การพัฒนาวัคซีนสำหรับโรคเลปโตสไปโรซิสโดยใช้แอนติเจน LipL32 ร่วมกับแอนติเจน Loa22 และให้ในรูปแบบ heterologous prime-boost

นางสาวสุภาวดี อุ้มทอง

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตร์มหาบัณฑิต
สาขาวิชาจุลชีววิทยาทางการแพทย์ (สหสาขาวิชา)
บัณฑิตวิทยาลัย จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2555

DEVELOPMENT OF LEPTOSPIROSIS VACCINE BY USING THE COMBINATION OF LIPL32 AND LOA22 ANTIGENS AND IMMUNIZATION IN A HETEROLOGOUS PRIME-BOOST REGIMEN

Miss Supawadee Umthong

A Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of Master of Science Program in Medical Microbiology

(Interdisciplinary Program)

Graduate School

Chulalongkorn University

Academic Year 2012

Thesis Title	DEVELOPMENT OF LEPTOSPIROSIS VACCINE BY USING THE COMBINATION OF LIPL32 AND LOA22 ANTIGENS AND IMMUNIZATION IN A HETEROLOGOUS PRIME-BOOST REGIMEN
By	Miss Supawadee Umthong
Field of Study	Medical Microbiology
Thesis Advisor	Associate Professor Tanapat Palaga, Ph.D.
Thesis Co-advisor	Assistant Professor Kanitha Patarakul, M.D., Ph.D.

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

.....Dean of the Graduate School
(Associate Professor Amorn Petsom, Ph.D.)

THESIS COMMITTEE

.....Chairman
(Associate Professor Ariya Chindamporn, Ph.D.)

.....Thesis Advisor
(Associate Professor Tanapat Palaga, Ph.D.)

.....Thesis Co-advisor
(Assistant Professor Kanitha Patarakul, M.D., Ph.D.)

.....Examiner
(Associate Professor Sanipa Suradhat, D.V.M., Ph.D.)

.....External Examiner
(Assistant Professor Thareerat Kalambaheti, Ph.D.)

สุภาวดี อุ้มทอง : การพัฒนาวัคซีนสำหรับโรคเลปโตสไปโรซิสโดยใช้แอนติเจน LipL32 ร่วมกับแอนติเจน Loa22 และให้ในรูปแบบ heterologous prime-boost. (DEVELOPMENT OF LEPTOSPIROSIS VACCINE BY USING THE COMBINATION OF LIPL32 AND LOA22 ANTIGENS AND IMMUNIZATION IN A HETEROLOGOUS PRIME-BOOST REGIMEN) อ.ที่ปรึกษาวิทยานิพนธ์หลัก : รศ.ดร. ธนาภัทร ปาลกะ, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม : ผศ.พญ.ดร. กนิษฐา ภัทรกุล, 152 หน้า.

เลปโตสไปโรซิสเป็นโรคติดต่อจากสัตว์สู่คน เกิดจากการติดเชื้อแบคทีเรียเลปโตสไปราชนิดก่อโรค ซึ่งมีความหลากหลายมากกว่า 250 ซีโรวาร์ งานวิจัยนี้มีวัตถุประสงค์ในการพัฒนาวัคซีนโดยใช้แอนติเจนสองชนิด คือ LipL32 และ Loa22 ร่วมกันเป็นวัคซีน และให้ในรูปแบบ heterologous prime-boost โดยให้วัคซีนครั้งแรก (prime) ในรูปของดีเอ็นเอวัคซีนที่บรรจุในอนุภาคนาโนไคโตซานซึ่งใช้เป็นระบบนำส่งดีเอ็นเอเข้าสู่เซลล์ และกระตุ้น (boost) ในรูปของรีคอมบิแนนท์โปรตีนของ LipL32 และ Loa22 พบว่าอนุภาคนาโนไคโตซานสามารถนำส่งดีเอ็นเอพลาสมิดที่มียีน *lipL32* และ *loa22* ทำให้มีการแสดงออกของยีนทั้งสองในเซลล์ไลน์ HEK293T ที่ได้รับการทรานสเฟคชัน ผลจากการปลูกภูมิคุ้มกันในหนูไมซ์ พบว่าการใช้ พลาสมิดนี้เป็นดีเอ็นเอวัคซีนที่มียีน *lipL32* ร่วมกับ *loa22* ในพลาสมิดเดียวกันให้ประสิทธิภาพในการกระตุ้นการสร้างแอนติบอดีได้ดีกว่าการใช้ดีเอ็นเอวัคซีนที่มีพลาสมิดของสองยีนแยกกันแต่ให้ร่วมกัน อีกทั้ง การใช้สองแอนติเจนร่วมกันในการปลูกวัคซีนไม่ได้ส่งผลเสริมกันหรือหักล้างกันในการกระตุ้นภูมิคุ้มกันทั้งในการสร้างแอนติบอดี การเพิ่มจำนวนของทีลิมโฟซัยท์และการหลั่ง ไซโตไคน์ เมื่อเปรียบเทียบกับการใช้เพียงแอนติเจนเดียว ดังนั้น การใช้แอนติเจน LipL32 และ Loa22 ร่วมกันและให้ในรูปแบบ heterologous prime-boost จึงมีศักยภาพในการนำไปศึกษาในสัตว์ทดลองที่เป็นแบบจำลองของโรคต่อไป

สาขาวิชา จุลชีววิทยาทางการแพทย์ ลายมือชื่อนิสิต .....

ปีการศึกษา 2555 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม .....

5387838720 : MAJOR MEDICAL MICROBIOLOGY

KEYWORDS : *lipL32-loa22* DNA vaccine/ LipL32/ Loa22/ heterologous prime-boost immunization/ humoral immune response/ cellular immune response

SUPAWADEE UMTHONG : DEVELOPMENT OF LEPTOSPIROSIS VACCINE BY USING THE COMBINATION OF LIPL32 AND LOA22 ANTIGENS AND IMMUNIZATION IN AHETEROLOGOUS PRIME-BOOST REGIMEN. ADVISOR : ASSOC. PROF. TANAPAT PALAGA, Ph.D., CO-ADVISOR : ASST. PROF. KANITHA PATARAKUL, M.D., Ph.D., 152 pp.

Leptospirosis is a zoonotic disease caused by infection with pathogenic leptospira bacteria which comprise more than 250 serovars. This research aimed at developing vaccine by combining two antigens which are LipL32 and Loa22 as a vaccine and using a heterologous prime-boost regimen for immunization. The immunization was performed by priming with DNA vaccine encapsulated in chitosan (CS) nanoparticle which was used as a DNA delivery system and boosting with LipL32 and Loa22 recombinant proteins. CS was able to deliver DNA plasmid harboring *lipL32* and *loa22* and resulted in the expression of both genes in transfected HEK293T cell line. The study in mice showed that using the DNA vaccine encoding *lipL32-loa22* genes in the same plasmid was better in stimulating antibody production than using co-administration of *lipL32* and *loa22* in different plasmids. Combination of two antigens for immunization provided neither synergistic nor antagonistic effect in induction of immune response including antibodies, lymphoproliferation, and cytokines production, compared with when using a single antigen. Therefore, combination of LipL32 and Loa22 and heterologous prime-boost regimen has potentials for further development which should be tested in an animal model of leptospirosis.

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

Academic Year : 2012 Advisor's Signature .....

Co-advisor's Signature .....

ACKNOWLEDGEMENTS

I would like to extend my sincere thanks to all those who participated in the success of this thesis. I am highly indebted to my advisor Associate Professor Dr. Tanapat Palaga (Department of Microbiology, Faculty of Science, Chulalongkorn University) and my co-advisor, Assistant Professor Dr. Kanitha Patarakul (Department of Microbiology, Faculty of Medicine, Chulalongkorn University) and for their primary supervision of this work, for critical comments, suggestions, the effective training of my logical ideation and efficient communication skills during the whole research period.

I appreciate Assistant Professor Dr. Thareerat Kalambaheti (Department of Microbiology and Immunology, Faculty of Tropical Medicine, Mahidol University) for her kindness to be my external examiner. I would like to express my gratitude towards Associate Professor Dr. Alain Jacquet (Recombinant vaccine unit, Department of Medicine, Faculty of Medicine) for his advice for protein purification. I would like to express my special thanks to Mr. Arun Buaklin for their helps on animal studies. I greatly thanks Associate Professor Dr. Supason Wanichwecharungruang (Department of Chemistry, Faculty of Science, Chulalongkorn University) for the kindness gift of chitosan used in this study. My thanks and appreciations also go to my colleague in 403 Molecular Immunology Laboratory (Faculty of Science, Chulalongkorn University), all members in Leptospirosis Laboratoy (Faculty of Medicine, Chulalongkorn University), and Dr. Jomkhwan Meerak who have willingly helped me out with their abilities and gave me a great lab advice and technical support. I would like to thank for the Development and Promotion of Science and Technology Talents Project (DPST) for financial support cover my living allowance, tuition fees, and other expenses during the study period.

Finally, I would like to thank my family for their encouragement and understanding.

CONTENTS

CHAPTER PAGE

CHAPTER PAGE

LIST OF TABLES

TABLE PAGE

LIST OF FIGURES

FIGURE PAGE

FIGURE PAGE

LIST OF ABBREVIATIONS

APS	Amminium peroxodisulfate
Bp	Base pair
BSA	Bovine serum albumin
°C	Degree Celsius
$C_3N_3NaO_3$	Sodium pyruvate
$C_8N_7N_3O_2$	3-Aminophthalhydraxide,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	Dithiothreitetraacetaol
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
g	Gram
GRAS	generally regarded as safe
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	Immunofluorescent test
IPTG	Isopropyl-D-thiogalactopyranoside
KCl	Patassium chloride
kDa	Kilodalton
KH_2PO_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-diphenyltettazolium bromide
MW	Molecular weight
NaCl	Sodium chloride
Na_2CO_3	Disodium carbonate
$NaHCO_3$	Sodium carbonate
Na_2HPO_4	Disodium hydrogen phosphate
NaN_3	Sodium azide
NaOH	Sodium hydroxide
Na_2SO_4	Sodium sulphate
ng	Nanogram
NH_2CH_2COOH	Glycine

NP-40	Terditol-type NP-40
OMPs	Outer membrane protein
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PMA	Phorbol 12-myristate 13-acetate
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
ZnSO4	Zinc Sulfate

CHAPTER I

INTRODUCTION

Leptospirosis is a zoonotic disease which is found worldwide [1]. It is caused by pathogenic *Leptospira*, which have more than 250 serovars [2]. Reservoir hosts of *Leptospira* include pets such as dogs, live stocks such as dogs, cattle, pigs, and and wildlife animals such as bats. Moreover, animals in the rodent group are major carriers of the disease since they harbor the bacteria in their kidneys but develop no symptoms. These animals can transmit bacteria to other hosts via releasing them in urine into the environment [3]. Infection to human results from the direct contact with urine of infected animals or indirect contact with urine-contaminated-environment [4]. Many symptoms appear after infection. Clinical manifestation may range from a mild flu-like illness to a severely fatal disease [4, 5].

