รายงานวิจัยฉบับสมบูรณ์

การใช้เทคนิคระดับโมเลกุลเพื่อจำแนกเอพิโทปที่มีความสำคัญในการกระตุ้นภูมิคุ้มกัน และเปปไทด์ที่เป็นส่วนสำคัญในการก่อพยาธิสภาพของโปรตีนลิปแอลสามสิบสองของเชื้อเลปโตสไปรา

Molecular techniques for identification of protective epitope and pathogenic peptides of LipL32 protein of *Leptospira* spp.

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26 พฤศจิกายน 2557

สัญญาเลขที่ MRG5380138

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ภาควิชาชีวโมเลกุลและพันธุศาสตร์โรคเขตร้อน
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สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย

Project Code : **MRG5380138**

ชื่อโครงการ: การใช้เทคนิคระดับโมเลกุลเพื่อจำแนกเอพิโทปที่มีความสำคัญในการกระตุ้นภูมิคุ้มกันและเปปไทด์ที่เป็นส่วนสำคัญในการก่อพยาธิสภาพของโปรตีนลิปแอลสามสิบสองของเชื้อเลปโตสไปรา

Project Title: Molecular techniques for identification of protective epitope and pathogenic peptides of LipL32 protein of *Leptospira* spp.

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Project Period: 2010-2012

Keywords: *Leptospira* spp., LipL32, mAbLPF1, therapeutic epitope, epitope mapping, sphingomyelinase H (SphH), hemolytic activity

Executive summary

Basic research entitled “**Molecular techniques for identification of protective epitope and pathogenic peptides of LipL32 protein of *Leptospira* spp.**” was financially supported by New Researchers Grant from Thailand Research Fund (TRF), Office of Higher Education Commission, and Mahidol University, Thailand (to Dr. Santi Maneewatchararangsri, No. MRG5380138, 2010-2012). The objectives of research are to identify and characterize LipL32 epitope bound by therapeutic LPF1 monoclonal antibody (mAbLPF1) and to identify LipL32 peptides/domains responsible for hemolytic activity of the protein. The research was conducted at Faculty of Tropical Medicine, Mahidol University and Laboratory for Research and Technology Development, Department of Parasitology, Faculty of Medicine Siriraj Hospital, Mahidol University. Benefits obtained from the project are summarized as the following:

1. Basic knowledge gained from the project are firstly, known neutralizing/protective epitope of *Leptospira* LipL32-specific mAb (mAbLPF1 epitope) and its properties including peptide sequence and molecular structure, epitope conservation among *Leptospira* spp. and the LipL32 protein superfamily. Secondly, presumptive known of antigenic determinants (key residues) involved in mAbLPF1 epitope-paratope interaction. Lastly, known potentially LipL32 peptides responsible for hemolytic activity.
2. Transformed *E. coli* clones carrying recombinant *lipl32* and *sphH* plasmids *(*encoding for LipL32Δ1-90*,* LipL32Δ1-170 and SphH) were established from the project and the *lipl32*-pET *E. coli* clone was used as material for molecular cloning practice in TMMO504 course, Department of Molecular Tropical Medicine and Genetics, Faculty of Tropical Medicine, Mahidol University.
3. One original paper publication (Maneewatch *et al*., 2014, published in PEDS journal, IF=2.58), 4 oral and 2 poster presentations from national and international conferences.
4. Original paper (Maneewatch *et al*., 2014) was submitted for Assistant Professor (Molecular Biology) position.

บทคัดย่อ

โมโนโคลนาลแอนติบอดีของหนูโคลน LPF1 (mAbLPF1) ที่มีความจำเพาะต่อโปรตีนลิปแอลสามสิบสองที่มีคุณสมบัติลบล้างการแตกของเม็ดเลือดแดงจากเชื้อเลปโตสไปราในหลอดทดลอง และให้ผลรักษาหนูแฮมสเตอร์ที่ติดเชื้อเลปโตสไปราได้ถูกผลิตในห้องปฏิบัติการ งานวิจัยนี้มีวัตถุประสงค์เพื่อจำแนกและศึกษาคุณลักษณะของโปรตีนลิปแอลสามสิบสองเอพิโทปของโมโนโคลนาลแอนติบอดีโคลน LPF1 ด้วยเทคนิคทางอิมมิวโนโลยี การจำแนกฟาจมิโมโทป LPF1 ด้วยการทำ multiple mimotope alignments และการวิเคราะห์สายเปปไทด์ที่มีเอพิโทปของโมโนโคลนาลแอนติบอดีโคลน LPF1 การวิเคราะห์โครงสร้างสามมิติ และหน้าที่ ตลอดจนจำแนกสายเปปไทด์ของโปรตีนลิปแอลสามสิบสองที่ทำให้เม็ดเลือดแดงแตก

ผู้วิจัยทำการวิเคราะห์และจำแนกหน้าที่ของเปปไทด์ของโปรตีนลิบแอลสามสิบสองแบ่งเป็นสามส่วน ได้แก่ เปปไทด์ส่วนต้นเป็น integral membrane (ลำดับกรดอะมิโน 1 ถึง 90) เปปไทด์ช่วงกลางกลุ่ม flavin utilizing monooxygenase (ลำดับกรดอะมิโนที่ 91 ถึง 170) และ เปปไทด์ด้านปลาย (ลำดับกรดอะมิโนที่ 171-272) เป็น periplasmic binding protein ทำการผลิตโปรตีนลิบแอลสามสิบสองท่อนสั้นลูกผสม คือ โปรตีนท่อนสั้น LipL32Δ1-90 (ตัดกรดอะมิโนลำดับที่ 1 ถึง 90) และ LipL32Δ1-170 (ตัดกรดอะมิโนลำดับที่ 1 ถึง 170) และถูกนำไปใช้ในการตรวจสอบความจำเพาะของโมโนโคลนาลแอนติบอดีโคลน LPF1 กรดอะมิโนของเอพิโทปแอนติบอดีโคลน LPF1 ประกอบด้วย P243, L244, I245, H246, L252, และ Q253 บนโครงสร้าง β13-turn และ amphipathic α6 helix ที่ปลายด้าน C-terminus ของโปรตีนลิบแอลสามสิบสองซึ่งประกอบด้วย hydrophobic patch ที่มีคุณสมบัติในการเกาะติดกับ phospholipid และโฮสท์เซลล์ และคุณสมบัติในการสอดเข้าในผนังเซลล์ อีกด้านหนึ่งของเอพิโทปแอนติบอดีโคลน LPF1 ประกอบด้วยกรดอะมิโนชนิด hydrophilic acidic และ basic เอพิโทปนี้เป็นอนุรักษ์เอพิโทปในเชื้อเลปโตสไปรากลุ่มสายพันธ์ก่อโรคและกลุ่ม intermediate และไม่พบเอพิโทปแอนติบอดี LPF1 ในโปรตีนกลุ่มลิปแอลสามสิบสองของจุลชีพอื่น

ผู้วิจัยทำการศึกษาความสามารถของโปรตีนลิบแอลสามสิบสองในการทำให้เม็ดเลือดแดงของมนุษย์แตก (hemolytic activity) โดยในหลอดทดลอง พบว่าโปรตีนลูกผสมลิปแอลสามสิบสองชนิดแต็มเส้น และโปรตีนท่อนสั้น LipL32Δ1-90 ให้ผลฮีโมไลซิสต่อเม็ดเลือดแดงมนุษย์ และปฏิกิริยาลดลงอย่างมีนัยสำคัญเมื่อทดสอบโดยโปรตีนท่อนสั้น LipL32Δ1-170 และไม่เกิดปฏิกิริยาฮีโมไลซิสโดยโปรตีน LipL32Δ166-245 ดังนั้น เปปไทด์ลิปแอลสามสิบสองโปรตีนที่ลำดับกรดอะมิโนที่ 166 ถึง 245 เป็นเปปไทด์ยาวน้อยสุดที่ก่อให้เกิดฮีโมไลซิส งานวิจัยนี้เป็นการค้นหา และจำแนกคุณลักษณะของเอพิโทปที่มีความสำคัญในการกระตุ้นภูมิคุ้มกันของโปรตีนลิปแอลสามสิบสองและจำแนกเปปไทด์ของโปรตีนลิปแอลสามสิบสองที่ก่อให้เกิดฮีโมไลซิสในระหว่างการติดเชื้อเลปโตสไปรา

Abstract

LipL32-specific mouse monoclonal antibody clone LPF1 (mAbLPF1) which neutralized *Leptospira*-mediated hemolysis *in vitro* and rescued hamsters from lethal *Leptospira* infection was previously produced (Maneewatch *et al*., 2008). This research was carried out in order to identify and characterize LipL32 epitope bound by therapeutic mAbLPF1 antibody by immunoassay and phage consensus mimotope identification and multiple alignments, and determining peptide epitopes locations, 3D structures and their functions and to identify LipL32 peptides responsible for hemolytic activity. Three functionally LipL32 domains, namely integral membrane domain (approximately residues 1 to 90), flavin utilizing monooxygenase superfamily (91-170) and periplasmic binding domain (171-272) from *lipl32* sequence, respectively were assigned. LipL32 N-terminally deleted LipL32 (LipL32Δ1-90, deleting aa. 1-90), LipL32Δ1-170 (containing C-terminus of LipL32, deleting aa. 1-170) protein truncations were produced and tested for antigenic specificity

The mAbLPF1 epitope consisted of P243, L244, I245, H246, L252, and Q253 on non-continuous β13-turn and C-terminal amphipathic α6 helix of LipL32 structure; containing hydrophobic patch, contributing to phospholipid/host cell adhesion and membrane insertion on one side, and hydrophilic, acidic and basic amino acid residues on another side. Epitope is highly conserved among the pathogenic and intermediately pathogenic *Leptospira* spp. and are absent from the LipL32 superfamily proteins of other microorganisms.

