การพัฒนาดีเอ็นเอวัคซีนของยีน *lipL32* สำหรับโรคเลปโตสไปโรซิสโดยใช้อนุภาคนาโนไคโทซานดัดแปร

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DEVELOPMENT OF *LIPL32* DNA VACCINE FOR LEPTOSPIROSIS USING MODIFIED CHITOSAN NANOPARTICLES

Miss Ruthairat Kerdkaew

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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โรคเลปโตสไปโรซิสเป็นโรคติดเชื้อจากสัตว์สู่คนซึ่งพบได้ทั่วโลกและพบว่าเป็นปัญหาสาธารณสุขที่สำคัญของประเทศไทย ในการศึกษานี้จึงได้มีการพัฒนาดีเอ็นเอวัคซีนโดยใช้อนุภาคนาโนไคโตซานดัดแปรเชื่อมต่อกับดีเอ็นเอวัคซีนของยีน *lipL32* ซึ่งเป็นพลาสมิดที่บรรจุยีน *lipL32* สมบูรณ์เต็มความยาว และทดสอบคุณสมบัติและประสิทธิภาพของวัคซีนทั้งในหลอดทดลองและในสัตว์ทดลอง ดีเอ็นเอวัคซีนของยีน *lipL32* สามารถทรานสเฟคเข้าสู่เซลล์ไลน์ HEK293T ได้ โดยสามารถตรวจพบการแสดงออกของโปรตีน LipL32 ทั้งในเซลล์และที่หลั่งออกมานอกเซลล์ โดยอนุภาคนาโนไคโตซานถูกดัดแปรให้เชื่อมต่อกับแมนโนสเพื่อให้สามารถจับอย่างจำเพาะกับเซลล์ที่มีการแสดงออกของตัวรับแมนโนสบนผิวเซลล์ เช่น เซลล์มาโครฟาจและเซลล์เดนไดรติกซึ่งเป็นเซลล์ที่นำเสนอแอนติเจนที่สำคัญ การศึกษานี้พบว่าอนุภาคนาโนไคโตซานและนาโนไคโตซานดัดแปรสามารถบรรจุพลาสมิดดีเอ็นเอวัคซีนของยีน *lipL32* ได้อย่างสมบูรณ์ เมื่อนำไปทำการทรานสเฟคชันเข้าสู่เซลล์ไลน์ของมาโครฟาจ RAW 264.7 พบว่าไม่เป็นพิษต่อเซลล์ และพบการแสดงออกของโปรตีน LipL32 ในเซลล์ได้ เมื่อทำการทดสอบประสิทธิภาพของ CS-pVITRO-*lipL32* และ MC-pVITRO-*lipL32* ในการกระตุ้นระบบภูมิคุ้มกันในสัตว์ทดลอง โดยฉีดดีเอ็นเอวัคซีนให้แก่หนูชนิด BALB/c พบว่าในสัปดาห์ที่ 8 กลุ่มที่ได้รับวัคซีน MC-pVITRO-*lipL32* มีการเพิ่มขึ้นของระดับแอนติบอดีรวมชนิด IgG ที่จำเพาะต่อโปรตีน LipL32 สูงกว่ากลุ่มที่ได้รับวัคซีน CS-pVITRO-*lipL32* และ pVITRO-*lipL32* อย่างมีนัยสำคัญทางสถิติ ($p<0.05$) นอกจากนี้ หนูที่ได้รับวัคซีน MC-pVITRO-*lipL32* มีการตอบสนองทางภูมิคุ้มกันแบบเซลล์มีแนวโน้มไปในทาง T-helper 1 แต่อยู่ในระดับที่ต่ำกว่ากลุ่มที่ได้รับวัคซีน CS-pVITRO-*lipL32* จากผลการศึกษานี้ แสดงให้เห็นว่าอนุภาคนาโนไคโตซานและอนุภาคนาโนไคโตซานดัดแปรสามารถเชื่อมต่อกับดีเอ็นเอวัคซีนของยีน *lipL32* และมีประสิทธิภาพในการนำส่งดีเอ็นเอวัคซีนของยีน *lipL32* อย่างจำเพาะและสามารถกระตุ้นระบบภูมิค้มกันได้ทั้งแบบสารน้ำและแบบเซลล์ซึ่งมีแนวโน้มไปในทาง T-helper 1

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RUTHAIRAT KERDKAEW : DEVELOPMENT OF *LIPL32* DNA VACCINE FOR LEPTOSPIROSIS USING MODIFIED CHITOSAN NANOPARTICLES. ADVISOR : ASST. PROF. KANITHA PATARAKUL, M.D., Ph.D., CO-ADVISOR : ASSOC PROF. TANAPAT PALAGA, Ph.D.,129 pp.

Leptospirosis is a global zoonotic disease and public health problem in Thailand. In this study, we have developed a DNA vaccine by using mannosylated chitosan nanoplaticle-encapsulating plasmid carrying *lipL32*. The efficiency of the system was evaluated *in vitro* and *in vivo*. After transfection of plasmid containing full-length *lipL32* gene (pVITRO-*lipL32*) into HEK293T cells, LipL32 was expressed in both the cell lysate and culture supernatant. The chitosan nanoparticles were modified by mannosylation to specifically target antigen presenting cells expressing mannose receptors, such as macrophages and dendritic cells. The chitosan and mannosylated chitosan nanoparticles were able to completely encapsulate the *lipL32* DNA vaccine (CS-pVITRO-*lipL32* and MC-pVITRO-*lipL32,* respectively). When RAW 264.7 cells were transfected with CS-pVITRO-*lipL32* or MC-pVITRO-*lipL32*, no cytotoxicity was observed, and expression of LipL32 was detected. To evaluate immunogenicity, BALB/c mice were immunized with CS-pVITRO-*lipL32* and MC-pVITRO-*lipL32*. Total IgG level of mice immunized with MC-pVITRO-*lipL32* was significantly higher than that of CS-pVITRO-*lipL32* and naked plasmid (pVITRO-*lipL32*) at 8 week of immunization ($p<0.05$). In addition, mice immunized with MC-pVITRO-*lipL32* vaccine tended to develop toward Th1 response but at the lower extent than that of chitosan-conjugated vaccine. Our results suggest that CS and MC nanoparticles are efficient delivery systems for *lipL32* DNA vaccine to specifically induce humoral and Th1-predilected cell-mediated immune responses.

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LIST OF ABBREVIATIONS

APS	Ammonium peroxodisulfate
Bp	Base pair
BSA	Bovine serum albumin
ºC	Degree celsius
$C_3H_3NaO_3$	Sodium pyruvate
$C_8H_7N_3O_2$	3-Aminophthalhydrazide, 5-amino-2,3-dihydro-1,4-phthalazinedione
$C_{24}H_{39}O_4Na$	Sodium deoxycholate
$CaCl_2$	Calcium chloride
CH_3COONa	Sodium acetate
$CuSO_4$	Copper sulfate
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
dNTPs	Deoxynucleotide triphosphates
DTT	Dithiothreitol
EDTA	Ethylenediamine tetraacetate acid
ELISA	Indirect enzyme-linked immunosorbent assay
EMJH	Johnson and Harris modification of the Ellinghausen and McCullough medium
et al.	et alii
FBS	Fetal bovine serum
$FeSO_4$	Ferrous sulfate
g	Gram
h	Hour
H_2O_2	Hydrogen peroxide
H_2SO_4	Sulfuric acid

HCl	Hydrochloric acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
$HOC_6H_4CH=CHCO_2H$	Trans-4-hydroxycinnamic acid
HRP	Horseradish peroxidase
IgG	Immunoglobulin G
IFA	Immunofluorecence test
IPTG	Isopropyl-D-thiogalactopyranoside
KCl	Potassium chloride
kDa	Kilodalton
KH_2 PO_4	Potassium dihydrogen phosphate
LB	Luria-bertani
LipL32	Leptospira lipoprotein 32 kDa
LPS	Lipopolysaccharide
MAT	Microscopic agglutination test
2ME	2-mercapto-ethanol
$MgCl_2$	Magnesium chloride
ml	Milliliter
mM	Millimolar
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MW	Molecular weight
NaCl	Sodium chloride
Na_2CO_3	Disodium carbonate
$NaHCO_3$	Sodium carbonate
Na_2HPO_4	Disodium hydrogen phosphate
NaN3	Sodium azide
NaOH	Sodium hydroxide
Na_2SO_4	Sodium sulphate
ng	Nanogram

NH_2CH_2COOH	Glycine
NP-40	Tergitol-type NP-40
OMP	Outer membrane protein
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PMA	Phorbol 12-myristate 13-acetate
Raw264.7	a mouse monocyte/macrophage cell line
SDS	Sodium lauryl sarcosine
SDS-PAGE	SDS- Polyacrylamide Gel Electrophoresis
TAE	Tris-acetate buffer
TBS	Triethanolamine-buffer-saline
TEMED	N,N,N,N,-Tetramethylethylenediamine
Tween 20	Polysorbate 20
Tween 80	Polysorbate 80
µg	Microgram
µl	Microliter
Vit B12	Vitamin B12
$ZnSO_4$	Zinc Sulfate

CHAPTER I

INTRODUCTION

Leptospirosis is a zoonotic disease that becomes an important human and veterinary health problem around the world [1, 2]. It is caused by pathogenic *Leptospira* species. The spiral shaped bacteria belong to the family Leptospiraceae, order Spirochaetales, genus *Leptospira*. The genus *Leptospira* has been separated into two major species, *L. interrogans* which includes all pathogenic strains and *L. biflexa* which are saprophytic strains isolated from environment [1]. Both *L. interrogans* and *L. biflexa* have been serologically classified into more than 24 serogroups and 250 serovars [2] based on agglutinating leptospiral lipopolysaccharides (LPS) [3]. It is about 0.1 μm in diameter and 6–20 μm in length. *Leptospira* are obligate aerobe. The cell walls of leptospires share characteristics of both Gram-positive and Gram-negative bacteria [4]. They are not stained with Gram staining and should be observed under dark-field microscopy. Leptospires are catalase and oxidase positive. They are slow-growing bacteria. Cultures should be checked for the presence of bacteria after 3–4 days and subcultured after 7–21 days [5]. Optimal growth temperature is between 28°C and 30°C. The minimal growth temperature for pathogenic and saprophytic species is in the range of 13°C-15°C and 5°C-10°C, respectively. The growth ability at 13°C of pathogenic species can be used to differentiate pathogenic from saprophytic species [6]. Leptospires are cultivated in media containing 10% rabbit serum [7] or 1% bovine serum albumin supplemented with long-chain fatty acids at pH 6.8–7.4 such as Ellinghausen-McCullough-Johnson-Harris (EMJH) [8].

Several animal species including pets and livestock such as dogs, bats, cattle, pigs can be reservoir animals, which do not show symptoms but excrete leptospires into urine. Pathogenic leptospires can grow outside the host in soil and water and create a biofilm, helping them to survive in environmental habitats for weeks or months [9]. Human is an accidental host. Leptospirosis is mainly transmitted through skin wounds or mucosa by contact with water or soil contaminated with urine of infected

animals. *Leptospira* can spread into the bloodstream and target organs such as kidneys, liver, heart, causing inflammation of various organs. The disease can cause severe conditions such as pulmonary haemorrhage syndrome, myocarditis, or meningitis with the mortality rate of up to 50% [10-12].

In Thailand, data from disease notification reports show an increase in the incidence rate. In 1996, 398 cases were reported and the number of cases increased to 14,285 cases and 2,868 cases in year 2000 and 2005, respectively [13, 14]. The major endemic area (up to 90% of reported cases) was in the Northeastern region [15]. The dominant serovars reported in Thailand are Autumnalis, Bratislava, Bataviae, Javanica, Hebdomadis, Grippotyphosa, Bangkok, and Pyrogenes [14, 16]. Paddy farmers, rat hunters, and canal dredgers were found to be high risk groups for leptospirosis in Thailand [15]. The prevalence of leptospirosis in the Thai-Myanmar border area was found to be 17% of patients presenting with fever [17]. The main outbreak season of leptospirosis in Thailand corresponds with the rainy season during July to October [15].

Humoral-mediated immunity has been shown to be utmost importance to confer resistance to leptospirosis [18-20]. Antibodies produced during leptospiral infection were agglutinating antibodies [21]. IgG antibodies against *Leptospira* was reported to be detected in patients up to 6 years after initial infection [22]. A monoclonal antibody directed against leptospiral LPS was found to transfer passive protection to newborn guinea pigs [21]. The anti-LPS antibody-mediated immunity was serovar specific [2]. However, cattle infected with *Leptospira borgpetersenii* serovar Hardjo showed high titers of anti-LPS antibody but did not result in protection [23]. Peripheral blood mononuclear cells (PBMC) from cattle immunized with whole-cell killed vaccine of *L. interrogans* serovar Hardjo were found to proliferate after stimulation with immunized antigens *in vitro* [24]. In addition, CD4+ and TCR$\gamma\delta$+ T cells derived from these vaccinated cattle produced gamma interferon (IFN-γ) suggesting a strong Th1 response [23-27] and possible role of cell-mediated immunity in protection against leptospirosis.

Commercially available vaccines for leptospirosis are heat-killed whole-cell vaccines that confer protection only against homologous serovars [28]. The

disadvantages of the vaccine are adverse side effects such as pain, nausea, fever; short-term immunity; and serovar-specific protection. Current trend in development of vaccine for leptospirosis have focused on subunit vaccine including recombinant protein and DNA vaccine using leptospiral components, such as lipoproteins [48-56] and outer-membrane proteins (OMPs) as vaccine candidates [29]. The ultimate goal for vaccine development is to identify a candidate gene or protein that protects against multiple pathogenic *Leptospira* species, induce long-term protective immunity, and cause no side-effects.

LipL32 is the outer membrane protein found in pathogenic *Leptospira*, accounted for approximately 75% of total OMPs [30-32]. The protein is expressed *in vitro* and during infection [31]. More than 95 % of patients with leptospirosis were shown to have antibody against LipL32 [33]. LipL32 has been used as a vaccine candidate for both recombinant protein and DNA vaccines. For example, immunization with LipL32 and LipL41 have been reported to stimulate partial protection against pathogenic leptospires in animal models [34]. Animals immunized with DNA vaccine construct containing gene *lipL32* in adenovirus vector showed a survival rate of 87% after challenge. However, using virus as a carrier may not be safe for humans [35, 36]. A study on pcDNA 3.1-*lipL32* used as a DNA vaccine demonstrated that it was able to partially prevent cross-serovar infection [36].

Several delivery systems have been utilized to improve efficacy of DNA vaccine. Chitosan is a natural and biodegradable polymer which is a deacetylated form of chitin under alkaline condition. It is found in the shells of crustaceans, such as lobsters, crabs, and shrimp; and many other organisms, including insects and fungi [37]. Chitosan has been used in various applications including medical and pharmaceutical substances, and agriculture. Chitosan is positively charged and can bind to molecules that are negatively charged, such as nucleic acids, mucosal surface, or plasma membrane [37,

38]. It is also able to alter tight junctions, thereby enhancing the cellular uptake of peptides and proteins [39, 40]. Chitosan conjugated with DNA can be used as an effective carrier to introduce DNA into cells [41, 42]. Chitosan micro- and nanoparticles has been reported to exhibit immune-stimulating activity, such as activation of macrophage release of nitric oxide (NO) and polymorphonuclear cells [43]. Chitosan can stimulate humoral as well as cell-mediated immune responses after subcutaneous vaccination [44, 45]. Chitosan nanoparticles were shown to promote both Th1 and Th2 response [46]. The modification of chitosan to mannosylated chitosan (MC) has been reported to have low cytotoxicity and enhance transfection efficiency into RAW 264.7 macrophage cell line [47] and mouse peritoneal macrophages [48]. The objective of mannosylated chitosan-based delivery system is to target vehicle binding to a cell-specific receptor found on antigen presenting cells (APCs) such as macrophages and dendritic cells leading to mannose receptor (MR)-mediated endocytosis to potentially enhance both innate and adaptive immune responses [49-51]. The mannosylation has been reported to enhance MHC class I- and MHC class II-restricted Ag presentation and T cell stimulation by up to 200-fold compared with non-mannosylated proteins [51-53].

Using chitosan nanoparticles for DNA vaccine delivery has never been reported in vaccine for leptospirosis. In this study, we proposed to develop a *lipL32* DNA vaccine using mannosylated chitosan nanoparticles as a delivery system to test its physical properties and to study immune response of mannosylated chitosan-conjugated *lipL32* DNA vaccine in mice.

CHAPTER II
OBJECTIVE

Hypothesis

1. Mannosylated chitosan can encapsulate and deliver *lipL32* DNA vaccine to macrophages.

2. Mannosylated chitosan-conjugated *lipL32* DNA vaccine (MC-pVITRO-*lipL32*) can induce better immune response than chitosan-conjugated (CS-pVITRO-*lipL32*) and naked *lipL32* (pVITRO-*lipL32*) DNA vaccines.

Objective

1. To develop *lipL32* DNA vaccine using mannosylated chitosan as a delivery system and to test its physical properties and transfection efficiency in macrophages.

2. To study immune response of mannosylated chitosan-conjugated *lipL32* DNA vaccine (MC-pVITRO-*lipL32*) in mice.

CHAPTER III

REVIEW OF RELATED LITERATURES

Leptospira and leptospirosis

Taxonomy and classification

Leptospira is a member of the phylum Spirochaetes, class Spirochaetes, order Spirochaetales, family Leptospiraceae and genus *Leptospira* [54]. Leptospires are spirochetes that curled about 6-20 μm in length, 0.1 μm in diameter and the wavelength is approximately 0.5 μm [55]. Leptospires are highly motile, obligate aerobes. The cell wall of leptospires is similar to that of Gram-positive bacteria and Gram-negative bacteria [4]. Either or both end of the spirochetes bends like a hook-shaped (Figure 1). These bacteria are poorly Gram-stained but may be stained with carbon fuchsin solution. The leptospires can be viewed by dark-field or phase-contrast microscopy.

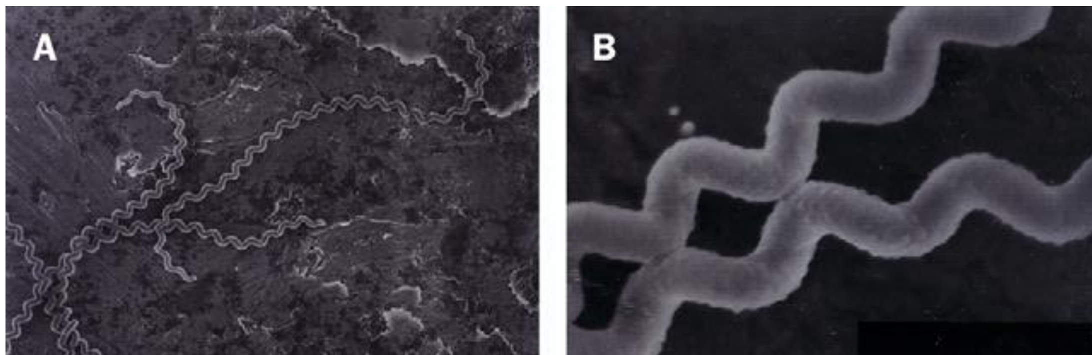


Figure 1. High-resolution scanning electron micrograph of *Leptospira interrogans* serovar Copenhageni. (A) Note characteristic hooked ends (B) At high magnification the surface of the spirochete seems ruffled and beaded [56].

Leptospires have a double membrane structure similar to other spirochetes, in which the cytoplasmic membrane and peptidoglycan layer are closely related and are

overlaid by the outer membrane [57]. The outer membrane of leptospires contains phospholipids, outer membrane proteins (OMPs) and lipopolysaccharide (LPS). LPS of leptospires has elements similar to other Gram-negative bacteria, but is less endotoxic [58]. Leptospires have two periplasmic flagella with polar insertions which are responsible for motility [59, 60]. The flagella of 11.3 nm in diameter are surrounded by two sheath layers. In addition, the core and the two sheath layers are composed of two layers of 34 and 36 kDa proteins that are homologous to flagellar proteins of other spirochetes [61]. Leptospires show two forms of movement in the direction of the straight end, and circular motion [10].

Based on serological classification, genus *Leptospira* can be divided into 2 species: pathogenic *Leptospira interrogans* which cause disease in humans and animals, and saprophytic *Leptospira biflexa* found in the environment [1]. More than 60 serovars of *L. biflexa* and over 250 serovars of *L. interrogans* have been reported [2, 10, 55].

Currently, the genomospecies are classified based on DNA hybridization, multilocus enzyme electrophoresis, and 16S rRNA sequences [62]. Pathogenic *Leptospira* genospecies is so far composed of 21 species based on 16S rRNA sequences [63, 64]. However, the genotypic classification of *Leptospira* does not correspond to serological classification since different serovars belong to the same genospecies, and vice versa [56]. Saprophytic and pathogenic *Leptospira* species whose genomes have been sequenced were shown in Table 1.

Table 1. Saprophytic and pathogenic *Leptospira* species whose genomes have been sequenced [65].

Leptospira species	Serovar	Strain	Typical reservoir [56]	Reference
Saprophytic				
L. biflexa	Patoc	Patoc1 (strains Paris and Ames)	Do not survive in hosts	[66]
Pathogenic				
L. interrogans	Lai	56601	Rat	[67]
L. interrogans	Copenhageni	Fiocruz L1-130	Rat	[68]
L. borgpetersenii	Hardjo	L550 JB197	Cattle and sheep	[69]

Epidemiology

Leptospirosis is the most common widespread zoonosis in the world [70]. It can be transmitted to human via either direct or indirect contact with urine of infected animals. The incidence of leptospirosis depends on environmental factors including rainfall and temperature. Leptospirosis is commonly found in the tropics during the rainy season to winter. In the temperate areas, leptospirosis is usually reported in the warm season. However, in tropical region leptospirosis tends to occur throughout the year, and increases when it rains [71].

Leptospires are able to enter through skin abrasion or mucous membrane. The cycle of *Leptospira* infection is shown in Figure 2. Water-born transmission was reported in several outbreaks of leptospirosis [56, 72]. Transmission directly between humans has been rarely reported due to the acidic pH of human urine which does not allow leptospires to survive after excretion. Infection via inhalation or ingestion of contaminated dust, the mucosal membranes of the respiratory and gastrointestinal tract, and animal bites are rare [10, 73-75].

Animals that can transmit the infection include rodents such as rat, followed by dogs, pigs, and cattle. Animals may be asymptomatic carriers of which leptospires colonize the renal tubules and are released into the urine for a long period of time [19]. Leptospires are able to survive in a warm, humid, and neutral or slightly alkaline pH environment. Humans are accidental hosts. The risk factors for infection include exposure to a variety of sources that may be associated with infection or exposure to animals, such as farmers, veterinarians, and slaughterhouse workers [10].

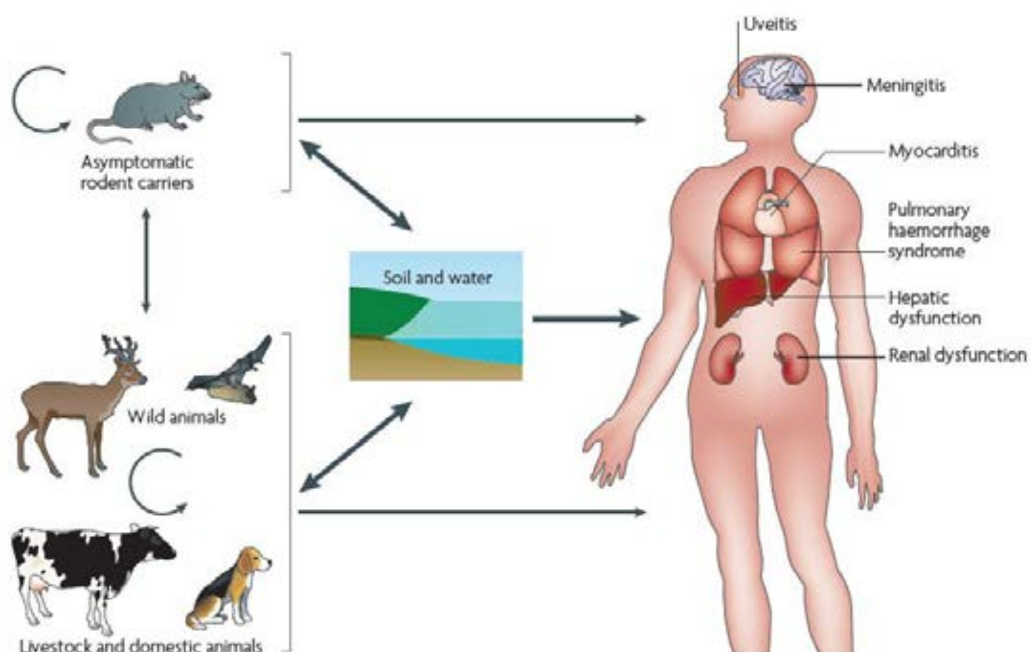


Figure 2. The cycle of *Leptospira* infection. The rodents are asymptomatic carriers or reservoir hosts of *Leptospira*. Leptospires can infect humans, wild animals, domestic and livestock animals by contact with contaminated soil and water [19].

Outer membrane proteins (OMPs) of *Leptospira*

In contrast to saprophytic species, pathogenic *Leptospira* have been shown to attach cultured mammalian cells indicating the association of their attachment ability and virulence [76, 77]. The initial step of infection requires the interaction of host cells and pathogens. Then, pathogens invade the host tissue and colonize in target organs. Therefore, the host-pathogen interaction is crucial for understanding the mechanisms of pathogenesis. OMPs of *Leptospira* are utilized as a part of the cells in direct contact with the environment and the host tissue including the immune system [78, 79]. Therefore, OMPs play an important role in the pathogenesis of leptospirosis.

Leptospiral OMPs have been categorized into three classes [80]; (i) lipoprotein, the most abundant class, such as LipL32, LipL41 and LipL21. (ii) transmembrane protein, such as OmpL1. (iii) peripheral membrane protein such as LipL45 (Figure 3). Several OMPs are up regulated *in vivo*, expressed only in pathogenic strains, and can stimulate protective host immune responses but their functions in pathogenic *Leptospira* are not well understood.

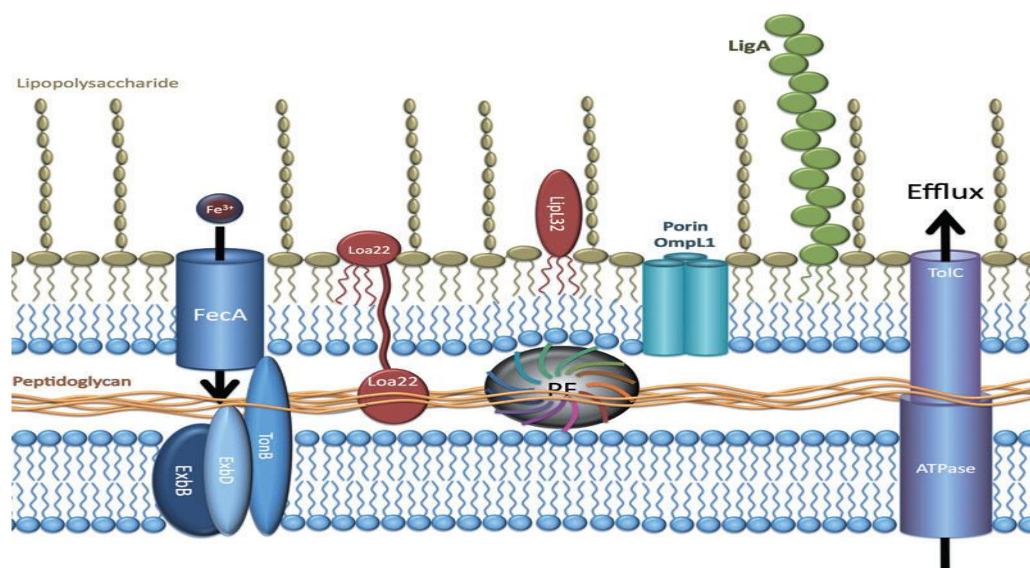


Figure 3. Structure of leptospiral cell wall containing the outer membrane, periplasm, peptidoglycan, and inner membrane [81].

Expression of leptospiral OMPs depends on environmental conditions, such as osmolarity and temperature and can be up or down regulated in the host to render leptospiral survival and pathogenesis. The OMPs of *Leptospira* can induce immune response of the host [82-85]. OMPs on the surface of *L. interrogans* serovar Copenhageni L1-130 strain is shown in Table 2. Proteomic analysis of virulent *L. interrogans* serovar Pomona revealed 86 leptospiral OMPs such as OmpL1, LipL21, LipL31, LipL32/Hap-1, LipL41, LipL 45, LipL46, LruA/LipL71, OmpA-like protein Loa22, and 8 novel hypothetical proteins which were absent in the saprophytic *L. biflexa* [86]. The genetic tool has been employed to reveal the role of putative virulence factors of pathogenic *Leptospira* in pathogenesis. For instance, Loa22 has been defined as a virulence factor using transposon mutagenesis approach since the virulence of *loa22*$^{-}$ mutant was attenuated in hamsters [87]. Hence, genetic tool will provide the essential information to understanding biology of pathogenic *Leptospira* and pathogenesis of leptospirosis.

Table 2. Surface-exposed outer membrane proteins of *Leptospira interrogans* serovar Copenhageni strain L1-130 [Lip, lipoprotein; TM, transmembrane] [81].

Name	Locus	Tag Type	Size (kD)	Copy Number	Knockout Virulent?	Putative Function(s)	References
LipL32	11352	Lip	32	38,050	Yes	Binds host ligands - fibronectin - collagen IV	[88, 89]
Loa22	10191	Lip	22	30,329	NO	Binds Host ligands - fibronectin - fibrinogen	[90, 91]
LipL41	12966	Lip	41	10,531	-	Binds hemin	
LipL21	10011	Lip	21	8,830	-	-	[92]
OmpL36	13166	TM	36	8,021	-	-	[93]
OmpL1	10973	TM	33	5,441	-	Porin	[94, 95]
LipL46	11885	Lip	46	5,276	-	-	[96]
LigB	10464	Lip	200	914	YES	Binds host ligands - fibronectin - fibrinogen - collagen I, IV - elastin	[97, 98]
LigA	10465	Lip	130	553	-	Binds host ligands - fibronectin - fibrinogen - collagen I, IV	[97]

LipL32

Protein LipL32 or haemolysis associated protein 1 (Hap1) is the major outer membrane protein of pathogenic *Leptospira*, accounted for approximately 75% of total OMPs [30, 31]. The size of this protein is approximately 32 kDa [30, 99]. It 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 [33, 100]. LipL32 was demonstrated to express at a high level in leptospires during acute lethal infection [101]. It is highly immunogenic [31, 102]. Immunohistochemical analysis has shown that LipL32 was expressed on the surface of *Leptospira* in the proximal tubule and the interstitium of infected hamster kidneys, suggesting its function in tubular colonization [31]. 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 [103, 104]. In addition, LipL32 has been shown to bind extracellular matrix (ECM) such as laminins, collagens, and fibronectins [89, 105]. The ECM-binding domain of LipL32 was characterized to be in the C-terminal region [106, 107]. LipL32 at amino acid residues 21–272 showed Ca2+ binding activity [108]. 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 [109]. Similarly, potential virulence genes such as *ligB* [110], *ligC, lenB and lenE* were not essential for virulence in animal models [111]. 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. However, vaccine construct of *lipL32* gene and

adenovirus vector showed a survival rate of 87% in mongolian gerbils [35, 36]. In addition, pcDNA 3.1-*lipL32* DNA vaccine showed partial cross-serovar protection [36].