Since *Leptospira* is an extracellular bacterium and the protective immunity to leptospirosis is serovar specific, humoral immune response is considered to be a main type of immune response against *Leptospira* infection [2]. Transferring of monoclonal anti-LPS antibody to newborn guinea pigs provides passive protection [6]. The recent study has shown that protection of guinea pigs from fatal pulmonary hemorrhages induced by serovar Copenhageni challenge resulted from passive immunization with Leptospiral LPS-specific agglutinating but not non-agglutinating monoclonal antibodies [11].

On the other hand, the study in cattle showed that Th1 response is essential as a protective response against *L. borgpetersenii* serovar hardjo infection. Cattle received pentavalent leptospiral vaccines (inactivated whole-cell vaccines containing *L. interrogans* serovars hardjo, canicola, Pomona, and icterohaemorrahagiae and *L. kirschneri* serovar grippotyphosa) failed to protect against *L. borgpetersenii* serovar Hardjo but can effectively prevent infection from other serovars [7]. The immune response conferred reveal that it generated high level of anti-LPS response against *L. borgpetersenii* serovar hardjo [8]. These results are in contrast to the protective result

obtained in cattle receiving a monovalent vaccine of *L. borgpetersenii* serovar *hardjo*that which can stimulate strong Th1 response [9, 10].

Thus far, available leptospirosis vaccines have been produced as whole cell-killed vaccine. Whole-cell leptospirosis vaccines have been used to protect against several serovars of *Leptospira*, including icterohaemorrhagiae, grippotyphosa and Pomona [7]. However, they still have limitations such as the inability to induce cross-protection among pathogenic serovars, generation of short-term immunity, and safety concerns. These shortcomings prevent them to be an effective and reliable vaccine in other areas which has variation in geographic distribution of serovars [12-14]. Subunit vaccines, including recombinant proteins and DNA vaccine, are also developed to overcome the serovar specific-protection problems. Unfortunately, the success of the subunit vaccines has been hampered by weak or short-term immunity, partial protection and unavailability of nontoxic, potent adjuvants [15].

Thus, new vaccine strategies are needed to broadly prevent leptospirosis. The complete genomic DNA sequences of pathogenic *Leptospira interrogans* serovar Lai [16] and Copenhageni [17] are useful tools for the design of novel vaccines, for the prediction of candidate antigens of outer-membrane lipoproteins. Many molecular and cellular studies have been carried out on potential virulence factors, the features of lipopolysaccharide, and the outer membrane proteins (OMPs). Studies of leptospiral OMPs [18] are powerful approach to identify novel vaccine candidates.

LipL32 and Loa22 are some of the candidate antigens for subunit vaccine development against *Leptospira* [19]. A full-length genome analysis of the strain Fiocruz L1-130 has been used to identify candidate antigens for leptospiral vaccine. A total of 206 genes had been predicted and 150 of them were expressed in *E. coli*, purified, and used for immunoblotting with leptospirosis patient sera. Patient sera can react with 16 proteins in immunoblotting including LipL32 and Loa22 [20]. This study indicated that LipL32 and Loa22 are expressed during infection and the host immune response recognized them.

LipL32 is the most abundant constituent of the *L. interrogans* serovar Lai outer-membrane proteome [21] and is shared by pathogenic *Leptospira* genomospecies but is not present in saprophytic genomospecies [22]. This protein is expressed both *in vitro* and *in vivo* during infection [23] and patient sera were found to recognize this protein [24]. Previous studies have shown that LipL32 induces significant protection against leptospiral challenge in a hamster model. Nevertheless, the immune response induced by LipL32 vaccine only confers partial protection with particular formulations [25-27].

Loa22 is a surface-exposed, outer membrane protein containing an OmpA domain [28]. It is conserved among pathogenic serovars and is expressed during both acute and chronic infection [29]. It is recognized by sera from human patients [30]. Loa22 is also essential for virulence in the hamster model of infection [31]. A role is compatible with a protective role in immunity; antibodies directed against Loa22 may bind to the protein, thereby neutralizing its role in pathogenesis. However, the precise function of Loa22 remains undefined. Forty two percents of partial protection by Loa22 (also known as Lp0222) vaccine against *Leptospira* in hamster model was also reported [32]. A complete protection against heterologous challenge in hamster model using live-attenuated LPS mutant of *L. interrogans* serovar Manilae as a vaccine revealed that Loa22 is a potential antigen recognized by serum of immunized animals that were protected [33].

DNA plasmids can directly transfect animal cells and corresponding proteins can be expressed *in vivo* [34]. Therefore, the immune response can be induced upon vaccination by DNA plasmid. DNA vaccines provide prolonged antigen expression, leading to amplification of the immune response. Furthermore, they have several advantages such as easy construction, low cost for mass production, high temperature stability, and the ability to elicit both humoral and cell-mediated immune responses [35, 36].

Chitosan (CS) which is a natural polymer containing polycationic charge from amine group is a good vaccine delivery system since it is non-toxic and generally regarded as safe (GRAS) and easy to prepare. Moreover, the polycationic charged

chitosan make it possible to encapsulate the negative-charged molecule like DNA. Chitosan-DNA complex can be condensed into nanoparticle. This formulation efficiently promote DNA plasmid uptake into the cells.

Moreover, in the field of vaccine development, one particular promising approach is the prime–boost strategy, which has been shown to generate high levels of T-cell memory in animal models [37]. The basic prime–boost strategy involves priming the immune system to immunize with one formulation of vaccine and then selectively boosting this immunity by re-administration of the antigens with another vaccine formulation. The key strength of this strategy is that greater levels of immunity are established by heterologous prime–boost than cannot be attained by a single vaccine administration or homologous boost strategies [38]. In leptospirosis vaccine, the study of lipL32-OmpL1-lipL41 DNA and recombinant protein vaccine revealed that immunization with heterologous prime-boost immunization maintains both humoral and cellular immune response better than when using homologous regimen [39].

Taken together, it can be summarized as follows: 1) partial protection of LipL32 as a vaccine 2) the characteristic of Loa22 as a good vaccine candidate 3) the ability of DNA vaccine in stimulating cell-mediated and humoral immune response 4) the capability of chitosan as a vaccine delivery system, and 5) the efficiency of heterologous prime-boost immunization strategy. In this study, we used the CS nanoparticle as a delivery vehicle synthesized by the complex coacervation method [40] of CS with either of *lipL32 or loa22* or combination of these two antigens and used as a DNA vaccine. We tested whether LipL32 and Loa22 combination and using heterologous prime-boost immunization improves the efficacy of the vaccine in term of inducing humoral and cellular immune responses, and whether LipL32 combining with Loa22 generates better immune response than using only one antigen.

CHAPTER II

OBJECTIVES

Hypotheses

1. Chitosan nanoparticle can be used as a delivery system for *lipL32* and *loa22* DNA vaccine.

2. The combination of LipL32 and Loa22 antigens generate better immune response in terms of antibody production, lymphocyte proliferation and cytokine profiles in response to recall antigen stimulation, when compared with LipL32 or Loa22 alone.

Objectives

1. To develop *lipL32* and *loa22* as a DNA vaccine combination for leptospirosis by using chitosan as a vaccine delivery system.

2. To compare the immune response induced by CS/pVITRO-*lipL32-loa22* with CS/pVITRO-*lipL32*+CS/pVITRO-*loa22* co-administration.

3. To compare the immune response induced by immunization of the combination vaccine of LipL32 and Loa22 with the single LipL32 or Loa22 vaccine by heterologous prime-boost immunization in mice.

CHAPTER III

REVIEW OF RELATED LITERATURE

Leptospira and leptospirosis

Leptospira spp.

Leptospira spp. includes both saprophytic and pathogenic species comprising the genus *Leptospira*, which belongs to the family Leptospiraceae, order Spirochaetales [41]. According to the classification of *Leptospira* spp. based on their genomospecies, pathogenic *Leptospira* spp. comprise 13 strains with more than 250 serovars and saprophytic of *Leptospira* spp. which include 7 species of non-pathogenic strains and contain more than 60 serovars (Table 1). Based on the specificity of epitopes depending on the component and orientation of sugar on lipopolysaccharide (LPS), the system of serovar classification was set [2].

Leptospires are gram-negative, thin helical bacteria with about 0.15 μm wide and 10 to 20 μm long. Leptospires are bacteria in the question mark shaped and have distinctive hooked ends (Figure 1). They have two internal flagella with polar insertion in the periplasmic spaces which promote active movement [42]. They are catalase and oxidase positive [43]. Like other gram negative-bacteria, Leptospires have a typical double membrane structure associated with cytoplasmic membrane and peptidoglycan cell wall. Comparing with other molecules within outer membrane, LPS is the main antigen for *Leptospira*. Nevertheless, other structural and functional proteins are found to be important parts of an outer membrane (Figure 2). Most of the outer membrane proteins are lipoprotein, for examples, LipL32, LipL31, and LipL41. The relative abundance of such proteins are LipL32> LipL21>LipL41 [43]. Besides, other integral membrane proteins such as the porin OmpL1 [44] and the type two secretion system

(T2SS) secretin GspD ([45], are located in the outer membrane of *Leptospira* and exhibit antigenicities.

Table 1 Current species of *Leptospira spp.* [modified from 41]

	Pathogenic *Leptospira spp.*	Non-pathogenic *Leptospira spp.*
	L. alexanderi	*L.biflexa*
	L. alstonii	*L.kmetyi*
	L.borgpetersenii	*L. meyeri*
	L. inadai	*L. yamagawae*
	L. interrogans	*L.wilbachii*
	L. fainei	*L.vanthielii*
	L. kirschneri	
	L. licerasiae	
	L. noguchi	
	L. santarosai	
	L. terpstrae	
	L. weilii	
	L. wolffii	
total	13	7

Leptospires grow at an optimal growth temperature of 28-30 °C, and they are obligate aerobic bacteria. Ellinghausen-McCullough-Johnson-Harris (EMJH) medium is a common medium used for leptospiral culture. Media containing serum or albumin at pH 6.8-7.4 are needed for *Leptospira* growth [46, 47]. Repeated subculture or storage in semisolid agar containing hemoglobin is a traditional method used to maintain *Leptospira*. Long-term storage in liquid nitrogen is useful method to keep its virulence.