Hemolytic activity of recombinant LipL32 proteins to human erythrocytes was examined *in vitro*. Hemolytic activity mediated by full-length LipL32 and LipL32Δ1-90 proteins to human erythrocyte was investigated while the activity was abolished in LipL32Δ1-170 and in LipL32Δ166-245. The minimum peptide essential for hemolytic activity was found to be amino acids 166-245 of LipL32. The experiments demonstrate not only protective epitope of LipL32 but also LipL32 peptides that mediate hemolysis during *Leptospira* infection.

Keywords: *Leptospira* spp., LipL32, mAbLPF1, therapeutic epitope, epitope mapping, sphingomyelinase H (SphH), hemolytic activity

Acknowledgements

This research was financially supported by New Researchers Grant (to Dr. Santi Maneewatchararangsri, No. MRG5380138, 2010-2012) from Thailand Research Fund (TRF), the Office of Higher Education Commission and Mahidol University. I would like to express my deepest gratitude and appreciation to my research consultant, Professor Dr. Wanpen Chaicumpa, Laboratory for Research and Technology Development, Department of Parasitology, Faculty of Medicine Siriraj hospital, Mahidol University for her supports and valuable RESEARCH supervision. Department of Tropical Hygiene, and Department of Molecular Tropical Medicine and Genetics, Faculty of Tropical Medicine, Mahidol University, Laboratory for Research and Technology Development, Department of Parasitology, Faculty of Medicine Siriraj Hospital, Mahidol University are acknowledged for research supports.

Background

Leptospirosis (โรคไข้ฉี่หนู) is a worldwide neglected infectious zoonotic disease frequently occurs in tropical and sub-tropical countries which afflicts million cases of human and animals annually **(Adler and de la Pena Moctezuma, 2010; Bharti *et al*., 2003; Hartskeerl *et al*., 2011; Palaniappan *et al*., 2007)**. The disease is caused by pathogenic spirochetes of the genus *Leptospira*. Members of the *Leptospira* spp. are classified into 20 genomospecies of pathogenic, intermediately pathogenic and saprophytic clades. Serologically, they were placed into more than 250 serovars **(Adler and de la Pena Moctezuma, 2010)**. *Leptospira* spp. has high adaptation from a broad species of rodent, marsupial and mammal reservoirs to environments and humans which are accidental host **(Faine 1994)**. In the latter, the bacteria inflict a variety of clinical manifestations **(Matthias *et al*., 2008; Segura *et al*., 2005)** ranging from asymptomatic to serious systemic inflammation, jaundice, kidney, pulmonary hemorrhage multi-organs failure and septic shock, known as Weil's syndrome with high fatality rate. In the reservoir host (chronic carriage such as rodents and cattle), the bacteria are shed with urine which contaminates the environments **(Matthias *et al*., 2008; Segura *et al*., 2005)**. Early and accurate differential diagnosis of leptospirosis from other febrile illnesses which share similar clinical pictures and a development of a broadly spectrum leptospirosis vaccines that elicit protective immune responses across sero-variants are needed.

Leptospiral LipL32 is regarded as a dominant lipoprotein located at the bacterial outer membrane. LipL32 sequence is highly conserved (98%) within the pathogenic and decreased to 70% of the intermediately pathogenic *Leptospira* clades **(Haake *et al*., 2000; Guerreiro *et al*., 2001; Murray *et al*., 2013)**. Pathogenic *Leptospira* expresses LipL32 constitutively in both *in vitro* culture and during infection in mammalian hosts **(Haake *et al*., 2000; Sakolvaree *et al*., 2007)**. The protein is highly immunogenic, *i.e.,* LipL32-specific IgG can be detected in acute and convalescing leptospirosis sera **(Guerreiro *et al*., 2001; Sakolvaree *et al*., 2007; Chalayon *et al*., 2011)**. Thus, LipL32 is a one of molecular targets for leptospirosis diagnosis **(Bomfim *et al*., 2005; Chalayon *et al*., 2011; reviewed by Toyokawa *et al*., 2011)**. It is also a potential immunogen of a developing leptospirosis universal vaccine **(Aviat *et al*., 2010; Branger *et al*., 2005; Seixas *et al*., 2007)**. Passive immunotherapy of experimental leptospirosis by using LipL32-specific mAbs has been demonstrated. **(Maneewatch *et al*., 2008; 2009)**.

Murine hybridoma clone LPF1 secreting monoclonal antibody (mAbLPF1) specific to *Leptospira* LipL32 protein was produced **(Maneewatch *et al*., 2008)**. The mAbLPF1 neutralized *Leptospira*-mediated hemolysis *in vitro*. The mouse mAbLPF1 as well as its variants including mouse single chain variable fragment (ScFv) and humanized-mouse ScFv mitigated morbidity and decreased mortality rates of hamsters experimentally infected with lethal dose of heterologous *Leptospira* spp. **(Maneewatch *et al*., 2008; 2009)**. Therefore, mAbLPF1 epitope has high potential for using as diagnostic and vaccine targets for leptospirosis.

In this report, LipL32 epitope peptide of mAbLPF1 (mAbLPF1 epitope) was identified and mapped to LipL32 structure. Characteristics of mAbLPF1 epitope including molecular structure, conservation among *Leptospira* spp. and LipL32 protein superfamily from other microorganisms were elucidated. Antigenic determinants of mAbLPF1 epitope and paratope [key residues in variable heavy (VH) and light (VL) domains] involved in mAbLPF1-LipL32 interaction was studied.

Several virulence determinants of pathogenic *Leptospira* have been identified. These are lipopolysaccharide **(Isogai *et al.,* 1986)**, glycolipoproteins **(Alves *et al.*, 1992)**, peptidoglycan **(Dobrina *et al.*, 1995)**, heat shock proteins **(Stamm *et al.*, 1991)**, flagellin **(Goldstein and Charon, 1988)**, sphingomyelinases, serine proteases, zinc-dependent proteases (Bulach *et al.*, 2006), hemolysins **(Lee *et al.*, 2000)**. *Leptospira* hemolysins have been divided into two groups based on their ability to hydrolyze sphingomyelin substrate, sphingomyelinase group including Sph1-4 and non-sphingomyelinase group including SphH, TlyA, Hlyx, HlpA, HlyC, and HlyA. The sphingomyelinase C (Sph) homologs (SphA-G) and SphH were identified among pathogenic *Leptospira* **(Serger *et al.*, 1990; Lee *et al.*, 2000)**. Sphingomyelinase H (SphH) is a putative pore-forming hemolysin, induced colloid osmolytic lysis of erythrocyte and showed β-hemolytic zone on sheep blood agar **(Lee *et al.*, 2000)**, also present cytotoxic activity on mammalian cells **(Lee *et al.*, 2002).**

LipL32 has been identified as a member of *Leptospira* adhesive matrices (MSCRAMMs), responsible for binding to the host extracellular matrix (ECM) molecules, including matrigel, laminin, collagens (I and IV), and intact as well as 30 and 45 kDa proteolytic fragments of fibronectin (FN) **(Hauk *et al.*, 2008; Hoke *et al.*, 2008)**. LipL32 also binds to zymogen plasminogen to generate plasmin **(Vieira *et al.*, 2010)** and adheres to proteoglycan of human cell surface receptors **(Breiner *et al.*, 2009)**, cultured mammalian cells **(Liu *et al.*, 2007; Maneewatch *et al.*, 2014)** and neutrophils **(Wang *et al.*, 1984)**. Moreover, binding function of LipL32 to host membrane components, *i.e.*, cholesterol and phosphatidylcholine as well as plasma membrane were demonstrated **(Maneewatch *et al.*, 2014)**.

LipL32 [Hemolysis-associated protein-1 (Hap-1)] mediated hemolytic activity on several erythrocytes *in vitro* has been demonstrated. LipL32 and SphH showed additively on hemolytic activity on human erythrocyte membrane. **(Hauk *et al.*, 2005; Lee *et al.*, 2000)**. However, LipL32 peptide/domain mediated membrane and hemolytic activity in hemolysis mechanism has not been elucidated leading to the objective of this research to **characterize LipL32 peptides related to the hemolytic activity.**

Research Objectives

1. To identify neutralizing mAbLPF1 epitope sequence
2. To characterize mAbLPF1 epitope, including structure, conservation among *Leptospira* spp. and LipL32 protein superfamily from other microorganisms
3. To identify and characterize peptide(s)/domain(s) of LipL32 responsible for hemolytic activity

Materials and Methods

Murine monoclonal antibodies (mAbs):

Mouse hybridomas secreting monoclonal antibodies used in this study included clones LPF1 and LPF2 which secrete IgG1 mAbs specific to *Leptospira* LipL32 **(Maneewatch *et al*., 2008)**, and clone 204D3 which secretes IgG3 mAb204D3 specific to antigen 9 in LPS of group D *Salmonella* Typhi **(Chaicumpa *et al*., 1988)** were kindly provided by Prof. Dr. Wanpen Chaicumpa. The hybridomas were individually cultured either in complete RPMI-1640 medium [10% (v/v) heat-inactivated fetal bovine serum (FBS), 2 mM L-glutaMAX, 100 units/ml penicillin and 100 µg/ml streptomycin] or in protein-free CD hybridoma medium (supplemented with 2 mM L-glutaMAX) at 37°C in humidified 5% CO_2.

