGAGCAAGGTCCTGTAGAAAGAAAGAAAAAGGAAAGAAGGA

AAGAAGGACGGAAGGAAGGAGGGAAGGAACAAAGGAAGGAAGGAAAAAAGGAAGG

AAGGAAAAGGGAGAGAGAAAGAAAGAGAGAGAAAGAGGGAGAAAGAAAGAAAGAA

AGAGAAAAGAAAGAAGAAAGAAAAAAGAAAGAAAGAAGAAGGAAAAAGAAAGAAAG

AAGAAAAGGAAGGCGGGGAGGAAGGAAGGAAGGAAGGAAAAGAAGGAAGGAAAGA

AGGAAGGAAGAAAGAAAGAGA

Sequence 9 found 1 in 16 clones (6.25%)

TTAGTCGGGGACGATTCAGCCTGACGCAGGCACTTACTTCCTATTCTACACCCTAGTA

GGCTCCCTTCCCCTACTCATCGCACTAATTTACACTCACAACACACTAGGCTCACTAA

ACATTCTACTACTCACTCTCACTGCCCAAGAACTATCAAACTCCTGAGCCAACAACTTA

ATATGACTAGCTTACACAATAGCTTTTATAGTAAAGATACCTCTTTACGGACTCCACTTA

TGACTCCCTAAAGCCCATGTCGAAGCCCCCATCGCTGGGTCAATAGTACTTGCCGCA

GTACTCTTAAAACTAGGCGGCTATGGTATAATACGCCTCACACTCATTCTCA

Sequence 10 found 1 in 16 clones (6.25%)

CACTCCTATAAAAAGTGACATTAAAAGTGACTGAATAAGTCTCTGAAGGACAGAAATCT

GTCCTTTCAGGAATTACTCGAGTGGTCACTGTTTTGAACTGGCTCAAACATCACTCTAC

ATGAATCCGAATACATCAGCCACTGGTCCACTTGCTAGAAGAACTGCGAGTGACCAA

GCACCACAGGAATTTGCCAAGACTTAGGAAGACTCACTAGGTAGAGGGCGAGGCAC

TGTGATCGCTCTGGTTATCTCATACGAGCAGGGAAAATGCATGACTACTCATCGAGAA

CTTTTACTGCAGATGTGATTTTACAGACGGGTGATTTGGGTGGCATCTTCAGAAACTGA

ATAGAGATGTGGCCCTCGGTAGCCTGCAGACCCGACTGTCACCCCCTTATAAATGGG

AGAGGCATTCTAGAAGAGGTAGTGTTTGAGGAAGAGTGGAGTTTTAGATCTTTCTTTCC

TGATTTTTAAAATCACTCCCTTTGGAGTGGTCACAATGACAAAACATCCCTACAACAGC

CAAACTTCTGCTACAAACAGAAGTTACACTGGAAGGCTTTTGGTCCCAGGACAGT
CTGCAGAGTA

Sequence 11 found 1 in 16 clones (6.25%)
TCAGCGTTGACTATTCTCTACAAACCACAAAGACATTGGAACACTATACCTATTATTCG
GCGCATGAGCTGGAGTCCTAGGCACAGCTCTAAGCCTCCTTATTCGAGCCGAGCTGG
GCCAGCCAGGCAACCTTCTAGGTAACGACCACATCTACAACGTTATCGTCACAGCCC
ATGCATTTGTAATAATCTTCTTCATAGTAATACCCATCATAATCGGAGGCTTTGGCAACT
GACTAGTTCCCCTAATAATCGGTGCCCCCGATATGGCGTTTCCCCGCATAAACAACAT
A

2. Complete sequence of proteins that interact with rLipL32 which were identified by T7 select® cDNA liver phage display library

Sequence 1 found 19 in 28 clones (68%)
GGCCAGGGTGCCGCAGACGCGGGGACGCTGGCTCGCTCCCTCCCTCCCTCCCTCC
GACGCTGTGAGTAGAGAAGCTAGGCCCCGAGCCGGGCGGGACTAGGGTGGTGGTT
GTGTTCTGCCCTCGCCCGTCGCCAACGCTGGGCTGACTGAAAACCTGGCCCTTTGGG
TCGCGCGTCTTCGACCTTCACCGCCATGCGGACATGGGGCCGTGGGAGAAACTCTG
GGCGGGATTACGGTGAAAATTCTTGTTTGGGCGGGCCCTGCGGCTTCTTTCTTCCGG
CTCGCCCTCGGCCCCGCCCCCGCCCCGCCCCCGCTCCAGCTCCAGCCGGGTCCTC
GCTCGGCCCCGCACGCGGCCCGCGGTAGACGGAGGGCCTGGGCACCCACCGTCT
CCATCCCGGCCCCAGCCCAGTCGAGCGTCCACTAGAAGGGCGCTCCCTGTCCGGC
CCTAATCCTCGCTCCTCACGGAGGCTCTTTGTCACAGCGAAGACTGACAGCCCGCAG
TCTTCTGGACTTCTTTTATGGGGCTCCTGGCGGTGCTATTCATTCAGTCATTGATTCGTC
TTGTAAATATGGAGCGCCTGCTCTGTACTCGACCCGGCACTGGGTACCGGAAGAACC
AGACAGCTCGGT

Sequence 2 found 2 in 28 clones (7%)

CCGGATGGTGCAGGAAGCGCCAGCTGCGCTGCCCACGGAGCCAGGCCCCAGCCCC

GTGCCTGCCTTCCTCGGCAAGCTATGGGCGCTGGTGGGGGACCCAGGCACAGACCA

CCTGATCCGCTGGAGCCCGAGCGGGACCAGTTTCCTCGTAAGCGACCAGAGCCGTT

TCGCCAAGGAAGTGCTGCCCCAGTATTTCAAGCATAGCAACATGGCGAGCTTCGTGC

GCCAACTCAACATGTACGGTTTTCGGAAGGTGGTGAGCATCGAGCAGGGCGGCCTG

CTTAGGCCGGAGCGCGACCACGTCGAGTTCCAGCACCCGAGCTTCGTGCGCGGCC

GCGAGCAGCTACTGGAGCGCGTGCGGCGCAAGGTGCCCGCGCTGCGCGGCGACG

ACGGCCGCTGGCGCCCGGAGGACCTGGGTCGACTACTGGGCGAGGTGCAGGCTTT

GCGGGGAGTGCAGGAGAGCACCGAGGCGCGGCTGCGGGAGCTCAGGCAGCAGAA

CGAGATCTTGTGGCGGGAGGTGGTGACACTTCGGCAGAGCCACGGTCAGCAGCACC

GGGTCATTGGCAAGCTGATCCAGTGTCTCTTTGGGCCACTTCAGGCGGGGCCGAGC

AATGCAGGAGGCAAGAGAAAGCTGTCCCTGATGCTGGATGAGGGGAGCTCATGCCC

AACACCTGCCAAGTTCAACACCTGCCCTCTACCTGGTGCCCTTCTGCAGGACCCCTA

CTTCATCCAGTCGCCTTC

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PUBLICATION	Attachment of pathogenic *Leptospira interogans* to Vero cells. Poster presentation in Pure and Applied Chemistry International Conference (PACCON2011), Miracle Grand Hotel, Bangkok, Thailand, January 2011: 131-134.