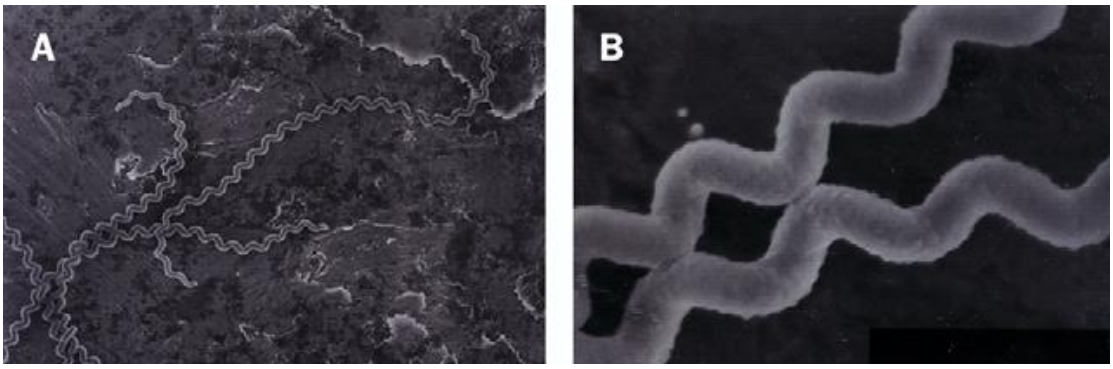


Figure 1 the characteristic of *Leptospira interrogans* serovar Copenhageni under high-resolution scanning electron microscope. (A) The spiral shaped with hooked ends (B) Surface of *Leptospira* under high magnification [1].

Genomics and molecular biology

Leptospiral genomes have a GC content between 35% and 41% and possess two circular chromosomes of around 4 Mb and 300 kb. Six leptospiral genome sequences have been published, including two serovars of *L. interrogans* (Lai and Copenhageni), two strains of *L.borgpetersenii* serovar Hardjo and two strains of *L. biflexa* serovar Patoc [16-17, 48]. *L interrogans* and *L. borgpetersenii* have two circulars. Contrastingly, the saprophyte *L. biflexa* possesses an extra third circular replicon of 74 kb, called p74, which does not present in the pathogenic strain [29].

Genomic comparison of the two pathogenic and one saprophytic species has identified 2052 genes which are common to all. This study provides an advantage to identify genes that are restricted to the pathogenic species. Nonetheless, many genes identified in this manner encode for unknown-functional proteins. For example, 627 genes are unique to *L. interrogans* and more than 80% (500 genes) of these encode for hypothetical proteins and for *L. borgpetersenii*, more than 200 out of 265 (75%) unique genes encode proteins of unknown function. Thus, these data indicate that *Leptospira* possesses unique virulence factors with an unknown function.

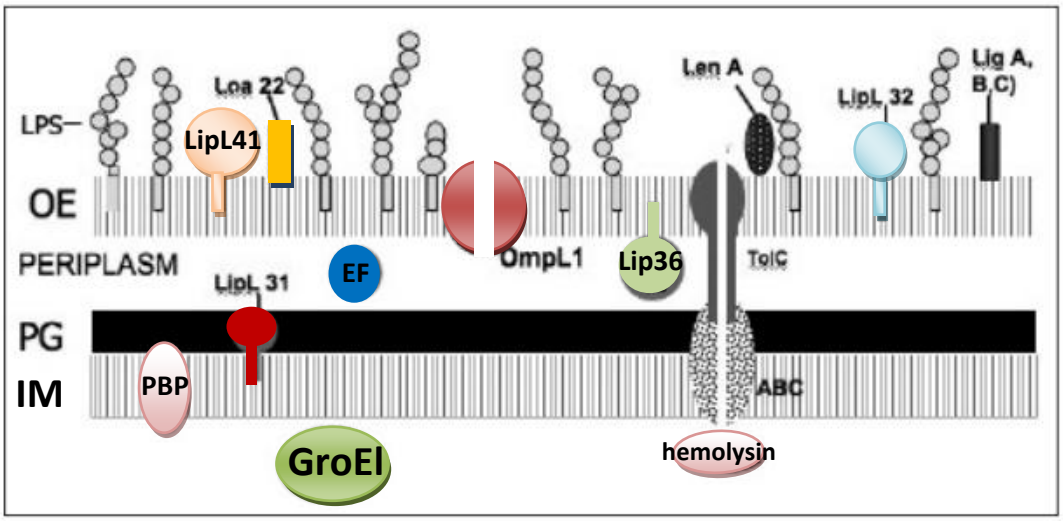


Figure 2. Structure and composition of leptospiral membranes. IM = inner membrane; PG = peptidoglycan closely associated with IM; OE = outer membrane; LPS = lipopolysaccharide. Subsurface proteins include GroEl, the periplasmic flagellum EF, lipoprotein LpL31, penicillin binding proteins PBP. The OE contains the transmembrane proteins including porin OMpL1. Type 1 efflux system is represented by TolC transmembrane protein, forming a complex with the ATP binding cassette transporter ABC to export cytoplasmic component such as hemolysin. Loa22, LipL41, LipL36, LipL32, Len A, Lig A, B, C, are the surface exposed proteins [modified from 52].

Leptospirosis

Leptospirosis is a zoonotic disease which is caused by infection with pathogenic leptospires. It is a worldwide health problem, especially in tropical countries. Human and domestic animal such as dogs, cattle, and swine (Table 2) can be infected and systemic disease can develop, manifested by fever, renal and hepatic failure, pulmonary and reproductive problem. Clinical signs are quite variable ranging from flu-like illness to severe multiple organ failure. Direct or indirect contact with urine or tissue

of infected animal is the main route of infection to susceptible hosts. Leptospirosis recovering-animals may turn to become carriers. They harbor bacteria in the renal tubules for a particular period and shed the contaminated urine into environment. Some species of mice and rats are reservoirs for their host-related serovars [1]. They usually have no symptoms, but harbor leptospires in their kidneys. This reservoir serves as a vital source of bacteria and plays a vital role of leptospirosis infection to human and others susceptible hosts.

Human is generally infected by directly receiving the bacteria from animal via being in a direct contact with urine or indirectly from leptospires polluted-water. The carriers of bacteria may be wild or domestic animals, especially rodents and small marsupials, cattle, pigs and dogs (Figure 3). Though, many organs of carrier or infected animal have leptospires, proximal renal tubules serve as a main source of infection. Once leptospires are excreted out with urine, the bacteria can reside in soil and water. *Leptospira* spp. may survive in environment by biofilm formation [53].

Table 2. Typical reservoir hosts of common leptospiral serovars [1]

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

The severity of the infection in human depending on the serovar of *Leptospira*, age, health status, and immunity of infected individual. Infection causes an acute febrile illness during the early 'leptospiraemic' phase. In this period, however, the infection may not be detected and sometimes misdiagnosis as it has no specific clinical manifestation. The progression of the disease in the late immune phase which may cause severe multisystem manifestations such as hepatic dysfunction and jaundice, acute renal failure, pulmonary haemorrhage syndrome, myocarditis and meningoencephalitis (Figure 3 and 5) and may lead to death [60].

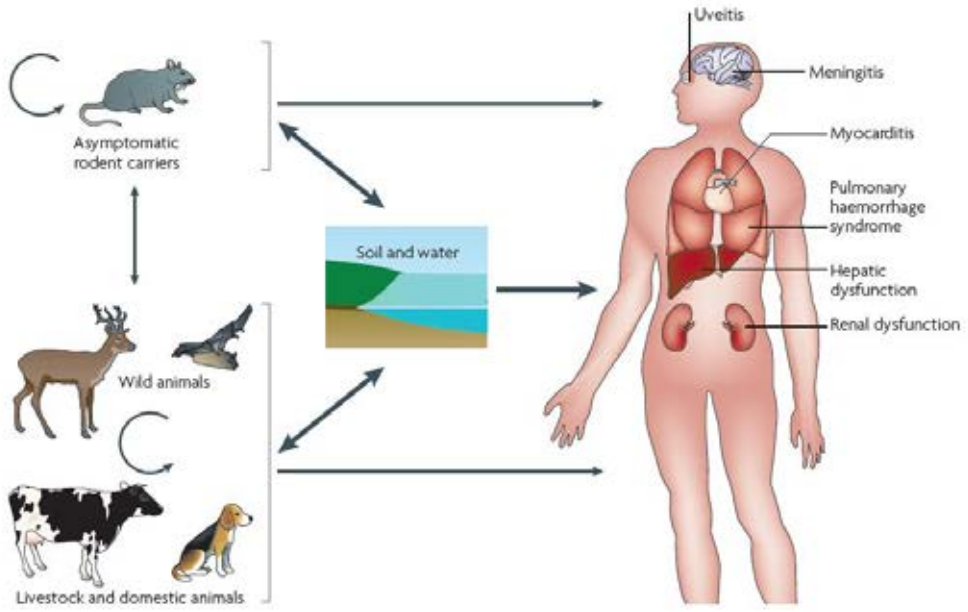


Figure 3. The transmission cycle of leptospirosis. Many animals serve as hosts and carriers of pathogenic *Leptospira* spp. *Leptospira* are secreted into environment via urinary shedding. Rodent species are asymptomatic but are important of transmission. Human acquire infection via direct contact with Leptospires from infected animal or accidentally receive bacteria from Leptospires polluted-environment. Leptospires enter into body via abraded skin or mucous membranes, circulate in blood and disseminate throughout the body tissue and finally cause multi-organ failure [3].

Epidemiology

Leptospirosis distributes worldwide, and the prevalence is higher in tropical regions than in temperate countries [54]. The warm and humidity conditions support longer survival of leptospires. Leptospirosis is not limited to developing countries. The incident rate is high in summer or fall in temperate regions and during rainy seasons or flood in warm climate regions [55].

. Occupation is an associated critical risk factor for humans [56]. Some occupation is at higher risk to be in contact with infected animal or *Leptospira*-contaminated environment than other careers such as farmers, veterinatrians, rodent control workers [57-59].

Increased in infection cases may result from bathing or accidental immersion in the contaminated water of liver or lake. Some leptospiral serovars are generally related with specific host. Therefore, the prevalence of different leptospiral serovars may occur in human depending on the reservoirs that contain different serovar of *Leptospira* [1]. Nevertheless, the association of specific serovars and the severity of the disease have not been described. [55, 61-62].

Protective clothing is useful to prevent the infection, however, it is difficult to implement and it is not practicable to suggest those workers to avoid contacting animals or environment contaminated by the urine of animals. Without vaccination, prevention of leptospirosis is much challenging; especially in the area that basic sanitation is limited and occupational hygiene insufficiency.