Antigenic specificity and Reactivity of LipL32 specific-mAbs were determined by using *Leptospira* whole cell homogenates and LipL32 antigens in Western blotting and indirect ELISA, respectively **(Saengjaruk *et al*., 2002)**. mAb204D3 was used as irrelevant antibody control.

Leptospira spp. and culture condition:

Leptospira strains (**Table 1**) were individual grown in liquid EMJH (Ellinghausen-McCullough-Johnson-Harris) medium supplemented with 10% (v/v) *Leptospira* enrichment at 30°C under aerobic condition. Growth and motility of spirochetes were examined under dark-field microscopy (20×, 40× magnifications). Bacterial contamination were checked by dropping 50 µl of individual culturing aliquots on bacterial culture agar, incubated at 37°C for 48 h.

Preparation of *Leptospira* whole cell homogenates:

Leptospiral homogenates were prepared **(Sakolvaree *et al.,* 2007)**. Leptospires were collected from the cultures by centrifugation at 10,000 ×*g*, 4°C for 15 min, washed twice with cold 5 mM $MgCl_2$-EMJH. Bacterial suspensions in PBS, pH 7.4 were subjected to sonication. Protein concentration was measured by Bradford assay, protein patterns was analyzed under SDS-PAGE and Coomassie Briliant Blue-G250 staining.

Recombinant LipL32 constructs:

Transformed *E. coli* carrying full-length *lipl32*-pET23a(+) plasmid was from **Maneewatch *et al*., 2008**. Recombinant truncated LipL32 proteins (N-terminally truncated LipL32Δ1-90, and LipL32Δ1-170) were prepared **(Maneewatch *et al*., 2014)**. Briefly, two DNA sequences containing of truncated *lipl32* (nt. 271-816 and 511-816) were individually amplified from *lipl32*-pET23a(+) DNA template by inverse polymerase chain reaction (iPCR) using the respective primers (**Table 2**). The forward and reverse primers amplified the DNA template in opposite directions to those of conventional PCR (**Fig. 1**). The 5'-end of the p23/R reverse primer sequence specific to *Nde*I of the pET23a(+) backbone amplified the template towards the T7 promotor while the forward primers amplified DNA sequence coding for the truncated LipL32Δ1-90 (L271/F) started at nt. 271 of the *lipl32*- insert towards the 3'-end of this DNA construct. The forward primer of the truncated LipL32Δ1-170 (L511/F) started the amplification at nt. 511 of the *lipl32*-insert towards the 3'-end. The *Nde*I endonuclease site was included in the primer sequences at the 5'-end of the forward and 3'-end of the reverse primers to facilitate subsequent gene cloning. PCR amplicons were purified, digested with *Nde*I endonuclease and subsequently ligated using T4 DNA ligase. The resulting plasmids, LipL32Δ1-90-pET23a(+) and LipL32Δ1-170-pET23a(+), were introduced separately into competent BL21(DE3) *E. coli* by heat-shock method. Positive *E. coli* transformants were screened by colony PCR using universal T7 primers.

Preparation of recombinant proteins:

Recombinant proteins were expressed from individually transformed *E. coli* and the respective recombinant proteins were purified using TALON metal affinity resin as previously described **(Maneewatch *et al*., 2008; 2014)**. Purified proteins were verified by SDS-PAGE and Coomassie Brilliant Blue-G250 stain and Western blot analysis, respectively.

LipL32 epitope mapping by Western blot analysis:

LipL32 subdomains bound by the mAbLPF1 (mAbLPF1 epitope) was determined by using Western blot analysis. The LipL32, LipL32Δ1-90 and LipL32Δ1-170 proteins were subjected to 15% SDS-PAGE and subsequently blotted the separated components onto nitrocellulose membrane (NC). The blotted NC was blocked with blocking reagent for 1 h at 25°C. After washing with PBST, membrane was placed in spent culture medium containing mAbLPF1, followed by goat anti-mouse IgG immunoglobulin-AP conjugate (1:3,000) for 1 h at 25°C for each steps. Immunoreactive bands were developed using BCIP/NBT substrate.

Identification of LipL32 mimotope bound by mAbLPF1:

mAbLPF1 mimotope sequence (12-mer) was searched from a Ph.D.-12^{TM} phage display library kit (NEB). The selected phage displayed peptide sequences (mimotope sequences) were multiply aligned using the MIMOX program **(Huang *et al.*, 2006)** with manual adjustment to identify the mAbLPF1 mimotope consensus sequence. The consensus mimotope was then aligned with the LipL32 amino acid sequence to locate the mAbLPF1 epitope on LipL32.

Protein modeling:

mAbLPF1 epitope structure was modeled from LipL32 structure template (2WFKA) *via* SWISS-MODEL Server **(Amold *et al.*, 2006)**. Alpha-helix conformation was analyzed by HELIQUEST server **(Gautier *et al.*, 2008)**.

Amino acid residues responsible for complementarily determining regions (CDRs) of heavy (H1-3), light (L1-3) chains and frameworks of mAbLPF1 **(Maneewatch *et al.*, 2009)** were identified by IMGT server **(Lefranc *et al.*, 2009)**. The solvent accessible estimations were calculated from NetSurf program and residues with >25% ASA were assigned to be exposed. Structures of heavy (VH) and light (VL) regions of MAbLPF1were homology modeled using 3gk8H and 1bafl as structural templates, respectively. The constructed models were verified by QMEAN Z-score calculation **(Benkert *et al.*, 2011)** and edited using POLYVIEW3D server **(Porollo and Meller, 2007)**.

Table 1. *Leptospira* spp. used in this study[1]

Species	Serogroup	Serovar/strain	Pathogenicity[2]
L. interrogans	Icterohaemorrhagiae	Copenhageni	Pathogenic
L. interrogans	Icterohaemorrhagiae	Icterohaemorrhagiae	Pathogenic
L. interrogans	Pomona	Pomona	Pathogenic
L. interrogans	Mini	Mini	Pathogenic
L. biflexa	Andamana	Andamana	Non-pathogenic
L. biflexa	Semaranga	Patoc	Non-pathogenic

[1] *Leptospira* spp. were kindly provided by Prof. Dr. Wanpen Chaicumpa, Laboratory for Research and Technology Development, Department of Parasitology, Faculty of Medicine Siriraj Hospital, Mahidol University. [2] Pathogenicity is classified based on 16S rRNA genetic relatedness (**Adler and de la Pena-Moctezuma, 2010)**

Table 2. List of oligonucleotide primers

Recombinant protein	Primer/ orientation	Sequence (5' to 3')	Annealing site
LipL32Δ1-90	p23/R	CCCGGGCATATGTATATCTCCTTCTTAAAGTT	205*
	L271/F	AATCCCCATATGGCTGAAATGGGAGTTCGTAT	271
LipL32Δ1-170	p23/R	CCCGGGCATATGTATATCTCCTTCTTAAAGTT	205*
	L511/F	AGGCCCCATATGAAAGAAGAGAGACACAACAAG	511
SphH	SphH/F	GGCGAGCTCATGCGAAACATTTTCCGAAAAA	1
	SphH/R	CCGGCAAGCTTACGATAAATTAAATCGTCTTTCC	1662

F, forward; R, reverse, *annealing at -64 bp before *lipl*32 start codon in recombinant *lipl*32-pET23a(+) plasmid (**Fig. 1**). Restriction endonuclease sites were underlined.

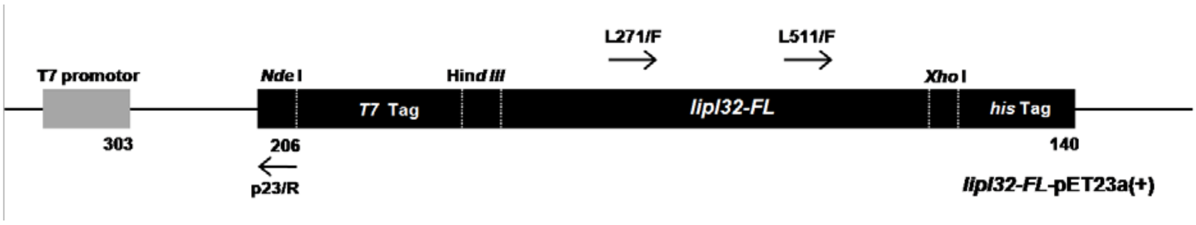


Fig. 1. Genetic map of *lipl32* constructs **(Maneewatch *et al.*, 2014)**

The *lipl32* coding sequence (nucleotides 1-816) was inserted into the pET23a(+) plasmid *via* *Hind*III and *Xho*I restriction endonucleases in the cloning region. The transcription start site of the recombinant plasmid was from *Nde*I, followed by coding sequences for T7 Tag, *lipl32* and C-terminal *his* tag. In iPCR, the 5'-end of p23/R reverse primer annealed to *Nde*I at nucleotide 260 and amplified the template towards the T7 promoter (lower arrow). The forward primers, L271/F and L511/F bound to nucleotides 271 and 511 of the *lipl32*, respectively, and amplified the template towards the *his* tag (upper arrows).

mAbLPF1 epitope sequence conservation and LipL32 subdomains prediction:

mAbLPF1 epitope sequence was multiply aligned to published LipL32 sequences from NCBI database as well as orthologous proteins of the LipL32 superfamily using BLASTP 2.2.26+ Server (http://www.ncbi.nlm.nih.gov/Entrez/) **(Altschul *et al.*, 1997)**. Conservation rates of conserved epitope sequence among *Leptospira* spp. and LipL32 protein superfamily were determined. Subdomains of LipL32 were predicted from conserved domain database.