Pathogenesis

The mechanism of leptospirosis remains unclear. Several leptospiral virulence factors that cause disease have been reported. However, the virulence factors remain largely unknown. The initial step of infection is leptospiral adherence to host cells, replication, followed by dissemination to target organs. Leptospires does not cause inflammation at the site of entry [10, 56]. Leptospires can stimulate the innate and adaptive immunity production. Motility of *Leptospira* in the host is probably important in initial infection for invasion and dissemination to target organ such as lung, liver, kidney. The study of whole genome sequencing of pathogenic *L. interrogans* serovar Lai revealed 4,768 predicted genes including about 50 hypothetical genes related to chemotaxis and motility [67, 112, 113].

Endotoxin of leptospires has been reported in numerous studies. Many serovars exhibit endotoxic activity [74, 114, 115]. Leptospiral endotoxin is lipopolysaccharide (LPS) which has been shown to be less toxic than that of other Gram-negative bacteria [74, 114, 115]. Moreover, LPS causes coagulation defects by reducing platelet number that leads to internal bleeding and tissue damage. Previous studies reported that O antigen of leptospiral LPS in target organs of chronically infected rat was higher than O antigen derived from those of acutely infected guinea pigs, indicating that the expression of O antigen is associated with acute or chronic infection of the host [116]. Several hemolysins have been described in pathogenic leptospires such as sphingomyelinase C, sphingomyelinase H and haemolysins-associated protein-1 (Hap-1, or LipL32) [32, 117-122]. Moreover, heamolytic, sphingomyelinase and phospholipase activities have been characterized *in vitro.* On the other hand, sphingomyelinase C and sphingomyelinase H did not show sphingomyelinase activity. They act as a cytotoxic pore-forming protein to many mammalian cells [121]. In serovar Lai, many hemolysins and sphingomyelinase-like proteins have been identified [32].

Pathogenic leptospires show attachment ability to endothelial, fibroblast, kidney epithelial, and monocyte/macrophage cell lines *in vitro* [123-125]. In addition, they adhere to renal epithelial cell *in vitro* [77]. A large number of adhesins of pathogenic *Leptospira* are mostly OMPs. They have been shown ECM binding ability *in vitro* [78]. Pathogenic strains of leptospires are able to adhere to ECM components such as collagen type I, type IV, laminin and fibronectin [85]. For example, LenA (or Lsa24), Lsa21, Lp49, LipL32, Lig proteins were shown to bind to laminin, collagen type IV, collagen type V, fibronectin in a dose-dependent manner [107, 126-129]. The attachment between endostatin-like proteins A (LenA) and human plasminogen was demonstrated [130]. Leptospiral fibronectin binding protein has been described and shown in the process of host infection. LipL32 was also described as an ECM binding protein [89]. All adhesins of pathogenic leptospires are expressed at the appropriate conditions such as temperature, pH, and osmolarity [78].

Laboratory diagnosis

Leptospires can be detected in blood, urine, tissue, cerebrospinal fluid (CSF) and peritoneal dialysis fluid of patients. Diagnosis of leptospirosis is difficult due to a variety of clinical symptoms and depends on laboratory assays.

Microscopic demonstration

Leptospires can be detected under a dark-field and immunofluorescence microscopes. The samples such as blood, urine, CSF and peritoneal dialysis fluid have been used. Minimal requirement of leptospires that is necessary for observation by dark-field microscope is approximately 10^4 cell/ml. Immunofluorescence and immunoperoxidase staining have been applied to increase sensitivity and specificity of direct microscopic examination. In addition, leptospires can be detected by histophatological examination in tissue by staining with silver staining or immunohistochemical staining [131].

Cultivation

The isolation of leptospries from blood, CSF, and tissue can be achieved during the first week of infection and from the urine and kidney during approximately 2nd and 3rd weeks [132]. This approach requires a long period of incubation, resulting in delayed diagnosis. It also requires a special enriched medium, which make the method complicated and expensive. Thus, culture is rarely used as a routine test for diagnosis.

Serological diagnosis

Serology is the common approach for leptospirosis diagnosis. The antibody can be detected about 5-7 days after the patient begins to show symptoms. Detection of antibody can be done in several ways, such as microscopic agglutination test (MAT) which is the standard method for diagnosis of leptospirosis. The MAT detects agglutinating antibodies in sera of patients. The standard criteria for positive MAT is four-fold antibody titer increase or a high single titer of 1/100 or above. The MAT is read under dark-field microscope. The method is quite complicated and requires experienced personnel.

There are several other serological tests, such as macroscopic slide agglutination test (MSAT) [133, 134], indirect hemagglutination test (IHA), latex agglutination test (LA) [135-138], indirect immunofluorescence test (IFA), complement fixation test (CF), and enzyme-linked immunosorbent assay (ELISA) [139-144].

Molecular diagnosis

DNA of leptospires can be detected in blood, cerebrospinal fluid, and urine by polymerase chain reaction (PCR) method, which is highly sensitive [145-149]. Various primer pairs have been used for PCR detection of specific target genes of leptospires including 16S rRNA or 23S rRNA gene [150-152]. Currently, a real-time quantitative Taqman PCR was utilized to identify 16S rRNA gene of leptospires in patient and environmental samples [153]. Moreover, real-time SYBR green PCR is used to detect *lipl32 gene* of pathogenic strains [100].

Immune response against leptospirosis.

Protective immune response against leptospirosis is unclear. Humoral-mediated immunity has been shown to be utmost important to protect against *Leptospira* infection [18-20]. The antibodies produced during an infection are agglutinating antibodies [21]. LPS is the immunodominant antigen to stimulate production of protective antibodies against *Leptospira*, but are serovar-specific [2, 154, 155]. The passive transfer of convalescent serum can induce protective immunity [156]. Anti-LPS antibody can prevent infection in animal studies such as hamsters and guinea pigs [21, 157, 158]. LPS of *Leptospira* can stimulate human macrophages through Toll-like receptor (TLR) 2 whereas it can stimulate murine macrophages via both TLR2 and TLR4 [159, 160]. Leptospiral components can stimulate the secretion of pro-inflammatory cytokines and induce production of chemokines, such as inducible nitric oxide (iNOS), tumor necrosis factor-α (TNF- α), and monocyte chemoattractant protein-1 (MCP-1) via NF-kB pathway in renal tubular cells [104, 161].

However, the role of cell-mediated immunity has been reported to be important in preventing the disease as well. For example, killed strain of *L. interrogans* serovar Hardjo was shown to stimulate cell-mediated immunity, particularly Th1 response, as

shown by interferon (IFN)-γ secretion by TCR$\gamma\delta$+ T cell and WC1+ gammadelta T cell memory population [23-27].

Leptospirosis in thailand

The incidence of leptospirosis in Thailand has been increased from 398 cases in 1996 to 14,285 cases in 2000, and 2,868 cases in 2005 [13]. The increasing number of cases during the year 1990-2010 is shown in Figure 4. Up to 90% of reported cases are in the Northeast region of Thailand. However, more cases have been reported in the southern region (Figure 5). Risk groups of leptospirosis in Thailand include agricultural workers, paddy farmers, and rat hunters [15]. Up to 17% of patients presented with fever in the hospitals at Thai-Myanmar border area was shown to be leptospirosis [17]. Most patients with leptospirosis are usually in the ages of 45-54 (Figure 6). In Thailand the disease usually occurs during the rainy season between June to October as shown in Figure 7 [162]. The most commonly reported serovars in Thailand are Autumnalis, Bratislava, Bataviae, Javanica, Hebdomadis, Grippotyphosa, Bangkok, and Pyrogenes [14, 16].

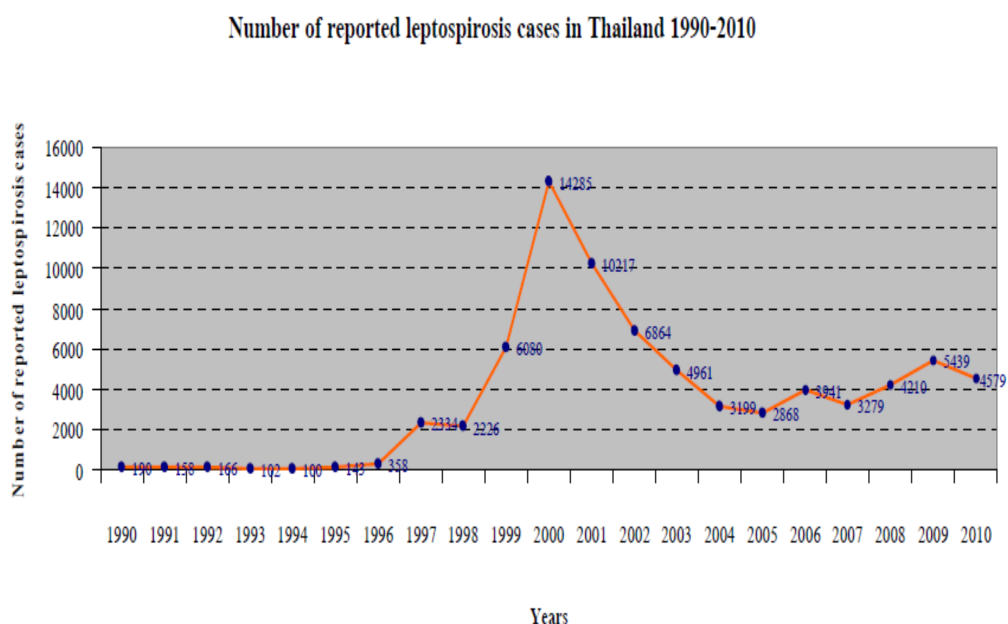


Figure 4. Reported cases of leptospirosis in Thailand during 1990-2010 [162].

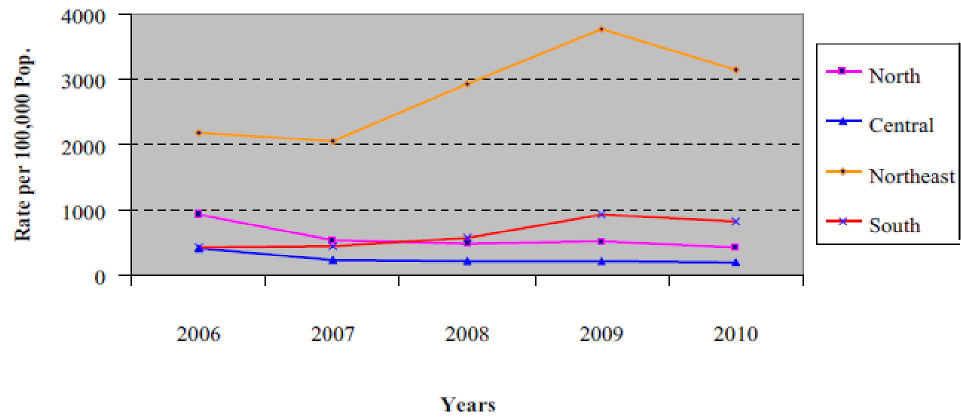


Figure 5. Reported cases of leoptospirosis per 100,000 populations by region of Thailand [162].

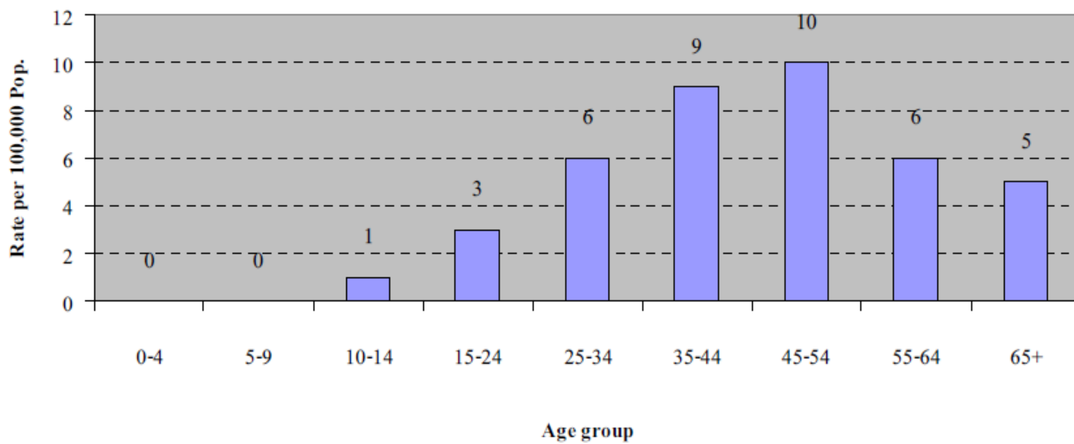


Figure 6. Reported cases of leptospirosis in Thailand per 100,000 populations by age-group in year 2010 [162].

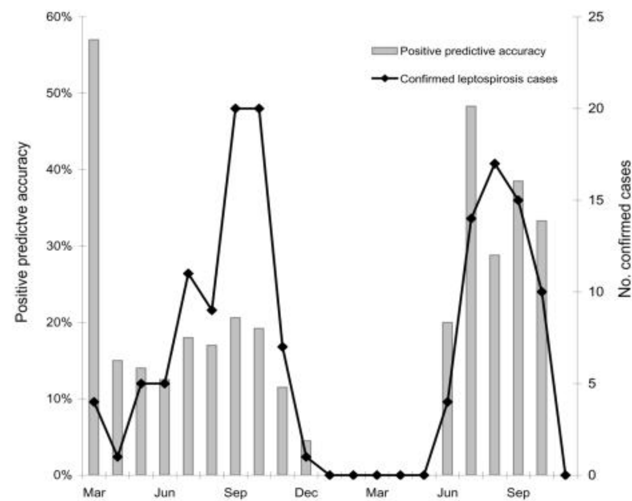


Figure 7. The results of laboratory confirmed leptospirosis in Thailand between March 2003-November 2004 [14].

Vaccines of Leptospirosis

Vaccine is a biological preparation that establishes or improves immunity to a particular disease. Vaccines can be used for prophylaxis to prevent the disease or for therapeutic purpose to ameliorate or cure the disease. Nevertheless, licensed human vaccines for leptospirosis are currently administered only in a few countries such as Cuba [163] and China [164]. Commercially available vaccines for leptospirosis are whole cell killed vaccine that currently restricted to be used only in animals and can prevent infection by homologous serovars (Table 3). Several types of vaccines for leptospirosis have been developed as follows [28].

1. Killed Vaccine

Killed vaccine is prepared by inactivation of whole-cell microorganisms by various means, such as chemicals, heat, or formalin kill. In addition, hydrostatic pressure-treated leptospires can be used as killed vaccine when treated with 2 kbar for 60 minutes [165, 166]. The vaccine can stimulate antigen-specific proliferation response in peripheral blood mononuclear cells (PBMC) of cattle after 2-month post-vaccination [167]. The killed whole-cell vaccine has several side effects including pain, nausea, fever; induce short-term immunity and serovar-specific protection; and may induce autoimmune disease, such as uveitis [28, 168, 169].

Table 3. List of commercial vaccines for use in animals.

Name	Manufacturer	Serovars incorporated	Amimal host
Leptavold H	Scherring – plough animal helath, U.K.	Hardjoprajitno and Hardjobovis	Cattle
Leptpferm - 5	Pfizer animal health	Canicola, Grippotyphosa, Hardjo, Pomona, and Icterohemorrhagiae	Pigs, Cattle
Farrowsure	Pfizer animal health	Pomona, Grippotyphosa, Icterohemorrhagiae, Hardjo, Canicola, and Porcine parvovirus, Erysipelas	Pigs
Suileptovac TPC1	Not known	Tarassovi, Canicola, Pomona, and Icterohemorrhagiae	Pigs
Nobivac	Intervet International	Canicola	Dogs

2. Live attenuated vaccine

Live attenuated vaccine is prepared from attenuated virulent or non-virulent strains. Leptospires are attenuated by passage through eggs, exposure to gamma irradiation (less than 70,000 rad) or subculture *in vitro* for a long time [170, 171]. The limitation of this vaccine is incomplete inactivation resulting in the risk of reversion to the virulent form and contamination during preparation [172]. Cattle immunized twice with live attenuated *L. interrogans* serovar Pomona was able to neutralize against homologous infection and reduce abortion rate [173]. In addition, live attenuated *L. interrogans* serovar Pomona vaccine protected hamsters and swine from death, leptospiruria and renal lesions [174, 175]. Amporn *et al*. showed that live, attenuated LPS mutant derived from *L. interrogans* serovar Manilae was used as a vaccine which was able to induce protection against infection with homologous and heterologous challenges [176].

3. Subunit vaccine

Subunit vaccine has been studied due to limitations of killed whole and live attenuated vaccines. The current development of leptospirosis vaccines have focused on identifying vaccine candidates such as LPS [10,40-47], lipoproteins [48-56], OMPs [52,53,57-62] and potential virulence factors [39,63-68]. The subunit vaccine has the advantage of ability to select only leptospiral components that are specific to and conserved for most pathogenic leptospires and strongly immunogenic as vaccine candidates. The main goal is to develop a vaccine that can prevent infection caused by several pathogenic *Leptospira* species, confer long-term immunity, and has no side effects [29].

3.1. Recombinant protein vaccines

Recombinant protein vaccines have been reported to induce a better immune response and cause fewer side effects than killed whole cell vaccines (Table 4). Reverse vaccinology approach has been utilized to search for novel vaccine candidates from whole genome sequences of *Leptospira* (Figure 8) [177]. So far there are several reports of recombinant protein vaccine formulas using different combination of antigens and adjuvants which yield different success rate of protection (Table 4). For example, LipL32 and LipL41 has been reported to induce partial immunity against leptospirosis in animal models [34]. However, immunization of hamsters with LipL32 and LigA stimulated antibody responses but found no protection after challenge with virulent *L. interrogans* [178].

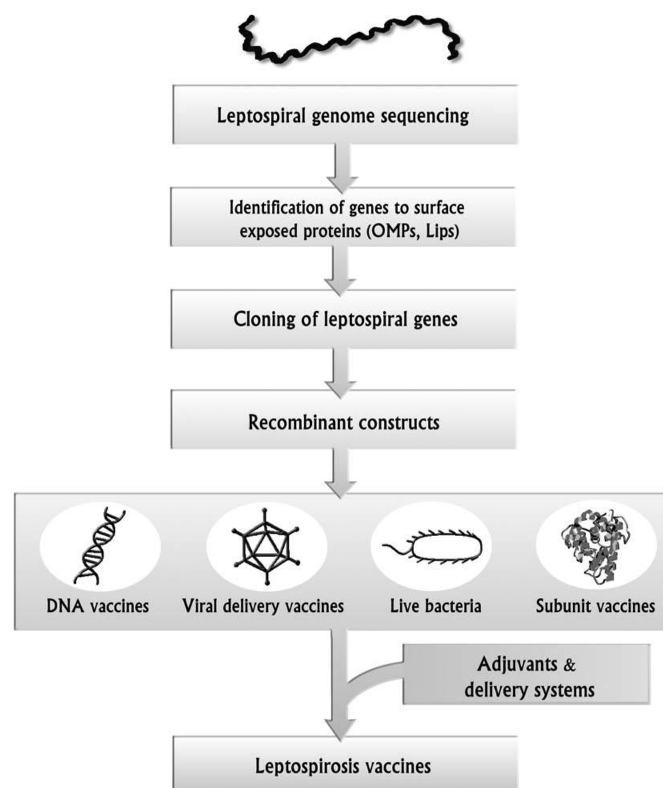


Figure 8. Schematic flow chart of reverse vaccinology approach to identify novel vaccine candidates in leptospiral vaccine development [177].

Table 4. Examples of recombinant protein vaccine formulas for leptospirosis [177].

Antigen (aa)	Serovar	Adjuvant Dose/Via	Challenge Serovar/Dose	%Survival Tested	%Survival Control	Ref.
LigA (68–1224) LigB (68–1191) LigA + LigB	Manilae	Freund's 3 x 10μg /SC/M	Manilae/10^6	90 90 100	- - -	[179]
LigA (32–626) + LigA (631–1225)	Pomona	Alum 2 x 50 μg/SC	Pomona/10^8	100	-	[180]
LigA (625–224)	Copenhageni	Freund's 80 + 40 μg/SC	Copenhageni/10^3	60-100	0	[181]
LigA (631–1225)	Pomona	Alum 2 x 10 μg/SC PLGA 1 x 20 μg/SC Liposomes 2 x 10 μg/SC	Pomona/10^5	50 85 87	0	[182]
LigB (31–630) LigB (630–1418) LigB (1418-1890) LigB + LigB + LigB	Pomona	Alum 2 x 50 μg/SC	Pomona/10^8	62–75 50–62 25–37 75–87	12–25	[183]
LigB (307–630) : (1014–1165) LigB (307–403) :	Pomona	Oil 2 x 50 μg/SC	Pomona/2.5 x10^2	50 50	0	[184]

(1014–1165) LigB (307–630) : (1014–165): LipL32 (185–272) LigB (307–403): (1014-1165):LipL32 (185–272) LigB (47–630)				50 50 34		
LipL32 (265–271)	Autumnalis	- 2 x 10^9 pfu/ IM/G	Canicoloa/10^4	87	51	[185]
LipL32 (23–273)	Copenhageni	- 2 x 106 cfu/IP	Copenhageni/102	12–56	0-20	[186]
LipL41 (53–408) OmpL1 (1–321) LipL41 + OmpL1	Grippotyphosa	- 3 x 50 µg/IP	Grippothyphosa/102	17-19 0-100 50-100	0-33	[187]
Lp0607 (20–267) Lp1118 (26–317) Lp1454 (32–359) Lp0607 + Lp1118 +	Pomona	Alum 2 x 50 µg/SC	Pom/10^8	66–100 66–75 66–71 87	0–50	[188]

Lp1454						
Lp0607 + Lp1118 + Lp1454	Pomona	Leptosomes 2 x 10 µg/SC	Pomona/10^8	75	0	[189]
Lp0607 + Lp1118 + Lp1454	Pomona	Smegmosomes 2 x 10 µg/SC	Pomona/10^8	75	0	[190]
LIC 10494 (28–100) LIC 12730 (19–126) LIC 12922 (47–166)	Copenhageni	Alum 3 x 50 µg/SC	Copenhageni/2 x 10^5	29–50 38–50 29–30	0–20	[191]
Lp4337 (2–429) Lp3685 (7–661) Lp0222 (7–196) [or Loa22]	Pomona	Oil 2 x 50 µg/SC	Pomona/10^8	67–83 50–67 33-50	17	[192]

3.2. DNA vaccine

DNA vaccines have been studied because of several advantages as shown in Table 5. Activation of immune system by plasmid immunization is a new way to produce vaccines that are convenient, easy to produce and safe since the plasmid is not infectious and does not increase the number of its own in the host that received the vaccine. In addition, DNA vaccine can be modified to induce immune system of both cell-mediated immunity (CMI) and humoral-mediated immunity (HMI). Comparative analysis of DNA vaccine with other vaccine types is shown in Table 6 [193]. Current licensed DNA therapies are shown in Table 7. Several DNA vaccines for leptospirosis have been reported in several studies (Table 8). For example, a *Mycobacterium bovis*

BCG vector expressing LipL32 protein protected 56% of immunized hamsters. *LipL32* DNA vaccine in BCG or Adenovirus constructs partially prevented leptospiral infection (38-72%) [185].

Table 5. Advantages of DNA vaccination [194, 195].

commendable qualities	Attributes
Design	• Synthetic and PCR methods allow simple engineering design modifications • Optimization of plasmids through codon • Brings the power of genomics to vaccine construction
Time to manufacture	• Rapid production and formulation • Reproducible, large-scale production and isolation
Safety	• Unable to revert into virulent forms, unlike live vaccines • No significant adverse events in any clinical trial any thousands vaccinated so far
Stability	• More temperature-stable than conventional vaccines • Long shelf life
Mobility	• Ease of storage and transport
Immunogenicity	• Induction of antigen-specific T and B cell responses similar to those elicited by live attenuated platforms

Table 6. Comparative analysis of various vaccine formulations [193].

		Live attenuated	Killed/protein subunit	DNA vaccine
Immune response				
Humoral	B cells	+++	+++	+++
Cellular	CD4+	+/- Th1	+/- Th1	+++ Th1[a]
	CD8+	+++	-	++
	Antigen presentation	MHC class I & II	MHC class II	MHC class I & II
Memory	Humoral	+++	+++	+++
	Cellular	+++	+/-	++
Manufacturing	Ease of development and production	+	++	++++
	Cost	+	+	+++
	Transport/Storage	+	+++	+++
Safety		++[c]	++++	+++[b]

[a] Th2 responses can be induced by gene gun immunization in mice.

[b] Data available only from Phase 1 trials.

[c] Live/attenuated vaccines may be precluded for use in immunocompromised patients and certain infections such as HIV.

Table 7. Current licensed DNA therapies [194].

Vaccine	Name	Company	Date licensed	Target
West Nile virus	West Nile Innovator	Centers for Disease Control and Prevention and Fort Dodge Laboratories	2005 USA	Horses
Infectious haematopoietic necrosis virus	Apex-IHN	Novartis	2005 Canada	Salmon
Growth hormone Releasing hormone	LifeTide-SW5	VGX Animal Health	2007 Australia	Swine and food animals
Melanoma	Canine Melanoma Vaccine	Merial, Memorial Sloan-Kettering Cancer Center and The Animal Medical Center of New York	2007 USA, conditional license	Dogs

Table 8. DNA vaccine candidates for leptospirosis [29].

Antigen	Adjuvant	Animal model	Serovar	Vaccine efficacy (%)	References
LipL41 and OmpL1	*Escherichia coli* OMVs	Hamsters	Grippotyphosa	40–100	[187]
LipL32	Adenovirus	Gerbils	Canicola	73–75	[185]
LigA and LigB	Freunds	Mice	Manilae	90–100	
LipL32	DNA	Gerbils	Canicola	39	[196]
LigA	Alum	Hamsters	Pomona	100	[180]
Carboxyl teminus of LigA	Freunds	Hamsters	Copenhageni	67–100	
LipL32	BCG	Hamsters	Copenhageni	50	[186]
LigA	DNA	Hamsters	Pomona	100	[197]
LigB	Alum	Hamsters	Pomona	67–86	[183]
C terminus of LigA	Liposomes	Hamsters	Pomona	88	[182]

The mechanism of immune response induction of DNA vaccines

DNA vaccine is constructed by cloning a gene of interest into the plasmid DNA. The vaccine is injected into the skin or muscle in combination with adjuvant or by technique that improves the efficiency of DNA vaccine delivery. After immunization, the plasmid uses host cellular machinery to stimulate the immune system both humoral and cellular immunity responses as follows (Figure 9).

i. Plasmid is transported into the nucleus of transfected cells, such as myocytes or keratinocytes as well as antigen presenting cells (APCs). The endogenous antigen will be processed and presented through MHC class I molecules and stimulate cytotoxic lymphocytes (CTLs).
ii. Plasmid is transported into the nucleus of transfected cells, such as myocytes or keratinocytes as well as antigen presenting cells (APCs). The gene is translated into proteins and excreted outside the cell to become exogenous antigens before it is phagocytosed and go through the endocytic pathway. Then, the antigen is presented as peptides via MHC class II molecules to T-cells leading to activation of cellular immunity, secretion of cytokines from stimulated T cells, and production of memory cells. The cytokines secreted from T cells can further stimulate immune cells, for example, IFN-γ can induce macrophages and IL-4 stimulates antibody production by B cells [194, 198].

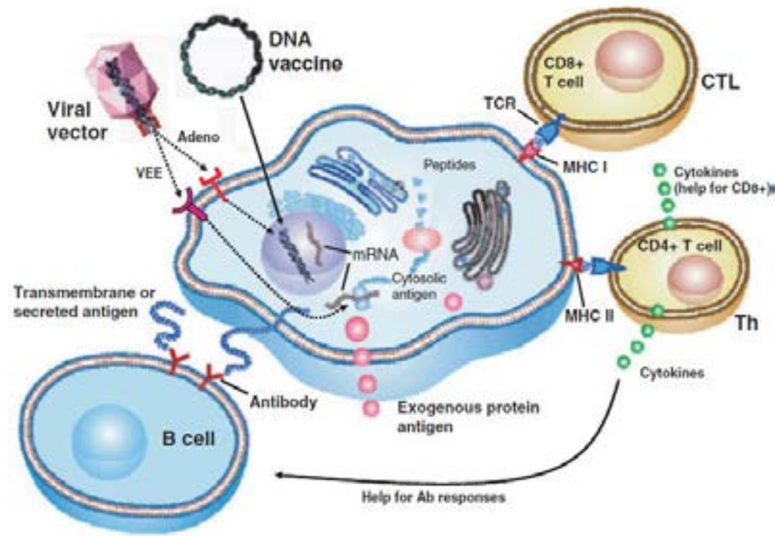


Figure 9. Pathways of DNA vaccine to induce humoral and cell-mediated immune responses [199].

Strategies to improve efficacy of DNA vaccine

There are several strategies to improve immunogenicity of DNA vaccine, such as plasmid optimization, gene optimization, improved RNA structural design, novel formulations and immune adjuvants, and more effective delivery approaches as shown in Figure 10 [194].

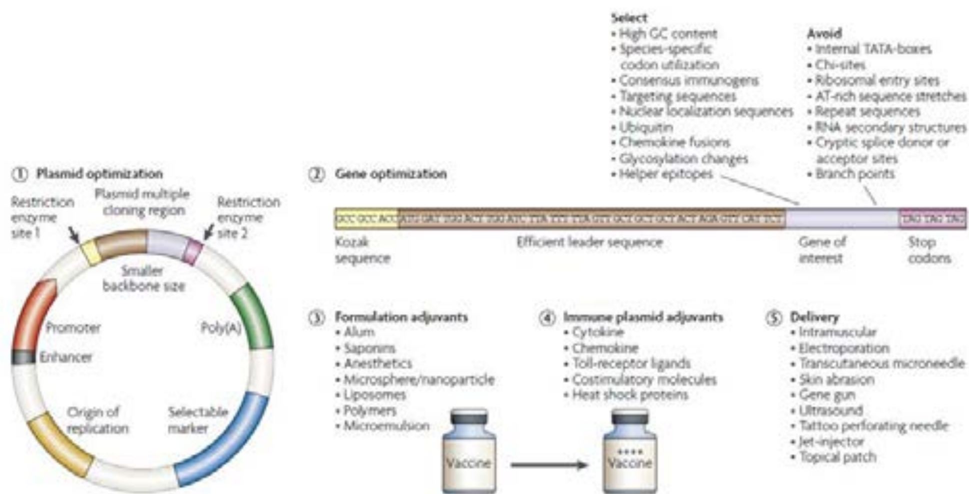


Figure 10. DNA vaccine optimization strategies to enhance immunogenicity [194].