Leptospirosis in Thailand

Leptospirosis is also an emerging health problem in Thailand. The reported incidences of leptospirosis have increased since 1996. The number of reported cases per year of leptospirosis was at 398 cases in 1996 but increased to 14,285 cases in 2000 and decreased but kept at high level at 10,217, 6,864, and 4,958 cases in 2001, 2002, and 2003, respectively (Figure 4A). Fifteen thousand cases with 400 deaths in the lastest outbreak (1997, 1999) is an important re-emerging period. Recently updated information from the Ministry of Public Health [63] show that between 1 Jan 2011 and 14 Dec 2011, there are a total of 3,699 cases and 66 fatalities from 69 provinces. The attack rate was 5.82 per 100,000 populations. The case fatality rate (CFR) was 0.10 percent. Between 1 Jan 2012 and 4 Aug 2012, a total of 1,779 cases and 27 fatalities were reported from 70 provinces. Generally, in September and October, the incidence peak increases correlating with rainy season (Figure 4B). Almost all cases (90%) were reported in the Northeast of Thailand with the fatality rate of 4.4%. Almost all of them are predominantly associated with male farmers who are around 15 to 45 years old. Rather than being admitted into the hospital, approximately 90% of them are outpatient.

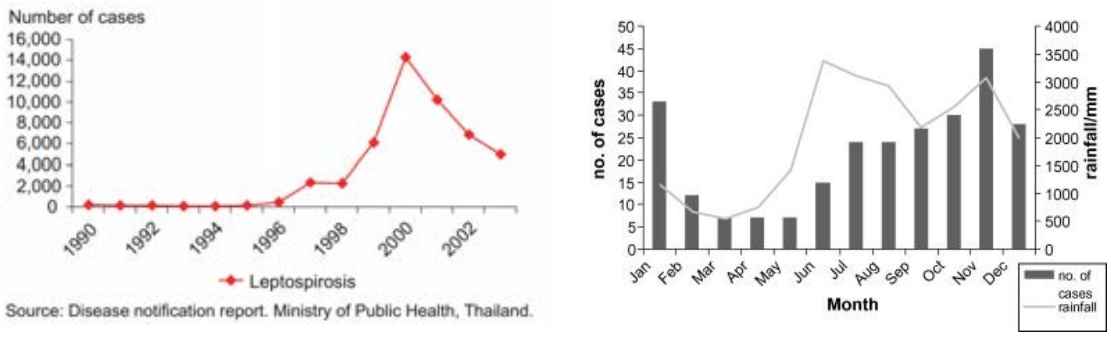


Figure 4. Number of leptospirosis cases in Thailand from 1990 to 2002 (A). Monthly distribution of confirmed Leptospirosis cases (B) [64, 65].

Pathogenesis

Knowledge about pathogenesis of leptospirosis is still unclear. Interaction of host-microbe via bacteria adhesions may facilitate for colonization of bacteria. Many pathogenic bacteria produce a surface layer or adhesions to use in the first step of interaction with the host [21, 66]. LPS of leptospiral outer membrane can stimulate host immune response [67-69]. After systemic dissemination, *Leptospira* spp. adheres and colonizes host cells resulting in tissue damage [1, 3].

During the penetrating steps, haemolysins, proteases and motility may promote pathogenic *Leptospira* spp. entering into host cell using those molecules to destroy connective tissue [70]. This mechanism was based on the evidence of the analysis of *L. interrogans* genome which was found to conatin nine-genes encoding for haemolysins, a pore-forming protein and a sphingomyelinase. These genes were not found in the saprophyte *L. biflexa* [29, 71]. Moreover, a microbial collagenase of *L. interrogans* may contribute in destroying host tissue.

Since the expression of proteins that relate to mammalian host infection are usually regulated by osmolarity and temperature [72, 82, 85], environmental-sensitive protein may be involved in the survival of *Leptospira* in the environment or in the infected hosts [48]. For instance, nineteen genes out of 25 genes in *L. interrogans* encode for hypothetical proteins that can be induced by salt [82].

Binding of extracellular matrix proteins of host cell is also a critical step contributing to bacterial pathogenesis. Previous studies show that many outer membrane proteins of pathogenic *Leptospira* spp. such as Loa22 [74], LipL32 [75], Lsa21 [76], LigA and LigB [77, 78] bind to extracellulat matrix of host cell such as fibronectin, fibrinogen, collagen and laminin. Mutagenesis provides useful tools to study

pathgenesis of leptospirosis. A *LipL32* mutant strain is still as virulent as the wildtype of *L. interrogans* [79]. LigA, LigB and LigC are immunoglobulin-like protein and surface exposed proteins [80, 81]. A LigB mutation did not reduce virulence of *L. interrogans* [83]. Both LigA and LigB bind fibronectin and their expressions are upregulated under conditions of physiological osmolarity [84]. Mutation in *Loa22* reduced virulence of *L. interrogans* in guinea pig and hamster model [31]. Thus, *Loa22* is the first gene that was identified as a true virulent factor of *Leptospira*. However, the function of Loa22 remains unknown and a Loa22 homologue is found in *L. biflexa*. More detail of the molecular mechanism studies point out that many proteins of *Leptospira* have redundancy in functions involving in adhesion, survival *in vivo* and renal colonization. These characteristics make it difficult to identify the virulence factors with a single gene inactivation.

Immune response against *Leptospira* infection

Humoral immune response

Humoral immune response is considered to be the primary mechanism of immunity to leptospirosis in humans and most animal species, including dogs, pigs, guinea pigs and hamsters [86]. Because they are extracellular bacteria and antibody produced upon infection is serovar specific, LPS is the main molecule for induction of host immune response [88]. After exposure, leptospiraemia produces in the first few days then followed by dissemination of leptospires to the target tissues of multiple organs. A fever develops in correlation with the present of agglutinating antibodies within 5–14 days after exposure. Leptospires are cleared from the bloodstream and organs because the titers of serum agglutinating antibodies increase (Figure 5). The

correlation between passive transfer immunity and agglutinating LPS-specific antibodies levels of transferred sera indicates that anti-LPS antibody may be a protective response [87]. Passive protection of naive animals from leptospirosis can be conferred by LPS-passive immunization with specific monoclonal antibodies [6]. However, it is not known whether antibody responses against leptospiral antigens other than LPS also confer protection. Outer membrane proteins or lipoproteins are the focus of intense interest to study their roles in protection.

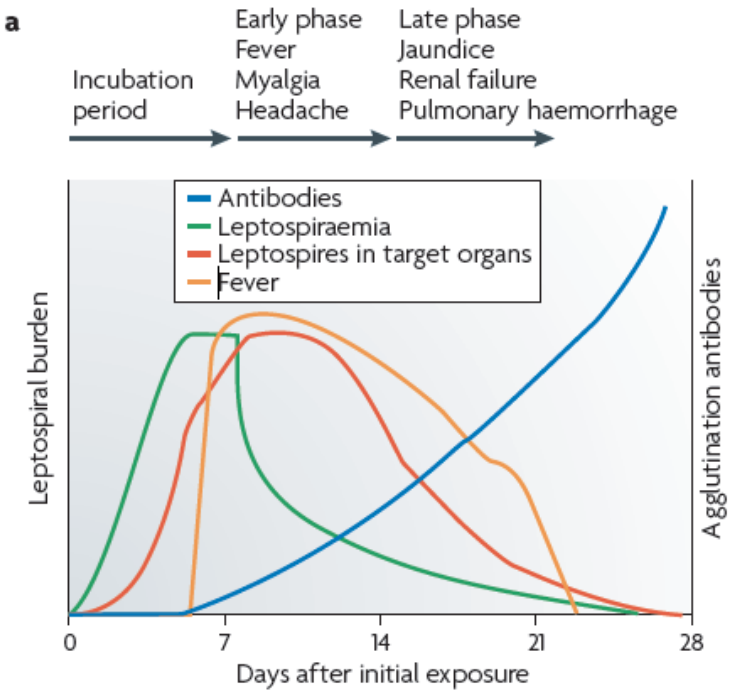


Figure 5. The correlation of antibody responses and the progression of the disease. After infection, leptospires circulate in blood and disseminate into target organs. However, increasing in the number of bacteria in blood and target organ is a negative correlation with the increasing of agglutinating antibody in host [3].

Cellular immune response

In contrast to the humoral immune response in human and many species of animals, cellular immunity is suspected to be important in cattle. Cattle receiving whole leptospire-based vaccines generate high levels of serum agglutinating antibodies but cannot be protected from challenging with the same serovar of *Leptospira* [7]. Surprisingly, the agglutinating antibodies from those susceptible cattle can transfer passive immunity to hamsters. Hence, protective immune mechanisms in different animal species are clearly different.

Recent studies pointed out a role of cell-mediate immune response in protection against leptospirosis in cattle. Th1 response mediated by IFN-γ release provides protection against *L. interrogans* serovar Hardjo [89]. Peripheral blood mononuclear cells (PBMCs) from cattle immunized with a killed *L. borgpetersenii* serovar Hardjo vaccine proliferated and produced IFN-γ after *in vitro* stimulation with leptospiral antigens [89]. $CD4^+$ T cells were the main source of IFN-γ, but $CD8^+$ and $\gamma\delta$ T cells also produced this cytokine [90]. PBMCs from non-vaccinated cattle responded to leptospiral antigens, but the responses were lower than those of vaccinated cattle. The low cell-mediated immune response in unvaccinated cattle correlated with the poor protection from chronic infection [91]. Thus, protective immunity against serovar Hardjo infection in cattle correlates with establishment of Th1 immunity.

Vaccine for leptospirosis

Killed-whole cell vaccine

The first evidence that killed leptospire vaccine provides protection against *Leptospira* infection was described in 1916 [92]. The bacteria-based vaccines have been generally used to immunize livestock, domestic animals, and human [5]. Almost all of the vaccines were prepared from whole leptospiral cells killed by various methods, including heat, formalin, phenol, irradiation etc [2, 93]. Commercial *Leptospira* vaccines are available globally for cattle, pigs and dogs but vaccination provides only partial effectiveness, because of the serovar restricted response induced by whole cell-based vaccine. Killed whole cell leptospiral vaccines for humans are available in some countries such as China, Japan, and Cuba [103-105]. In all cases, repeated annual revaccination is recommended to maintain immunity. The efficacy rates of whole cell vaccines were about 60-100%. Nonetheless, it needs to be reformulated if the new serovar is present [94]. The limitation in cross protection of whole cell-based vaccine is a major obstacle since there are more than 250 serovars among the pathogenic leptospires. The local variability in serovars of endemic leptospiral strains makes it impossible to develop whole cell vaccine that can be used worldwide [2, 106]. Moreover, the side effects of the whole cell vaccines were reported [73]. In addition to side-effects, the whole cell vaccine may induce autoimmune diseases, such as uveitis [107]. To reduce side effect, outer envelope vaccine for leptospirosis was studied in China [108]. The results of the study showed a good protection with less side effects and higher agglutinating titer than those in a whole cell-based vaccine.