Hemolytic activity assay:

Hemolytic activity of recombinant LipL32 proteins was examined as previously described **(Lee *et al*., 2002; Carvalho *et al*., 2009)** in V-bottom microtiter plate. Briefly, sample (50 μl) was mixed with 50 μl of 1% (w/v) human erythrocyte suspension in PBS, pH 7.4, followed by incubation at 25°C or 37°C. 1% SDS solution (50 μl) was used as positive hemolysis (100% hemolysis, PC). Thereafter, supernatant was collected and the absorbance was measured at 540 nm against spontaneous hemolysis (blank, NC).

Percent hemolysis was calculated using the formula: % hemolysis = $[(OD_{Test} - OD_{NC})/OD_{PC}] \times 100$, whereas NC and PC are the negative and positive hemolysis controls. Hemolysis unit was determined as the highest dilution that caused 100% hemolysis.

Physiochemical chemical of *Leptospira* hemolysins was determined by treating of *Leptospira* lysates (100°C, 5 min), divalent cations (1 mM of $Ca^{2+,}$ Mg^{2+}), cation chelator (1 mM EDTA), serine protease inhibitor (1 mM PMSF). All experiments were performed three times.

Results

Assignments of LipL32 protein subdomains:

Conserved domains of LipL32 *Leptospira interrogans* serovar Lai (NP_712818) was searched from Conserved Domain Database (CCD), NCBI and the result showed significant LipL32 protein superfamily (aa. 50-240). Three annotated functionally subdomains, namely integral membrane domain (approximately aa. 1-90), flavin utilizing monooxygenase superfamily (aa. 91-170) and periplasmic binding domain (aa. 171-272) of LipL32$_{1-272}$, respectively were assigned. (**Fig. 2**)

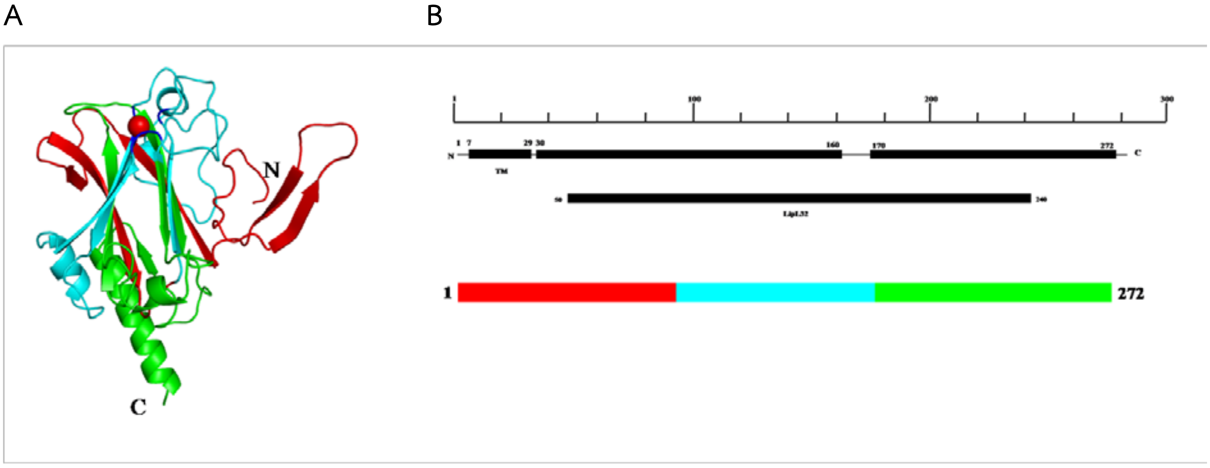


Fig. 2. LipL32 subdomains.

- **(A)** Ribbon representation of LipL32 residues 21-90 (Red), 91-170 (Cyan), and 171-272 (Green) of LipL32 monomer. Red dot represents Ca^{2+}. The model was constructed from template [PDB ID: 2WFK **(Tung *et al*., 2010)**]. Graphic representation was generated by POLYVIEW3D.
- **(B)** Schematic illustration of LipL32 domain assignment. Each assigned domain model is presented as black bar with region indicated. The significant LipL32 superfamily and subdomains was model as black bar. The lower E-value indicating subdomains are colored correspond as in **A**. Amino acid scale numbers are indicated.

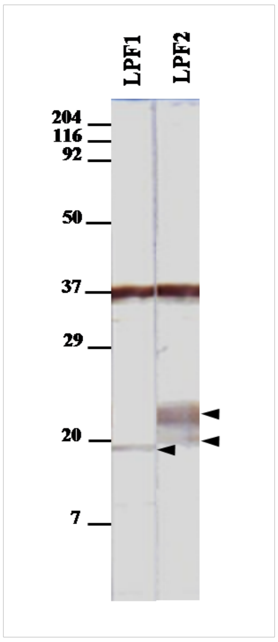
Characterization of mAbLPF1 antibody:

Mouse monoclonal antibody clone LPF1 (mAbLPF1) specific to *Leptospira* LipL32 protein was produced **(Maneewatch *et al*., 2008)**. **Table 3** summarizes mAbLPF1 characteristics compared to LipL32 specific-mAb clone LPF2 (mAbLPF2). Antigenic specificity of the mAbLPF1 was determined by indirect ELISA against whole cell homogenates of pathogenic and two non-pathogenic *Leptospira* strains and recombinant LipL32 protein (**Table 4**). It was found that the mAbLPF1 gave positive ELISA result (OD_{405nm} >0.05) to all whole cell homogenates of pathogenic *Leptospira* strains and LipL32 but were negative to homogenate of *L.* Patoc (non-pathogenic) and weakly positive to *L.* Andamana (intermediately pathogenic). The mAb204D3 antibody control did not react to any of the *Leptospira* antigens (data not shown).

Table 3. Summary of LipL32 specific-mAbs

	mAbLPF1	**mAbLPF2**
Ig isotype	IgG1/κ	IgG2a/κ
Reciprocal titer[2]	10,24	32
MAT titer	<1:10	<1:10
Antigenic specificity (kDa) in WB[2]	major band (27)	major band (27) diffuse band (15)
Neutralizing activity against the *Leptospira* mediated-hemolysis[3] (%)	56.84 and 30.37	Nd
Immunoprotective activity[4]	yes	Nd

[1] The titers of antibody produced in vitro culture hybridoma cell culture supernatants was measured by indirect ELISA against *Leptospira* whole homogenate. [2] WB analysis for determining mAbs antigenic specificities against SDS-PAGE separated-whole-cell homogenate of various pathogenic *Leptospira* strains. [3] Inhibition of the 1% human RBC hemolysis caused by 1.4 (5 ×10^7), and 2.8 (1 ×10^8) hemolytic dose 50 (HD50) of *L. interrogans* sv. Pomona by 2 μg of individual mAbs preparations.[4] Passive protection of hamsters from lethal dose 5LD50 of *L. interrogans* sv. Pomona by mAb. Nd, not determine.



Antigenic specificity of LipL32 specific-mAbs (LPF1 and LPF2 clones) to SDS-PAGE separated *Leptospira* homogenate revealed different immunoblot patterns apparent at lower molecular sizes (arrows) indicated the difference of epitope recognition within LipL32 molecule. (**Fig. 3)**

Fig. 3. Immunoblot patterns of mAbs-LPF1 and -LPF2 (Maneewatch *et al.*, 2008) against SDS-PAGE separated *Leptospira interrogans* sv. Icterohaemorrhagiae homogenate. The principal reactive band of the intact LipL32 is seen at ∼37 kDa. The mAb LPF1 also reacted to a lower molecular band at ∼18 kDa, whereas mAbLPF2 bound to a diffuse band at ∼22-23 kDa and a band at ∼20 kDa (arrow heads) of the *Leptospira* homogenate. The differences in the reactive band numbers and patterns at the lower molecular sizes indicated that the mAbLPF1 and mAbLPF2 may have different epitope specificities. The mAb204D3 did not react to *Leptospira* antigen (data not shown).

Table 4. MAbLPF1 antibody reactivity against whole cell homogenates of various *Leptospira* spp. and recombinant LipL32 protein by indirect ELISA.

Antigen used for coating the ELISA well[1]	**$OD_{405\ nm}$**	**Reactivity**[2]
L. interrogans sv. Icterohaemorrhagia	0.344	reactive
L. interrogans sv. Pomona	0.190	reactive
L. interrogans sv. Wolffi	0.196	reactive
L. biflexa sv. Andamana	0.07	Weakly reactive
L. biflexa sv. Patoc	0.009	negative
LipL32	0.832	reactive
BSA	Blank	

[1] whole cell homogenates were prepared according to Saengjaruk *et al*., 2002. They were used to coate the ELISA wells (1 μg/100 μl of carbonate-bicarbonate buffer, pH 9.6/well). [2] positive ELISA signal = $OD_{405nm} > 0.05$ (subtracted from BSA control)

Production of recombinant truncated *lipl32* constructs:

Transformed BL21(DE3) *E. coli* clones carrying recombinant plasmids [LipL32Δ1-90-pET23a(+), and LipL32Δ1-170-pET23a(+) encoding N-terminally truncated LipL32Δ1-90 (contained amino acid residues 91-272) and LipL32Δ1-170 (contained amino acids 171-272, respectively] were produced by inverse polymerase chain reaction (iPCR). **Fig. 3** illustrates PCR amplicons of LipL32Δ1-90 (lanes no. 2, 3) and LipL32Δ1-170 (lanes no. 4, 5) with expected sizes at 4.2 and 3.9 kb, respectively.