Nanoparticles for vaccine delivery systems

Properties of delivery systems are crucial for its efficacy for vaccine delivery and antigen uptake, such as particle size, shape, surface charge, hydrophobicity and hydrophilicity, as well as receptor [200]. Nanoparticles are solid colloidal particles from 1 to 1000 nm in size which is the size range for efficient uptake of antigens by APCs [201] as shown in Figure 11. The role of particle size in vaccine design affects not only on antigen uptake but also on processing and antigen transport. Due to their small size, mobility of nanoparticles into the cells is enhanced resulting in higher accumulation at the inflammatory site. Uptake of antigens by APCs, particularly B cells and dendritic cells, is the key step in the generation of potent immune responses [202].

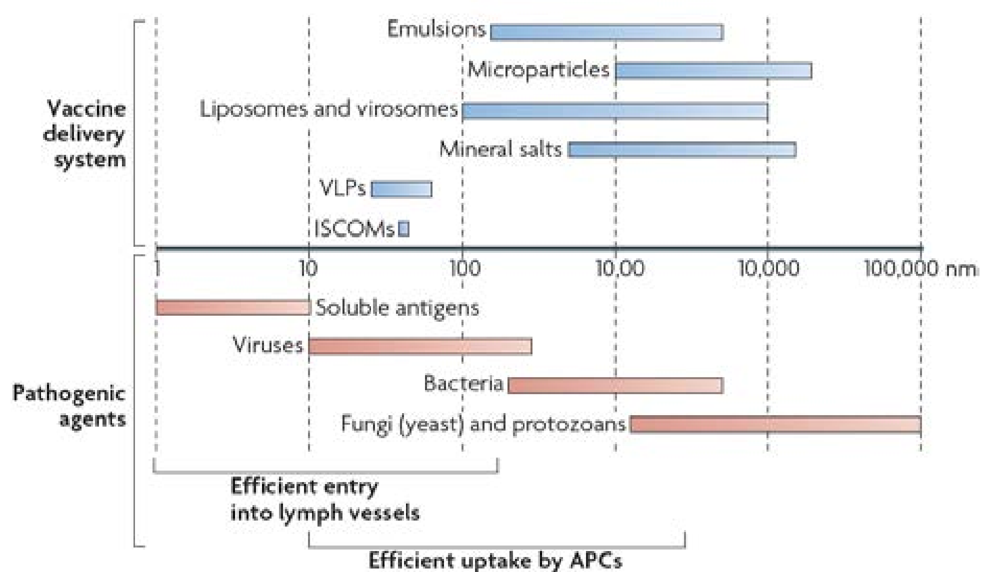


Figure 11. The size ranges of various adjuvant delivery systems and the dimensions of different pathogenic agents are indicated on a nanometre log scale [202].

Chitosan for delivery system

Chitosan is a natural and biodegradable polymer. Chitosan is a deacetylated form of chitin under alkaline condition. It is composed of D-glucosamine and N-acetyl-D-glucosamine containing β-(1-4) glycosidic bonds (Figure 12).

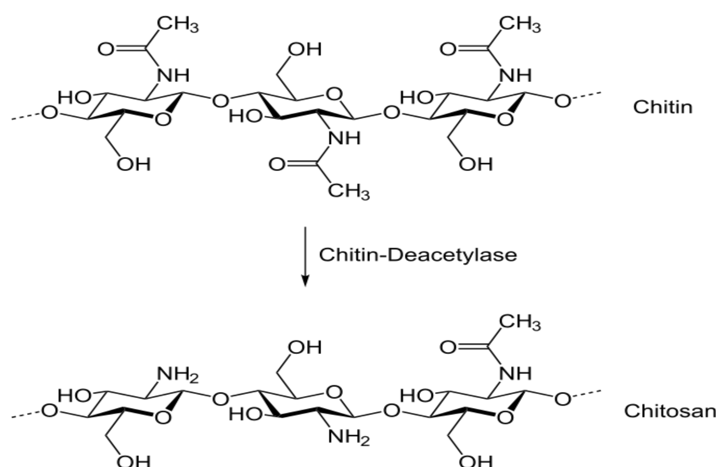


Figure 12. Chemical structure of chitin and chitosan.

Chitosan is found in the shells of crustaceans, such as lobsters, crabs, shrimp, and many other organisms, including insects and fungi as shown in Table 9. It is one of the most abundant biodegradable materials in the world. Chitosan is degraded through enzymatic hydrolysis by chitinases, chitosanases, and other non-specific enzymes such as lysozymes [203]. Chitosan has been used in various applications, such as medical and pharmaceutical substances, treatment of industrial wastewater, beauty products, Industry, agriculture, plants (Table 10 and 11). Chitosan is not toxic and poorly allergenic biological polymers [37, 204]. Chitosan is positive charged due to the weakly basic amino groups of the N-acetyl-D-glucosamine monomers. This is an advantage since it can interact with negatively charged sites on cell surfaces [38]. It is also able to alter tight junctions, thereby enhancing the paracellular uptake of peptides and proteins

[39, 40]. Chitosan can be formulated into micro- or nanoparticles by precipitation [43]. Chitosan suspensions and chitosan micro- and nanoparticles have been reported to exhibit immune-stimulating activity, such as increasing accumulation and activation of macrophages and polymorphonuclear cells, suppressing tumour growth, and enhancing cytotoxic T lymphocyte (CTL) responses and delayed type hypersensitivity (DTH). Chitosan has been used for mucosal delivery due to enhanced binding to the mucosa such as buccal, nasal, intestinal, and vaginal mucosa [205, 206]. Currently safer gene delivery systems including chitosan have been developed to replace viral vectors. Chitosan has positive charges that can bind to negatively charged molecules, such as nucleic acids, mucosal surface or plasma membrane [37]. Chitosan conjugated with DNA is found to be effective in introducing DNA into cells through endocytosis by binding to receptors on the cell surface [41, 42]. Chitosan nanoparticles can promote both Th1 and Th2 responses as shown by *in vitro* stimulatory effect on both macrophage nitric oxide (NO) production and chemotaxis [46]. Chitosan solution was demonstrated to enhance both humoral and cell-mediated immune responses after subcutaneous vaccination [44, 45]. It has also been used in cancer immunotherapy. MC/plasmid encoding murine IL-12 complex was introduced into BALB/c mice bearing CT-26 carcinoma cells resulting in suppression of tumor growth and angiogenesis, and significant induction of cell cycle arrest and apoptosis [207]. The goal of using mannosylated chitosan complexes is to target vehicle binding to a cell-specific receptor including mannose receptor (MR)-mediated endocytosis potentially leading to enhancement of both innate as well as adaptive immune responses [49]. A variety of approaches involving delivery of antigens to the MR have demonstrated effective induction of potent cellular and humoral immune responses [50].

Table 9. Sources of chitin and chitosan [208].

Source structure	Type
Chitin	
Insects	
Cuticle	α -Chitin
Ovipositors	α -Chitin
Beetle cocoon	γ-Chitin
Crustaceans	
Crab shell	α -Chitin
Shrimp shell	α -Chitin
Squid	
Ommastrephes pen	β -Chitin
Loligo stomach wall	γ-Chitin
Centric Diatoms	
Thalassiosira fluviatilis	β -Chitin (100% N-acetylated)
Algae	β -Chitin
Fungi	
Mucor rouxi	β -Chitin
Aspergillis nidulans	β -Chitin
Fungi	
Mucor rouxi	60–92% deacetylated
Deacetylated chitin	
Shrimp shell	Mw up to 1.6 × 106 Da.

Table 10. Principal properties of chitosan in relation to its use in biomedical applications.

Potential Biomedical applications	Principal characteristics
Surgical sutures Biocompatible	• Surgical sutures Biocompatible
Dental implants Biodegradable	• Dental implants Biodegradable
Artificial skin Renewable	• Artificial skin Renewable
Rebuilding of bone Film forming	• Rebuilding of bone Film forming
Corneal contact lenses Hydrating agent	• Corneal contact lenses Hydrating agent
Time release drugs for animals and humans	• Nontoxic, biological tolerance
Encapsulating material	• Hydrolyzed by lyzosyme • Wound healing properties • Efficient against bacteria, viruses, fungi

Table 11. Principal applications for chitosan.

Agriculture	• Defensive mechanism in plants • Stimulation of plant growth • Seed coating, Frost protection • Time release of fertilizers and nutrients into the soil
Water & waste treatment	• Flocculant to clarify water (drinking water, pools) • Removal of metal ions • Ecological polymer (eliminate synthetic polymers) • Reduce odors
Food & beverages	• Not digestible by human (dietary fiber) • Bind lipids (reduce cholesterol) • Preservative • Thickener and stabilizer for sauces • Protective, fungistatic, antibacterial coating for fruit
Cosmetics & toiletries	• Maintain skin moisture • Treat acne • Improve suppleness of hair • Reduce static electricity in hair • Tone skin • Oral care (toothpaste, chewing gum)
Biopharmaceutics	• Immunologic, antitumoral • Hemostatic and anticoagulant • Healing, bacteriostatic

Chitosan nanoparticles for gene delivery

Chitosan is able to bind to DNA through the electrostatic interactions between positively charged glucosamine of chitosan and negatively charged phosphates of DNA. Bowman et al. showed that chitosan nanoparticles can be used as a carrier for Factor VIII DNA delivery via the oral route in hemophilia A mice [209]. In 2009, Li et al reported that admistration of chitosan-DNA nanoparticles encoding house dust mite allergen (Derp2) via oral delivery was able to induce an immune response against the allergy in BALB/c mice [210]. Chitosan nanoparticles are effective in drug and gene delivery. Their advantages include slow and controlled release of the particles in solution, which allows better and more stable delivery of conjugated particles including DNA vaccine. Due to small particles they can easily pass biological barriers *in vivo* such as blood-brain barrier. In addition, chitosan nanoparticle acts as a gene delivery system by enhancing its movement from intracellular endosomes and then releasing conjugated DNA to its target location in the nucleus or cytoplasm or cell membrane leading to improvement of gene targeting [203, 211] as illustrated in Figure 13.

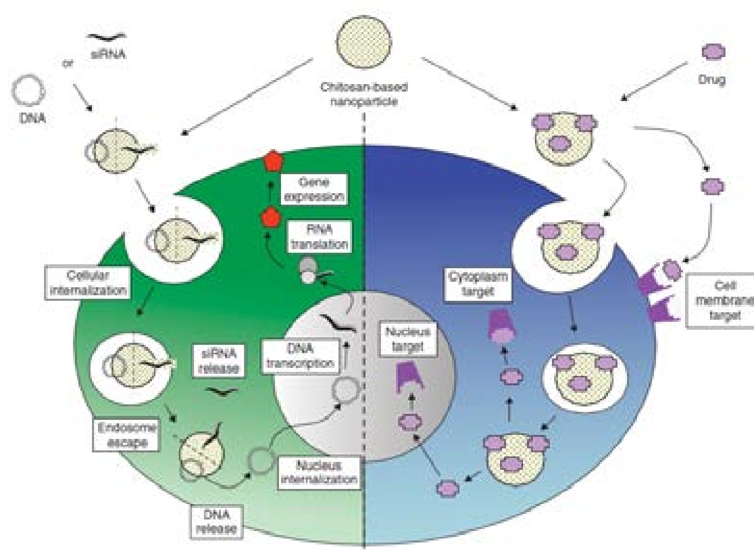


Figure 13. Schematic representation of DNA or siRNA (left) and drug (right) delivery using nanocarriers [203].

Mannose receptor (MR)-mediated gene delivery

Mannose receptor (MR), a C-type lectin, is expressed on the surface of macrophages and dendritic cells [212]. The mannose receptors bind to mannose molecules of a variety of microorganisms such as, LPS of *Escherichia coli*, lipoarabiomannan from *M. tuberculosis* and manan from *Cannida albicans*. It may play a role in innate immunity, such as activation of lectin pathway of the complement system, opsonization and inducing phagocytosis or endocytosis by macrophages (Figure 14) [52, 213, 214]. The mannose receptors are expressed on hepatic and lymphatic sinusoidal endothelial cells, interstitial cells of secretory organs, mucosal sites, and APCs such as tissue macrophages (Kupffer, dermal, peritoneal and alveolar), and macrophages of red pulp of the spleen and subcapsular sinus of lymph nodes [215].

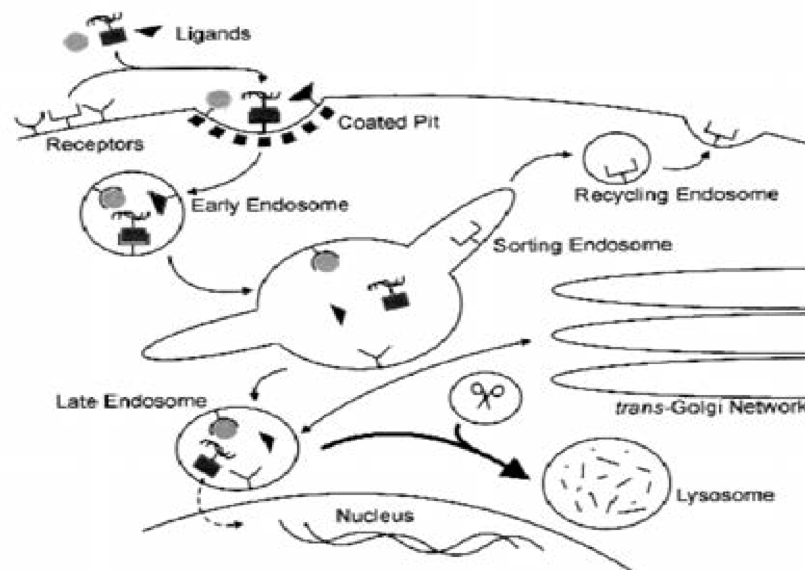


Figure 14. Receptor-mediated trafficking for ligands and gene delivery vectors [216].

New approach to vaccine development is mannose conjugation which helps to target mannose receptors on the cell surface with high affinity and specificity [217]. Mannose conjugation can be achieved by a variety of chemical reactions resulting in the

connection between a mannose and a protein or multiple carriers such as chitosan (Figure 15), liposome. Previous report showed that mannosylation of antigen (Ags) helps stimulating Ag presentation by MHC class I and MHC class and enhancing T cell stimulation more than 200-fold compared with the non-mannosylated Ags [51-53]. Mannose-targeted system has been used in the development of vaccines against pathogens and cancer and drug delivery [217]. Mice immunized with mannosylated vaccines were shown to induce Th1 cytokines and stimulate Ag-specific cytotoxic T lymphocytes (CTL) [50]. Moreover, mannosylated chitosan (MC) was shown to have low cytotoxicity and enhance transfection efficiency into the RAW 264.7 macrophage cell line [47] and mouse peritoneal macrophages [48]. Therefore, mannosylated chitosan is a promising delivery system for DNA vaccine.

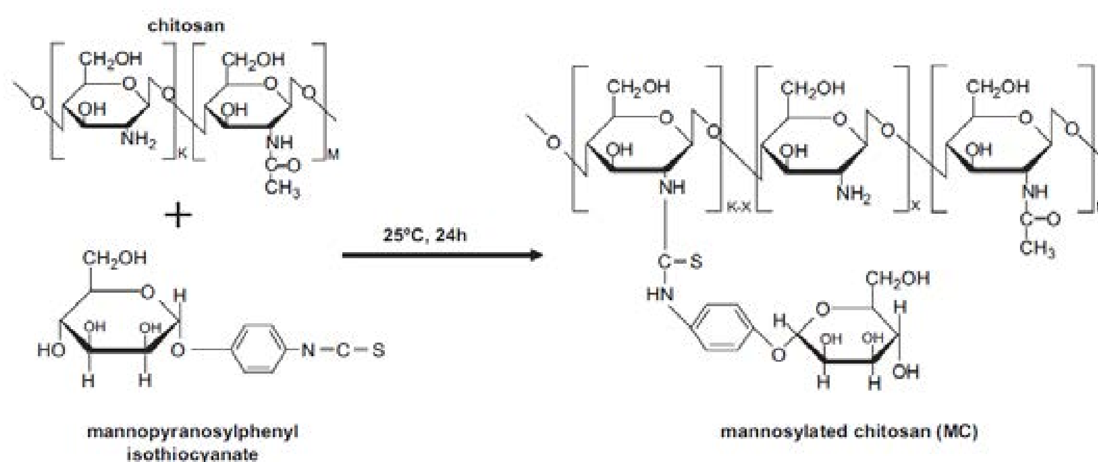
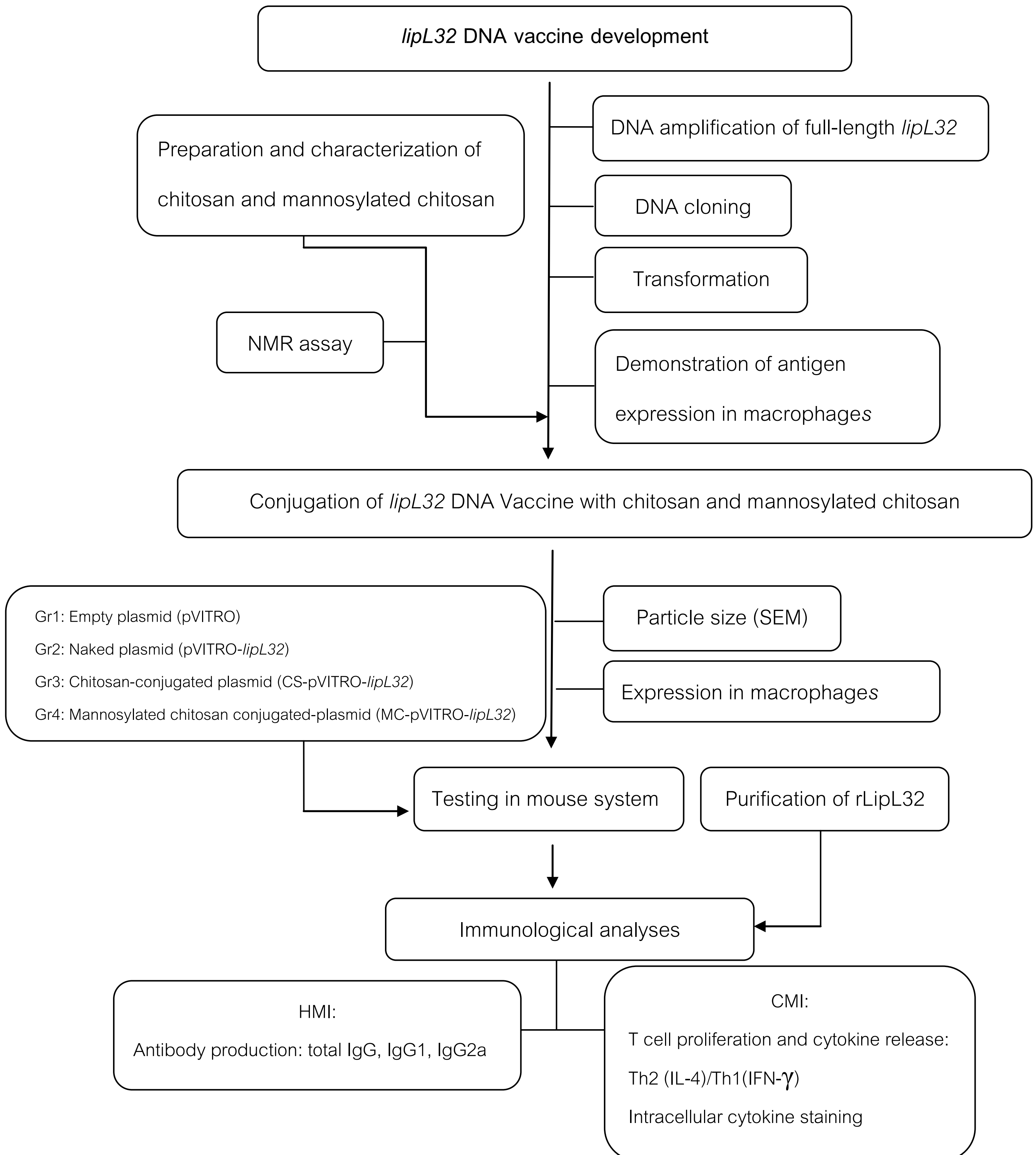


Figure 15. Reaction scheme of mannosylated chitosan (MC) production [218].

CHAPTER IV

MATERIALS AND METHODS

Expermental Flow Chart



Bacterial cultivation

Leptospira cultivation

L. interrogans serovar Pomona was obtained from Khon Kaen University. *Leptospira* was cultivated in Ellinghausen-McCullough-Johnson-Harris (EMJH) enrichment culture media at 30°C for 5 to 7 days until cell density reached approximately 10^8 cells/ml and then harvested by centrifugation [219]. The cell numbers were counted under dark-filed microscopy.

Escherichia coli

E. coli strain BL21(DE3) pLysS (Novagen), *E. coli* strain DH5α and *E. coli* strain ER 2925 were cultivated in Luria-Bertani (LB) broth at 37 °C under shaking condition or on LB agar in incubator at 37°C with appropriate antibiotics.

Cell Culture

Human embryonic kidney 293T (HEK293T) cell line (ATCC No. CRL-1573 – LGC) and RAW 264.7 cell line (ATCC No. TIB-71) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 units/ml penicillin, and 100 µg/ml streptomycin at 37°C under humidified air containing 5% CO_2.

Animals

Female BALB/c mice (6-8 weeks old) were purchased from the National Laboratory Animal Centre, Mahidol University. They were housed at the Department of Pathology, Chulalongkorn University. All procedures involving manipulations of animals in this project have been approved by Chulalongkorn University Animal Ethics Committee under the protocol 15/54.

Polymers and reagents

Water-soluble chitosan (WSC, average molecular weight of 150-300 kDa and 75-90% deacetylation) was obtained from Kittolife Co. Ltd. (Korea) and D-mannopyranosylphenyl isothiocyanate was purchased from Sigma Aldrich (USA). All other materials were obtained from Sigma Aldrich (USA) and were of analytical reagent grade.

Preparation of vaccine

Preparation of *Leptospira* genomic DNA

The genomic DNA of *L. interrogans* serovar Pomona was prepared by phenol chloroform method. Ten millimeters of 10^8 cells/ml *L. interrogans* serovar Pomona were harvested by centrifugation at 8,000 x g for 15 minutes. The pellet was resuspended with TE buffer containing 0.5% SDS and proteinase K (100 µg/ml), mixed, and then incubated at 37 °C for 1 hour. After incubation, 0.25 M NaCl was added to the solution followed by vortex mixing. A 500 µl of phenol:chloroform:isoamyl alcohol (25:24:1) was added, mixed by inverting a tube and then centrifuged at 12,000 rpm for 5 minutes. Upper aqueous solution was transferred to a new tube and 2 volumes of chloroform:isoamyl alcohol (24:1) were added and centrifuged at 12,000 rpm for 5 minutes. Upper phase was transferred to a new tube and 2 volumes of ice-cold absolute ethanol were added, followed by incubation at -20°C for 1 hour. After centrifugation at 12,000 rpm for 10 minutes, DNA pellet was washed with 70% ethanol and allowed to dry at 37°C overnight. DNA was resuspended in sterile distilled water.

PCR amplification of *lipL32*

The extracted DNA of *L. interrogans* serovar Pomona was used as a template for amplification of *lipL32* gene encoding mature protein, from amino acid 1 to 272. The primers for gene amplification were designed to include 19 amino-acid signal peptide, using forward primer: 5' GGATCCGGAATGAAAAAACTTTCGAT 3' and reverse primer: 5' TAACGTACGTTACTTAGTCGCGTCAG 3' containing restriction sites of *Bsp*EI and *Bsi*WI (underlined sequences) in forward and reverse primers, respectively. The PCR amplification was performed in a total volume of 50 µl using 50 ng of leptospiral DNA under the following conditions: primary denaturation at 94 °C for 5 minutes, followed by 30 cycles of denaturation at 94°C for 1 minute, annealing at 53° C for 1 minute, extension at 72 °C for 1 minute; and final extension at 72 °C for 5 minutes. After the reaction, PCR products were analyzed using 1.5 % agarose gel electrophoresis.

Cloning

The 819-bp PCR product amplified from the genomic DNA of *L. interrogans* serovar Pomona was cloned into the pTZ57R/T TA cloning vector (Fermentas, Germany). pTZ57R/T-*lipL32* was digested, and subcloned into pVITRO plasmid at *Bsp*EI and *Bsi*WI restriction sites. The pTZ57R/T-*lipL32* and pVITRO1 vectors were digested with *Bsp*EI and *Bsi*WI restriction enzymes (New England Biolabs) under the following conditions; 1µg of PCR products and 2 µg of pVITRO1 was separately digested with 10 units of *Bsp*EI in NEB buffer 3 (New England Biolabs) at 37°C for 4 hours and then was heat inactivated at 65°C for 10 minutes. Next, 10 units of *Bsi*WI was added and incubated at 55°C for 4 hours followed by heat inactivation at 65°C for 10 minutes. Digested DNA was purified with Nucleospin® Extract II (Macherey-Nagel) according to manufacturer's protocol as follows; 2 volume of NT buffer was mixed with 1 volume of digested products and then loaded onto the Nucleospin® Extract II column, centrifuged at 11,000xg for 1 minute, and the flow through was discarded. A 700 µl of buffer NT3 was loaded onto the column, centrifuged at 11,000xg for 1 minute, re-spun for 2 minutes, and the flow through was discarded. Finally, the column was placed in a

1.5 ml microcentrifuge tube and 20 µl buffer NE was added, incubated at room temperature for 5 minutes, and then centrifuged at 11,000xg for 2 minutes to collect purified DNA.

Ligation reaction was performed by T4 DNA ligase (Fermentas, Germany) under the following conditions; 5 units of T4 ligase, 0.048 pmole of digested *lipL32* and 1.52 pmole digested pVITRO (1:4) were incubated overnight at 16 °C followed by heat inactivation at 65°C for 10 minutes. After incubation, ligation mixture was transformed into *E. coli* strain DH5α by heat shock method as follows; 10 µl of ligation mixture was added to a microcentrifuge tube containing 50 µl of *E. coli* DH5α competent cells and then incubated on ice for 30 minutes. Next, the solution was placed in a 42°C water bath for 90 seconds and immediately incubated on ice for 2 minutes. A 450 µl of SOC medium was added to the solution, mixed, and incubated for 1.5 hours at 37°C under shaking condition. The transformants were plated onto LB agar containing 50 µg/ml of kanamycin, and incubated at 37 °C overnight.

Colony screening

Restriction enzyme screening method

To screen for plasmid containing *lipL32* (pVITRO-*lipL32*), transformant colonies were selected by sterile toothpick and transferred onto the master plate (LB agar containing 50 µg/ml kanamycin) and then incubated at 37 ºC overnight. The selected colony was cultured in 5 ml of LB broth containing 50 µg/ml of kanamycin. The plasmid DNA was extracted by QIAprep Miniprep (Qiagen, Germany) and digested with *Bsp*EI and *Bsi*WI restriction enzymes (New England Biolabs). The restricted DNA pattern was analyzed by 1.5 % agarose gel electrophoresis.

Colony PCR screening

Alternatively, *E. coli* colonies containing *lipL32* (pVITRO-*lipL32*) were screened by PCR reaction using specific primers for *lipL32*. The colonies from the master plate were picked and resuspended in PCR reaction mixture. The expected PCR products were observed by 1.5 % agarose gel electrophoresis.

Endotoxin-free plasmid preparation

Large-scale extraction of plasmid (pVITRO-*lipL32*) and an empty control vector (pVITRO) was performed with an endotoxin-free giga QIAGEN kit (Qiagen, Germany) according to the manufacture's protocol. The extracted plasmids were used for *in vitro* transfection and vaccination studies in mice. Plasmid stocks were stored at -20°C until use.

DNA sequencing

Colonies containing *lipL32* were cultured in LB broth containing 50 µg/ml of kanamycin at 37°C overnight and then plasmid was extracted using QIAGEN plasmid kit according to the manufacturer's protocol. The plasmid sequences were determined by First BASE Laboratories, Malaysia. DNA sequencing results were compared to *lipL32* sequence of *L. interrogans* serovar Pomona obtained from GenBank database.

Transfection and visualization of expressed antigens

HEK293T cells were transfected with plasmid pVITRO-*lipL32* in 24-well plate using Lipofectamine™2000 (Invitrogen, USA) according to the manufacturer's protocol. First, 1.5 µL Lipofectamine™ 2000 and 36 µL serum-free medium was mixed and incubated for 5 minutes at room temperature and 500 ng DNA was added to the Lipofectamine™ 2000 mixture and incubated for 20 minutes. Then, another 500 µL serum-free medium was added, and the final mixture was added to a 24-well plate and

incubated at 37°C. After 72 hours, the transfected cells were washed twice with ice-cold PBS and lysed in RIPA buffer [50mM Tris-HCl, pH 7.4, 150 mM NaCl, 1.0% NP-40, 0.5% $C_{24}H_{39}O_4Na$, 0.1% SDS, protease inhibitor (Roche Diagnostics, Sweden)] and the culture supernatants were collected and stored for 10 minutes on ice. Cell lysates and culture supernatants were boiled and centrifuged at 10,000 g at 4°C for 10 minutes. The transfection efficiency was evaluated by Western blot analysis.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis

Equal proportion of lysates or supernatants of transfected HEK293T cells was boiled at 95 ºC for 10 minutes. Twenty microliters of samples (15 µg of total proteins) was loaded onto a 15% sodium dodecyl sulfate (SDS)-polyacrylamide gel. Proteins were then transferred from SDS-PAGE onto polyvinylidene fluoride membrane (PVDF, Millipore) by Semidry system (Bio-RAD, USA) using transfer buffer (48 mM Tris pH 9.2, 39 mM glycine, 1.3 mM SDS, 20% methanol). Next, the membrane was incubated with blocking buffer (1X Tris buffered saline (TBS) + 3 % skim milk) at room temperature for 1 hour followed by washing 3 times, 10 minutes each with washing buffer (1X TBS + 0.1% Tween 20). The membrane was then incubated with polyclonal mouse anti-LipL32 antiserum (generated by immunization of female BALB/c mice with purified 6His tag-LipL32 fusion proteins) (1:10,000 in blocking buffer) at room temperature for 1 hour followed by washing 3 times, 10 minutes each with washing buffer (1X TBS + 0.1% Tween 20). The membrane was incubated in horseradish peroxidase (HRP)-conjugated goat anti-mouse antiserum (KPL, 1:5,000 in blocking buffer) at room temperature for 1 hour and then washed 3 times, 10 minutes each with washing buffer (1X TBS + 0.1% Tween 20). The blot was detected by enhanced chemiluminescence (ECL) system (Amersham Pharmacia Biotech). The membrane was incubated with the ECL reagents for 1 minute and then exposed to ChemiDoc System (Bio-RAD, USA).