Live attenuated vaccine

Variation of carbohydrate composition of lipopolysaccharide (LPS) results in the antigenic diversity among pathogenic leptospires. The protective immunity conferred by leptospiral LPS as an immunogen is generally serovar specific. The administration of *L. biflexa* LPS preparation in hamsters was protective against a challenge with virulent *L. interrogans* serovar Manilae without any side effects [134]. However, this type of vaccine takes a risk in term of reverse mutation to be virulent if it is incomplete inactivation.

Subunit vaccine

Owning to the drawbacks in protection across serovars, adverse side effect, duration of protection, and safety concern of whole cells vaccine and live-attenuated vaccine, subunit vaccines become promising new vaccine formulation against leptospirosis. The identification of proteins, which are conserved among pathogenic leptospires that can generate cross-protection against various serovars, has become a major focus of leptospirosis vaccine research. The immunogenic proteins, especially the outer membrane surface proteins, of pathogenic *Leptospira,* may be effective agents. Subunit vaccines have fewer side effects than the killed-whole cell vaccine. Sera from patients with leptospirosis have antibodies against several protein antigens [24]. Protein extracts prepared from a pathogenic *Leptospira* can induce protective immunity against challenge with a heterologous serovar strain in an experimental animal model [109]. These data emphasizes the potentiality of leptospiral proteins that could apply for cross serovar protective vaccines.

Subunit vaccines that have been developed and tested for the protective efficacy in animal models are summarized in Table 3. These data include DNA vaccine and recombinant protein vaccine. Most studies are based on using candidate protein that located on the outer membrane (Figure 2). The studies can be divided into four broad groups; 1) LipL32, 2) LipL41-OmpL1, 3) LigA and LigB, 4) OmpA family

lipoprotein, and others new recombinant protein. The protection depends on a particular formulation, serovars and innoculum dose of leptospires used for challenge.

Table 3. Subunit vaccine candidate for Leptospirosis [Modified from 3]

Antigen	Adjuvant	Animal model	Inocula	serovar	%Protection	Ref.
LipL41-OmpL1	*E. coli* OMVs	Hamsters	10^2	Grippotyphosa	40-100	95
LipL32	Adenovirus	Gerbils	10^4	Canicola	73-75	27
LigA and LigB	Freünd's	Mice	10^6	Manilae	90-100	80
LipL32	DNA	Gerbils	10^7	Canicola	39	25
LigA	Alum	Hamsters	10^8	Pomona	100	96
C-terminus of LigA	Freünd's	Hamsters	250	Copenhagini	67-100	97
LipL32	BCG	Hamsters	10^2	Copenhageni	50	26
LigA	DNA	Hamsters	10^8	Pomona	100	98
LigB	Alum	Hamsters	10^5	Pomona	67-86	99
C-terminus of LigA	Liposome	Hamsters	10^5	Pomona	88	15

LipL32	LTB	Hamsters	10^2	Copenhageni	80-85	101
Lp4337	EMULSIGEN-D	Hamsters	2.5×MLD_{50}	Pomona	75	19
Lp3685	EMULSIGEN-D	Hamsters	2.5×MLD_{50}	Pomona	58	19
Loa22	EMULSIGEN-D	Hamsters	2.5xMLD_{50}	Pomona	42	101
LIC10325	Alum	Hamsters	10^2	Copenhageni	33.3	101
LIC13059	Alum	Hamsters	10^2	Copenhageni	33.3	102
LigA domain 10, 13	Freünd's	Hamsters	10^3	Copenhageni	100	102

Candidate antigens used in this study

LipL32/Hap-1

LipL32 is one of the most abundant proteins in *Leptospira*, accounting for 75% of the outer membrane proteome [21, 23]. LipL32 is an outer membrane lipoprotein that is conserved, both genetically and immunologically, in the various pathogenic leptospires [110], whereas there are no orthologues in the saprophytic *L. biflexa* [29]. LipL32 was long thought to be a putative virulence factor. Higher levels of LipL32 are expressed in leptospires during acute lethal infections than during *in vitro* culture [22]. In addition, the C-terminus of LipL32 binds *in vitro* to laminin, collagen I, collagen IV, collagen V and plasma fibronectin [75, 111] which play a role in pathogenesis. Furthermore, the crystal structure of LipL32 was recently elucidated and was shown to have structural homologies with proteins such as collagenase that bind to components of the extracellular matrix [112]. However, a LipL32-mutant strain, obtained by *Himar1* insertion mutagenesis, was found to be as efficient as the wild-type strain in causing acute disease and chronic colonization in experimental animals [79]. Hence, LipL32 not play role in *Leptospira* virulence. LipL32 antigen induces antibodies production in patients with leptospirosis. A recombinant LipL32 antigen has good sensitivity and specificity when used in an ELISA for detecting human leptospirosis IgG [113]. LipL32 is also called haemolysis associated protein-1 (Hap-1) because *E. coli* harboring the plasmid encoding this gene showed some haemolytic activity on sheep red blood cells, but not on human erythrocytes [114]. LipL32 stimulates the expression of both *MCP-1* and *iNOS* mRNAs and augments the nuclear binding of NF-kB and AP-1 transcription factors in cultured mouse proximal tubule cells [115]. Vaccination using an adenovirus vector encoding the *lipL32/hap-1* gene induced cross-protection in the gerbil model of leptospirosis [27]. The *lipL32/hap-1* gene derived from *L. interrogans* serovar

Autumnalis conferred protective immunity against a heterologous challenge with *L. interrogans* serovar Canicola. However, OmpL1, either alone or in combination with LipL32/Hap-1, had no protective activity. In the recent study based on immunofluorescence, it was shown that LipL32 is sub-surface protein not surface exposed protein [116]. Even there are many studies relating to LipL32, the protection of LipL32 vaccine seems to be controversial [117]

Loa22

Loa22 is a surface-outer membrane protein, lipoprotein 22 kDa with a C-terminal OmpA domain [28]. Linking between the outer membrane and the peptidoglycan layer is the function of OmpA, which play a role in the maintenance of the structure [38, 118]. *Loa22* is curretly the only gene that follow Koch's molecular postulates. Mutation by insertion of the transposon *Himar1* into a gene encoding Loa22 results in reduction in virulence of *L. interrogans* in guinea pig and hamster models. In addition, complementation of the mutantation restored the virulence of *L. interrogans* in both animal models [31]. Besides, Loa22 increase expression during acute infection of guinea pigs [22]. Loa22 was found in urine from chronically infected rats and was recognized by serum from patients [30]. However, gene *loa22* has ortholog with saprophytic *L. biflexa* [29]. Differential expressions of this gene in non-pathogenic and pathogenic may be dependent on pathogen-specific sialic acid modification pathway [3]. The indirect correlation of Loa22 in cross protection was demonstrated by live attenuated-LPS mutant (M1352) vaccine. Serum from hamsters which provide 100% protection after vaccinated with M1352 recognized only three proteins, including Loa22. This study clearly indicates that Loa22 may correlate with a complete protection against homologous and heterologous challenge [33]. Nevertheless, no study has ever shown

the protective efficacy of Loa22 in a DNA vaccine format. Furthermore, there are no direct evidences of cross protective efficacy of Loa22 against heterologous challenge in animal model. Up until now, there is only one study describing the immune protection of Loa22 recombinant protein vaccine which provides protective efficacy of 42% in hamster challenged with *L. interrogans* serovar Pomona [19]. Thus, efficacy of protection of Loa22 needs further investigations.

Immunization strategies used in this study

1. **Induction of both humoral and cellular immune response by DNA vaccine**

Not only good candidate antigens contribute to the protection of vaccine, the appropriate immune response generated by a particular vaccine formulation is also needed. From the available knowledge in the literatures, it can be assumed that immune responses needed for protection against leptospiral infection is mainly humoral immune response. However, the study in cattle also demonstrated the indispensible effect of Th1 immunity in protection against the disease. DNA vaccine has ability in induction of both humoral and cell mediated immune responses (Figure 6) and have benefits in terms of saving cost and the easiness in preparation when compared to live attenuated and protein subunit vaccine (Table 4). The mechanism of induction of humoral and cellular immune response by a DNA vaccine was described in Figure 6.

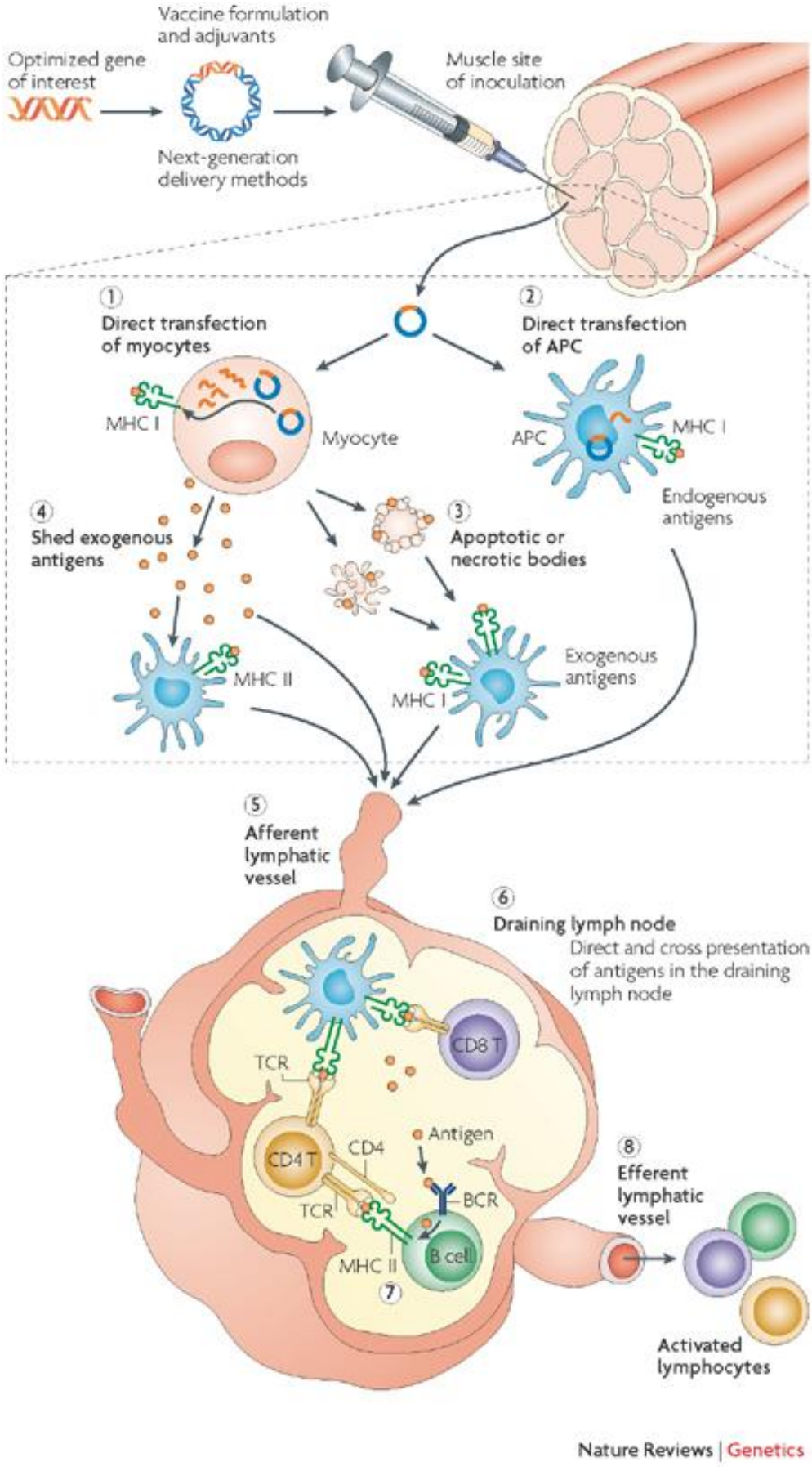
Table 4. Comparative analysis of various vaccine formulations [131]

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

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

[b] Data available only from Phase 1 trials.