Preparation of recombinant LipL32 proteins:

Recombinant LipL32 proteins, *i.e*., full-length LipL32 (LipL32, amino acids 1-272, 29.61 kDa); N-terminally deleted LipL32 proteins including LipL32Δ1-90 (N-terminal deletion amino acids 1-90, ∼20 kDa), and LipL32Δ1-170 (N-terminal deletion amino acids 1-170, ∼12 kDa) with C-terminal hexahistidine tag (6×His) were successfully expressed from transformed *E. coli* (**Fig. 4B**) and localized of both soluble and insoluble *E. coli* fractions (data not shown). Purified recombinant LipL32 proteins were analyzed under 13% SDS-PAGE and Western blotting (**Fig. 4A** and **Fig. 4B**, respectively).

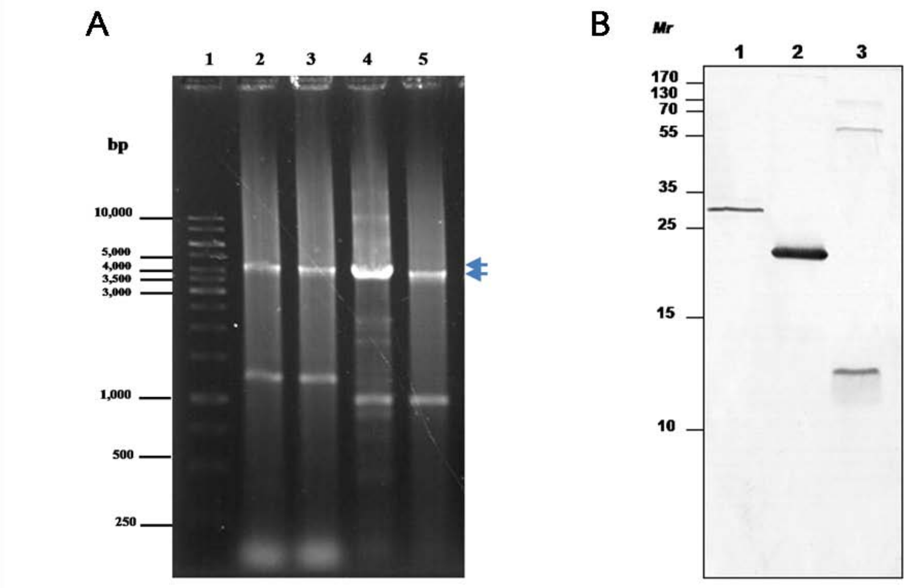


Fig. 4. Production of recombinant LipL32 proteins

(A) PCR amplicons encoding for LipL32Δ1-90 (lanes no. 2 and 3, 4.2 kb) and LipL32Δ1-170 (lanes no. 3 and 4, 3.9 kb) were amplified by iPCR reactions (Arrows)

(B) Preparation of recombinant LipL32 proteins, *i.e.*, LipL32, LipL32Δ1-90, and LipL32Δ1-170 (lane no. 1, 2, and 3, respectively) at molecular sizes 29, 20, 12 kDa, respectively**.** Numbers at the left are relative protein molecular masses (*Mr*)**.**

Determination of LipL32 epitope of the mAbLPF1:

Western blot analysis revealed reactive bands of mAbLPF1 bound to full length LipL32 as well as to the two truncated LipL32 proteins (**Fig. 5B**, **Table 5**) at the same molecular sizes to control (**Fig. 5A**). The mAbLPF1 reacted to all recombinant proteins, indicating that the epitope of mAbLPF1 is located within residues 171-272 of LipL32 molecule. Irrelevant mAb204D3 was used as antibody control and do not showed reactive band (data not shown).

Reactivity of mAbLPF1 to various recombinant LipL32 proteins was also examined by indirect ELISA. Reactivity of mAb to *L. interrogans* was included in the assay and served as internal control. Percent of median reactivity of mAbs LPF1 and LPF2 against various recombinant LipL32 proteins by indirect ELISA in comparison to LipL32 (100%).

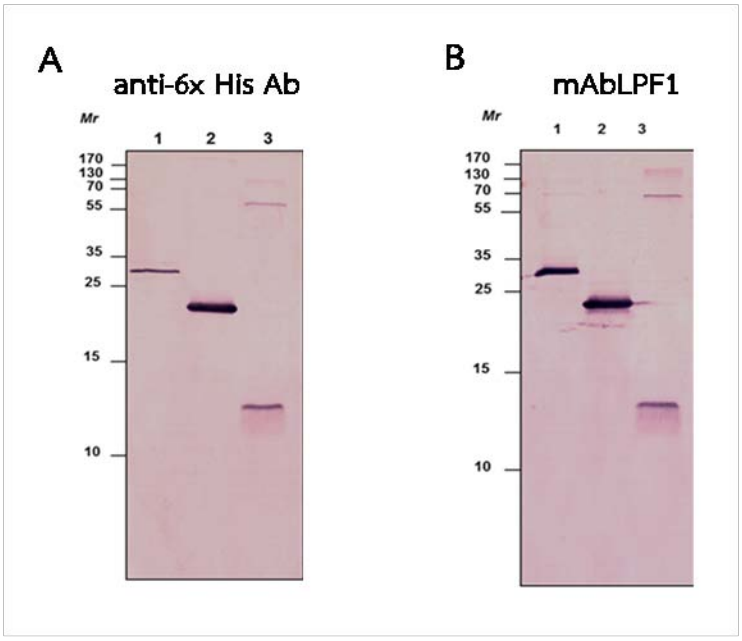


Fig. 5. Western blot results of mAbLPF1 antigenic specificity to LipL32 proteins.

Recombinant LipL32, LipL32-1, and LipL32-2 proteins (lanes 1-3, respectively) were fractionated by 15% SDS-PAGE under denaturation condition (5%, 2-mercaptoethanol and boil for 5 min), the blotted nitrocellulose membrane membranes were individual probed with mouse anti-6x His antibody (**A**), mAbLPF1 (**B**). Antigen-antibody reactive bands were developed by BCIP/NBT substrate. Position of standard molecular mass marker (KDa) are shown on the left.

Table 5. Reactivity of anti-LipL32 mAbs against different recombinant LipL32 proteins.

mAb	Reactivity to rLipL32 (WB[1]/ ELISA[2])						Reactivity of mAbs to *L. interrogans*[3]
	LipL32		LipL32Δ1-90		LipL32Δ1-170		
	WB	ELISA	WB	ELISA	WB	ELISA	
LPF1	+	100	+	100	+	141*	0.427 ± 0.59
LPF2	+	100	+	86	-	9.2	0.447 ± 0.32

[1] +, react; -, not react. The mAbsLPF9 (Maneewatch *et al*., 2008) and the mAb204D3 which were used as the mAb controls did not react to any of the recombinant LipL32 proteins in Western blotting. [2] Percent of median reactivity of mAbs LPF1 and LPF2 against various recombinant LipL32 proteins by indirect ELISA in comparison to LipL32 (100%). The cut-off value ($OD_{405\ nm}$ =0.04) is 18.5. The P3x-63-Ag8.653 myeloma culture medium was used as blank. * significant difference (P <0.05) [3]ELISA signal (mean ± SD) of mAbs reactivity to immobilized *L. interrogans* serovar Icterohaemorrhagiae by surface ELISA, irrelevant mAb204D3 was 0.06 ± 0.15.

mAbLPF1 specific-phage mimotopes:

From the 15 randomly picked phage clones that appeared on the selective agar plate spread with mAbLPF1 bound-phages, four mimotope types (KPHSIPLLDLTR, KPPQMPLYNLSA, NVYPTPESSLSL and NVTLHASMLNMD) were found in two phage clones each (**Table 6**), while the other mimotope types were found in each one of the phages (data not shown). The consensus peptide of the 4 mimotopes was -----PLLNLS-. Consensus mimotope peptide was aligned with LipL32 sequence (accession no. YP_001316.1). It was found that the presumptive epitope of LipL32 matched the consensus mimotope of mAbLPF1 and was located at residues P243, L244, I245, H246, L252, and Q253.

Table 6. Sequences of phage mimotopes that bound to mAbLPF1, consensus mimotope sequences, percent inhibition of the mAb binding to LipL32 by phage mimotopes and presumptive LipL32 epitope.

mAb	Frequency of peptide[1]	Phage mimotope sequence	% Inhibition of the mAb binding to LipL32[2]
LPF1	2	KPHSIPLLDLTR	75.8
	2	KPPQMPLYNLSA	79.1
	2	NVYPTPESSLSL	84.1
	1	NVTLHASMLNMD	79.1

```
Mimotope consensus sequence:    -----PLLNLS-
Epitope on LipL32:    232-LLFPPGIPGVSPLIHSNPEELQKQAIAAEESLKKA-267
                                     PLLN-----LS-
                                     **::     *.
```

[1] Among 15 randomly picked phage clones. [2] Average of % ELISA inhibition from triplicate wells of each phage mimotope type. (*****), identical amino acid; (**:**), conserved amino acid substitution; and (**.**), semi-conserved amino acid. The LipL32 sequence was from accession no YP_001316.1.