Expression and purification of LipL32 proteins

The obtained BL21(DE3)pLysS containing pRSETC-*lipL32* with insert of signal sequence-deficient full-length *lipL32* was cultivated in LB broth with 35 µg/ml chloramphenicol and 100 µg/ml ampicillin at 250 rpm at 37°C. Next, the overnight culture was added to 25 ml the fresh media 500 ml before incubation until OD_{600} reached 0.35 at which isopropyl-β-D thiogalactopyranoside (IPTG, Fermentas, USA) was added at a final concentration of 1 mM. The culture was incubated at 37°C with shaking at 200 rpm for 3 hours before centrifugation at 8000xg for 15 minutes. The harvested cells were resuspended, washed, and sonicated using High intensity ultrasonic processor VC/VCX 750 sonicator, 40% amplitude for 12 minutes on ice. After that, the lysate was centrifuged at 16,000xg for 20 minutes at 4°C and the supernatant was transferred to a new tube. Next, the supernatant was directly loaded onto a Hi-Trap chelating column (Amersham Pharmacia Biotech) equilibrated with phosphate buffered saline (PBS). Proteins were eluted with stepwise gradient of 60 to 250 mM imidazole. Then, the solution was centrifuged at 16000xg for 20 minutes at 4°C and the supernatant was transferred to a fresh tube. The supernatant was analyzed by 15% SDS-PAGE, followed by staining with Coomassie brilliant blue, and Western blot analysis [219]. The concentration of purified protein was measured by *RC DC* protein assay (Bio-RAD, USA) as manufacturer's instruction. First, Reagent A was prepared by adding 5 µl of *DC* Reagent S to each *DC* Reagent A which contains 250 µl followed by preparing 7 dilution of BSA ; 0.05, 0.1, 0.2, 0.5, 0.75, 1.0 and 1.5 mg/ml for standard curve. Next, each 25 µl of standards or samples was added into microcentrifuge tubes. Then, 125 µl of *RC* Reagent I was added into each tube, vortexed, and incubated at room temperature for 1 minute. After incubation, 125 µl of *RC* Reagent II was added into each

tube and vortexed. The tubes were centrifuged at 15,000Xg for 5 minutes. After centrifugation, 1 ml of *DC* Reagent B was added into each tube followed by immediate mixing. Afterward, tubes were incubated at room temperature for 15 minutes. The concentration of protein was measured by reading the absorbance at 750 nm. The purified recombinant LipL32 was used for stimulating splenocytes *in vitro* and coating plates for enzyme-linked immunosorbent assay (ELISA).

Preparation and characterization of chitosan (CS) and mannosylated chitosan(MC)

Preparation of chitosan (CS)

CS was dissolved in milli-Q water and stirred at 25 ºC for 1-2 hours. The CS composition was determined by proton Nuclear Magnetic Resonance spectroscopy (NMR; Varian model mercury +400, CA, USA). It was diluted with 5 mM sodium acetate buffer, and filtered through a 0.22-µm filter before use.

Preparation of mannosylated chitosan (MC)

MC was prepared by previously reported method [220] with minor modifications. Fifty milligrams of CS were dissolved in 1 ml of milli-Q water before mixing with fifty milligrams of α-D-Mannopyranosyl-phenyl-isothiocyanate (Sigma Aldrich, USA) in 1 ml of dimethylsulfoxide (DMSO) and stirred for 24 hours at room temperature. MC was precipitated by adding 10 volumes of isopropanol and centrifuged at 10,000 rpm at 4°C for 30 minutes. After repeating this process five times, the pellets were dried in a vacuum oven at 50 °C for 3 hours and kept in desiccators for 1 day. The MC composition was determined by proton NMR spectroscopy (NMR; Varian model mercury +400, CA, USA). It was diluted with 5 mM sodium acetate buffer and filtered through a 0.22 µm-filter before use.

CS- and MC-DNA encapsulation

MC was dissolved in sterile water to a concentration of 1 mg per ml. Preparation of MC/DNA complex at varying charge (N/P) ratios was performed. The N/P ratio is the charge ratio of amine groups of CS or MC and the phosphate moieties of DNA [220]. CS or MC was mixed with plasmid DNA solution at varying N/P ratios in a total volume of 500 µl. Uniform particles were obtained by mixing CS, pH 5.5 at 55ºC with plasmid DNA (500 µg/ml in 50 mM sodium acetate) at high-speed vortexing. The complexes were allowed to stand at room temperature for 30 minutes. Complex formation was confirmed by electrophoresis on a 1% agarose gel electrophoresis in Tris-acetate running buffer (1X TAE buffer, 40 mM Tris-acetate, 1 mM EDTA) at 100 V for 40 minutes. DNA was visualized by staining with ethidium bromide (0.2 g/ml) and images were acquired on an UV transilluminator. Protection of encapsulated DNA in the complexes was determined via agarose gel electrophoresis. The morphology and size of CS-conjugated plasmid carrying *lipL32* (CS-pVITRO-*lipL32*) and MC-conjugated plasmid (MC-pVITRO-*lipL32*) were observed by scanning electron microscope (SEM). The complex solution prepared at the N/P ratio of 10:1 for CS and 20:1 for MC was dropped on the opposite charged mica and incubated for 2 minutes. Then, the mica was rinsed with distilled water, and dried at room temperature.

Transfection of CS and MC-DNA encapsulation

RAW264.7 cells were prepared on a glass slide as a monolayer by plating 5 $x10^4$ cells on a glass slide placed at the bottom of each well in 0.5 ml of complete growth medium. The next day, transfection using various formulations of copolymer/DNA complex was performed by treating RAW264.7 cells with each ratio of CS-pVITRO-*lipL32* and MC-pVITRO-*lipL32* followed by incubation at 37ºC in a CO_2 incubator for 5 days. Cells transfected with lipofectamine (lipofectamine-pVITRO-*lipL32*) were considered as the positive control and untransfected RAW264.7 cells were acted as the negative control. Next, expression of LipL32 in transfected cells was determined by

indirect immunofluorescence assay (IFA). Transfected cells were fixed with acetone and permeabilized with methanol. Staining was performed with a polyclonal mouse anti-LipL32 (1:10,000) followed by a fluorescein isothiocynate (FITC)-conjugated goat anti-mouse IgG (1:64, Sigma, USA) in PBS with 0.1% bovine serum albumin (BSA; Amresco, USA). The cell preparations were washed with PBS containing 0.1% BSA and mounted with mounting medium (USA). Fluorescent signals and transmitted light was visualized with a Nikon C1si spectral imaging confocal system (Nikon, Tokyo, Japan) fitted with lasers emitting light at 488 nm for FITC excitation.

Cytotoxicity assay

RAW264.7 cells were seeded at a density of 1×10^4 cells per well in 100 µl growth medium in 96-well plates and incubated for 24 hours. Growth medium was replaced by fresh serum-free media containing free polymer. Untreated cells were used as a negative control. After incubation, 10 µl of a 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) solution (5 mg/ml in PBS) was added, and incubated for additional 4 hours at 37 °C for MTT formazan formation. Then, 0.04 N HCl in isopropanol was added to stop the reaction. The plates were mildly shaken for 10 minutes to ensure the dissolution of formazan. The absorbence values were measured by using microplate reader at a wavelength of 450 nm. Three replicates were counted for each sample. The mean value of the three replicates was used as the final result.

$$\% \text{ viability} = \frac{\text{OD test-OD blank} \times 100}{\text{OD control -OD blank}}$$

Evalution of immunogenicity of *lipL32* DNA vaccine in mice

To evaluate immunogenicity in mouse model after immunization, 18 female BALB/c mice (6–8 weeks old, each weighing 20-25 g) were divided into 4 experimental groups as follows.

Group 1. Empty vector control (pVITRO empty vector)	N=3
Group 2. Naked plasmid (pVITRO-*lipL32*)	N=5
Group 3. Chitosan-conjugated plasmid (CS/pVITRO-*lipL32*)	N=5
Group 4. Mannosylated chitosan-conjugated plasmid (MC/pVITRO-*lipL32*)	N=5

Immunization protocol

One week before commencing the experiments, blood samples were taken from individual mice before the first immunization and serum samples were collected (day 0) by retro-orbital bleeding. Each group of mice was anesthetized with inhalation of anesthetic AERRANE (isoflurane, Baxter, USA). Mice were vaccinated by intramuscular injection (IM) for a total of 4 times at week 0, 2, 4, and 6 (Figure 18). Each mouse was intramuscularly immunized with 50 µg of DNA.

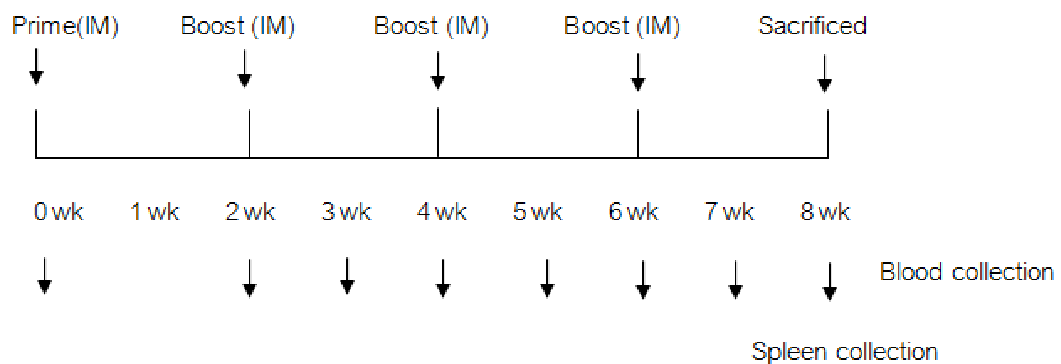


Figure 16. Schedule of vaccination with *lipL32* DNA vaccine and specimen collection after immunization.

Blood sample collection

The blood samples were collected from mice by retro-orbital puncture on day 0 before immunization (pre-immune serum) at day 14, 28 42 and 56 of immunization. After incubation for 30 minutes to 1 hour at room temperature, the clotted blood was centrifuged at 4°C for 25–30 minutes and serum was collected and stored at –20°C until use. The sera were tested for immunogenicity by ELISA assay. Endpoint antibody titers from mice in each group were compared before and after vaccination to detect LipL32-specific IgG1 (Th2 response) and IgG2a (Th1 response).

Determining of total IgG, IgG1, and IgG2a antibody responses to LipL32

The anti-lipL32-specific total IgG, IgG1, and IgG2a antibodies in mouse sera before and after immunization were determined by ELISA assay as described previously [28]. Briefly, 96-well plates (Maxisorp, Nunc, Denmark) were coated with recombinant LipL32 (rLipL32, 500 ng per well) in coating buffer (0.1 M Na_2CO_3, 0.1 M $NaHCO_3$, pH=9.5) at 4 °C overnight. The wells were washed 5 times with 200 µl of washing buffer (1x PBS, 0.05% Tween-20). After washing, the plates were blotted on the paper towel to remove any residual buffer. The plates were blocked for 1 hour at 37°C with 200 µl blocking buffer (1% BSA, 1x PBS, 0.05% Tween-20). One hundred microliters of four-fold serially diluted sera were added into appropriate wells and incubated for 1 hour at 37 ºC. Polyclonal anti-LipL32 (1:10,000) was used as a positive control. After washing for 5 times with 200 µl of washing buffer, one hundred microliters of 1:1,000 dilution of biotinylated rat anti-mouse IgG1 and IgG2a antibody (BD Biosciencers, USA) and 1:1,000 dilution of HRP-conjugated goat anti-mouse IgG (KPL, USA) (for total IgG) was added into appropriate wells. The wells were washed 5 times with 200 µl of washing buffer. One hundred microliters of horseradish peroxidase-conjugated streptavadin (SAv-HRP) (1:1,000, BD Biosciencers, USA) was added to appropriate wells and incubated for 30 minutes at room temperature. After washing, one hundred microliters of 3,3’,5,5’-tetramethylbenzidine substrate (TMB substrate) solution (BD Biosciencers,

USA) was added to each well and incubated at room temperature for 15 minutes in the dark. Finally, fifty microliters of stop solution (0.5 M H_2SO_4) was added to each well. The absorbance was read within 30 minutes at 450 nm using microplate manager (Bio-Rad, USA). The specific IgG titers of all samples were assayed in triplicates.

Lymphocyte proliferation assay

Proliferation assays

The animals from each group were sacrificed and spleens were removed aseptically. The spleens were suspended in RPMI2ME (RPMI-1640 medium containing 5% FBS and 2-mercaptoethanol (2-ME, Gibco, Grand Island, NY) by homogenizing spleens using cell strainer to prepare single cell suspension. Splenocytes were washed twice in RPMI and finally suspended in RPMI2ME and cell viability was determined by staining with 0.4% trypan blue. Splenocytes were seeded in 96-well flat bottom plates at a concentration of 5×10^5 cells in 200 µl of RPMI2ME and stimulated with varying concentrations of purified recombinant LipL32 (5, 10, and 20 µg/ml) for 72 hours at 37˚C with 5% CO_2. Splenocytes stimulated with Concanavalin A (Con A) at a final concentration of 10 µg/ml (Sigma, USA) were considered as the positive control and those with medium alone were acted as the negative control. DNA synthesis in induced and control cells was measured by staining with cell proliferation dye eFluor® 670 (e-Bioscience). The cells were washed twice, and fixed in 1% paraformaldehyde for FACS analysis. Flow cytomerty was done by FACS Calibur™ (BD Biosciencers, USA) and data were analyzed using Summit version 4.2 software. The percentage of positive cells in the experimental groups was obtained by subtracting percentage of cell control.

Cytokine assays

Splenocytes were seeded in 96-well flat bottom plates at a concentration of 5×10^5 cells in 200 µl of RPMI2ME and stimulated with varying concentrations of purified recombinant LipL32 (5, 10, and 20 µg/ml) for 72 hours at 37˚C with 5% CO_2. Splenocytes stimulated with Concanavalin A (Con A) at a final concentration of 10 µg/ml (Sigma, USA) were considered as the positive control and those with medium alone were considered as the negative control. Culture supernatants were collected after 72 hours to determine the cytokine response (IFN- γ and IL-4) by using a capture ELISA kit (BD Biosciences, USA) according to the manufacture's protocol. The specific cytokine response of all samples was assayed in triplicates.

Detection of T cell response by Flow cytometry

For the analysis of specific CD4+ T cell proliferation, spleen was aseptically removed and mechanically homogenized with a 3-ml syringe plunger. Complete RPMI 1640 media (RPMI-1640) was used to cultivate the cells. Cell viability was determined by trypan blue staining. Cells with > 95% cell viability were re-suspended in RPMI supplemented with 50 µM 2 ME, adjusted to 2 x 10^6 cells/well in 24-well plate, and re-stimulated *in vitro* with 20 µg/ml of rLipL32. Cells stimulated with 10 µg/ml concanavalin A (ConA) were used as a positive control. Cells were incubated at 37°C with 5% CO_2 for 72 hours. The cells were stained using Fixation/Permeabilization Kit (Qiagen, Germanyaccording to the manufacturer's instruction and using antibody directed against mouse differentiation markers (e-Bioscience); Alexa Fluor® 488 anti-mouse CD4 (clone GK1.5), Peridinin Chlorophyl Protein (PerCP)/ cyanine dye (Cy5.5™), PerCP/Cy5.5 anti-mouse IFN-γ (clone XMG1.2), Phycoerythrin (PE) anti-mouse IL-2(clone JES6-5H4), and Allophycocyanin (APC) anti-mouse IL-4 (clone 11B11). After 72-hour incubation, cells were incubated with BD GolgiPlug™ Protein Transport Inhibitor (Qiagen, Germany) containing Brefeldin A, phorbol myristate acetate (PMA, 50 ng/ml), and ionomycin (1mM) at 37°C with 5% CO_2 for 4 hours. Next, cells were washed with 1

ml staining buffer and ~10^6 cells were stained in 50 μl of staining buffer (0.09% (w/v) sodium azide, 1% heat inactivated FBS, 1XPBS) containing the appropriate amount of a fluorochrome-conjugated monoclonal antibody specific for a cell surface (Alexa Fluor® 488 anti-mouse CD4), washed twice with staining buffer, and then fixed and permeabilized using fixation/permeabilization solution. Then, cells were stained for intracellular cytokines using PerCP/Cy5.5 anti-mouse IFN-γ, PE anti-mouse IL-2, and APC anti-mouse IL-4. Negative control staining was performed by using isotype controls including APC and PerCP/Cy5.5–rat $IgG_{1,k}$ (clone RTK2071) for APC-conjugated anti-mouse IL-4, PerCP/Cy5.5 anti-mouse IFN-γ, and rat $IgG_{2b,k}$ (clone RTK4530) for both PE-anti-mouse IL-2 and anti-mouse CD4 (e-Bioscience). The cells were washed twice, and fixed in 1% paraformaldehyde for subsequent FACS analysis on a FACSCalibur. Flow cytomerty was done by FACS Calibur™ (BD Biosciencers, USA) and data were analyzed using Summit v 4.2 software. The percentage of positive cells in the experimental groups was obtained by subtracting the result of the isotype control.

Statistical analysis

Results are expressed as means ± SD. The one-way ANOVA analysis of variance with Tukey–Kramer's post-hoc test was used to determine the statistical significance for all pairwise multiple-comparison procedures. A P-value of $p<0.05$ was considered as statistical significance. All experiments were performed in triplicates. Data were analyzed and plotted as graphs using GraphPad Prism software.

CHAPTER V

RESULTS

Cloning of *lipL32* in mammalian expression vector

The genomic DNA of *L. interrogans* serovar Pomona was used as a template for amplification of the 819 bp insert sequence of full-length *lipL32* gene encoding 272 amino acids of LipL32 (Figure 17, A). The pTZ57R/T-*lipL32* (3,705 bp) and pVITRO of 6,295 bp (Figure 17B lane 1 and 3) were digested with *Bsp*EI and *Bsi*WI, and yielded products of *lipL32* insert (819 bp) as shown on agarose gel (Figure 17B, lane 4).

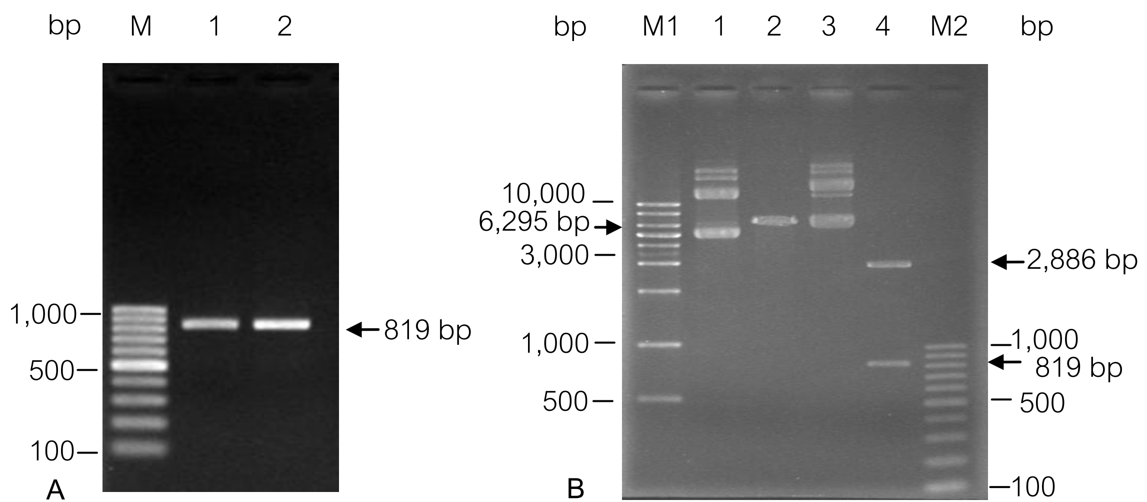


Figure 17. PCR products of *lipL32* gene and pVITRO expression vector on agarose gel. *LipL32* amplicons of *L. interrogans* serovar Pomona (A); lane M, 100 bp DNA ladder; lane 1, and 2 show a single band of PCR products at 819 bp in size. The pTZ57R/T-*lipL32* and pVITRO vector after digestion by *Bsp*EI and *Bsi*WI (B); lane M1, 1 kb DNA ladder; lane 1 uncut pVITRO, and 2 digested pVITRO (6,295 bp); lane 3 uncut pTZ57R/T-*lipL32*, and 4, digested pTZ57R/T-*lipL32*, lane M2, 100 bp DNA ladder.

Transformants grown on antibiotic agar plates were screened for *lipL32* gene insertion by PCR amplification (Figure 18). Three positive clones were randomly selected for sequencing of the inserted gene. Two sequences were the same as *lipL32* gene of *L. interrogans* serovar Pomona as reported in GenBank database (EU871716.1). Clones containing *lipL32* with correct sequences and in framed with the expression vector were further used for protein expression in mammalian cells.

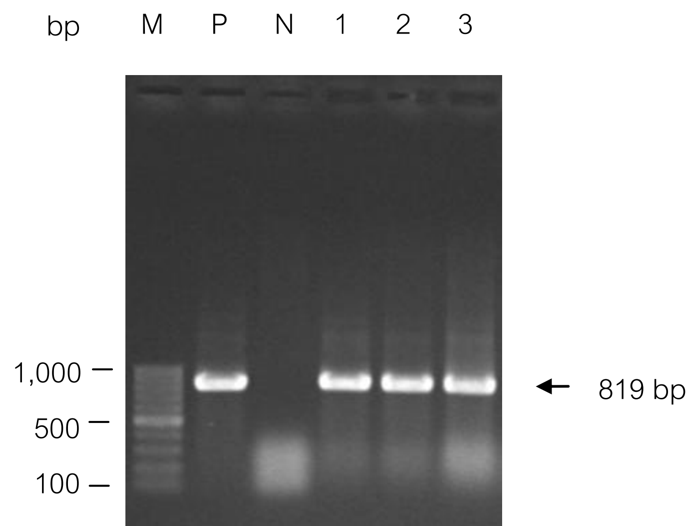


Figure 18. PCR amplification of *lipL32* insert in transformants. Gel electrophoresis shows a single band of PCR products of 819 bp. Lane M, 100 bp DNA ladder; P, positive control (genomic DNA of *L. interrogans* serovar Pomona); N, negative control; lane 1-3, PCR products of selected colonies.

The expression and secretion of LipL32 in transfected Human Embryonic Kidney (HEK293T) cell line

The expression and secretion of LipL32 in HEK293T cells transfected with *lipL32* plasmid construct (pVITRO-*lipL32*) was confirmed by Western blotting. LipL32 was expressed in both the cell lysate and culture supernatant with an apparent molecular weight of about 32 kDa (Figure 19), suggesting that LipL32 was produced in the transfected cells and then secreted outside the cells. In addition, this finding demonstrated that the bacterial or natural leader sequence of LipL32 was functional in mammalian cells. LipL32 was not detected in HEK293T cells transfected with pVITRO control (Figure 19, lane 2) as expected. However, secreted LipL32 (lane 4) has higher molecular weight (MW) than intracellular LipL32 (lane 3) and recombinant LipL32 produced by *E. coli* (lane 5).

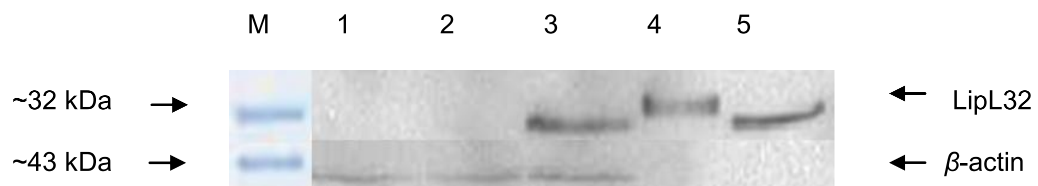


Figure 19. Expression of *lipL32* DNA vaccine construct in HEK293T cells. After 72-hour post-transfection, expression of *lipL32* was analyzed by Western blot. Lane M, pre-stained protein MW marker; lane 1, lysate of untransfected cells; lane 2, lysate of cells transfected with pVITRO; lane 3, lysate of cells transfected with pVITRO-*lipL32*; lane 4, culture supernatant of cells transfected with pVITRO-*lipL32*; lane 5, rLipL32. LipL32 was detected with mouse polyclonal anti-LipL32 antibody.

Protein Extraction and Purification

Expression of LipL32 in *E. coli* strain BL21(DE3)pLysS was induced by 1 mM IPTG at 37ºC for 3 hours. The rLipL32 was expressed at a predicted size of 32 kDa in soluble parts and then purified with metal-chelation affinity chromatography as shown on SDS-PAGE and Western blot (Figure 20). The purified rLipL32 was used for ELISA and lymphoproliferation assay

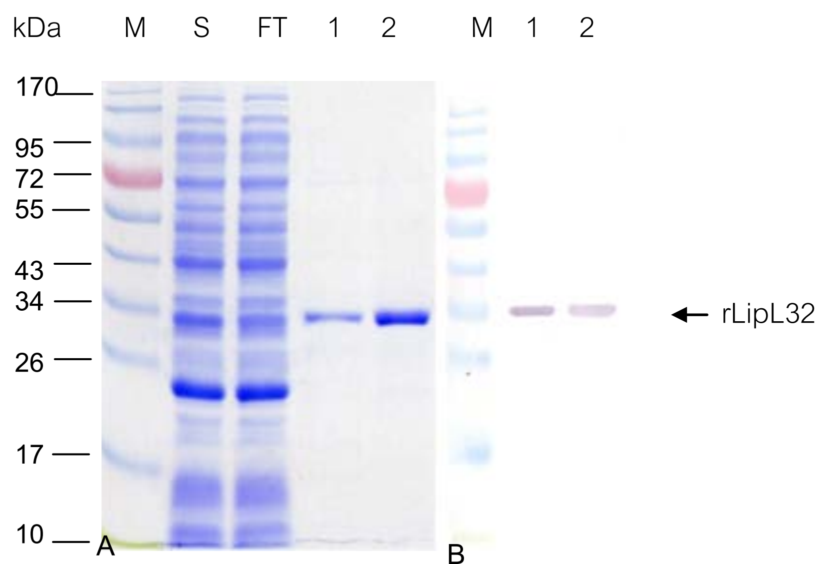


Figure 20. Detection of purified rLipL32 by SDS-PAGE and Western blot. The rLipL32 expression was detected by Coomassie blue staining of SDS-PAGE (A); lane M, pre-stained protein MW marker; S, Proteins from soluble part; FT, flowthrough; the purified rLipL32 eluted fractions from Ni^{2+} column using 100 mM immidazone (lane 1, and 2). Western blotting of purified rLipL32 (B); lane M, pre-stained protein MW marker; lane 1, protein detected by anti-rLipL32 antibody and anti-His antibody (lane 2). Arrow indicates the position of rLipL32 protein.

Characterization of chitosan (CS) and mannosylated chitosan (MC)

The water soluble chitosan was conjugated with mannose by using D-mannopyranosylphenyl isothiocyanate. The products from this step were characterized by nuclear magnetic resonance (NMR) analysis and found that 83.33% of mannose was conjugated with chitosan (Figure 21).

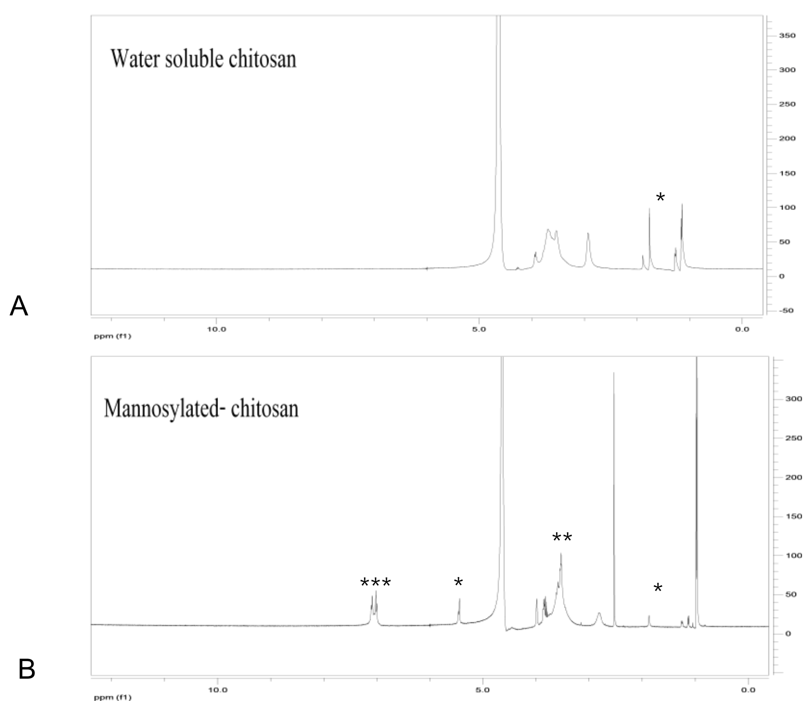


Figure 21. Representative 1H NMR spectra of chitosan (CS) (A) and mannosylated chitosan (MC) (B). * indicates the position of chitosan in D_2O (-CH3- group); ** indicates the position of mannosylated chitosan (MC), *** indicates the position of mannose in D_2O (-CH- group).

Agarose gel electrophoresis assay for plasmid DNA binding with chitosan (CS) and mannosylated chitosan (MC)

The incorporation of pVITRO-*lipL32* into chitosan or mannosylated chitosan nanoparticles was monitored by agarose gel electrophoresis assay (Figure 22). Unconjugated plasmid was demonstrated as bands in agarose gel due to the fluorescence emission of ethidium bromide intercalated into the DNA double helix while conjugated DNA remained in the wells. The N/P ratio is the ratio of positive charge (amine group) of CS or MC to negative charge (phosphate group) of DNA. The finding suggested that DNA was able to be encapsulated inside the nanoparticles. Chitosan was able to conjugate with pVITRO-*lipL32* at the N/P ratios of 6:1 to 20:1 (Figure 22, A). However, mannosylated chitosan was demonstrated to conjugate with pVITRO-*lipL32* at the N/P ratios of 20:1 to 50:1 (Figure 22, B), which are the ratios of total chitosan before mannosylation. After mannosylation, 1.2 amine groups of chitosan were replaced with mannose, resulting in the reduction of positive charges of amine groups in the chitosan, The N/P ratios of chitosan/DNA complexes at 6:1, 8:1, 10:1, 20:1, 30:1, 40:1, and 50:1 are corresponding to 2.4:1, 3.2:1, 4:1, 8:1, 12:1, 16:1, and 20:1 of mannosylated chitosan/DNA complexes, respectively. Therefore, chitosan was able to conjugate with pVITRO-*lipL32* at a lower N/P ratio than mannosylated chitosan.