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



Nature Reviews | Genetics

Figure 6. Immune responses induced by DNA vaccine [130]

I. Optimization of interested gene, antigenic, adjuvant. Gene of interested was amplified and cloned into expression vector, purified, and then delivered to the body via i.m. or s.c. Usually, intramucuscularly injection allow plasmid to enter to the nucleus of myocytes (1) and of resident antigen presenting cells (APCs) (2)

II. In the resident APCs, inserted gene in the plasmid was transcribed, and protein was produced in the cytoplasm. As a foreign antigen, protein was processed via endogenous pathway and present through MHC class I and MHC class II. APCs generally present endogenous peptides on MHC class I. Apoptotic transfected cells can be subjected to MHC class I presentation by APC as well via a cross priming mechanism (3).

III. Transfected myocytes secret protein outside the cells and this exogenous protein was captured, processed within the endocytic pathway and presented the peptide on MHC class II by APCs (4).

IV. Antigen-loaded APCs migrate to the draining lymph node (DLN) via the afferent lymphatic vessel (5) and react with the naive T cells via MHC molecule and T cell receptor with the presentation of co-stimulatory molecules, giving the co signals to initiate an immune response and expansion of T cells (6).

V. Stimulation of T cells via MHC and costimulatory molecules allow CD4+ T helper cells to secrete cytokines to contact to B cells. CD4+ T helper cells have an interaction with B cell through B cells co-stilulartory molecule and B cells are activated. Shaded antigen can be captured by BCR in the DLN; these then present processed antigen to CD4+ T helper cells, thereby facilitating the induction of an effective B cell response.

In theory, once migrating T cells have been primed in the DLN they could be restimulated and further expanded at the site of immunization by presentation of the peptide–MHC complexes displayed by transfected muscle cells. These processes coordinately elicit specific immunity against plasmid-encoded antigen by activating both T and B cells, which, now they are 'armed'. Activated lymphocytes can travel through the efferent lymphatic system and provide a surveillance system. Together, the two arms of the immune system, which are induced specifically following DNA vaccination, can create a powerful defense against most infectious diseases (8).

2. **Improvement of vaccine efficacy by nanoparticle delivery system.**

More than 80 years ago, the efficiency of biodegradable microparticles to induce immune response specific to vaccine has been discovered. Early studies have demonstrated that the vaccine efficacy may be promoted by using nanoparticle formulation that facilitates its uptake by dendritic cells (DCs) [135]. This process also provides the induction of cellular immunity specific to vaccine [136].

The size of nanoparticles usually demonstrates the advantageous effect as a vaccine delivery system because of the sub-micron size which can be easily taken up by APCs (Figure 7). The nanoparticles promote the uptake of antigen by M-cells, in mucosa-associated lymphoid tissue (MALT), *i.e.*, gut-associated, nasal-associated and bronchus-associated lymphoid tissue, initiating sites of vigorous immunological responses [137]. However, the mechanism of immune promotion by nanoparticles is currently unclear. Possible explanations postulated that nanoparticles induce epithelial cells to secret cytokines, maintain the balance of Th1/Th2 response, activate macrophages and natural killer cells (NK) and improve the delayed-type hypersensitive reaction, or simply by increased absorption of antigens [138].

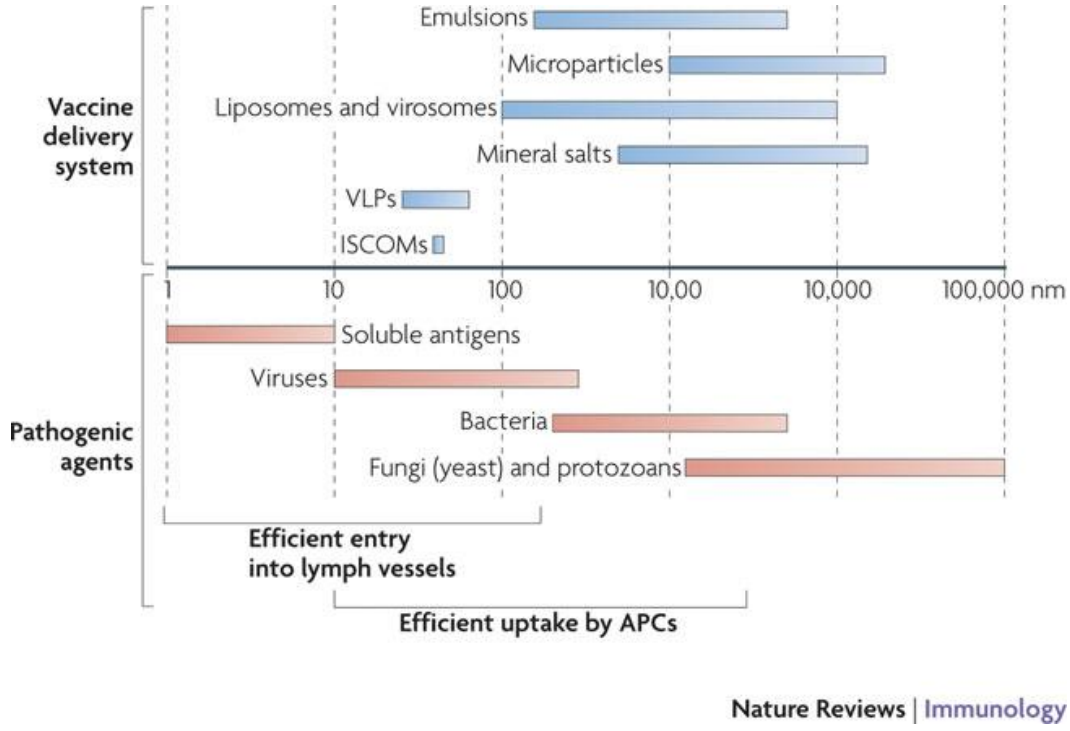


Figure 7. The sizes of particles for vaccine delivery systems and pathogenic agents. Ranges in size of various adjuvant delivery systems and the dimension of different pathogenic agents indicated on nonometre log scale. The range of particle sizes that efficiently uptake into APCs and entry into lymph vessel are shown. ISCOMs, immunostimulation complexes; VLP, virus-like particles [139].

Chitosan nanoparticle for DNA vaccine delivery

Chitosan is a natural nontoxic biopolymer produced by the deacetylation of chitin (Figure 8), a major component of the shells of crustaceans such as crab, shrimp, and crawfish. Recently, chitosan has received much attention for its commercial applications in the biomedical products, food, and chemical industries [141-143]. The ability of chitosan to form nanopaticle makes it beneficial than chitin. Chitosan nanoparticles have been synthesized as drug and vaccine delivery carriers as reported in previous studies [144,145]. Chitosan nanoparticle is able to condense with DNA via polycationic charges of amine groups of chitosan and poly anionic charges of phosphate groups of DNA. Encapsulation of DNA by chitosan can be condensed into a CS/DNA nanoparticle form that can be easily taken up into the cells (Figure 8). Because of their bioadhesive, biocompatible and biodegradable properties, chitosan nanoparticles are effectively taken up by phagocytic cells and this uptake, in turn, result in strong systemic and mucosal immune responses against particular antigens [140, 146-147]. Furthermore, chitosan and its nanoparticles may also stimulate the immune system. Chitosan has been shown to have immune activating ability such as increasing accumulation of macrophages and polymorphonuclear cells, inducing cytokines release after intravenous administration [148-149]. Therefore, using chitosan is not only efficient in delivery of DNA into cells, but it also improves the immunogenicity of DNA vaccine.

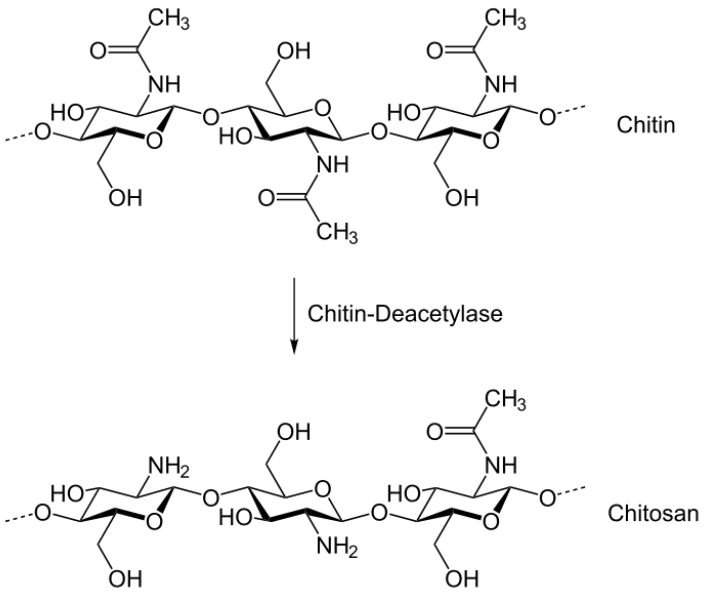


Figure 8. Polymer of chitin and chitosan. Chitosan is derived from the deacetylation of chitin. Polycationic charge of amine group makes it possible to bind with polyanionic charged molecule like DNA [49].