Locations and structures of the mAbLPF1 epitope:

The mAbLPF1 specific-epitope including P243, L244, I245, H246, L252 and Q253, was located at the β13 region and C-terminal α-helix (α6) of the LipL32 structures **(Vivian *et al.*, 2009)** (**Fig. 6A**). The α6 helix contained potential internal lysine acetylation (K-Ac) sites, *i.e*. K254, K264, and K265. Further analysis of this helix by using HELIQUEST server revealed that the helix had surface exposed hydrophilic residues (Q253, Q255, I257, S262), electro-positive (K254, K264 and K265) and -negative (E250, E251, E260, E261) patches on one side, and hydrophobic residues (P249, L252, A256, A259, A266) on another side (**Fig. 6B**). The amphipathic helix is important in membrane binding and insertion **(Thiyagarajan *et al.*, 2004)**. mAbLPF1 epitope (red) are shielded by nearby residues **(Fig. 6C).**

Prevalence of the mAbLPF1 epitope:

The epitope peptide sequences of the mAbLPF1 (PLIH-----LQ) was multiply aligned with 70 LipL32 peptide sequences of *Leptospira* spp. belonging to 11 genomospecies: *L. interrogans* (serovars Australis, Autumnalis, Balico, Canicola, Copenhageni, Grippotyphosa, Hardjo, Hebdomadis, Icterohaemorrhagiae, Jalna, Javanica, Kremastos, Lai, Manilae, Mini, Paidjan, Pomona, Pyrogenes, and Wolffi), *L. noguchii* (serovar Pomona)*, L. kirschneri* (serovar Bim)*, L. borgpetersenii* (serovars Ballum, Hardjo-bovis, Javanica, Mini, and Tarassovi), *L. weilii* (serovar Manhao II), *L. santarosai* (serovar Shermani), *L. alexanderi* (serovar Manhao III)*, L. licerasiae* (serovar Varillal)*, L. inadai* (serovar Lyme), *L. broomii*, and *L. kmetyi* (serovar Malaysia), deposited in the NCBI database (accessed Aug 2012). It was found that epitope peptide of mAbLPF1 was highly conserved **(Table 7**). There are four variants of the mAbLPF1 epitope (variants 1-4) among the 70 LipL32 NCBI database sequences (**Table 7**). Variant-1 (PLIH-----LQ) is predominant (62/70) among the pathogenic *Leptospira* spp., variant-2 (PAVH-----LQ) was found in one strain of pathogenic *L. interrogans*, variant-3 (PIVH-----LQ) was found in two species of intermediately pathogenic *Leptospira* spp. (*L. broomii* and *L. inadai*), and variant-4 (PILH-----LE) was found in two strains of the intermediately pathogenic *L. licerasiae*.

Multiple alignments were also performed between the epitope sequences and orthologous proteins of the LipL32 superfamily: three outer membrane proteins (OMPs) of bacteria of the genus *Pseudoalteromonas*: *P. tunicate*-D2 **(Holmstrom *et al*., 1998)**, *P. rubra* (ATCC29570) and *P. spongiae* (UST010723-006), two proteins of the genus *Cellulophaga*: *C. lytica* (DSM7489) and *C. algicola* (DSM14237) (Bowman 2000), LipL32 proteins from *Moritella* sp. (PE36), *Marinomonas mediterranea* (MMB-1), unidentified *Eubacterium* (SCB49), and the outer membrane protein (OMP) of *Treponema brennaborense* (DSM12168). The LipL32 epitope sequence was not found in any of the orthologous proteins of the LipL32 superfamily derived from other microorganisms (**Fig. 7**).

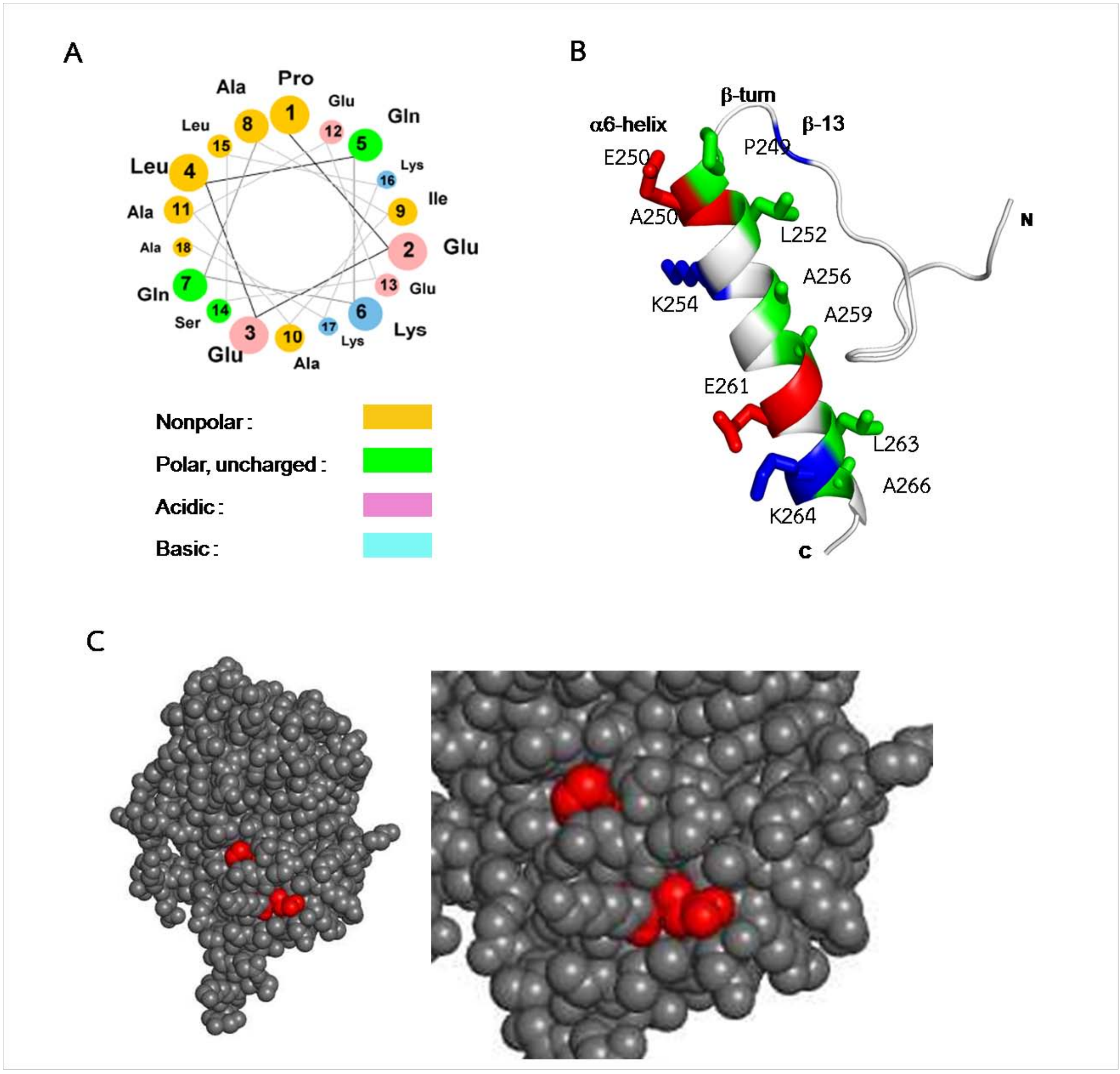
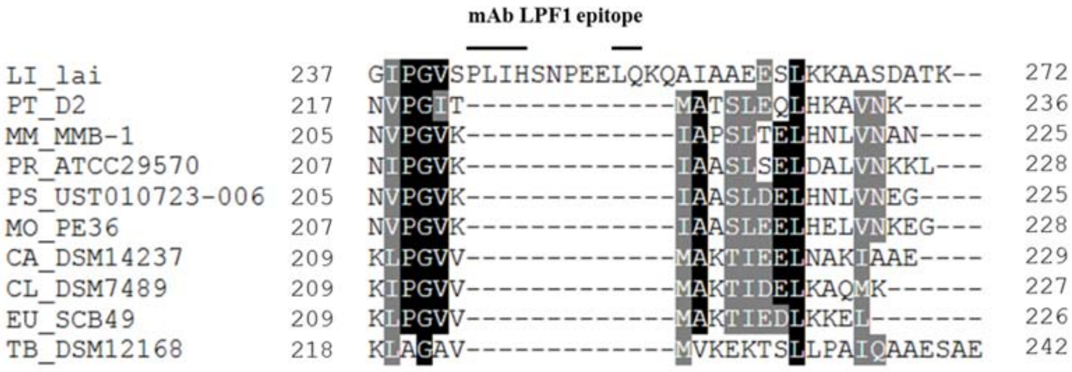


Fig. 6. Structure and location mAbLPF1 epitope on LipL32 structure.

(A) A helical wheel projection of C-terminal α6 (249-PEELQKQAIAAEESLKKAA-267) of LipL32. The helix revealed a cluster of hydrophobic patches P249, L252, A256, A259, L263, A266) patch on one side and the surface exposed hydrophilic and charged residues (K265K254E261E250I257K264Q253E260), on another side suggests an amphipathic configuration.

(B) Close-up of carboxy-terminal loop formed by β13-turn and α6 helix of LipL32 structure. Side chains of hydrophobic patch (green), acidic (red) and basic (blue) residues are shaded.