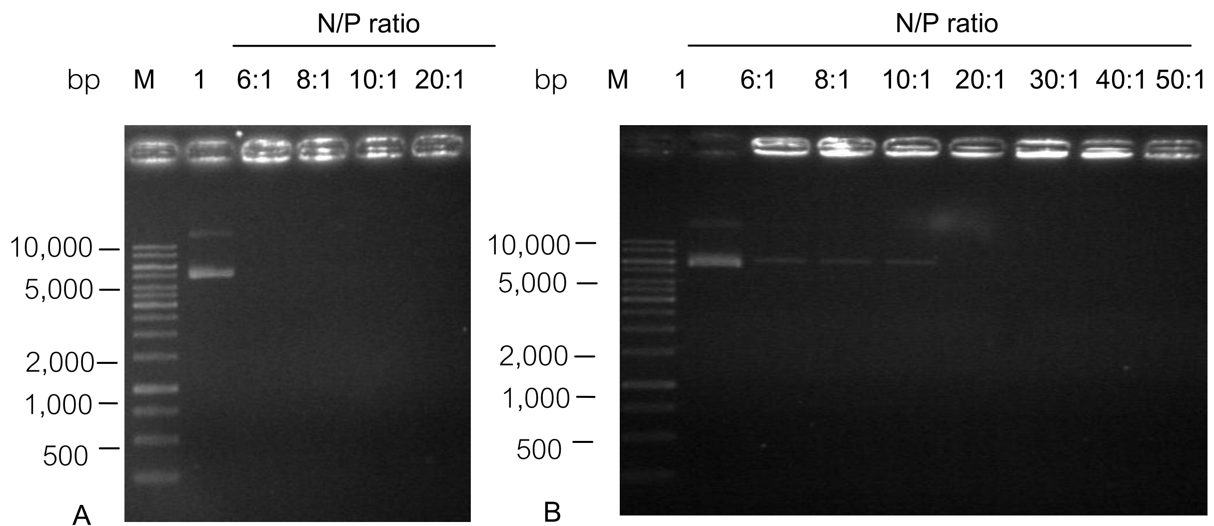


Figure 22. The incorporation of pVITRO-*lipL32* into chitosan or mannosylated chitosan by agarose gel electrophoresis. Chitosan-conjugated plasmid carrying *lipL32* (CS-pVITRO-*lipL32*) at varying N/P ratios (A); lane 1, naked DNA control (pVITRO-*lipL32*); lane 2–5, CS-pVITRO-*lipL32* prepared at N/P ratios of 6:1, to 20:1 respectively. mannosylated chitosan-conjugated plasmid (MC-pVITRO-*lipL32*) at varying N/P ratios(B); lane 1, pVITRO-*lipL32*; lane 2–8, MC-pVITRO-*lipL32* prepared at N/P ratios of 6:1, to 50:1 respectively.

The effect of chitosan-conjugated plasmid carrying *lipL32* (CS-pVITRO-*lipL32*) and mannosylated chitosan-conjugated plasmid (MC-pVITRO-*lipL32*) at varying N/P ratios on the cell viability of murine macrophages cell line (RAW 264.7) was investigated by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenylte-bromide (MTT) assay. As shown in Figure 23, both CS-pVITRO-*lipL32* and MC-pVITRO-*lipL32* showed no significant cytotoxicity at any N/P ratios when compared with unstimulated control.

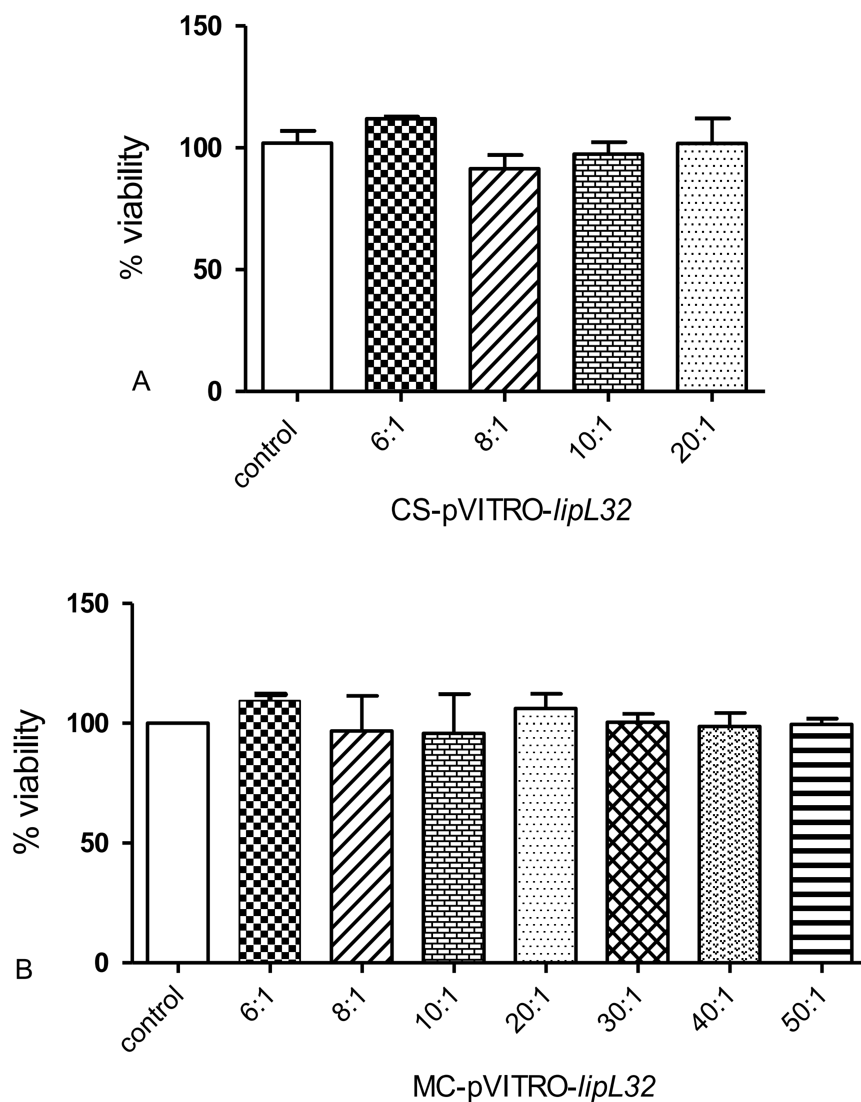


Figure 23. Cytotoxicity of CS-pVITRO-*lipL32* (A) and MC-pVITRO-*lipL32* (B) copolymer at various concentrations in RAW264.7 cells (n=3, error bars represent standard deviation). Results are representative of three independent experiments with similar trends.

Morphology of chitosan and mannosylated chitosan

The nanoparticle morphology of CS, CS-pVITRO-*lipL32* at the N / P ratio of 10:1, MC, and MC-pVITRO-*lipL32* at the N/P ratio of 20:1 was investigated by scanning electron microscopy (SEM). Figure 24 shows that after conjugation with DNA, CS and MC remained nanoparticles in size of approximately 500 nm. Different shapes of CS and MC particles were observed after conjugation with plasmid as shown in Figure 26B and D. The chitosan and mannosylated chitosan particles became more oblong after conjugation with plasmid compared to more spherical morphology of unconjugated chitosan or mannosylated chitosan.

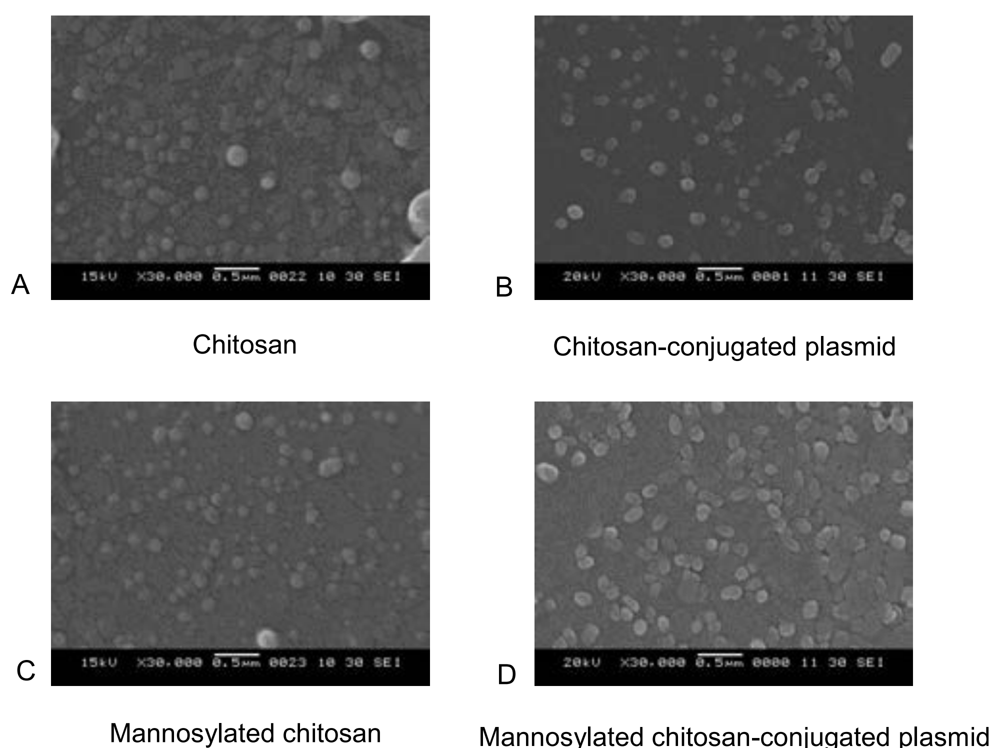
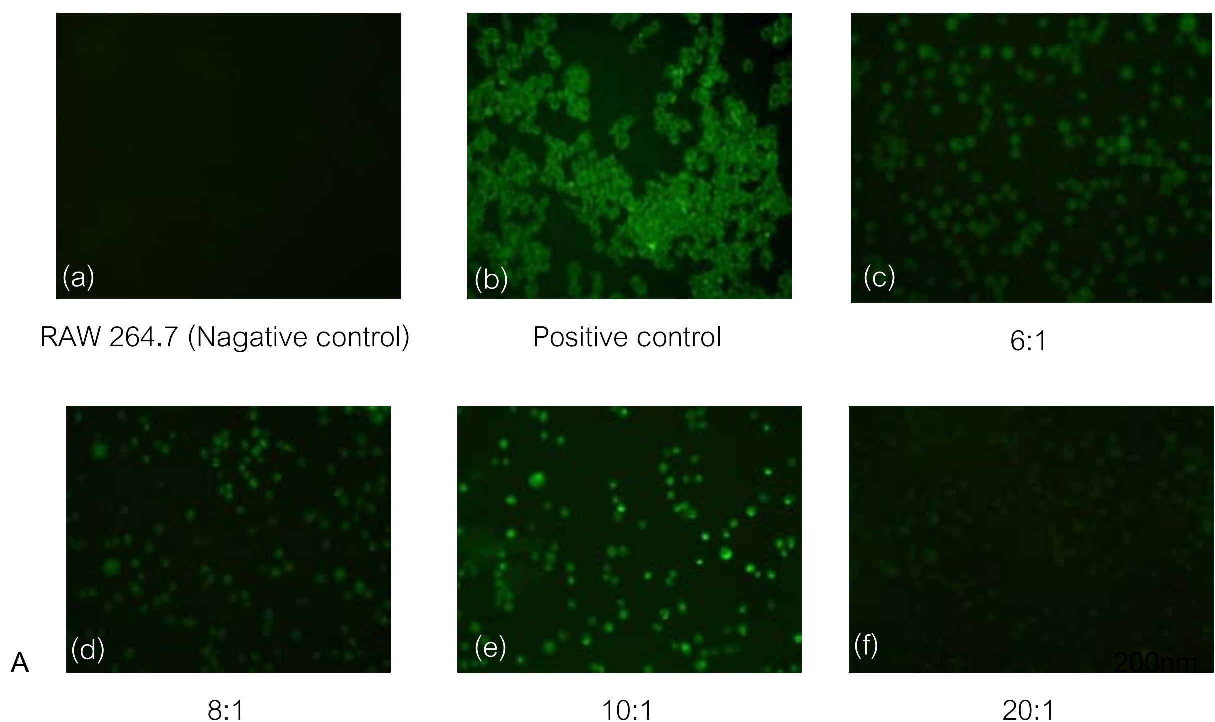


Figure 24. SEM images of different polymer nanoparticles: CS (A), CS-pVITRO-*lipL32* at the N/P ratio of 10:1 (B), MC (C), and MC-pVITRO-*lipL32* at the N/P ratio of 20:1 (D). Results are representative of 2 independent experiments with similar trends.

In vitro transfection efficiency of chitosan and mannosylated chitosan-conjugated plasmid at varying N/P ratios

Transfection efficiency of RAW264.7 cells by chitosan and mannosylated chitosan-conjugated plasmid containing *lipL32* at varying N/P ratios were analysed by expression of LipL32 using indirect immunofluorescence assay (IFA). Untransfected cells were used as a negative control and cells transfected with pVITRO-*lipL32* by lipofectamine were used as a positive control (Figure 25). The transfection efficiency of MC-pVITRO-*lipL32* was comparable to that of CS-pVITRO-*lipL32* but at a lower N/P ratio. The highest LipL32 expression was detected in cells transfected with CS-pVITRO-*lipL32* at the N/P ratio of 10:1 (Figure 25A, e) whereas the highest fluorescence intensity was obtained in cells transfected with MC-pVITRO-*lipL32* at the N/P ratio of 20:1 (Figure 25B, f). Therefore, these N/P ratios were utilized for DNA vaccine preparation for immunization in mice. However, transfection efficiency as a result of pVITRO-*lipL32* same as untransfected cells (data not shown), CS-pVITRO-*lipL32* at the N/P ratio of 10:1 (3.5%) and MC-pVITRO-*lipL32* at the N/P ratio of 20:1 (5.5%) was lower than that obtained by lipofectamine (positive control, 58%).



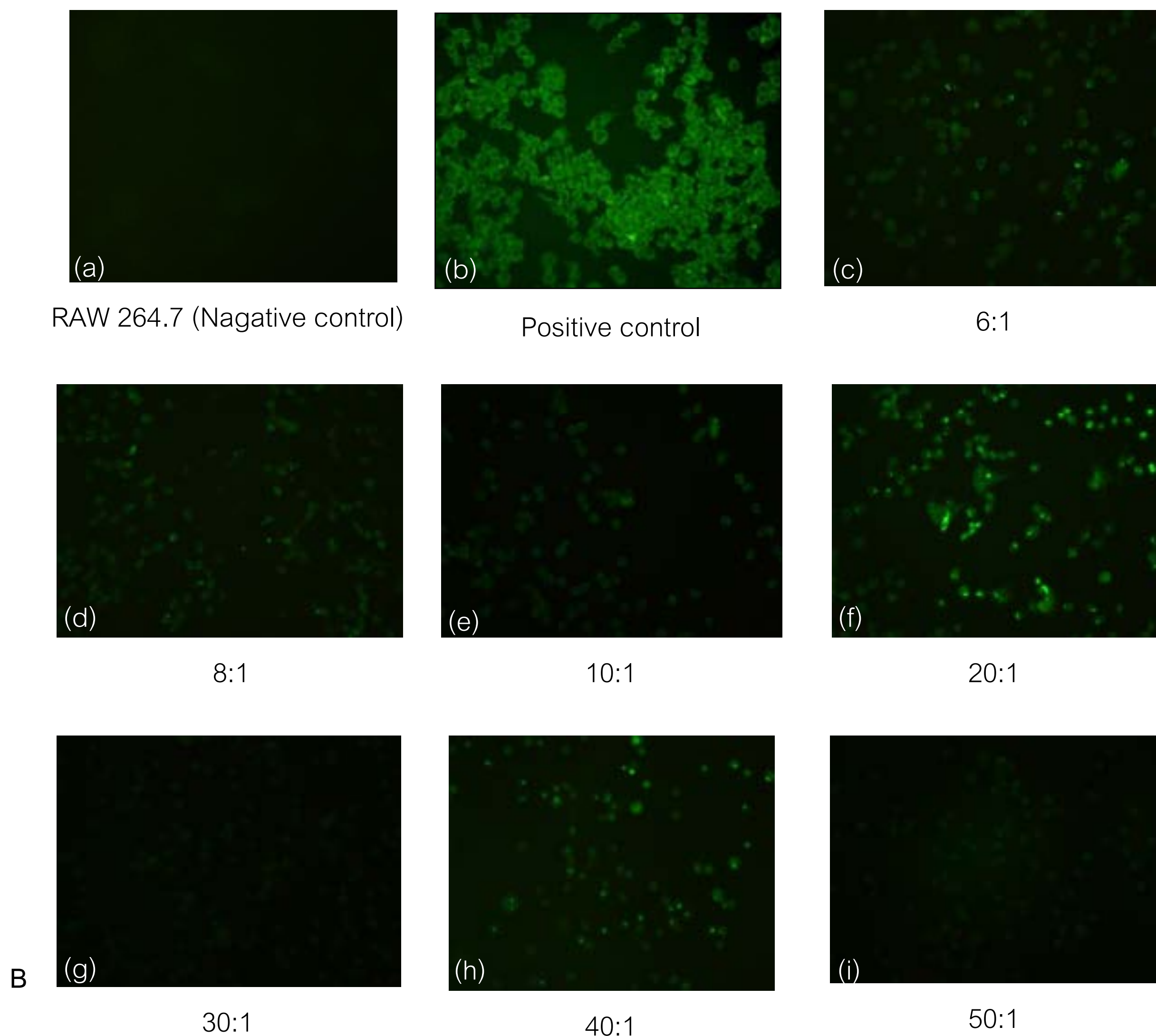


Figure 25. Immunofluorsent stains of LipL32 in RAW 264.7 cells transfected with CS-pVITRO-*lipL32* (A) and MC-pVITRO-*lipL32* (B) at varying N/P ratios was detected with anti-LipL32 and FITC-conjugated rabbit anti-mouse IgG; untrasfected RAW 264.7 (negative control) (a); positive control (b); 6:1 (N/P) (c); 8:1 (N/P) (d); 10:1 (N/P) (e); 20:1 (N/P) (f); 30:1 (N/P) (g); 40:1 (N/P) (h); 50:1 (N/P) (i).

Antibody response to *lipL32* DNA vaccine immunization

At six weeks after immunization, the level of LipL32-specific total IgG was significantly increased ($p<0.05$) in mice immunized with pVITRO-*lipL32*, CS-pVITRO-*lipL32* at the N/P ratio of 10:1, and MC-pVITRO-*lipL32* at the N/P ratio of 20:1 when compared with pVITRO (Figure 26). However, the antibody level was not significantly different between mice immunized with CS-pVITRO-*lipL32* and MC-pVITRO-*lipL32* ($p>0.05$) but significantly higher than that of mice immunized with pVITRO-*lipL32* ($p<0.05$). The antibody response of mice immunized with only pVITRO-*lipL32* tended to develop at a slower rate than that of mice immunized with the CS-pVITRO-*lipL32* at the N/P ratio of 10:1 and MC-pVITRO-*lipL32* at the N/P ratio of 20:1. In addition, this study found that after third boost (8 weeks) LipL32-specific total IgG significantly increased in the group immunized with MC-pVITRO-*lipL32* when compare with those immunized with CS-pVITRO-*lipL32*, pVITRO-*lipL32*, and pVITRO ($p<0.05$). The mean level of anti-LipL32 antibodies gradually increased up to the end of the experiment as shown in Table 12.

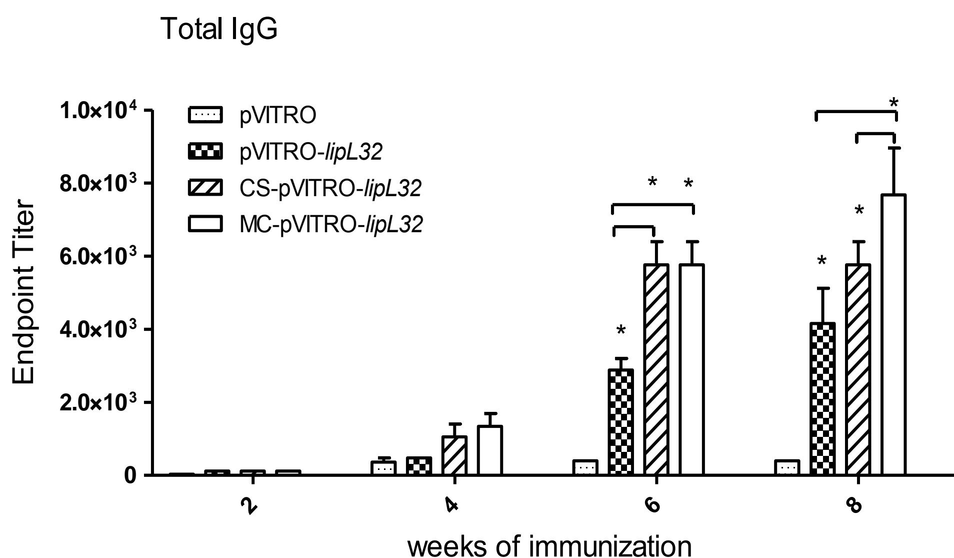


Figure 26. The level of LipL32-specific total IgG after vaccination measured by ELISA. Serum samples from BALB/c mice immunized intramuscularly (IM) with 50 μg of

pVITRO, pVITRO-*lipL32*, CS-pVITRO-*lipL32* at the N/P of 10:1, and MC-pVITRO-*lipL32* at the N/P of 20:1. Data are plotted as a mean of triplicate wells. *$^*P < 0.05$* compared to plasmids control.

Table 12. The mean values of specific total IgG against LipL32 protein in serum of vaccinated mice.

Week of immunization	Immunized with			
	pVITRO (n=3)	pVITRO-*lipL32* (n=5)	CS-pVITRO-*lipL32* (n=5)	MC-pVITRO-*lipL32* (n=5)
Week 1	nd	nd	nd	nd
Week 2	1:100	1:100	1:100	1:100
Week 3	1:400	1:400	1:400	1:400
Week 4	1:400	1:400	1:960	1:1440
Week 5	1:400	1:200	1:400	1:800
Week 6	1:400	1:2880	1:5760	1:5760
Week 7	1:400	1: 2880	1:4160*	1:4800
Week 8	1:400	1:4160	1:5760	1:7680

nd = No determination, * = 1 mouse died during the experiment

At eight weeks of immunization, the level of LipL32-specific IgG isotype (IgG1 or IgG2a) significantly increased in mice immunized with pVITRO-*lipL32*, CS-pVITRO-*lipL32* at the N/P of 10:1, and MC-pVITRO-*lipL32* at the N/P of 20:1 when compared with pVITRO ($p<0.05$). The LipL32-specific IgG2a isotype production was significantly higher in mice immunized with CS-pVITRO-*lipL32* at the N/P ratio of 10:1 than that immunized with MC-pVITRO-*lipL32* at the N/P ratio of 20:1 ($p<0.05$, Figure 27A). However, LipL32-specific IgG1 isotype production was not significant difference in mice immunized with pVITRO, CS-pVITRO-*lipL32* at the N/P ratio of 10:1, and MC-pVITRO-*lipL32* at the N/P ratio of 20:1 (Figure 27B). The antibody response of the vaccinated group tended to develop toward Th1 response (IgG2a) rather than Th2 response (IgG1) as shown in Figure 27.

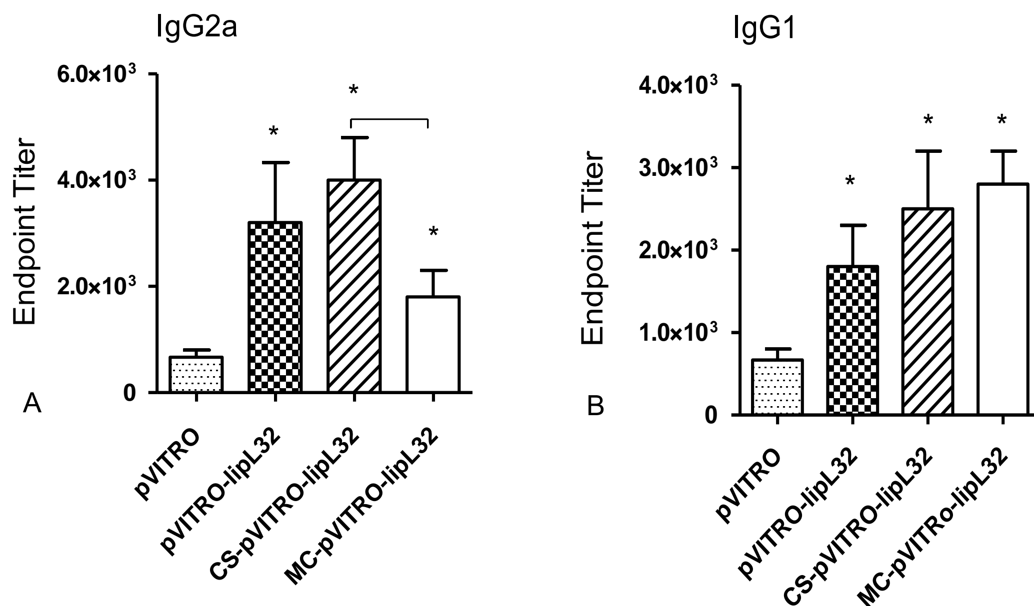
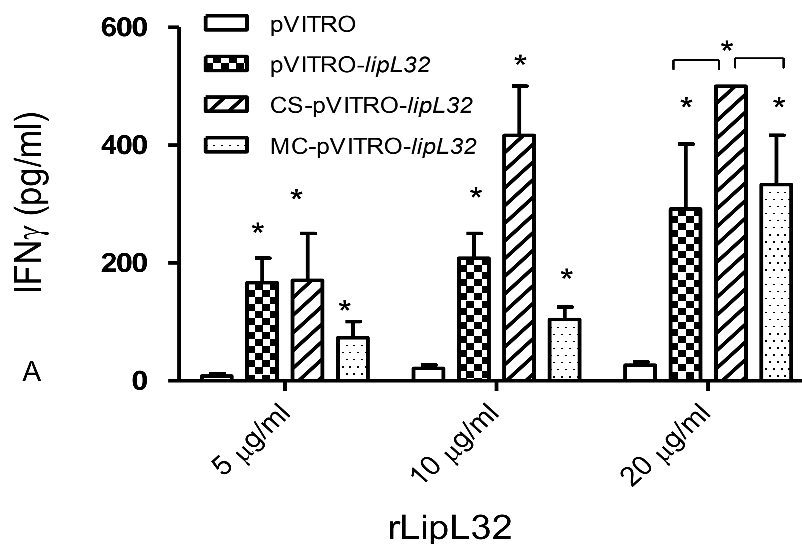


Figure 27. Endpoint titers of LipL32-specific IgG2a isotype (A) and IgG1 isotype (B) at eight weeks of vaccination measured by ELISA. Serum samples from BALB/c mice immunized intramuscularly (IM) with 50 µg of pVITRO, pVITRO-*lipL32*, CS-pVITRO-*lipL32* at a N/P of 10:1, and MC-pVITRO-*lipL32* at a N/P ratio of 20:1. Data are plotted as a mean of triplicate wells. $^*P< 0.05$ compared to the plasmid control.

Cytokine response to *lipL32* DNA vaccine immunization

LipL32-specific IFN-γ production significantly increased in mice immunized with pVITRO-*lipL32*, CS-pVITRO-*lipL32* at the N/P ratio of 10:1, and MC-pVITRO-*lipL32* at the N/P ratio of 20:1 when compared with that of mice immunized with pVITRO ($p<0.05$). However, when splenocytes were stimulated with 20 µg/ml of rLipL32 the IFN-γ level was significantly higher in mice immunized with CS-pVITRO-*lipL32* than that of mice immunized with pVITRO-*lipL32*, and MC-pVITRO-*lipL32* ($p<0.05$, Figure 28A). The LipL32-specific IL-4 production significant increased in mice immunized with pVITRO-*lipL32*, CS-pVITRO-*lipL32* at a N/P of 10:1, and MC-pVITRO-*lipL32* at a N/P of 20:1 when compared with pVITRO ($p<0.05$). However, in response to rLipL32 the IL-4 level was significantly higher from splenoytes of mice immunized with CS-pVITRO-*lipL32* at the N/P of 10:1 than that of pVITRO-*lipL32*, and MC-pVITRO-*lipL32* at the N/P of 20:1 ($p<0.05$, Figure 28B). The cytokine responses of the vaccinated group tended to develop toward Th1 response (IFN-γ) rather than Th2 response (IL-4) as shown in Figure 28.