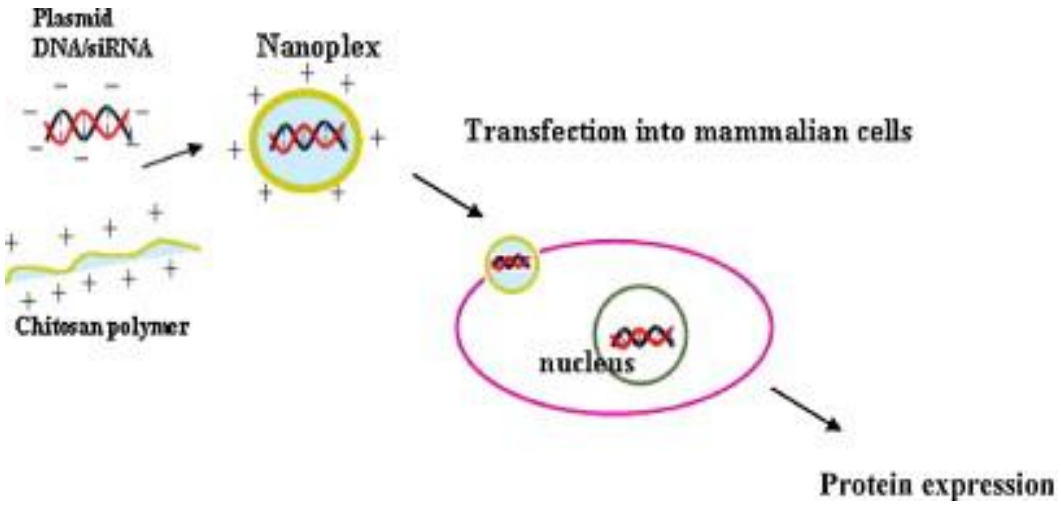


Figure 9. Enhancement of DNA vaccine uptake into cell by chitosan nanoparticle. Encapsulation of chitosan and DNA form nanocomplex. DNA is packaged in to chitosan nanoparticle. CS/DNA complex in nanoparticle can easily take up into cells. DNA is slowly release and enter into nucleus, then following with transcription and translation to produce protein of specific antigen [modified from 140]

3. Improvement of vaccine efficacy by immunization strategy

Usually, an effective vaccines need re-immunization (booster) in order to maintain an immune response and boost activity of memory cells. When the same formulation of vaccine is used to re-boost the immune response, this regimen is called homologous prime-boost immunization. Homologous protein based immunization is generally used and it is highly efficient in generating strong humoral immune responses by induction of antibody production, but it is often insufficient in boosting the cellular immunity which is required for resistance against intracellular infection. Unlike protein based vaccine, DNA vaccines have potentiality in induction of both humoral and cellular immune responses in mice but so far it still exhibits weak responses in non-human primates and humans.

Heterologous prime-boost immunization

Recently, many studies have been recommended that repeating immunization should be performed by giving different formulations of vaccines that contain the same antigen in different forms. This strategy is called heterologous prime-boost regimen. Such method of administration is proposed because the improvement of immunogenicity of vaccine by this type of vaccination is usually observed.

The concept of heterologous prime-boost immunization is practicable by priming with DNA vaccine and boosting with either recombinant proteins or recombinant viral vector. This sequential of vaccination is effective in promoting immune response (Table 5).

Table 5. Frequently used heterologous prime–boost vaccinations [152]

Prime immunization	Boost immunization	References
DNA	Recombinant protein	126, 151
	Inactivated vaccine	128, 153
	Viral vector	125, [154-158]
	BCG	159
Viral vector	Recombinant protein	160
BCG	Viral vector	127

The outcome of heterologous prime-boost vaccination is the generation of antigen-specific memory T cells by priming followed by amplification of these cells after boosting with different vaccine formulation (Figure 10). DNA vaccines are effective priming component since they are internally processed by APCs, and antigens are presented on both MHC class I and class II and it, therefore, stimulates both CD4+ and CD8+ T cells and promotes B cell response (Figure 6). Successful boosting agents include recombinant proteins and recombinant viral vectors. Heterologous prime-boost strategies have been shown to promote the induction of cellular immunity in various animal and disease models, including HIV [125, 126], tuberculosis [127], influenza [128], malaria [161], hepatitis C virus [162], herpes simplex virus [163] and hepatitis B virus [164].

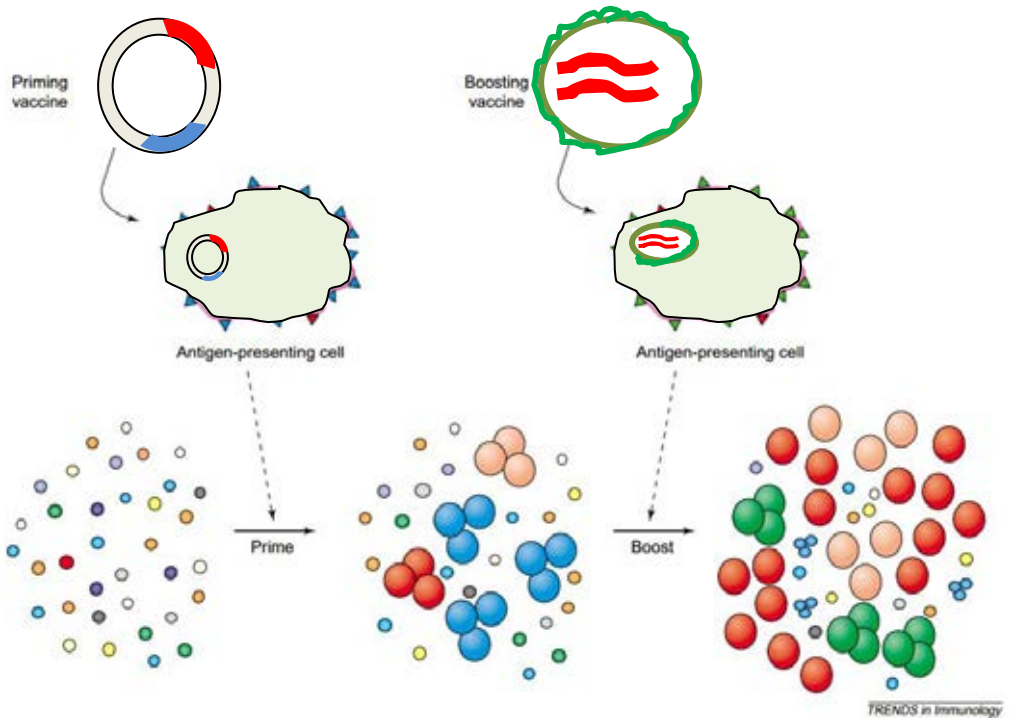


Figure 10. The result of heterologous prime boost regimen on improvement of the immunogenicity by T cell amplification. In priming, antigens (Ag) of interest (red) encoded in a recombinant DNA vector. APCs uptake the vector and present antigen including both the target antigens (red triangles) and vector antigens (blue triangles). APCs stimulate naïve T cells via antigens presentation and drive the expansion of both target-specific T cells (red cells, high avidity cells are represented by the darker red) and vector-specific T cells (blue cells). Subsequent boosting with a second vaccine (recombinant protein) results in the re-presentation of the target antigen (red triangles) and antigens from the second vector (green triangles) on APCs. These APCs then drive the expansion of target-specific memory T cells (red cells) and vector- specific naive T cells (green cells). Following heterologous booster immunization, the number of memory T cells against the desired vaccine antigens gets further expanded. The activation of T cell specific for the target antigen and the selection of T cells that have greater avidity for the antigen are promoted [modified from 150].

4. Improvement of vaccine efficacy by adjuvant.

More than 80 years ago, adjuvant for vaccine was established. Since then, it was traditionally used for enhancing immune responses against non-live-based vaccines. Most of them are focused on recombinant protein subunit vaccine. Because of the low toxicity, less side effect, and practical preparation, this type of vaccine is more advantageous than the heat inactivated or live-attenuated vaccine. However, such subunit antigens are generally lack immunogenicity when vaccinated without adjuvant or immunomodulation [51]. Thus, adjuvant is essential for advocating the immune response against specific antigens. The beneficial functions of adjuvant are describes in Table 6.

Table 6. The role of adjuvants in vaccines [165]

1. Increasing antibody responses – bactericidal, virus neutralizing
2. Inducing cell-mediated immunity, e.g. TH1 cytokines (interferon-γ)
3. Decreasing the dose of antigen in the vaccine
4. Decreasing the number of doses of vaccine necessary
5. Overcoming competition between antigens in combination vaccines
6. Enhancing immune responses in the young or elderly, who often respond poorly to vaccines

Based on their key mechanism of action, adjuvants for vaccine can be mainly divided into two groups; antigen delivery systems, or immune potentiators (Table 7).

Table 7. A simplified classification system for vaccine adjuvants [165]

Antigen delivery systems	Immune potentiators
Alum	MPL and synthetic derivatives
Calcium phosphate	MDP and derivative
Liposomes	CpG oligonucleotides
Virosomes	Alternative bacterial or viral components – flagellin etc.
Emulsions	Lipopeptides
Microparticles/nanoparticles	Saponins
Iscoms Virus-like particles	Small molecule immune potentiators, e.g. Resiquimod

Unfortunately, beneficial effects in promoting immune response of strong adjuvant usually come with its toxicity and negative side effects. The critical point to be considered to new and improved vaccine adjuvants are appropriate immune induction and safety. Freund's complete adjuvant (FCA) is a well-known adjuvant that is potent in induction of humoral and cellular immune responses [166]. Nonetheless, FCA contain *M. tuberculosis* agent that also provide adverse reactions and is too toxic to be use in human. Aluminum compounds (Alum), however, is the only licensed adjuvant approved by Food and Drug Administration (FDA) for human use in the United States [167]. In contrast to its non-toxic property, its ability to induce an immune response is weak, especially when it is used with recombinant subunit protein. Additionally, Alum is a Th2

biased adjuvant that generally induces IgG1 production, but not as potent in inducing IgG2a or Th1 response [168]. Besides, Alum is relatively poor in stimulating cell-mediated response and sometimes prevents the activation and differentiation of cytotoxic T-lymphocytes [169]. Therefore, the principle requirement of a new effective adjuvant is the safe and ability to induce both types of cellular and humoral immune responses.

With the development in the field of adjuvant for vaccine, MF59 adjuvant was introduced to the European market in 1997 for an influenza vaccine [170]. MF59 is an oil in water emulsion of squalene oil, which is a naturally occurring substance found in plants, animals, and human. It is biodegradable and biocompatible. MF59 contains two nonionic surfactants, polysorbate 80 and sorbitan trioleate 85, which are included to optimally stabilize the small emulsion droplets. MF59 adjuvant has also been shown to be safe and efficacious in induction of antibody response and T cell response in human [170]. The mechanism of action of MF59 is shown in Figure 11.