(C) Illustration of mAbLPF1 epitope (red) on LipL32 molecule (grey)



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LI_lai            237  GIPGVSPLIHSNPEELQKQAIAAEESLKKAASDATK--  272
PT_D2             217  NVPGIT-------------MATSLEQLHKAVNK-----  236
MM_MMB-1          205  NVPGVK-------------IAPSLTELHNLVNAN----  225
PR_ATCC29570      207  NIPGVK-------------IAASLSELDALVNKKL---  228
PS_UST010723-006  205  NVPGVK-------------IAASLDELHNLVNEG----  225
MO_PE36           207  NVPGVK-------------IAASLEELHELVNKEG---  228
CA_DSM14237       209  KLPGVV-------------MAKTIEELNAKIAAE----  229
CL_DSM7489        209  KIPGVV-------------MAKTIDELKAQMK------  227
EU_SCB49          209  KLPGVV-------------MAKTIEDLKKEL-------  226
TB_DSM12168       218  KLAGAV-------------MVKEKTSLLPAIQAAESAE  242
```

Fig. 7. Alignment of the mAbs peptide epitopes among orthologous proteins of the LipL32 superfamily.

LipL32 peptide epitope of the LPF1 was aligned through ClustalW2.1 server with orthologous LipL32 sequences including LI, *L. interrogans* Lai; PT, *Pseudoalteromonas tunicate* D2 (Holmstrom *et al*., 1998); MM; *Marinomonas mediterranea* MMB-1; PR, *Pseodoalteromonas rubra* ATCC29570; PS, *Pseudoalteromonas spongiae* UST010723-006; MO, *Moritella* sp. PE36; CA, *Cellulophaga algicola* DSM14237 (Bowman, 2000); CL, *Cellulophaga lytica* DSM7489; EU; *Eubacterium* sp. SCB49; T, *Treponema brennaborense* DSM12168. The mAbLPF1 epitope peptide was not found among orthologous LipL32 proteins. Black shades, identical amino acids; grey shade, conserve/semi-conserve amino acids; dash lines, deleted amino acids.

Table 7. Variants of LipL32 epitope at the location that interacted with the mAbLPF1 among different pathogenic and intermediately pathogenic *Leptospira* spp.

Epitope variant	Amino acids at the mAbLPF1 epitope (underlined)	Frequency[1]	Accession no.	*Leptospira* spp.	Clade[2]
1	236 PGIPGVSPLIHSNPEELQ 253	62	62 different accession no.	Pathogenic species	P
2	236 –----CG-AV-------- 253	1	ADC80912.1	*L. interrogans*	P
3	236 --T--L--IV----V--E 253	2	ZP_10531657.1	*L. broomii*	I
			ZP_10529670.1	*L. inadai*	I
4	236 –-V--L--IL-A--A--- 253	2	ZP_09259456.1	*L. licerasiae*	I
			EID99865.1	*L. licerasiae*	I
	`*::*     *:`				

[1] from 70 LipL32 sequences of the database (accessed on Aug 2012). There were three LipL32 sequences that had C terminal deletions (Δ243-272, Δ248-272 and Δ252-272). [2] clade **P**, pathogenic; clade **I**, intermediately pathogenic (based on 16S rRNA genetic relatedness). Dash indicates amino acid identity. (*****), identical amino acid; (**:**), conserved amino acid; and (**.**), semi-conserved amino acid.

Analysis of antigenic determinants involved in mAbLPF1 paratope-LipL32 epitope interaction

The antigenic determinants of mAbLPF1 epitope peptide and, heavy (VH) and light (VL) variable regions of MAbLPF1 were studied. Common amino acids in LPF1 antigen binding sites (CDRs) were analyzed. In LPF1 variable domain sequence of contains negatively charged and aromatic residues dominated distributed of 6 CDR loops (8 and 11 residues, respectively) which identified as common residues in paratope (MacCallum *et al*., 1996). In both paratope and epitope, the negatively charged residues dominated **(Fig. 8)**.

Several of the identified residues involved in antigen-antibody complex are located in central of H3 and L3 loops, and 2 positively charged in H3 (R105, R106). Potential residue in CDRs interact to mAbLPF1 epitope (C-terminal LipL32) was consider by 3 criterias, surface exposed in CDRs, central of CDRs regions and most involved in Ag-Ab complex identified by McCullum, 1996. In heavy chain, in light chain displayed two negatively charged, D51 and E91 in L2 and L3, respectively, and only positively charge R30 in L1. A total 11 aromatic residues display on the surface of mAbLPF1 include tyrosine, tryptophan and threonine. The CDRs residues are colored as follows: CDR1-(27-GYIF----TSYD-38) in red, CDR2-(56-IFPG—EGST-65) in yellow, CDR3-(105-ARGDYYRRYFDW-117) in purple.

Effects of ionic strength and charged amino acids on mAbLPF1 binding to immobilized LipL32 are shown in **Fig. 9**. The optical density of mAbLPF1 to immobilized LipL32-FL in the presence (0-500 mM) of NaCl, and DL-Lysine (K^+) monohydrochloride was not significantly different whereas ELISA signal was increased in the presence of aspartate (D^-) amino acid suggesting positively charged amino acids in variable domains involved in LipL32-mAbLPF1 interaction.

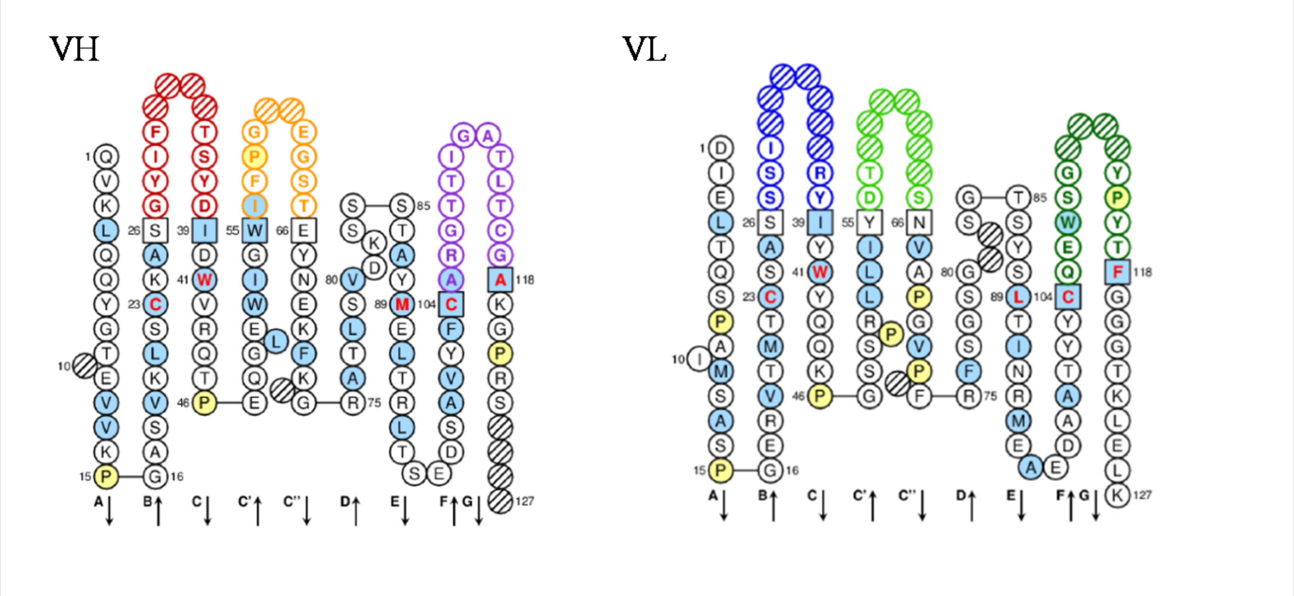

Fig 8. Graphic representation of mAbLPF1 variable (VH) and (VL) domain sequences.

IMGT Collier de Perles illustrating of 2D-represntation of 114 amino acids of variable domain of mAbLPF1. Immunoglobulin sequence was numbering according to IMGT numbering (Lefranc *et al*., 2009). Frameworks (FRs) and complementarity determining regions (CDRs) residues were identified by IMGT/V-QUEST. Five conserved residues of a V domain: 1st-CYS 23, CONSERVED-TRP 41, hydrophobic 89, 2nd-CYS 104, and J-TRP118 are shown in red and bold letters. Arrows indicate the β strands (from A to G) and their direction.

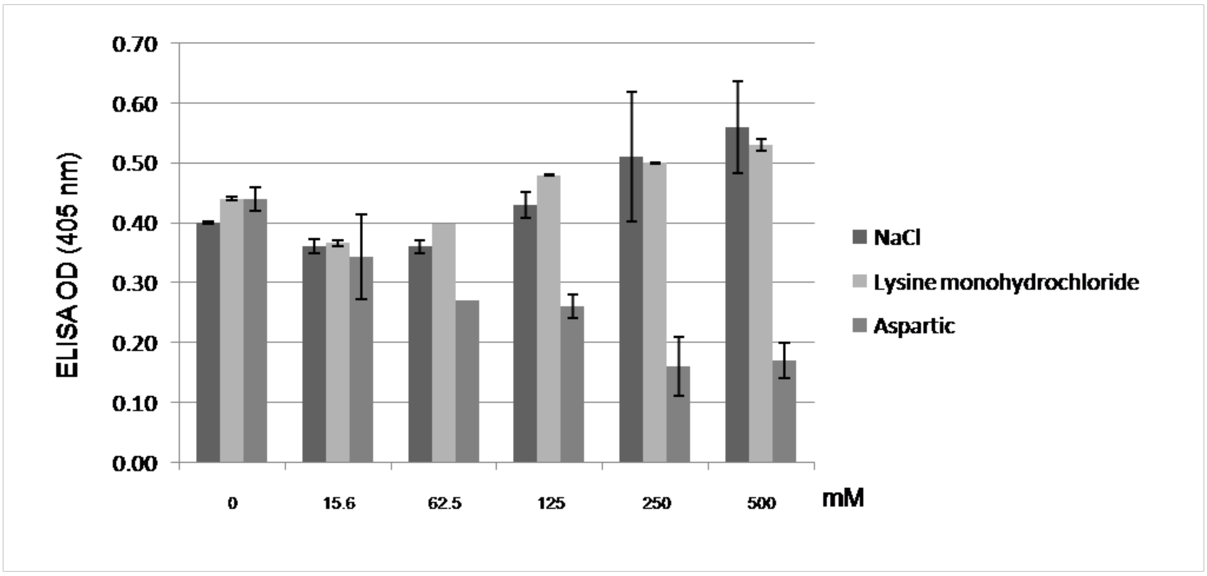

Fig 9. Effects of ionic strength and charged amino acids on MAbLPF1 binding to LipL32.

Indirect ELISA assay of mAbs LPF1 to immobilized LipL32-FL was performed in increasing concentrations of NaCl, DL-Lysine (K^+) monohydrochloride and Aspartate (D^-) amino acid from 0-500 mM in the assay buffer (0.15 M PBS). Bound immunoglobulin was measured by the optical density at 405 nm. Bar represent the mean absorbance values ± S.D. of three replicates, statistically significant differences are shown in * ($P < 0.05$).

Hemolytic activity mediated by *Leptospira* hemolysins and LipL32 proteins

Leptospira lysates (sv. Copenhageni, Andamana) were tested for hemolytic activity to human erythrocyte *in vitro*. At 2 h incubation, *Leptospira* lysates and all recombinant LipL32 proteins exhibited hemolytic activity and increased hemolysis titers at 12 h incubation (**Table 8)**. Its hemolytic activity was abolished after heated, treated with Mg^{2+}, and EDTA at 12 h incubation.

Hemolytic activity of recombinant LipL32 proteins to human erythrocytes was examined *in vitro* at 24 h of incubation. Hemolytic activity mediated by full-length LipL32 and LipL32Δ1-90 proteins to human erythrocyte was investigated at 20% and 50%, respectively while the activity was not observed in LipL32Δ166-245 (**Table 9)**. The minimum peptide essential for hemolytic activity was found to be amino acids 166-245 of LipL32.

Table 8. Hemolytic activity of *Leptospira* lysates and recombinant LipL32 proteins.

Sample	Treatment[2]	Hemolytic unit[3]		Percent hemolysis[4]	
		2 h	**12 h**	**12 h**	**24 h**
Copenhageni[1]	Non-treated	4	16	100	100
Andamana[1]	Non-treated	2	8	Nd	Nd
Copenhageni	Heat	Nd	Nd	10	10
Copenhageni	Ca^{2+}	Nd	Nd	50	100
Copenhageni	Mg^{2+}	Nd	Nd	100	100
Copenhageni	EDTA	Nd	Nd	100	100
LipL32	Non-treated	undiluted	8	Nd	20
LipL32Δ1-90	Non-treated	2	16	Nd	50
LipL32Δ1-170	Non-treated	<2	8	Nd	0

[1] Lysates of pathogenic strain, *L. interrogans* sv. Copenhageni and saprophytic *L. biflexa* sv. Andamana