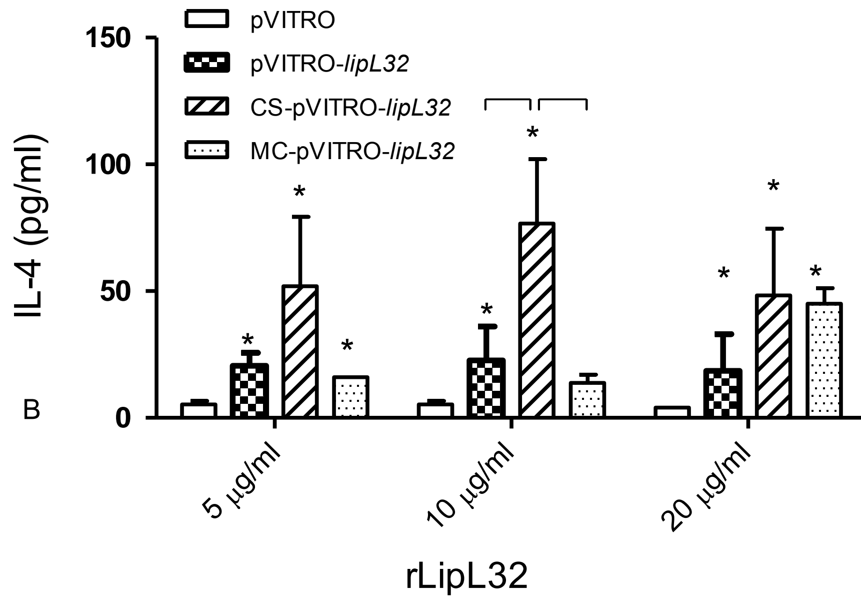
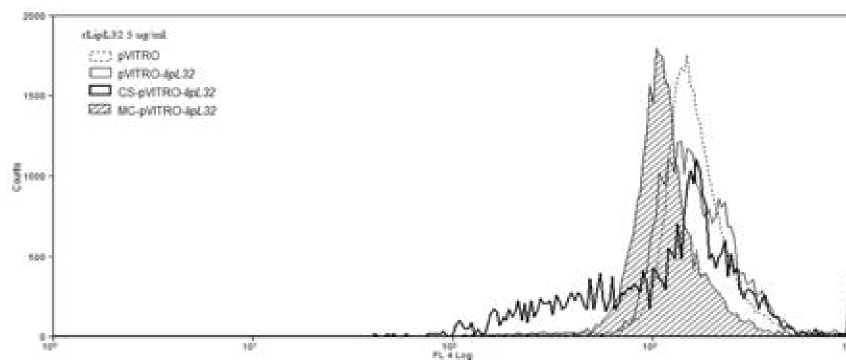


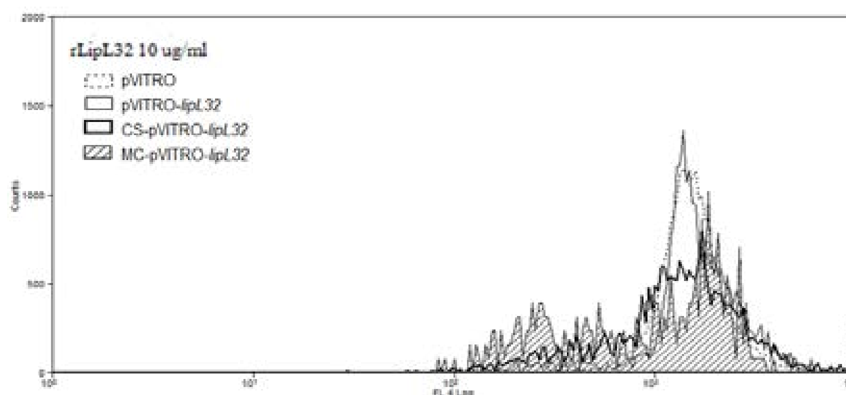
Figure 28. Cytokine responses after DNA vaccine vaccination were measured by ELISA. BALB/c mice were immunized intramuscularly (IM) with pVITRO, pVITRO-*lipL32*, CS-pVITRO-*lipL32* at a N/P of 10:1, and MC-pVITRO-*lipL32* at a N/P of 20:1. The splenocytes were stimulated with rLipL32 (5–20 µg/ml) for 72 hours. The LipL32-specific IFN-γ production (A), and LipL32 specific IL-4 production (B). Results are plotted as mean of triplicate wells. *$P < 0.05$ compared to plasmids control.

Lymphocyte proliferation

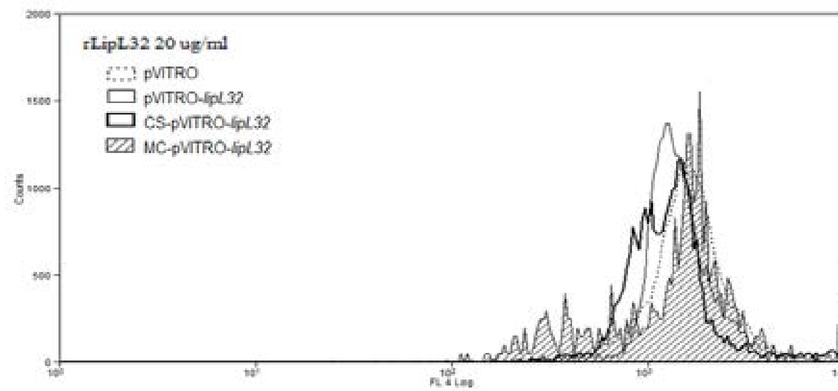
Lymphocytes were stimulated *in vitro* with varying concentrations of rLipL32 (5–20 μg/ml). Lymphocyte derived from BALB/c mice immunized with CS-pVITRO-*lipL32* at the N/P of 10:1, and MC-pVITRO-*lipL32* at the N/P of 8:1 proliferated in response to 10 and 20 μg/ml of antigen (Figure 29B and C). The CS-pVITRO-*lipL32* at the N/P of 10:1, and MC-pVITRO-*lipL32* at the N/P of 20:1 showed a stronger proliferative response to LipL32 antigen than pVITRO-*lipL32* and pVITRO (Figure 29).



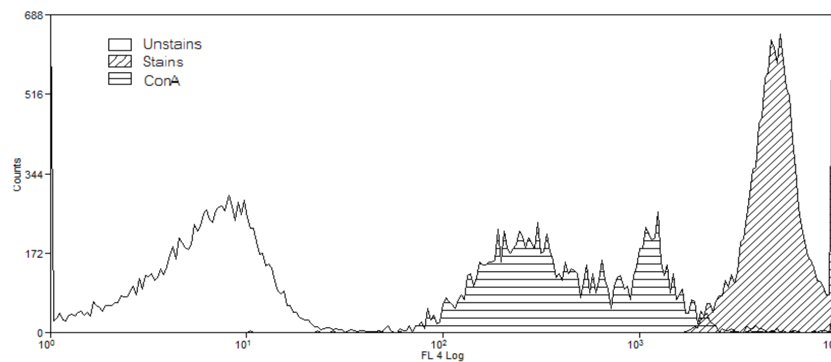
A rLipL32 5μg/ml



B rLipL32 10μg/ml



C rLipL32 20μg/ml



D controls

Figure 29. Cell proliferation after *lipL32* DNA vaccine immunization were analyzed by flow cytometry. BALB/c mice were immunized intramuscularly (IM) with pVITRO, pVITRO-*lipL32*, CS-pVITRO-*lipL32* at the N/P of 10:1, and MC-pVITRO-*lipL32* at the N/P of 20:1. The splenocytes were stimulated with rLipL32; (A) 5 μg/ml, (B) 10 μg/ml, (C) 20 μg/ml for 72 hours. (D) unstained cells and unstimulated cells (negative control), and ConA stimulated cells (positive control).

After stimulation with rLipL32 for 72 hours, the CD4+ T cells derived from mice immunized with pVITRO-*lipL32*, CS-pVITRO-*lipL32* at the N/P of 10:1, and MC-pVITRO-*lipL32* at the N/P of 20:1 produced cytokines (IFN-γ, IL-2, and IL-4) at a significantly higher level than that of cells derived from mice immunized with pVITRO ($p<0.05$). However, IFN-γ level produced from cells obtained from mice immunized with CS-pVITRO-*lipL32* was significantly higher than that of pVITRO-*lipL32*, and MC-pVITRO-*lipL32* ($p<0.05$) (Figure 30) suggesting that CS-pVITRO-*lipL32* mediated stronger immune response toward Th1 response.

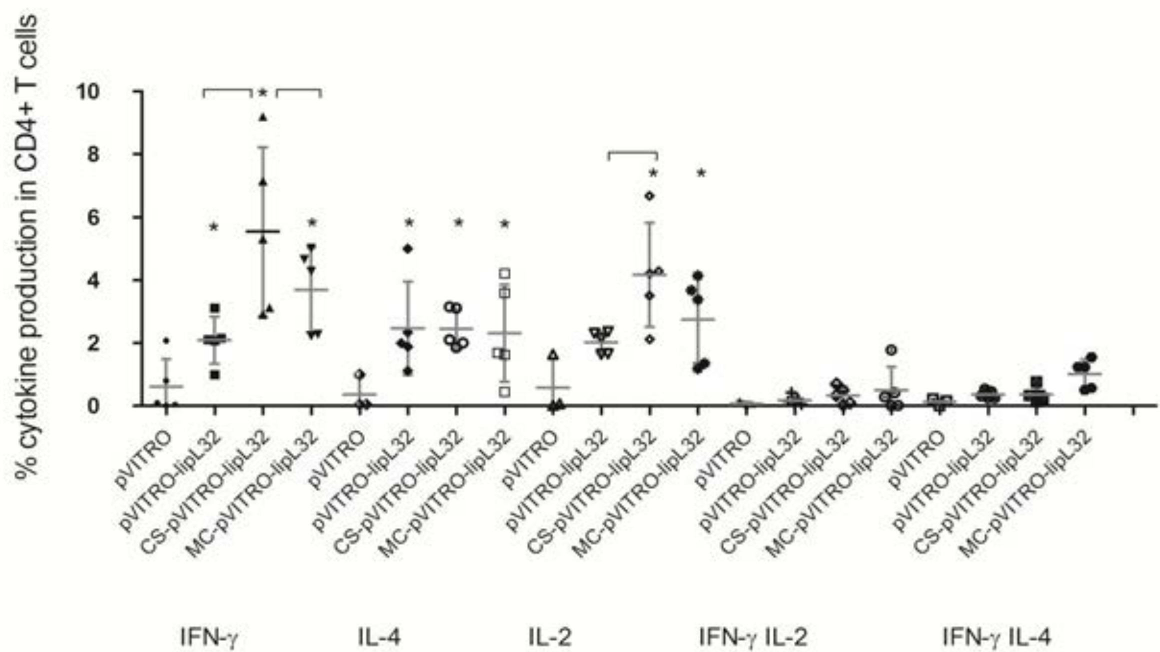


Figure 30. CD4+ T cells produced cytokine in response to *lipL32* DNA vaccine immunization were analyzed by flow cytometry. BALB/c mice were immunized intramuscularly (IM) with pVITRO, pVITRO-*lipL32*, CS-pVITRO-*lipL32* at the N/P of 10:1, and MC-pVITRO-*lipL32* at the N/P of 20:1. The splenocytes were stimulated with 20 μg/ml of rLipL32, isotype control (negative control), and ConA (positive control) for 72 hours.

CHAPTER VI
DISCUSSIONS

Leptospirosis is the most widespread zoonosis in the world. Currently available vaccines for leptospirosis, the heat-killed whole-cell vaccine preparations, stimulate protective immunity only against homologous serovars [28]. This type of vaccine has disadvantages including several side effects such as, pain, nausea, and fever; inducing only short-term immunity and serovar-specific protection. Therefore, the development of new vaccines for leptospirosis has been emphasized on identification of vaccine candidates that are conserved in *Leptospira*, which is expected to be able to prevent infection caused by all pathogenic serovars. Current vaccines are developed in many forms including recombinant protein and DNA vaccines, but so far none has the efficacy to completely prevent the disease and protect against heterologous serovars.

Humoral-mediated immunity (HMI) is important to confer resistance to leptospirosis [18-20]. The monoclonal antibodies directed against leptospiral lipopolysaccharides (LPS) were able to transfer passive protection in neonatal guinea pig [21]. The LPS antibody has been shown to prevent infection caused by only homologous serovar [2]. Cell-mediated immunity (CMI) was demonstrated to be critical for protection of cattle immunized with killed leptospiral vaccine by induction of Th1 response, as demonstrated by interferon (IFN)-γ secretion by CD4+ and TCR$\gamma\delta$+ T cells [23-25]. Therefore, stimulation of appropriate immune response, both humoral-mediated immunity (HMI) and cell-mediated immunity (CMI) possibly in the predilection of Th1 response, may be important for protection against leptospirosis.

LipL32 is the major outer membrane protein found in pathogenic *Leptospira*. Previous studies have evaluated immunization strategies against leptospirosis using LipL32 as a vaccine antigen. For example, combination of LipL32 and LipL41 was reported to stimulate partial immunoprotection against leptospirosis in animal models [34]. Recombinant protein vaccine comprised of LipL32 and LigA was unable to protect hamsters against challenge with virulent leptospires [178]. However, *Mycobacterium bovis* bacille Calmette-Guérin (BCG) and adenovirus vectors expressing LipL32

induced significant protection against pathogenic *Leptospira* challenge [36, 185]. BCG is a strong Th1 stimulator which may be important for protection against leptospiral infection as discussed earlier. These findings indicate that LipL32 can induce protective immunity against leptospiral infection only under certain conditions or formulations.

This study aimed to optimize the leptospirosis vaccine using *lipL32* as a model antigen. DNA vaccine is the type of vaccine of our interest due to its several advantages including easy preparation, stability, and ability to be modified to stimulate both HMI and CMI [193]. We constructed a plasmid containing full-length *lipL32* with natural leader sequence. After transfection, LipL32 was detected in both cell lysate and culture supernatant suggesting that LipL32 was able to be expressed in and secreted outside the mammalian cells. Secreted proteins may act as exogenous antigens and improve the ability to stimulate the immune system for antibody production [193]. LipL32 secreted into the culture supernatant was shown to have higher molecular weight than intracellular LipL32. It is possible that secreted LipL32 was post-translationally modified after transfection since its sequence contains predicted glycosylation site (at amino acid position 44, NET).

Chitosan is a polymer of positively charged N-acetyl-D-glucosamine [37, 204] which can bind to negatively charged DNA [38]. The slow release of DNA from chitosan particle makes it useful for gene delivery [221, 222]. Previous report showed that chitosan particles were effective in induction of local as well as systemic immunity [223]. Chitosan was selected as the delivery system for our *lipL32* DNA vaccine due to its low cost, low cell toxicity, and ability to be modified for cell targeting. Chitosan nanoparticles were used to conjugate *lipL32* DNA vaccine due to its stability and ability to enter cells through endocytosis or pinocytosis leading to enhancing the rate of transfection [224, 225].

Receptor-mediated endocytosis offers the potential to target specific cells and enhance the uptake. In this study, chitosan nanoparticles were modified by mannosylation for cell targeting. Active targeting using receptor-mediated interactions has been shown to be effective in gene delivery [226-229]. Kawakami et al. showed that

mannosylated liposomes exhibited high transfection activity due to recognition by mannose receptors both *in vitro* and *in vivo* [230]. Kim et al. demonstrated that mannosylated chitosan (MC) induced mannose receptor-mediated endocytosis of IL-12 gene directly into dendritic cells resided within the tumor leading to suppression of cancer growth in BALB/c mice bearing CT-26 carcinoma cells [207]. Therefore, mannosylated chitosan nanoparticle should be able to target our DNA vaccine to target cells expressing mannose receptor on their surface, such as macrophages and dendritic cells.

Several studies have shown that nanoparticles are able to induce higher intracellular uptake than that of microparticles [224]. In addition, nanoparticles can induce more inflammatory response than larger particles because the level of inflammatory response depends on the total surface area of particles [231, 232]. After encapsulation with *LipL32*-containing plasmid, chitosan (CS-pVITRO-*lipL32* at the NP ratio of 10:1) and mannosylated chitosan (MC-pVITRO-*lipL32* at the NP ratio of 20:1) remained nanoparticle in size of approximately 500 nm. The chitosan and mannosylated chitosan nanoparticles became more oblong after conjugation with plasmid compared to more spherical morphology of their unconjugated counterparts.

Our results showed that both chitosan and mannosylated chitosan was able to completely encapsulate *lipL32* DNA vaccine construct but at different N/P ratios. DNA-conjugated chitosan has been shown to enhance transfection efficiency in several cell lines [225, 233]. Our results showed that the highest transfection efficiency of MC-pVITRO-*lipL32* was obtained at the higher N/P ratio (20:1) than that of CS-pVITRO-*lipL32* (10:1). Hence, the modification of chitosan with mannose enhanced the transfection efficiency in macrophages (RAW 264.7). The result of this study is consistent with those from previous studies showing that mannose receptor-mediated gene delivery enhanced transfection efficiency [230]. Hashimoto et al. showed that CS- and MC-conjugated DNA improved transfection of mouse peritoneal macrophages through the process of phagocytosis [48]. Chitosan was previously shown to be relatively low cytotoxic on different cell lines [218, 223]. The present study revealed no

cytotoxicity of CS-pVITRO-*lipL32* and MC-pVITRO-*lipL32* since cell viability of transfected cells was similar to that of untransfected control.

After immunization of BALB/c mice, CS-and MC-conjugated plasmid containing *lipL32* significantly increased LipL32-specific total IgG production in comparison to the plasmid control ($p<0.05$). The highest level of total IgG level of mice immunized with MC-pVITRO-*lipL32* was obtained at 8 week of immunization, which was significantly higher than that from mice immunized with CS-pVITRO-*lipL32* and unconjugated plasmid (pVITRO-*lipL32*). The antibody response of the vaccinated group tended to be toward Th1 response (IgG2a) rather than Th2 response (IgG1). In addition, the CS-pVITRO-*lipL32* and MC-pVITRO-*lipL32* was able to stimulate LipL32-specific T cell proliferation and induce mainly IFN-γ cytokine release suggesting a predilection of Th1 stimulation, which is consistent with previous findings of antibody production. The group vaccinated with CS-pVITRO-*lipL32* induced cytokine release and cell proliferation at a higher level than the group immunized with MC-pVITRO-*lipL32* ($p<0.05$).

Our results in this study strongly support the efficacy of chitosan nanoparticles as an adjuvant-carrier system for the effective induction of both humoral and cell-mediated immune responses and should be further tested for its protection efficacy in animal models of leptospirosis.

CHAPTER VII
SUMMARY

The aim of this study was to develop *lipL32* DNA vaccine using mannosylated chitosan nanoparticles as a vaccine delivery system and determine its properties and ability to stimulate humoral (HMI) and cell-mediated (CMI) immune responses in mice. We found that chitosan and mannosylated chitosan nanoparticles were able to encapsulate *lipL32* DNA vaccine and enhance its transfection efficiency into RAW 264.7 macrophage cell line resulting in expression of LipL32 inside the cells with no cytotoxicity in comparison to naked *lipL32* DNA vaccine. At 8 week of immunization, total IgG level of mice immunized with mannosylated chitosan-conjugated *lipL32* DNA vaccine (MC-pVITRO-*lipL32*) was significantly higher than that of chitosan-conjugated plasmid (CS-pVITRO-*lipL32*) and naked plasmid (pVITRO-*lipL32*) suggesting its ability to induce specific HMI. In addition, mannosylated chitosan-conjugated vaccine was capable to stimulate CMI toward Th1 response but at the lower extent than that of chitosan-conjugated vaccine.

Our results demonstrated that the chitosan or mannosylated chitosan nanoparticles can be used as a DNA delivery system for *lipL32* DNA vaccine and can induce both specific humoral and Th1-predilected cell-mediated immune responses. The chitosan- and mannosylated chitosan-conjugated *lipL32* DNA vaccine will be further investigated for their efficacy to protect leptospirosis in animal models.

REFFRENCES

[1] R.C. Johnson. *Leptospira*. (1996).

[2] R.U.M. Palaniappan, *et al.* Leptospirosis: Pathogenesis, immunity, and diagnosis. Current Opinion in Infectious Diseases 20 (2007): 284-292.

[3] B. Adler, A. de la Pena Moctezuma. *Leptospira* and leptospirosis. Vet Microbiol 140 (2010): 287-296.

[4] D.A. Haake. Spirochaetal lipoproteins and pathogenesis. Microbiology 146 (Pt 7) (2000): 1491-1504.

[5] R.C. Johnson, P. Rogers. METABOLISM OF LEPTOSPIRES: II. THE ACTION OF 8-AZAGUANINE. Canadian Journal of Microbiology 13 (1967): 1621-1629.

[6] R.C. Johnson, P. Rogers. Differentiation of Pathogenic and Saprophytic Leptospires with 8-Azaguanine. J Bacteriol 88 (1964): 1618-1623.

[7] N.W. Charon, S.F. Goldstein, Genetics of motility and chemotaxis of a fascinating group of bacteria: The spirochetes 36, 2002, pp. 47-73.

[8] A.R. Bharti, *et al.* Leptospirosis: A zoonotic disease of global importance. Lancet Infectious Diseases 3 (2003): 757-771.

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

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

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

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

[13] สำนักระบาดวิทยา. สรุปรายงานการเฝ้าระวังโรคเลปโตสไปโรซิส. สำนักระบาดวิทยา, กระทรวงสาธารณสุข (2553).

[14] V. Wuthiekanun, *et al.* Clinical diagnosis and geographic distribution of leptospirosis, Thailand. Emerg Infect Dis 13 (2007): 124-126.

[15] W. Tangkanakul, *et al.* Leptospirosis: an emerging health problem in Thailand. Southeast Asian J Trop Med Public Health 36 (2005): 281-288.

[16] K. Laras, *et al.* The importance of leptospirosis in Southeast Asia. Am J Trop Med Hyg 67 (2002): 278-286.

[17] R.D. Ellis, *et al.* Causes of fever in adults on the Thai-Myanmar border. American Journal of Tropical Medicine and Hygiene 74 (2006): 108-113.

[18] P.N. Levett. Leptospirosis. Clinical Microbiology Reviews 14 (2001): 296-326.

[19] A.I. Ko, *et al.* *Leptospira*: the dawn of the molecular genetics era for an emerging zoonotic pathogen. Nat Rev Microbiol 7 (2009): 736-747.

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

[21] B.H. Jost, *et al.* A monoclonal antibody reacting with a determinant on leptospiral lipopolysaccharide protects guinea pigs against leptospirosis. J Med Microbiol 22 (1986): 269-275.

[22] P. Cumberland, *et al.* Persistence of anti-leptospiral IgM, IgG and agglutinating antibodies in patients presenting with acute febrile illness in Barbados 1979-1989. Eur J Epidemiol 17 (2001): 601-608.

[23] R.L. Zuerner, *et al.* A *Leptospira borgpetersenii* serovar Hardjo vaccine induces a Th1 response, activates NK cells, and reduces renal colonization. Clin Vaccine Immunol 18 (2011): 684-691.

[24] R.A. Brown, *et al.* Comparison of three different leptospiral vaccines for induction of a type 1 immune response to *Leptospira borgpetersenii* serovar Hardjo. Vaccine 21 (2003): 4448-4458.

[25] B.M. Naiman, *et al.* Protective killed *Leptospira borgpetersenii* vaccine induces potent Th1 immunity comprising responses by CD4 and gammadelta T lymphocytes. Infect Immun 69 (2001): 7550-7558.

[26] G.R. Klimpel, *et al.* *Leptospira interrogans* activation of human peripheral blood mononuclear cells: Preferential expansion of TCR$\gamma\delta$+ T cells vs TCR$\alpha\beta$+ T cells. Journal of Immunology 171 (2003): 1447-1455.

[27] S.L. Blumerman, *et al.* WC1+ gammadelta T cell memory population is induced by killed bacterial vaccine. Eur J Immunol 37 (2007): 1204-1216.

[28] N. Koizumi, H. Watanabe. Leptospirosis vaccines: past, present, and future. J Postgrad Med 51 (2005): 210-214.

[29] A.I. Ko, *et al. Leptospira*: The dawn of the molecular genetics era for an emerging zoonotic pathogen. Nature Reviews Microbiology 7 (2009): 736-747.

[30] P.A. Cullen, *et al.* Global analysis of outer membrane proteins from *Leptospira interrogans* serovar Lai. Infect Immun 70 (2002): 2311-2318.

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

[32] S.H. Lee, *et al.* Identification and partial characterization of a novel hemolysin from *Leptospira interrogans* serovar lai. Gene 254 (2000): 19-28.

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

[34] C.Y. Feng, *et al.* Immune strategies using single-component LipL32 and multi-component recombinant LipL32-41-OmpL1 vaccines against *Leptospira*. Brazilian Journal of Medical and Biological Research 42 (2009): 796-803.

[35] C. Branger, *et al.* Identification of the hemolysis-associated protein 1 as a cross-protective immunogen of *Leptospira interrogans* by adenovirus-mediated vaccination. Infection and Immunity 69 (2001): 6831-6838.

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

[37] H.C. Arca, *et al.* Chitosan-based systems for the delivery of vaccine antigens. Expert Review of Vaccines 8 (2009): 937-953.

[38] Ü. Guliyeva, *et al.* Chitosan microparticles containing plasmid DNA as potential oral gene delivery system. European Journal of Pharmaceutics and Biopharmaceutics 62 (2006): 17-25.

[39] M. Amidi, *et al.* Preparation and characterization of protein-loaded N-trimethyl chitosan nanoparticles as nasal delivery system. J Control Release 111 (2006): 107-116.

[40] J. Berger, *et al.* Pseudo-thermosetting chitosan hydrogels for biomedical application. Int J Pharm 288 (2005): 197-206.

[41] K. Corsi, *et al.* Mesenchymal stem cells, MG63 and HEK293 transfection using chitosan-DNA nanoparticles. Biomaterials 24 (2003): 1255-1264.

[42] T. Sato, *et al.* In vitro gene delivery mediated by chitosan. Effect of pH, serum, and molecular mass of chitosan on the transfection efficiency. Biomaterials 22 (2001): 2075-2080.

[43] S.A. Agnihotri, *et al.* Recent advances on chitosan-based micro- and nanoparticles in drug delivery. J Control Release 100 (2004): 5-28.

[44] D.A. Zaharoff, *et al.* Chitosan solution enhances both humoral and cell-mediated immune responses to subcutaneous vaccination. Vaccine 25 (2007): 2085-2094.

[45] Z.S. Wen, *et al.* Chitosan nanoparticles act as an adjuvant to promote both Th1 and Th2 immune responses induced by ovalbumin in mice. Marine Drugs 9 (2011): 1038-1055.

[46] G. Peluso, *et al.* Chitosan-mediated stimulation of macrophage function. Biomaterials 15 (1994): 1215-1220.

[47] H.L. Jiang, *et al.* Mannosylated chitosan-graft-polyethylenimine as a gene carrier for Raw 264.7 cell targeting. Int J Pharm 375 (2009): 133-139.

[48] M. Hashimoto, *et al.* Gene transfer by DNA/mannosylated chitosan complexes into mouse peritoneal macrophages. Biotechnol Lett 28 (2006): 815-821.

[49] C.M. Varga, *et al.* Receptor-mediated targeting of gene delivery vectors: insights from molecular mechanisms for improved vehicle design. Biotechnol Bioeng 70 (2000): 593-605.

[50] T. Keler, *et al.* Mannose receptor-targeted vaccines. Expert Opin Biol Ther 4 (2004): 1953-1962.

[51] T. Keler, *et al.* Mannose receptor-targeted vaccines. Expert Opinion on Biological Therapy 4 (2004): 1953-1962.

[52] A.J. Engering, *et al.* The mannose receptor functions as a high capacity and broad specificity antigen receptor in human dendritic cells. European Journal of Immunology 27 (1997): 2417-2425.

[53] Y. Hattori, *et al.* Enhancement of immune responses by DNA vaccination through targeted gene delivery using mannosylated cationic liposome formulations following intravenous administration in mice. Biochemical and Biophysical Research Communications 317 (2004): 992-999.

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

[55] K.V. Evangelista, J. Coburn. *Leptospira* as an emerging pathogen: a review of its biology, pathogenesis and host immune responses. Future Microbiol 5 (2010): 1413-1425.

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

[57] P.A. Cullen, *et al.* Outer membrane proteins of pathogenic spirochetes. FEMS Microbiol Rev 28 (2004): 291-318.

[58] J. Pillot, A. Ryter. [Structure of spirochetes. 1. Study of the genera Treponema, Borrelia and *Leptospira* by the electron microscope]. Ann Inst Pasteur (Paris) 108 (1965): 791-804.

[59] A.A. Varpholomeeva, E.S. Stanislavsky. [Electron microscopy of Leptospira morphology]. Ann Inst Pasteur (Paris) 94 (1958): 361-366.

[60] J.W. Czekalowski. Electron microscope study of *Leptospira*. Antonie Van Leeuwenhoek 29 (1963): 29-34.

[61] G.A. Trueba, *et al.* Characterization of the periplasmic flagellum proteins of *Leptospira interrogans*. J Bacteriol 174 (1992): 4761-4768.

[62] D.J. Brenner, *et al.* Further determination of DNA relatedness between serogroups and serovars in the family Leptospiraceae with a proposal for *Leptospira alexanderi* sp. nov. and four new *Leptospira* genomospecies. Int J Syst Bacteriol 49 Pt 2 (1999): 839-858.

[63] P. Ramadass, *et al.* Genetic characterization of pathogenic *Leptospira* species by DNA hybridization. Int J Syst Bacteriol 42 (1992): 215-219.

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

[65] T.R. Fraga, *et al.* Leptospirosis: aspects of innate immunity, immunopathogenesis and immune evasion from the complement system. Scand J Immunol 73 (2011): 408-419.

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

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

[68] A.L. Nascimento, *et al.* Genome features of *Leptospira interrogans* serovar Copenhageni. Braz J Med Biol Res 37 (2004): 459-477.

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

[70] Leptospirosis worldwide, 1999. Wkly Epidemiol Rec 74 (1999): 237-242.

[71] A.A. Adesiyun, *et al.* Human leptospirosis in the Caribbean, 1997-2005: characteristics and serotyping of clinical samples from 14 countries. Rev Panam Salud Publica 29 (2011): 350-357.

[72] M.A. St John, *et al.* Leptospirosis occurring in two children after fresh water immersion. West Indian Med J 49 (2000): 340-343.

[73] R.M. Barkin, *et al.* Infection by *Leptospira ballum*: a laboratory-associated case. South Med J 67 (1974): 155 passim.

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

[75] J.H. Gollop, *et al.* Rat-bite leptospirosis. West J Med 159 (1993): 76-77.

[76] Y. Liu, *et al.* Pathogenesis of leptospirosis: interaction of *Leptospira interrogans* with in vitro cultured mammalian cells. Med Microbiol Immunol 196 (2007): 233-239.

[77] S.A. Ballard, *et al.* Interactions of virulent and avirulent leptospires with primary cultures of renal epithelial cells. J Med Microbiol 21 (1986): 59-67.

[78] M. Cinco. New insights into the pathogenicity of leptospires: evasion of host defences. New Microbiol 33 (2010): 283-292.

[79] M.J. Kuehn, N.C. Kesty. Bacterial outer membrane vesicles and the host-pathogen interaction. Genes Dev 19 (2005): 2645-2655.

[80] C.Y. Feng, *et al.* Immune strategies using single-component LipL32 and multi-component recombinant LipL32-41-OmpL1 vaccines against *Leptospira*. Braz J Med Biol Res 42 (2009): 796-803.

[81] D.A. Haake, J. Matsunaga. *Leptospira*: a spirochaete with a hybrid outer membrane. Mol Microbiol (2010).

[82] J. Matsunaga, *et al.* Osmolarity, a key environmental signal controlling expression of leptospiral proteins LigA and LigB and the extracellular release of LigA. Infect Immun 73 (2005): 70-78.

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

[84] J. Matsunaga, *et al.* Osmotic regulation of expression of two extracellular matrix-binding proteins and a haemolysin of *Leptospira interrogans*: differential effects on LigA and Sph2 extracellular release. Microbiology 153 (2007): 3390-3398.

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

[86] M.L. Vieira, *et al.* Proteome Analysis of *Leptospira interrogans* Virulent Strain. Open Microbiol J 3 (2009): 69-74.

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

[88] P. Hauk, *et al.* Structure and Calcium-Binding Activity of LipL32, the Major Surface Antigen of Pathogenic *Leptospira* sp. Journal of Molecular Biology 390 (2009): 722-736.

[89] D.E. Hoke, *et al.* LipL32 is an extracellular matrix-interacting protein of *Leptospira* spp. and Pseudoalteromonas tunicata. Infection and Immunity 76 (2008): 2063-2069.

[90] N. Koizumi, H. Watanabe. Molecular cloning and characterization of a novel leptospiral lipoprotein with OmpA domain. FEMS Microbiol Lett 226 (2003): 215-219.

[91] P. Ristow, *et al.* The OmpA-like protein Loa22 is essential for leptospiral virulence. PLoS pathogens 3 (2007).

[92] P.A. Cullen, *et al.* LipL21 is a novel surface-exposed lipoprotein of pathogenic *Leptospira* species. Infection and Immunity 71 (2003): 2414-2421.

[93] M. Pinne, D.A. Haake. A comprehensive approach to identification of surface-exposed, outer membrane-spanning proteins of *Leptospira interrogans*. PLoS One 4 (2009).

[94] D.A. Haake*, et al.* Molecular cloning and sequence analysis of the gene encoding OmpL1, a transmembrane outer membrane protein of pathogenic *Leptospira* spp. Journal of Bacteriology 175 (1993): 4225-4234.

[95] E.S. Shang*, et al.* The rare outer membrane protein, OmpL1, of pathogenic *Leptospira* species is a heat-modifiable porin. Infection and Immunity 63 (1995): 3174-3181.

[96] J. Matsunaga*, et al.* LipL46 is a novel surface-exposed lipoprotein expressed during leptospiral dissemination in the mammalian host. Microbiology 152 (2006): 3777-3786.

[97] H.A. Choy*, et al.* Physiological osmotic induction of *Leptospira interrogans* adhesion: LigA and LigB bind extracellular matrix proteins and fibrinogen. Infection and Immunity 75 (2007): 2441-2450.

[98] Y.P. Lin*, et al.* Calcium binds to leptospiral immunoglobulin-like protein, LigB, and modulates fibronectin binding. Journal of Biological Chemistry 283 (2008): 25140-25149.

[99] P.A. Cullen*, et al.* Surfaceome of *Leptospira* spp. Infect Immun 73 (2005): 4853-4863.

[100] P.N. Levett*, et al.* Detection of pathogenic leptospires by real-time quantitative PCR. J Med Microbiol 54 (2005): 45-49.

[101] J.E. Nally*, et al.* Characterization of the outer membrane proteome of *Leptospira interrogans* expressed during acute lethal infection. Infect Immun 75 (2007): 766-773.

[102] D.A. Haake*, et al.* Molecular evolution and mosaicism of leptospiral outer membrane proteins involves horizontal DNA transfer. J Bacteriol 186 (2004): 2818-2828.

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

[104] C.W. Yang, *et al.* The *Leptospira* outer membrane protein LipL32 induces tubulointerstitial nephritis-mediated gene expression in mouse proximal tubule cells. J Am Soc Nephrol 13 (2002): 2037-2045.

[105] P. Hauk, *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. Infection and Immunity 76 (2008): 2642-2650.

[106] P. Hauk, *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 (2008): 2642-2650.

[107] D.E. Hoke, *et al.* LipL32 is an extracellular matrix-interacting protein of *Leptospira* spp. and Pseudoalteromonas tunicata. Infect Immun 76 (2008): 2063-2069.

[108] P. Hauk, *et al.* Structure and calcium-binding activity of LipL32, the major surface antigen of pathogenic *Leptospira* sp. J Mol Biol 390 (2009): 722-736.

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

[110] J. Croda, *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 (2008): 5826-5833.

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

[112] R. Lux, *et al.* Chemotaxis in pathogenic spirochetes: directed movement toward targeting tissues? J Mol Microbiol Biotechnol 2 (2000): 355-364.

[113] K. Yuri, *et al.* Chemotaxis of leptospires to hemoglobin in relation to virulence. Infect Immun 61 (1993): 2270-2272.

[114] E. Isogai, *et al.* Biological activities of leptospiral lipopolysaccharide. Zentralbl Bakteriol Mikrobiol Hyg A 261 (1986): 53-64.

[115] T. Masuzawa, *et al.* Biological activities and endotoxic activities of protective antigens (PAgs) of *Leptospira interrogans*. Zentralbl Bakteriol 274 (1990): 109-117.

[116] J.E. Nally, *et al.* Changes in lipopolysaccharide O antigen distinguish acute versus chronic *Leptospira interrogans* infections. Infect Immun 73 (2005): 3251-3260.

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

[118] G. del Real, *et al.* Cloning of a hemolysin gene from *Leptospira interrogans* serovar hardjo. Infect Immun 57 (1989): 2588-2590.

[119] Y.X. Zhang, *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 (2005): 453-461.

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

[121] S.H. Lee, *et al.* Cytotoxic activities of *Leptospira interrogans* hemolysin SphH as a pore-forming protein on mammalian cells. Infect Immun 70 (2002): 315-322.

[122] R.P. Segers, *et al.* Molecular analysis of a sphingomyelinase C gene from *Leptospira interrogans* serovar hardjo. Infect Immun 58 (1990): 2177-2185.

[123] F. Merien, *et al.* Invasion of Vero cells and induction of apoptosis in macrophages by pathogenic *Leptospira interrogans* are correlated with virulence. Infect Immun 65 (1997): 729-738.

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

[125] L. Li, *et al.* Comparison of invasion of fibroblasts and macrophages by high- and low-virulence *Leptospira* strains: colonization of the host-cell nucleus and induction of necrosis by the virulent strain. Arch Microbiol 188 (2007): 591-598.

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

[127] B. Stevenson, *et al.* *Leptospira interrogans* endostatin-like outer membrane proteins bind host fibronectin, laminin and regulators of complement. PLoS One 2 (2007): e1188.

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

[129] H.A. Choy, *et al.* Physiological osmotic induction of *Leptospira interrogans* adhesion: LigA and LigB bind extracellular matrix proteins and fibrinogen. Infect Immun 75 (2007): 2441-2450.

[130] A. Verma, *et al.* Leptospiral endostatin-like protein A is a bacterial cell surface receptor for human plasminogen. Infect Immun 78 (2010): 2053-2059.

[131] P. Vijayachari, *et al.* Evaluation of darkground microscopy as a rapid diagnostic procedure in leptospirosis. Indian J Med Res 114 (2001): 54-58.

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

[133] N. Andreescu. 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 (1990): 223-227.

[134] W. Lilenbaum, *et al.* Evaluation of a rapid slide agglutination test for the diagnosis of acute canine leptospirosis. Rev Latinoam Microbiol 44 (2002): 124-128.

[135] T. Senthilkumar, *et al.* Rapid serodiagnosis of leptospirosis by latex agglutination test and flow-through assay. Indian J Med Microbiol 26 (2008): 45-49.

[136] S. Dey, *et al.* Recombinant antigen-based latex agglutination test for rapid serodiagnosis of leptospirosis. Vet Res Commun 31 (2007): 9-15.

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

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

[139] B. Adler, *et al.* Detection of specific anti-leptospiral immunoglobulins M and G in human serum by solid-phase enzyme-linked immunosorbent assay. J Clin Microbiol 11 (1980): 452-457.

[140] M. Mailloux, *et al.* Application of an immuno-enzyme technique to titration of antibodies in leptospirosis: ELISA (enzyme-linked immunosorbent assay). Zentralbl Bakteriol Mikrobiol Hyg A 257 (1984): 511-513.

[141] O. Surujballi, C. Elmgren. 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 (2000): 149-159.

[142] R. Sharma, *et al.* Application of rapid dot-ELISA for antibody detection of leptospirosis. J Med Microbiol 56 (2007): 873-874.

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

[144] S. Dey, *et al.* Recombinant antigen-based dipstick ELISA for the diagnosis of leptospirosis in dogs. Vet Rec 160 (2007): 186-188.

[145] P. Renesto, *et al.* rpoB gene analysis as a novel strategy for identification of spirochetes from the genera Borrelia, Treponema, and *Leptospira*. J Clin Microbiol 38 (2000): 2200-2203.

[146] M.C. Ooteman, *et al.* Evaluation of MAT, IgM ELISA and PCR methods for the diagnosis of human leptospirosis. J Microbiol Methods 65 (2006): 247-257.

[147] M.C. Ooteman, *et al.* Potential application of low-stringency single specific primer-PCR in the identification of *Leptospira* in the serum of patients with suspected leptospirosis. Can J Microbiol 50 (2004): 1073-1079.

[148] M.A. Oliveira, *et al.* Low-stringency single specific primer PCR for identification of *Leptospira*. J Med Microbiol 52 (2003): 127-135.

[149] A.E. Bal, *et al.* Detection of leptospires in urine by PCR for early diagnosis of leptospirosis. J Clin Microbiol 32 (1994): 1894-1898.

[150] F. Merien, *et al.* Polymerase chain reaction for detection of *Leptospira* spp. in clinical samples. J Clin Microbiol 30 (1992): 2219-2224.

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

[152] R. Murgia, *et al.* Oligonucleotides specific for pathogenic and saprophytic leptospira occurring in water. FEMS Microbiol Lett 148 (1997): 27-34.

[153] L.D. Smythe, *et al.* A quantitative PCR (TaqMan) assay for pathogenic *Leptospira* spp. BMC Infect Dis 2 (2002): 13.

[154] C. Sonrier, *et al.* Evidence of cross-protection within *Leptospira interrogans* in an experimental model. Vaccine 19 (2000): 86-94.

[155] A.J. Chapman, *et al.* Antigens recognised by the human immune response to infection with *Leptospira interrogans* serovar hardjo. J Med Microbiol 25 (1988): 269-278.

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

[157] B.H. Jost, *et al.* Experimental immunisation of hamsters with lipopolysaccharide antigens of *Leptospira interrogans*. J Med Microbiol 29 (1989): 115-120.

[158] T. Masuzawa, *et al.* Immunochemical characteristics and localization on cells of protective antigen (PAg) prepared from *Leptospira interrogans* serovar lai. Microbiol Immunol 40 (1996): 237-241.

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

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

[161] C.W. Yang, *et al.* *Leptospira* outer membrane protein activates NF-kappaB and downstream genes expressed in medullary thick ascending limb cells. J Am Soc Nephrol 11 (2000): 2017-2026.

[162] สำนักระบาดวิทยา. สรุปรายงานการเฝ้าระวังโรคเลปโตสไปโรซิส. สำนักระบาดวิทยา, กระทรวงสาธารณสุข (2551).

[163] R. Martínez, *et al.* Efficacy and safety of a vaccine against human leptospirosis in Cuba. Eficacia y seguridad de una vacuna contra la leptospirosis humana en Cuba 15 (2004): 249-255.

[164] Y. Yan, *et al.* An evaluation of the serological and epidemiological effects of the outer envelope vaccine to *leptospira*. Journal of the Chinese Medical Association 66 (2003): 224-230.

[165] G.M. Painter, H.C. Ellinghausen, Jr. Immunising potency of *Leptospira interrogans* serotype canicola after heat inactivation at different temperatures. J Med Microbiol 9 (1976): 487-492.

[166] C.C. Silva, *et al.* Effects of hydrostatic pressure on the *Leptospira interrogans*: high immunogenicity of the pressure-inactivated serovar hardjo. Vaccine 19 (2001): 1511-1514.

[167] B.M. Naiman, *et al.* Protective killed *Leptospira borgpetersenii* vaccine induces potent Th1 immunity comprising responses by CD4 and γδ T lymphocytes. Infection and Immunity 69 (2001): 7550-7558.

[168] E. Shenberg, M. Torten. A new leptospiral vaccine for use in man. I. Development of a vaccine from *Leptospira* grown on a chemically defined medium. J Infect Dis 128 (1973): 642-646.

[169] S.R. Rathinam. Ocular leptospirosis. Curr Opin Ophthalmol 13 (2002): 381-386.

[170] W.T. Hubbert, J.N. Miller. Studies on immunity in experimental leptospirosis: the immunogenicity of *Leptospira icterohemorrhagiae* attenuated by gamma-irradiation. Journal of Immunology 95 (1965): 759-764.

[171] B. Babudieri, *et al.* Comparative tests with formolized and irradiated vaccines against leptospirosis. Bull World Health Organ 48 (1973): 587-590.

[172] H.O. Alpar, *et al.* Strategies for DNA vaccine delivery. Expert Opin Drug Deliv 2 (2005): 829-842.

[173] S.G. Kenzy, *et al.* Comparison of *Leptospira* pomona bacterin and attenuated live culture. Journal of the American Veterinary Medical Association 139 (1961): 452-454.

[174] N.A. Fish, B. Kingscote. Protection of gilts against leptospirosis by use of a live vaccine. Canadian Veterinary Journal 14 (1973): 12-15.

[175] O.H. Stalheim. Vaccination against leptospirosis: protection of hamsters and swine against renal leptospirosis by killed but intact gamma-irradiated or dihydrostreptomycin-exposed *Leptospira* pomona. American Journal of Veterinary Research 28 (1967): 1671-1676.

[176] A. Srikram, *et al.* Cross-protective immunity against leptospirosis elicited by a live, attenuated lipopolysaccharide mutant. Journal of Infectious Diseases 203 (2011): 870-879.

[177] A.D. Odir, *et al.* Recombinant vaccines against leptospirosis. Human Vaccines 7 (2011): 1215-1224.

[178] D.S. Deveson Lucas, *et al.* Recombinant LipL32 and LigA from *Leptospira* are unable to stimulate protective immunity against leptospirosis in the hamster model. Vaccine 29 (2011): 3413-3418.

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

[180] R.U. Palaniappan, *et al.* Immunoprotection of recombinant leptospiral immunoglobulin-like protein A against *Leptospira interrogans* serovar Pomona infection. Infect Immun 74 (2006): 1745-1750.

[181] E.F. Silva, *et al.* The terminal portion of leptospiral immunoglobulin-like protein LigA confers protective immunity against lethal infection in the hamster model of leptospirosis. Vaccine 25 (2007): 6277-6286.

[182] S.M. Faisal, *et al. Leptospira* immunoglobulin-like protein A variable region (LigAvar) incorporated in liposomes and PLGA microspheres produces a robust immune response correlating to protective immunity. Vaccine 27 (2009): 378-387.

[183] W. Yan, *et al.* Immunogenicity and protective efficacy of recombinant *Leptospira* immunoglobulin-like protein B (rLigB) in a hamster challenge model. Microbes Infect 11 (2009): 230-237.

[184] Y. Cao, *et al.* Evaluation of novel fusion proteins derived from extracellular matrix binding domains of LigB as vaccine candidates against leptospirosis in a hamster model. Vaccine 29 (2011): 7379-7386.

[185] C. Branger, *et al.* Identification of the hemolysis-associated protein 1 as a cross-protective immunogen of *Leptospira interrogans* by adenovirus-mediated vaccination. Infect Immun 69 (2001): 6831-6838.

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

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

[188] Y.F. Chang, *et al.* Immunogenicity of the recombinant leptospiral putative outer membrane proteins as vaccine candidates. Vaccine 25 (2007): 8190-8197.

[189] S.M. Faisal, *et al.* Leptosome-entrapped leptospiral antigens conferred significant higher levels of protection than those entrapped with PC-liposomes in a hamster model. Vaccine 27 (2009): 6537-6545.

[190] S.M. Faisal, *et al.* Immune response and prophylactic efficacy of smegmosomes in a hamster model of leptospirosis. Vaccine 27 (2009): 6129-6136.

[191] M.V. Atzingen, *et al.* Characterization of leptospiral proteins that afford partial protection in hamsters against lethal challenge with *Leptospira interrogans*. J Med Microbiol 59 (2010): 1005-1015.

[192] W. Yan, *et al.* Identification and characterization of OmpA-like proteins as novel vaccine candidates for Leptospirosis. Vaccine 28 (2010): 2277-2283.

[193] S. Gurunathan, *et al.* DNA vaccines: immunology, application, and optimization*. Annu Rev Immunol 18 (2000): 927-974.

[194] M.A. Kutzler, D.B. Weiner. DNA vaccines: ready for prime time? Nat Rev Genet 9 (2008): 776-788.

[195] D.J. Shedlock, D.B. Weiner. DNA vaccination: antigen presentation and the induction of immunity. J Leukoc Biol 68 (2000): 793-806.

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

[197] S.M. Faisal, *et al.* Evaluation of protective immunity of *Leptospira* immunoglobulin like protein A (LigA) DNA vaccine against challenge in hamsters. Vaccine 26 (2008): 277-287.

[198] D.F. Hoft, *et al.* Optimizing vaccine development. Cell Microbiol 13 (2011): 934-942.

[199] M.A. Liu. DNA vaccines: an historical perspective and view to the future. Immunol Rev 239 (2011): 62-84.

[200] F. Ahsan, *et al.* Targeting to macrophages: Role of physicochemical properties of particulate carriers - Liposomes and microspheres - On the phagocytosis by macrophages. Journal of Controlled Release 79 (2002): 29-40.

[201] R. Singh, J.W. Lillard Jr. Nanoparticle-based targeted drug delivery. Experimental and Molecular Pathology 86 (2009): 215-223.

[202] M.F. Bachmann, G.T. Jennings. Vaccine delivery: A matter of size, geometry, kinetics and molecular patterns. Nature Reviews Immunology 10 (2010): 787-796.

[203] N. Duceppe, M. Tabrizian. Advances in using chitosan-based nanoparticles for in vitro and in vivo drug and gene delivery. Expert Opinion on Drug Delivery 7 (2010): 1191-1207.

[204] C. Eatmon, A. Loxley. Chitosan: Sources, chemistry, properties & pharmaceutical uses. Drug Delivery Technology 9 (2009): 18-23.

[205] L. Illum, *et al.* Chitosan as a novel nasal delivery system for vaccines. Advanced Drug Delivery Reviews 51 (2001): 81-96.

[206] S. Şenel, *et al.* Enhancing effect of chitosan on peptide drug delivery across buccal mucosa. Biomaterials 21 (2000): 2067-2071.

[207] T.H. Kim, *et al.* Mannosylated chitosan nanoparticle-based cytokine gene therapy suppressed cancer growth in BALB/c mice bearing CT-26 carcinoma cells. Mol Cancer Ther 5 (2006): 1723-1732.

[208] S.M. Hudson, D.W. Jenkins, Chitin and Chitosan, Encyclopedia of Polymer Science and Technology, John Wiley & Sons, Inc., 2002.

[209] K. Bowman, *et al.* Gene transfer to hemophilia A mice via oral delivery of FVIII-chitosan nanoparticles. Journal of Controlled Release 132 (2008): 252-259.

[210] G. Li, *et al.* Induction of Th1-type immune response by chitosan nanoparticles containing plasmid DNA encoding house dust mite allergen der p 2 for oral vaccination in mice. Chinese Journal of Cellular and Molecular Immunology 6 (2009): 45-50.

[211] J.J. Wang, *et al.* Recent advances of chitosan nanoparticles as drug carriers. International journal of nanomedicine 6 (2011): 765-774.

[212] J.M. Irache, *et al.* Mannose-targeted systems for the delivery of therapeutics. Expert Opin Drug Deliv 5 (2008): 703-724.

[213] A.J. Engering, *et al.*, Mannose receptor mediated antigen uptake and presentation in human dendritic cells 417, 1997, pp. 183-187.

[214] P.R. Taylor, *et al.* The mannose receptor: Linking homeostasis and immunity through sugar recognition. Trends in Immunology 26 (2005): 104-110.

[215] E.J. McKenzie, *et al.* Mannose receptor expression and function define a new population of murine dendritic cells. Journal of Immunology 178 (2007): 4975-4983.

[216] C.M. Varga, *et al.* Receptor-mediated targeting of gene delivery vectors: Insights from molecular mechanisms for improved vehicle design. Biotechnology and Bioengineering 70 (2000): 593-605.

[217] J.M. Irache, *et al.* Mannose-targeted systems for the delivery of therapeutics. Expert Opinion on Drug Delivery 5 (2008): 703-724.

[218] H.L. Jiang, *et al.* The potential of mannosylated chitosan microspheres to target macrophage mannose receptors in an adjuvant-delivery system for intranasal immunization. Biomaterials 29 (2008): 1931-1939.

[219] S. Chaemchuen, *et al.* Identification of candidate host proteins that interact with LipL32, the major outer membrane protein of pathogenic *Leptospira*, by random phage display peptide library. Veterinary Microbiology.

[220] T.H. Kim, *et al.* Mannosylated chitosan nanoparticle-based cytokine gene therapy suppressed cancer growth in BALB/c mice bearing CT-26 carcinoma cells. Molecular Cancer Therapeutics 5 (2006): 1723-1732.

[221] K. Romoren, *et al.* Immersion delivery of plasmid DNA. II. A study of the potentials of a chitosan based delivery system in rainbow trout (Oncorhynchus mykiss) fry. J Control Release 85 (2002): 215-225.

[222] C. Prego, *et al.* Efficacy and mechanism of action of chitosan nanocapsules for oral peptide delivery. Pharm Res 23 (2006): 549-556.

[223] K. Khatri, *et al.* Plasmid DNA loaded chitosan nanoparticles for nasal mucosal immunization against hepatitis B. Int J Pharm 354 (2008): 235-241.

[224] D. Moinard-Checot, *et al.* Nanoparticles for drug delivery: review of the formulation and process difficulties illustrated by the emulsion-diffusion process. J Nanosci Nanotechnol 6 (2006): 2664-2681.

[225] K. Corsi, *et al.* Mesenchymal stem cells, MG63 and HEK293 transfection using chitosan-DNA nanoparticles. Biomaterials 24 (2003): 1255-1264.

[226] G.Y. Wu, C.H. Wu. Receptor-mediated in vitro gene transformation by a soluble DNA carrier system. J Biol Chem 262 (1987): 4429-4432.

[227] J.C. Perales, *et al.* An evaluation of receptor-mediated gene transfer using synthetic DNA-ligand complexes. Eur J Biochem 226 (1994): 255-266.

[228] P. Stahl, *et al.* Receptor-mediated pinocytosis of mannose glycoconjugates by macrophages: characterization and evidence for receptor recycling. Cell 19 (1980): 207-215.

[229] T.C. Gust, M. Zenke. Receptor-mediated gene delivery. ScientificWorldJournal 2 (2002): 224-229.

[230] S. Kawakami, *et al.* Mannose receptor-mediated gene transfer into macrophages using novel mannosylated cationic liposomes. Gene Ther 7 (2000): 292-299.

[231] D.M. Brown, *et al.* Size-dependent proinflammatory effects of ultrafine polystyrene particles: a role for surface area and oxidative stress in the enhanced activity of ultrafines. Toxicol Appl Pharmacol 175 (2001): 191-199.

[232] K. Inoue, *et al.* Size effects of latex nanomaterials on lung inflammation in mice. Toxicol Appl Pharmacol 234 (2009): 68-76.

[233] M. Thanou, *et al.* Quaternized chitosan oligomers as novel gene delivery vectors in epithelial cell lines. Biomaterials 23 (2002): 153-159.

APPENDICES

APPENDIX A
BUFFER AND REAGENT

Reagent for EMJH media

1. Albumin fatty acid supplement stock solution

$CaCl_2$+ $MgCl_2$.6 H_2O	0.076	g	Store at -20 ºC
$ZnSO_4$.7H_2O	0.04	g	Store at -20 ºC
$CuSO_4$. 5H_2O	0.03	g	Store at 4 ºC
Vit B12	0.002	g	Store at -20 ºC
Tween 80	1	g	Store at -20 ºC
Glycerol	1	g	Store at -20 ºC

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

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

BSA	5	g
$CaCl_2$+ $MgCl_2$.6 H_2O	750	µl
ZnSO4.7H2O	500	µl
$CuSO_4$. 5H2O	50	µl
Vit B12	500	µl
Tween 80	6.25	ml
Glycerol	500	µl
$FeSO_4$	0.025	g
Sodium pyruvate	0.02	g

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 ºC.

3. Basal Media

Bacto Leptospira Media Base EMJH dehydrated 0.23 g

Dissolve in distilled water and adjust volume with distilled water to make 90 ml.

Sterilize the solution by autoclaving at 121 ºC for 15 minute.

4. EMJH media

Basal media	90	ml
Albumin fatty acid supplement solution	10	ml

Mix the solution and store at 4 ºC.

Reagent for DNA Extraction

1. 0.5 M EDTA pH 8.0

Disodium ethylenediamine tetraacetate	18.66	g

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

2. TE buffer

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

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

3. 10% Sodium lauryl sarcosine (SDS)

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

Mix the solution and store at room temperature.

4. 5 M NaCl(100 ml)

NaCl	14.61	g

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

5. 25:24:1(v/v) Phenol:Chloroform:Isoamyl alcohol

Saturated phenol	50	ml
Chloroform	48	ml
Isoamyl alcohol	2	ml

Mix the reagent vigorously, and store at 4 ºC in dark.

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

Chloroform	48	ml
Isoamyl alcohol	2	ml

Mix the reagent vigorously, and store at 4 ºC in dark.

Reagent for agarose gel electrophoresis

1. 50X Tris-Acetate buffer (TAE)

This base	420	g
Glacial acetic acid	57.1	ml
0.5 M EDTA pH 8.0	100	ml

Dissolve in distilled water and adjust volume to 1000 ml. Sterilize the solution by autoclaving at 121 ºC for 15 minute.

2. Running buffer for agarose gel electrophoresis

50X TAE was diluted to a final concentration of 1X in 500 ml of deionized water.

3. 1% Agarose gel

Agarose gel	1	%
1X TAE	20	ml

The solution was dissolved by heating in microwave oven and occasional mix until on granules of are present.

4. 10 mg/ml Ethidium bromide

Ethidium bromide	1.0	g
Distilled water	100	ml

Mix the reagent vigorously, and store at 4 ºC in dark.

Reagent for cloning

1. 1 M Glucose (10ml)

Glucrose	1.8	g

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

2. 2 M $MgCl_2$(10ml)

$MgCl_2$	1.9	g

Dissolve in distilled water and adjust volume to 1000 ml. Sterilize the solution by autoclaving at 121 ºC for 15 minute.

3. SOB (100 ml)

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

Dissolve in distilled water and adjust volume to 1000 ml. Sterilize the solution by autoclaving at 121 ºC for 15 minute.

4. SOC (10ml)

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

Mix the solution and store at 4 ºC.

5. Ampiclilin stock (100 mg/ml)

Ampiclilin	1	g

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

6. Choramphinicol stock (35 mg/ml)

Choramphinicol	140	mg

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

7. Kanamycin stock (50 mg/ml)

Kanamycin	0.5	g

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

8. Lauria –Bertani (LB) Medium

Bacto tryptone	10	g
Yeast extracts	5	g
NaCl	10	g

Dissolve in distilled water and adjust volume to 1000 ml. Sterilize the solution by autoclaving at 121 ºC for 15 minute.

9. LB- Ampiclilin plates

Bacto tryptone	10	g
Yeast extracts	5	g
NaCl	10	g
Agar	15	g

Dissolve in distilled water and adjust volume to 1000 ml. Sterilize the solution by autoclaving at 121 ºC for 15 minute. After autoclaved, allowed media cool down, added 1ml ampicilin stock, poured and stored plates at 4 ºC.

10. LB- Ampiclilin- Choramphinicol plates

Bacto tryptone	10	g
Yeast extracts	5	g
NaCl	10	g
Agar	15	g

Dissolve in distilled water and adjust volume to 1000 ml. Sterilize the solution by autoclaving at 121 ºC for 15 minute. After autoclaved, allowed media cool down, added 1ml ampicilin stock and Choramphinicol stock, poured and stored plates at 4 ºC.

11. LB- Kanamycin plates

Bacto tryptone	10	g
Yeast extracts	5	g
NaCl	10	g
Agar	15	g

Dissolve in distilled water and adjust volume to 1,000 ml. Sterilize the solution by autoclaving at 121 ºC for 15 minute. After autoclaved, allowed media cool down, added 1ml Kanamycin stock, poured and stored plates at 4 ºC.

Reagent for SDS- Polyacrylamide Gel Ectrophoresis (SDS-PAGE)

1. 1 M Tris-HCl pH 8.8

Tris base	12.11	g

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

2. 0.5 M Tris-HCl pH 6.8

Tris base	6.055	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 ºC for 15 minute.

3. 2X Laemmli buffer (SDS-dye) 10 ml

1M Tris-HCl pH 6.8	1	ml(final concentration 100 mM)
10% SDS	4	ml(4%v/v)
99.5% glycerol	2.01	ml (20% v/v)
HPLC water	2.989	ml
Bromphenol blue	0.001	g

Mix the reagent vigorously, and store at -20 ºC.

4. 4X Tris HCl/SDS pH 8.8 (100 ml)

This base	18.21	g
SDS	0.4	g

Dissolve in distilled water and adjust pH to 8.8 with HCl (conc). stored at 4 ºC.

5. Running buffer

This base	15.1	g
Glycine	72	g
SDS	5.0	g

Dissolve in distilled water and adjust volume to 10 ml. Store at room temperature.

6. 6X sample buffer with DTT

4X Tris-HCl pH 8.8	7	ml
Glycerol	3	ml
SDS	1	g
DTT	0.93	g
Bromphenol Blue	1.2	mg

Dissolve in distilled water and adjust volume to 10 ml. Store at room temperature.

7. 10% Ammonium Persulfate (APS)

APS	1	g
Distilled water	10	ml

Mix the solution and store at -20 ºC

8. 10% Sodium laury sacosine (SDS)

Sodium laury sacosine (SDS)	1	g
Distilled water	10	ml

Mix the solution and store at -20 ºC.

9. 30% Acrymide/0.8 % Bisacrylamide

Acrylamide	30	g
Bis-acrylamide	0.8	g

Dissolve in distilled water and adjust volume to 10 ml. Sterilize the solution by filtration. Store in the dark at room temperature.

10. 15% SDS-PAGE

Separating gel (15ml)

Distilled water	4	ml
1.5 M Tris-HCl pH 8.8	3.75	ml
40% Acrylamide and Bis-acrylamide solution	6	ml
10% SDS	0.15	ml
10% APS	75	µl
TEMED	7.5	µl

Stacking gel

Distilled water	2.7	ml
1.5 M Tris-Hcl pH 6.8	0.5	ml
40% Acrylamide and Bis-acrylamide solution	0.67	ml
10% SDS	40	µl
10% APS	40	µl
TEMED	4.0	µl

Reagent for Western blot

1. TBS

1 M Tris base pH 7.5	20	ml
NaCl	29.22	g

Dissolve in distilled water and adjust volume to 1,000 ml. Sterilize the solution by autoclaving at 121 ºC for 15 minutes.