[2] Heat, 100°C, 5 min; divalent cations (Ca^{2+}, Mg^{2+}) and Ca^{2+} chelator (EDTA) 1 mM

[3] Hemolysis unit (at 2, and 12 h) was determined as the highest dilution that caused 100% hemolysis.

[4] Percent hemolysis (at 12, and 24 h) was calculated using the formula: % hemolysis = $[(OD_{Test} - OD_{NC})/OD_{PC}] \times 100$, whereas NC and PC are the negative and positive hemolysis controls.

RBC, group O washed human erythrocyte

Nd; not determined

Table 9. Hemolytic activity of recombinant LipL32 proteins.

LipL32 proteins		**Hemolysis (%)**[2]
LipL32	1=============================272	20
LipL32Δ1-90	91==================272	50
LipL32Δ1-170	171=======272	0
LipL32Δ166-245[1]	1=================165=246======272	0

[1] LipL32Δ166-245 was constructed by deleting at residue 166 to 245 of 272 residues of LipL32

[2] Percent hemolysis was calculated using the formula: % hemolysis = $[(OD_{Test} - OD_{NC})/OD_{PC}] \times 100$, whereas NC and PC are the negative and positive hemolysis controls.

RBC, group O washed human erythrocyte

Discussion

Penicillin is a primary drug of choice for the treatment of leptospirosis. Occasionally, Jarisch-Herxheimer reaction (JHR) occurs in antibiotic-treated cases due to the massive amounts of toxic materials released from pathogen (Watt and Warrell, 1995; Pound and May, 2005). In a previous study, two mouse mAbs specific to LipL32 of pathogenic *Leptospira*, their respective single chain antibody fragments (ScFv), and their humanized-ScFv were produced. When passively given to hamsters experimentally infected with heterologous *Leptospira* spp., the antibodies were able to rescue the animals from lethal infection. They also inhibited pathogenic *Leptospira*-mediated human erythrocyte lysis *in vitro* (Maneewatch *et al*., 2008; 2009). Thus, the antibodies, particularly the humanized-ScFv, have a high potential for further development as a safe adjunctive, if not sole, remedy for use in the treatment of leptospirosis, especially in antibiotic-allergic subjects. In this study, we sought to reveal the locations, structural characteristics, and bio-functions, of peptide epitopes of both mAbs, which elucidated the molecular mechanisms of the mAb in mediating *Leptospira's* neutralizing effects.

Recombinant full-length LipL32 (272 amino acids) was produced and purified. The protein was found to have an inherent molecular matrix adhesive property of the *Leptospira* native counterpart, *i.e.*, it bound readily to the previously reported host components, including plasma fibronectin, matrigel ECM and heparin sulfate (Hauk *et al*., 2008; Hoke *et al*., 2008). This study also noted the binding of recombinant protein to cholesterol and phosphatidylcholine, which are the predominant membrane lipid components of mammalian cells, and to intact MDCK cells. The most important prerequisite step in the pathogenesis of many microorganisms is of the ability to bind to the host component/cell/tissue, and *Leptospira* spp. is no exception (Patti *et al*., 1994; Cinco, 2010).

To analyze the peptides of the mAbLPF1 epitope, conventional protein-protein interaction by Western blotting and phage mimotope from the random 12-mer peptide phage display library were conducted (Perera *et al*., 2008; Thanongsaksrikul *et al*., 2010; Pissawong *et al*., 2013; Yodsheewan *et al*., 2013). Two recombinant N-terminally Δ90- and Δ170-LipL32 proteins were produced for determining the LipL32 portions bound by mAbLPF1. The results of Western blot analysis suggested that mAbLPF1 bound to peptide located between amino acids 171-272, which has been shown to be immunogenic and important for LipL32 binding to host components (Hauk *et al*., 2008; Hoke *et al*., 2008).

Mapping epitope peptide, by searching for phage mimotope peptide consensus sequence and multiple alignments with the LipL32 sequence, not only confirmed the location of the mAb epitope guided by the Western blot result, but also revealed the amino-acid residues and features of the epitopes. The epitope sequence of mAbLPF1 (PLIH-----LQ) was likely conformational, consisting of many hydrophobic residues (243PLIN/H246) in the β13-turn or P249, L256A, and I263L of the α6 hydrophobic patch (Hauk *et al*., 2009; Vivian *et al*., 2009). A previous study showed that the amphipathic α helix is involved in membrane binding and membrane insertion (Thiyagarajan *et al*., 2004), and LipL32 protein has been found to have hemolytic activity (Lee *et al*., 2000; Hauk *et al*., 2005). Thus, this epitope may have a functional role in the hydrophobic interaction and/or the hemolytic activity of LipL32. It has also been previously shown that mAbLPF1 inhibited *Leptospira*-mediated hemolysis and rescued *Leptospira* infected hamsters from death. It is therefore plausible to conclude that mAbLPF1 exerted the neutralizing/therapeutic activity by interfering with the pathogenic activities of the LipL32 epitope.

There were four mAbLPF1 epitope variants among the pathogenic and intermediately pathogenic clades of *Leptospira* spp. Most of the LipL32 sequences (62/70) of the database carried the variant-1 epitope. Few strains, however, had amino acids L244A and I245V of the variant-2, and L244I and I245V of the variant-3. Nevertheless, these amino acid substitutions are conserved non-polar residues, which retain the hydrophobic activity of the amino acids 244LIH246 of variant-1. The overall findings indicate that the mAbLPF1 epitope is highly conserved among the pathogenic and intermediately pathogenic species of *Leptospira* spp.

LipL32 [Hemolysis-associated protein-1 (Hap-1)] mediated hemolytic activity on several erythrocytes *in vitro* has been demonstrated. LipL32 and SphH showed additively on hemolytic activity on human erythrocyte membrane. **(Hauk *et al.*, 2005; Lee *et al.*, 2000)**. In the present study, hemolytic activity to human erythrocyte that mediated by LipL32 peptides was investigated *in vitro*. Hemolytic activity was abolished in LipL32Δ1-170 and in LipL32Δ166-245. The minimum peptide essential for hemolytic activity was found to be amino acids 166-245 of LipL32.

Conclusion

The mAbLPF1 epitope consisted of P243, L244, I245, H246, L252, and Q253 on the LipL32 protein; it is mapped on the surface exposed region of non-continuous β13-turn and C-terminal amphipathic α6 helix of LipL32 structure. Epitope of mAbLPF1 is highly conserved among the pathogenic and intermediately pathogenic *Leptospira* spp., and are absent from proteins of the LipL32 superfamily of other microorganisms. Hemolytic activity of recombinant LipL32 proteins to human erythrocytes was examined *in vitro*. Hemolytic activity mediated by full-length LipL32 and LipL32Δ1-90 proteins to human erythrocyte was investigated while the activity was significantly decreased in LipL32Δ1-170 and abolished in LipL32Δ166-245. The minimum peptide essential for hemolytic activity was found to be amino acids 166-245 of LipL32. The experiments demonstrate not only protective epitope of LipL32 but also LipL32 peptides that mediate hemolysis during *Leptospira* infection.

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