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

TBS	500	ml
Tween-20	500	µl

Mix the solution and store at room temperature.

3. Bloting buffer

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.

4. Blocking solution

PBST	200	ml
Non-fat dry milk	3	%

Mix the solution and store at 4 Cº.

5. 5X Running buffer

This-base	15.2	g
Glycine	94	g
SDS	5	g
Deionized water	1000	ml

Mix the solution and store at room temperature.

6. Transfer buffer for Western blot

This-base	5.08	g
Glycine	2.9	g
SDS	0.37	g
Deionized water	800	ml
Absolute methanol	200	ml

7. ECL substrate of HRP

Coumaric acid (90 mM) was dissolved in DMSO in total volume 10 ml. Then, the solution aliquots were keep at -20 ºC.

Luminol (250mM) was dissolved in DMSO in total volume 10 ml. Then, the solution aliquots were keep at -20 ºC.

Solution A

100 mM Tris-HCl pH 8.5 (stored at 4 ºC)	4	ml
90 mM coumaric acid	17.6	µl
250 mM luminal	40	µl

Solution B

100 mM Tris-HCl pH 8.5 (stored at 4 ºC)	4	ml
30% H_2O_2	2.4	ml

Reagent for Protein purification

1. Buffer A

0.5 M NaCl	2.922	g
20 mM $C_3H_4N_2$	0.136	g
20 mM Na_2HPO_4-$2H_2O$	0.356	g
20 mM NaH_2PO_4-H_2O	0.276	g

Dissolve in distilled water and add 100 ml. Store at -20 ºC.

2. Buffer B (Vary concentration of imidazole)

60 mM imidazole

0.5 M NaCl	2.922	g
60 mM $C_3H_4N_2$	0.408	g
20 mM Na_2HPO_4-$2H_2O$	0.356	g
20 mM NaH_2PO_4-H_2O	0.276	g

100 mM imidazole

0.5 M NaCl	2.922	g
100 mM $C_3H_4N_2$	0.681	g
20 mM Na_2HPO_4-$2H_2O$	0.356	g
20 mM NaH_2PO_4-H_2O	0.276	g

250 mM imidazole

0.5 M NaCl	2.922	g
250 mM $C_3H_4N_2$	1.7	g
20 mM Na_2HPO_4-$2H_2O$	0.356	g
20 mM NaH_2PO_4-H_2O	0.276	g

500 mM imidazole

0.5 M NaCl	2.922	g
500 mM $C_3H_4N_2$	3.4	g
20 mM Na_2HPO_4-$2H_2O$	0.356	g
20 mM NaH_2PO_4-H_2O	0.276	g

Dissolve in distilled water and add 100 ml. Store at -20 ºC.

Reagent for chitosan and mannosylated chitosan

1. 25mM Na_2SO_4

Na_2SO_4	0.35	g

Dissolve in distilled water and add 100 ml. Sterilize the solution by autoclaving at 121 ºC for 15 minute.

2. 5mM Sodium acetate pH 5.5

Sodium acetate	0.04	g

Dissolve in distilled water and add 100 ml. Sterilize the solution by autoclaving at 121 ºC for 15 minute.

Reagent for cell culture

1. Complete RPMI 1640 100 ml

RPMI 1640	90	%
FBS	10	%
Penicillin	100	U/ml
Streptomycin	0.4	mg/ml
Sodium pyruvate	1	%
HEPES	1	%

2. Complete DMEM 100 ml

DMEM	90	%
FBS	10	%
Penicillin	100	U/ml
Streptomycin	0.4	mg/ml
Sodium pyruvate	1	%
HEPES	1	%

3. Freezing media 10 ml

Complete media	90	%
DMSO	10	%

4. FBS inactivation

Before using FBS, FBS must be inactivated at 56 ºC for 30 minutes using water bath.

5. RIPA buffer for protein extraction 10 ml

50mM Tris-HCl, pH 7.4	1	ml
150 mM NaCl	1.5	ml
1.0% NP-40	100	µl
0.5% $C_{24}H_{39}O_4Na$	1	ml
0.1% SDS	100	µl

Adjusted volume to 10 ml using deionized water.

6. 1XPBS pH 7.4

NaCl	8	g
KCl	0.2	g
Na_2HPO_4	1.44	g
KH_2PO_4	0.24	g
Deionized water	1000	ml

autoclaved at 121ºC and pressure 15 psi for 15 min.

Reagent for Indirect immunofluforsent

1. 4% Paraformaldehyde

Paraformaldehyde (4g) was dissolved in 100 ml of PBS. After addition of a few drops of 1N NaOH, the solution was heated at 65 ºC in a chemical hood. Then, the solution was cooled to room temperature and, the pH adjusted to 7.4.

Reagent for MTT assay

1. MTT 5mg/ml in PBS

MTT	50	mg
Sterile PBS	10	ml

MTT was dissolved in sterile PBS and filtered though a 0.22 µm acrodisc syringe filter. Aliquot in 1.5 ml microcentrifuge tubes and kept at 4 ºC.

2. 0.04 N HCl in Isopropanol

Isopropanol	80	ml
HCl	0.331	ml

Adjust volume to 100 ml using isopropanol in volume metric flask.

Reagent for ELISA (IgG, IgG_1, IgG_{2a})

1. Coating buffer

$NaHCO_3$	7.13	g
Na_2CO_3	1.59	g

Dissolve in distilled water to 1,000 ml and adjust pH to 9.5 with 10N NaOH. Store at room temperature.

2. Blocking buffer

1XPBS	100	ml
Tween 20	50	µl
BSA	1	g

Mix the solution and store at 4 °C.

3. Washing buffer

1XPBS	100	ml
Tween 20	100	µl

Mix the solution and store at room temperature.

4. Stop reaction solution

0.5 M H_2SO_4	2.67	ml
DW	97.33	ml

Mix the solution and store at room temperature.

5. 3,3',5,5'-tetramethylbenzidine (TMB) Substrate

Reagent for ELISA (IFN- γ, IL-4)

IL-4	IFN-γ
1. Capture Antibody Anti-mouse IL-4 monoclonal antibody 2. Detection Antibody Biotinylated anti-mouse IL-4 monoclonal antibody 3. Enzyme Reagent Streptavidin-horseradish peroxidase	1. Capture Antibody Anti-mouse IFN-γ monoclonal antibody (clone AN-18) 2. Detection Antibody Biotinylated anti-mouse IFN-γ monoclonal antibody 3. Enzyme Reagent Streptavidin-horseradish peroxidase

conjugate (SAv-HRP)	conjugate (SAv-HRP)
4. Standards Recombinant mouse IL-4, lyophilized (33 ng/ml)	4. Standards Recombinant mouse IFN- γ, lyophilized (26 ng/ml)

5. Coating buffer

$NaHCO_3$	7.13	g
Na_2CO_3	1.59	g

Dissolve in distilled water to 1,000 ml and adjust pH to 9.5 with 10N NaOH. Store at room temperature.

6. Blocking buffer

1XPBS	90	ml
Tween 20	50	µl
FBS (heat inactivated)	10	µl

Mix the solution and freshly prepare or use within 3 days of preparation, with 2-8°C storage.

7. Washing buffer

1XPBS	100	ml
Tween 20	100	µl

Mix the solution and store at room temperature.

8. Stop reaction solution

0.5 M H_2SO_4	2.67	ml
DW	97.33	ml

Mix the solution and store at room temperature.

9. 3,3',5,5'-tetramethylbenzidine (TMB) Substrate.

Reagent for cell proliferation and Intracellular cytokines staining (ICS)

1. RPMI2ME

RPMI 1640	100	ml
2ME	35	µl

Mix the solution and store at 4 ºC.

2. 1XPBS

NaCl	8	g
KCl	0.2	g
Na_2HPO_4	1.44	g
KH_2 PO_4	0.24	g
Deionized water	1000	ml

Autoclave at 121ºC and pressure for 15 min.

3. Staining buffer

1XPBS	98	ml
1% heat inactivated FBS	1	ml
0.09% (w/v) sodium azide	1	ml

Adjust buffer pH 7.4-7.6, filter (0.2 µm pore membrane), and store at 4 ºC.

APPENDIX B

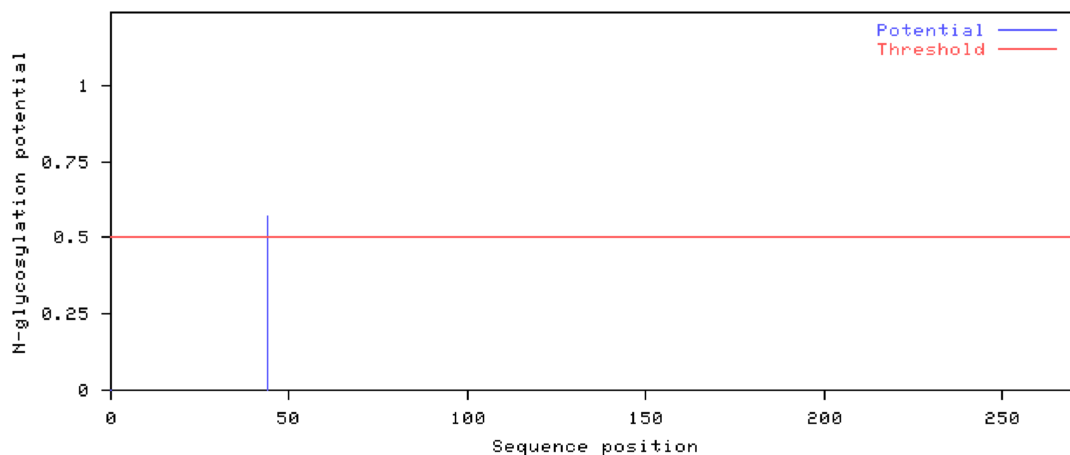
1. Complete sequence of *lipL32* gene: *Leptospira interrogans* serovar Pomona major outer membrane protein (LipL32) gene, complete cds GenBank: EU871716.1

ATGAAAAAACTTTCGATTTTGGCTATCTCCGTTGCACTCTTTGCAAGCATTACC
GCTTGTGGTGCTTTCGGTGGTCTGCCAAGCCTAAAAAGCTCTTTTGTTCTGAGCGAGG
ACACAATCCCAGGGACAAACGAAACCGTAAAAACGTTACTTCCCTACGGATCTGTGAT
CAACTATTACGGATACGTAAAGCCAGGACAAGCGCCGGACGGTTTAGTCGATGGAAA
CAAAAAAGCATACTATCTCTATGTTTGGATTCCTGCCGTAATCGCTGAAATGGGAGTTC
GTATGATTTCCCCAACAGGCGAAATCGGTGAACCAGGCGACGGAGACTTAGTAAGCG
ACGCTTTCAAAGCGGCTACCCCAGAAGAAAAATCAATGCCACATTGGTTTGATACTTG
GATCCGTGTAGAAAGAATGTCGGCGATTATGCCTGACCAAATCGTCAAAGCTGCGAA
AGCAAAACCAGTTCAAAAATTGGACGATGATGATGATGGTGACGATACTTATAAAGAA
GAGAGACACAACAAGTACAACTCTCTTACTAGAATCAAGATCCCTAATCCTCCAAAAT
CTTTTGACGATCTGAAAAACATCGACACTAAAAAACTTTTAGTAAGAGGTCTTTACAGA
ATTTCTTTCACTACCTACAAACCAGGTGAAGTGAAAGGATCTTTCGTTGCATCTGTTGG
TCTGCTTTTCCCACCAGGTATTCCAGGTGTGAGCCCGCTGATCCACTCAAATCCTGAA
GAATTGCAAAAACAAGCTATCGCTGCTGAAGAGTCTTTGAAAAAAGCTGCTTCTGACG
CGACTAAGTAA

2. Predicted glycosylation sites

Amino acid sequence of *lipL32* gene: *Leptospira interrogans* serovar Pomona major outer membrane protein (LipL32) gene, complete cds GenBank: EU871716.1 by using NetNGlyc 1.0 Server – prediction program.

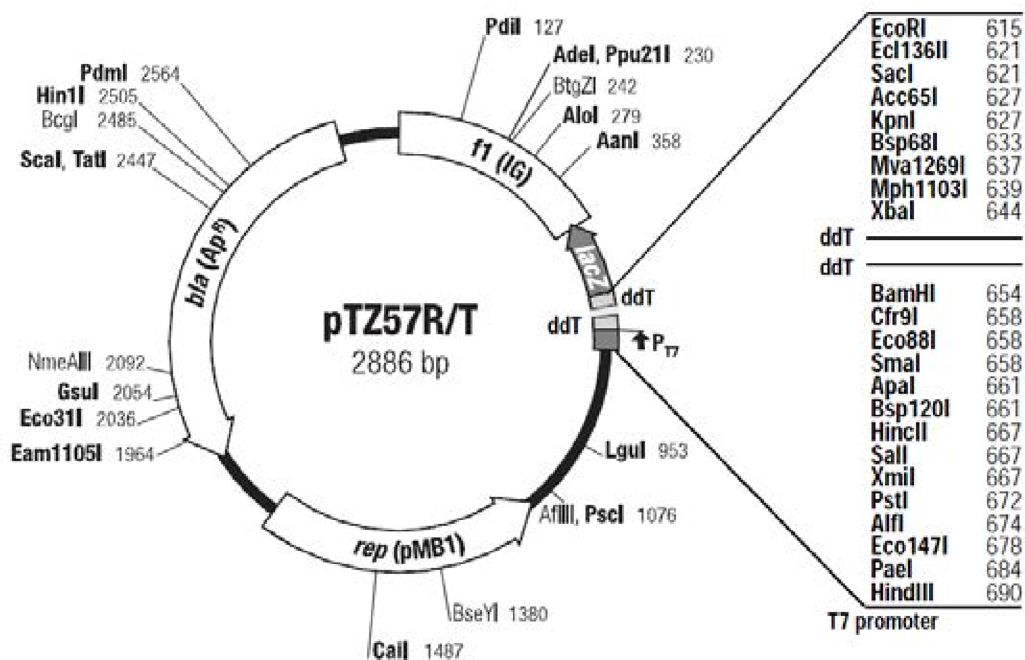
```
Asn-Xaa-Ser/Thr sequons in the sequence output below are highlighted in blue.
    Asparagines predicted to be N-glycosylated are highlighted in red.

Name:  Sequence    Length:  272
MKKLSILAISVALFASITACGAFGGLPSLKSSFVLSEDTIPGTNETVKTLLPYGSVINYYGYVKPGQAPDGLVDGNKKAY     80
YLYVWIPAVIAEMGVRMISPTGEIGEPGDGDLVSDAFKAATPEEKSMPHWFDTWIRVERMSAIMPDQIVKAAKAKPVQKL     160
DDDDDGDDTYKEERHNKYNSLTRIKIPNPPKSFDDLKNIDTKKLLVRGLYRISFTTYKPGEVKGSFVASVGLLFPPGIPG     240
VSPLIHSNPEELQKQAIAAEESLKKAASDATK
...........................................N....................................     80
................................................................................     160
................................................................................     240
................................                                                     320

(Threshold=0.5)
----------------------------------------------------------------------
SeqName      Position  Potential   Jury    N-Glyc
                                agreement  result
----------------------------------------------------------------------
Sequence       44 NETV   0.5716    (7/9)    +
----------------------------------------------------------------------
```



3. pVITRO_*lipL32* (Blast sequence of *lipL32*)

```
                    1       10        20        30        40        50        60        70        80        90        100       110       120       130
                    |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
             lipl32
                 F_ GGAATTTTTGCCCCTTTTTGAGTTTTGGAGCGAGGGTTAATTTTCAAGCCTTTTTAGCGGGTTCAAAGGTATTTTTTAAACCCGTTTGCCAGGTGTTGTGAAAGCCACCGCTAATTCAAAGCAATCCGGA
_pVITRO_lipL32_C1_Bs
          Consensus ..................................................................................................................................

                    131     140       150       160       170       180       190       200       210       220       230       240       250       260
                    |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
             lipl32 ATGAAAAAACTTTCGATTTTGGCTATCTCCGTTGCACTCTTTGCAAGCATTACCGCTTGTGGTGCTTTCGGTGGTCTGCCAAGCCTAAAAAGCTCTTTTGTTCTGAGCGAGGACACAATCCCAGGGACAA
                 F_ ATGAAAAAACTTTCGATTTTGGCTATCTCCGTTGCACTCTTTGCAAGCATTACCGCTTGTGGTGCTTTCGGTGGTCTGCCAAGCCTAAAAAGCTCTTTTGTTCTGAGCGAGGACACAATCCCAGGGACAA
_pVITRO_lipL32_C1_Bs                      GNTGCACCCATTCCTCT---TGCA-GCATTACCGCTTGTGGTGCTTTCGGTGGTCTGCCAAGCCTAAAAAGCTCTTTTGTTCTGAGCGAGGACACAATCCCAGGGACAA
          Consensus atgaaaaaactttcgattttgGcTatctCCgTTgCaCTcttTGCAaGCATTACCGCTTGTGGTGCTTTCGGTGGTCTGCCAAGCCTAAAAAGCTCTTTTGTTCTGAGCGAGGACACAATCCCAGGGACAA

                    261     270       280       290       300       310       320       330       340       350       360       370       380       390
                    |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
             lipl32 ACGAAACCGTAAAAACGTTACTTCCCTACGGATCTGTGATCAACTATTACGGATACGTAAAGCCAGGACAAGCGCCGGACGGTTTAGTCGATGGAAACAAAAAAGCATACTATCTCTATGTTTGGATTCC
                 F_ ACGAAACCGTAAAAACGTTACTTCCCTACGGATCTGTGATCAACTATTACGGATACGTAAAGCCAGGACAAGCGCCGGACGGTTTAGTCGATGGAAACAAAAAAGCATACTATCTCTATGTTTGGATTCC
_pVITRO_lipL32_C1_Bs ACGAAACCGTAAAAACGTTACTTCCCTACGGATCTGTGATCAACTATTACGGATACGTAAAGCCAGGACAAGCGCCGGACGGTTTAGTCGATGGAAACAAAAAAGCATACTATCTCTATGTTTGGATTCC
          Consensus ACGAAACCGTAAAAACGTTACTTCCCTACGGATCTGTGATCAACTATTACGGATACGTAAAGCCAGGACAAGCGCCGGACGGTTTAGTCGATGGAAACAAAAAAGCATACTATCTCTATGTTTGGATTCC

                    391     400       410       420       430       440       450       460       470       480       490       500       510       520
                    |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
             lipl32 TGCCGTAATCGCTGAAATGGGAGTTCGTATGATTTCCCCAACAGGCGAAATCGGTGAACCAGGCGACGGAGACTTAGTAAGCGACGCTTTCAAAGCGGCTACCCCAGAAGAAAAATCAATGCCACATTGG
                 F_ TGCCGTAATCGCTGAAATGGGAGTTCGTATGATTTCCCCAACAGGCGAAATCGGTGAACCAGGCGACGGAGACTTAGTAAGCGACGCTTTCAAAGCGGCTACCCCAGAAGAAAAATCAATGCCACATTGG
_pVITRO_lipL32_C1_Bs TGCCGTAATCGCTGAAATGGGAGTTCGTATGATTTCCCCAACAGGCGAAATCGGTGAACCAGGCGACGGAGACTTAGTAAGCGACGCTTTCAAAGCGGCTACCCCAGAAGAAAAATCAATGCCACATTGG
          Consensus TGCCGTAATCGCTGAAATGGGAGTTCGTATGATTTCCCCAACAGGCGAAATCGGTGAACCAGGCGACGGAGACTTAGTAAGCGACGCTTTCAAAGCGGCTACCCCAGAAGAAAAATCAATGCCACATTGG

                    521     530       540       550       560       570       580       590       600       610       620       630       640       650
                    |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
             lipl32 TTTGATACTTGGATCCGTGTAGAAAGAATGTCGGCGATTATGCCTGACCAAATCGTCAAAGCTGCGAAAGCAAAACCAGTTCAAAAATTGGACGATGATGATGATGGTGACGATACTTATAAAGAAGAGA
                 F_ TTTGATACTTGGATCCGTGTAGAAAGAATGTCGGCGATTATGCCTGACCAAATCGTCAAAGCTGCGAAAGCAAAACCAGTTCAAAAATTGGACGATGATGATGATGGTGACGATACTTATAAAGAAGAGA
_pVITRO_lipL32_C1_Bs TTTGATACTTGGATCCGTGTAGAAAGAATGTCGGCGATTATGCCTGACCAAATCGTCAAAGCTGCGAAAGCAAAACCAGTTCAAAAATTGGACGATGATGATGATGGTGACGATACTTATAAAGAAGAGA
          Consensus TTTGATACTTGGATCCGTGTAGAAAGAATGTCGGCGATTATGCCTGACCAAATCGTCAAAGCTGCGAAAGCAAAACCAGTTCAAAAATTGGACGATGATGATGATGGTGACGATACTTATAAAGAAGAGA

                    651     660       670       680       690       700       710       720       730       740       750       760       770       780
                    |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
             lipl32 GACACAACAAGTACAACTCTCTTACTAGAATCAAGATCCCTAATCCTCCAAAATCTTTTGACGATCTGAAAAACATCGACACTAAAAAACTTTTAGTAAGAGGTCTTTACAGAATTTCTTTCACTACCTA
                 F_ GACACAACAAGTACAACTCTCTTACTAGAATCAAGATCCCTAATCCTCCAAAATCTTTTGACGATCTGAAAAACATCGACACTAAAAAACTTTTAGTAAGAGGTCTTTACAGAATTTCTTTCACTACCTA
_pVITRO_lipL32_C1_Bs GACACAACAAGTACAACTCTCTTACTAGAATCAAGATCCCTAATCCTCCAAAATCTTTTGACGATCTGAAAAACATCGACACTAAAAAACTTTTAGTAAGAGGTCTTTACAGAATTTCTTTCACTACCTA
          Consensus GACACAACAAGTACAACTCTCTTACTAGAATCAAGATCCCTAATCCTCCAAAATCTTTTGACGATCTGAAAAACATCGACACTAAAAAACTTTTAGTAAGAGGTCTTTACAGAATTTCTTTCACTACCTA

                    781     790       800       810       820       830       840       850       860       870       880       890       900       910
                    |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
             lipl32 CAAACCAGGTGAAGTGAAAGGATCTTTCGTTGCATCTGTTGGTCTGCTTTTCCCACCAGGTATTCCAGGTGTGAGCCCGCTGATCCACTCAAATCCTGAAGAATTGCAAAAACAAGCTATCGCTGCTGAA
                 F_ CAAACCAGGTGAAGTGAAAGGATCTTTCGTTGCATCTGTTGGTCTGCTTTTCCCACCAGGTATTCCGGGTGTGAGCCCGCTGATCCACTCAAATCCTGAAGAATTGCAAAAACAAGCTATCGCTGCTGAA
_pVITRO_lipL32_C1_Bs CAAACCAGGTGAAGTGAAAGGATCTTTCGTTGCATCTGTTGGTCTGCTTTTCCCACCAGGTATTCCAGGTGTGAGCCCGCTGATCCACTCAAATCCTGAAGAATTGCAAAAACAAGCTATCGCTGCTGAA
          Consensus CAAACCAGGTGAAGTGAAAGGATCTTTCGTTGCATCTGTTGGTCTGCTTTTCCCACCAGGTATTCCaGGTGTGAGCCCGCTGATCCACTCAAATCCTGAAGAATTGCAAAAACAAGCTATCGCTGCTGAA

                    911     920       930       940       950       960       970       980       990       1000      1010      1020      1030      1040
                    |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
             lipl32 GAGTCTTTGAAAAAAGCTGCTTCTGACGCGACTAAGTAA
                 F_ GAGTCTT---AAACGTCTNCC
_pVITRO_lipL32_C1_Bs GAGTCTTTGAAAAAAGCTGCTTCTGACGCGACTAAGTAACGTACGCCTAGGAGCAGGTTTCCCCAATGACACAAAACGTGCAACTTGAAACTCCGCCTGGTCTTTCCAGGTCTAGAGGGGTAACACTTTG
          Consensus GAGTCTTtgaAAAaagCTgCttctgacgcgactaagtaa..........................................................................................

                    1041 1050       1060      1070      1080      1090      1100      1110      1120      1130      1140      1150      1160      1170
                    |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
             lipl32
                 F_
_pVITRO_lipL32_C1_Bs TACTGCGTTTGGCTCCACGCTCGATCCACTGGCGAGTGTTAATAACACCACTGTTGCTTCCTAACGGAACATGACGGCCGTGGGAACTCCTCCTTGGTAACAAGGACCCACGGGGCCAAAAGCCACGCCC
          Consensus ..................................................................................................................................

                    1171 1180       1190      1200      1210      1220      1230      1240      1250      12601264
                    |--------+---------+---------+---------+---------+---------+---------+---------+---------+---|
             lipl32
                 F_
_pVITRO_lipL32_C1_Bs CACACGGGCCCCGTCATGGTTTGCAACCCCAACAGGGNACTTTACTGGNNAACCCCCTTAANNTNNNNTTGAAACTGGGGACCCCCCCNCCCNG
          Consensus ..............................................................................................
```

4. Map of pTZ57R/T vector



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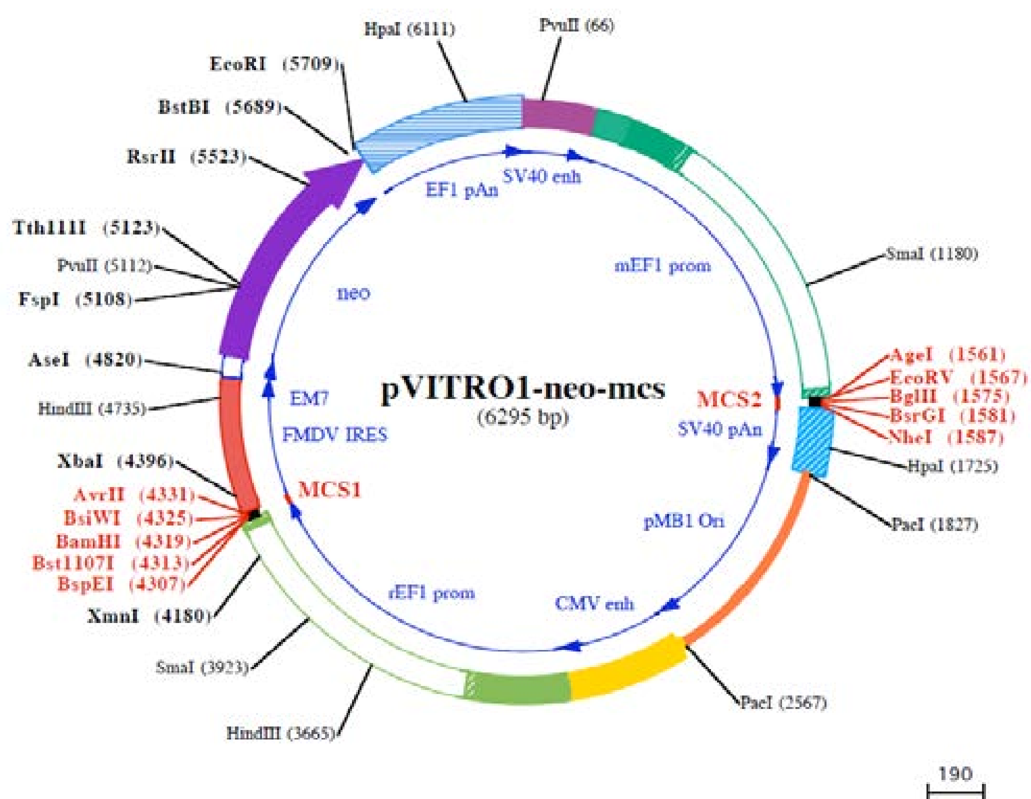
5. Map of pVITRO1- neo-mcs vector



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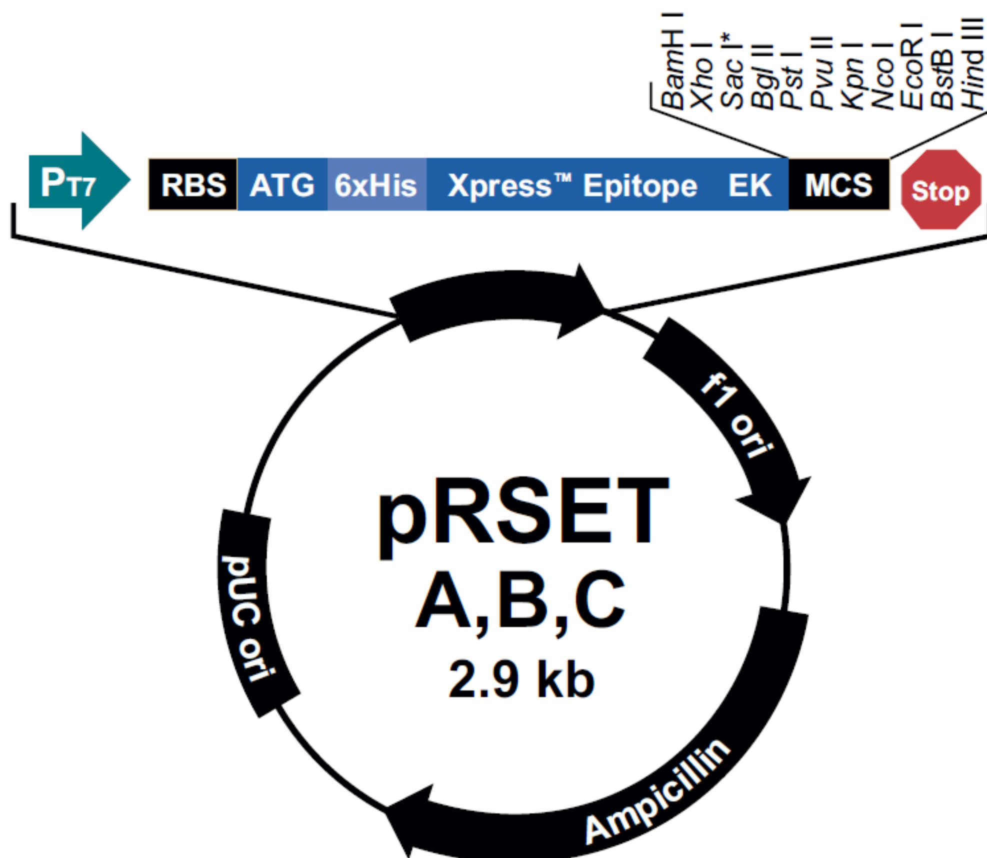
6. Map of pRSETC vector



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PUBLICATION :	Preparation of Chitosan for Leptospirosis Vaccine. Poster presentation in The Eighth Asian Congress for Microcirculation (ACM-2011) Bangkok Convention Center, Centara Grand, Bangkok, Thailand, October 2011:115-118.