AddaVax™ for recombinant vaccine adjuvants

In this study, we introduced Addavax™ to be used for recombinant protein subunit vaccine. Since AddaVax™ is a squalene-based oil-in-water nano-emulsion based on the formulation of MF59 that has been licensed in Europe for adjuvanted flu vaccines. Like MF59, AddaVax™ comprise of two components: i.e. Sorbitan trioleate (0.5% w/v) in squalene oil (5% v/v) and Tween 80 (0.5% w/v) in sodium citrate buffer (10 mM, pH 6.5). The mechanism of action of Addavax™ is based on MF59. Squalene is oil more readily metabolized than the paraffin oil used in Freund's adjuvants [172]. AddaVax™ promotes a significant increase in antibody titers with reportedly more balanced Th1/Th2 responses than those obtained with alum [172]. AddaVax™ is believed to act through a depot effect, enhancement of antigen persistence at the injection site, recruitment and

activation of antigen presenting cells, and direct stimulation of cytokines and chemokines production by macrophages and granulocytes [173]. Antibody responses typically obtained with AddaVax™ are shown in Figure 12.

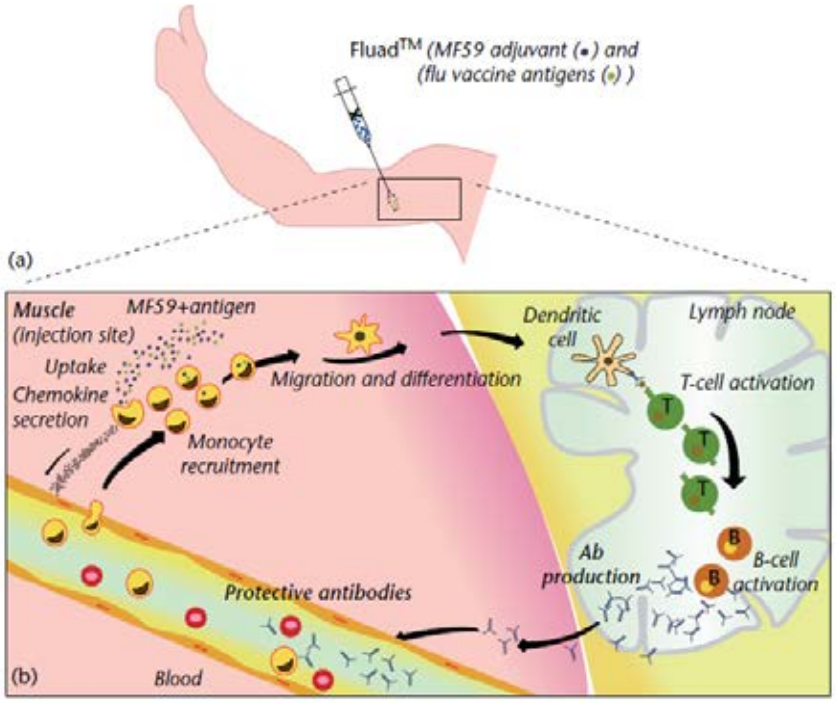


Figure 11. The immunological mechanism of action of MF59 adjuvant in flu vaccine. After injection through muscle cells, macrophages, monocytes and other cell types are activated after taking up MF59 adjuvant. Monocytic cells secrete chemokines to recruit other cells to the injection site. Activated macrophages move to draining lymph nodes and act as APCs to present antigen to T cells. Moreover, MF59 also enhances monocyte differentiation into dendritic cells, professional APCs. Such APCs also migrate from the tissues to the lymph nodes and they present antigens to naive T cells, resulting in activation of these cells. Activated T cells also contribute to B cells activation. Activated B cells produce antibody against specific antigen. Thus, MF59 directly enhances the numbers of activated flu antigen-specific T cells and also results in the secretion of higher levels of flu-specific antibodies into the blood. [165,171]

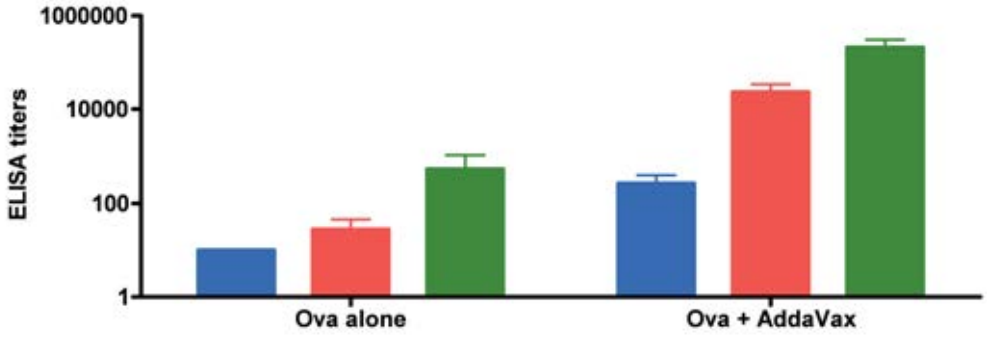


Figure 12. Anti-Ova mIgG levels after immunization for 15, 30 and 45 days. Mice were immunized s.c at 0, 2 and 3 weeks with 1 μg of EndoFit™ Ovalbumin alone or 1 μg of EndoFit™ Ovalbumin/AddaVax™ (1:1, v/v) in a final volume of 100 μl. Serum anti-OVA mIgG was monitored by ELISA (coated with ovalbumin at 10 μg/ml in PBS).

CHAPTER IV

MATERIALS AND METHODS

Experimental Flow Chart

***In vitro* study**

lipL32 and *loa22* DNA vaccine construction and preparation

- *lipL32* and *loa22* full-length PCR amplification
- cloning into pVITRO mammalian expression vector
- demonstrate antigen expression in HEK293T
- pVITRO/*lipL32*, pVITRO/*loa22*, and pVITRO/*lipL32-loa22* endotoxin free DNA vaccine giga preparation.
-

Optimization of the ratio of N/P of chitosan and DNA for encapsulation

- transfection efficiency observed by fluorescent microscope and flow cytometry
- Gel retarding analysis

Encapsulation of CS/pVITRO-*lipL32*, CS/pVITRO-*loa22*, and CS/pVITRO-*lipL32-loa22*

- Demonstrate the expression in vitro by immunofluorescent staining
- Morphology and particle size measurement by SEM
- Cytotoxicity test by using MTT assay
- Induction of expression in *E. coli* expression system

Recombinant LipL32 and Loa22 proteins eapression and purification

- His-tag column chromatography purification
- Hydrophobic column chromatography purification
- Purity checking by SDS-PAGE

***In vivo* study**

Testing immunogenicity of CS/pVITRO-*lipL32*, CS/pVITRO-*loa22*, and CS/pVITRO-*lipL32-loa22* vaccine in mice

Immunological analysis

- Humoral immune response
  - Antibody production in serum
    - Total IgG
    - Th1: IgG1
    - Th2: IgG2a
- Cellular immune response
  - T cells proliferation assay
  - Cytokines detection by ELISA
    - Th1: IFN-γ, IL-2
    - Th2: IL-4, IL-10
  - Intracellular cytokines staining
    - Th1: IFN-γ, IL-2
    - Th2: IL-4

Bacterial cultivation

Escherichia coli

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

Cell culture

Human embryonic kidney 293T (HEK293T) cell line (ATCC No.CRL-1573-LGC) was 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 ºC in 5% CO_2 incubator.

Animals

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

Polymers and reagents

Acid soluble low molecular weight chitosan from shrimp (average molecular weight 22-24 kDa and deacetylation rate of 75-90%) was obtained as a kind gift from Dr.Supasorn Wanichwecharungruang (Department of Chemistry, Faculty of Science, Chulalongkorn University). All other materials were obtained from Sigma Aldrich (USA) and were of analytical reagent grade.

Preparation of recombinant plasmids and recombinant proteins

Preparation of Leptospirosis genomic DNA

Ten milliliters of 10^8 cells/mL of *L. interrogans* serovar Pomona were harvested by centrifugation at 8,000 x g for 15 minutes. The genomic DNA of *L. Interrogans* serovar Pomona was extracted by DNA purification kit (Roche applied science) according to the manufacturer protocol. DNA was resuspended in TE buffer. The concentration of genomic DNA was measure by nanodrop2000 (Thermo Scientific)

PCR amplification of OmpA-family lipoprotein *loa22* gene (ACCESSION No. EKN96269.1, REGION: 195327-195914)

There are four plasmids which are used as DNA vaccines (pVITRO, pVITRO-*lipL32*, pVITRO-*loa22*, and pVITRO-*lipL32-loa22*). We obtained pVITRO-*lipL32* plasmid which contains full-length *lipL32* gene from Rithairat Kerdkaew, Faculty of Medicine, Chulalongkorn University. *lipL32* was insert into (multiple cloning site (MCS) 1 of pVITRO between *Bsp*EI and *Bsi*WI restriction sites. To construct plasmid pVITRO-*loa22* and pVITRO-*lipL32-loa22, loa22* gene was amplified to insert into pVITRO and pVITRO-

lipL32, respectively. Extracted genomic DNA of *L. interrogans* serovar Pomona was used as a template for PCR amplification of the full-length *loa22* gene encoding mature protein, from amino acid 1 to 196. The primers for gene amplification were designed to include signal peptide, using forward primer: 5' GCGCCACCGGTATGGTCAAAAAGATTTTG 3' and reverse primer: 5' CGGCTAGCTTATTGTTGTGGTGCGGAAGT 3' containing restriction sites of *Age*I and *Nhe*I (underlined sequences) in the forward and reverse primers, respectively. The PCR amplification was performed in a total volume of 20 μl using 100 and 200 ng of *L. interrogans* serovar Pomona genomic DNA under the following condition: primary denaturation at 95 ºC for 5 minutes, followed by 30 cycles of denaturation at 94 ºC for 1 minute, annealing at 58 or 60 or 62 ºC for 30 seconds, extension at 72 ºC for 1.5 minute; and final extension at 72 ºC for 10 minutes. After the reaction, PCR products were analyzed using 1% agarose gel electrophoresis.

Cloning

The PCR product (588-bp) of *loa22* including *Leptospira* leader signal sequence amplified from the genomic DNA of *L. interrogans* serovar Pomona was directly cloned into multiple cloning site2 (MCS2) of pVITRO (InvivoGen) and pVITRO-*lipL32* to obtain the plasmid pVITRO-loa22 and pVITRO-*lipL32-loa22* (Figure 13). The PCR product of *loa22,* pVITRO, and pVITRO-*lipL32* were cut at *Age*I (*Bsh*TI) and *Nhe*I restriction sites The *Nhe*I cut product was run on 1% agarose gel electrophoresis and then purified by QIAquick gel extraction kit (Qiagen, Germany). The obtained purified *Nhe*I digested-*loa22*, *Nhe*I digested-pVITRO, *Nhe*I digested-pVITRO-*lipL32* ware digested again with *Age*I (*Bsh*TI). The purified *Age*I and *Nhe*I digested-*loa22* and

digested-backbone vector of pVITRO and pVITRO-*lipL32* products were diluted in TE buffer and store at -20 ºC for ligation process.

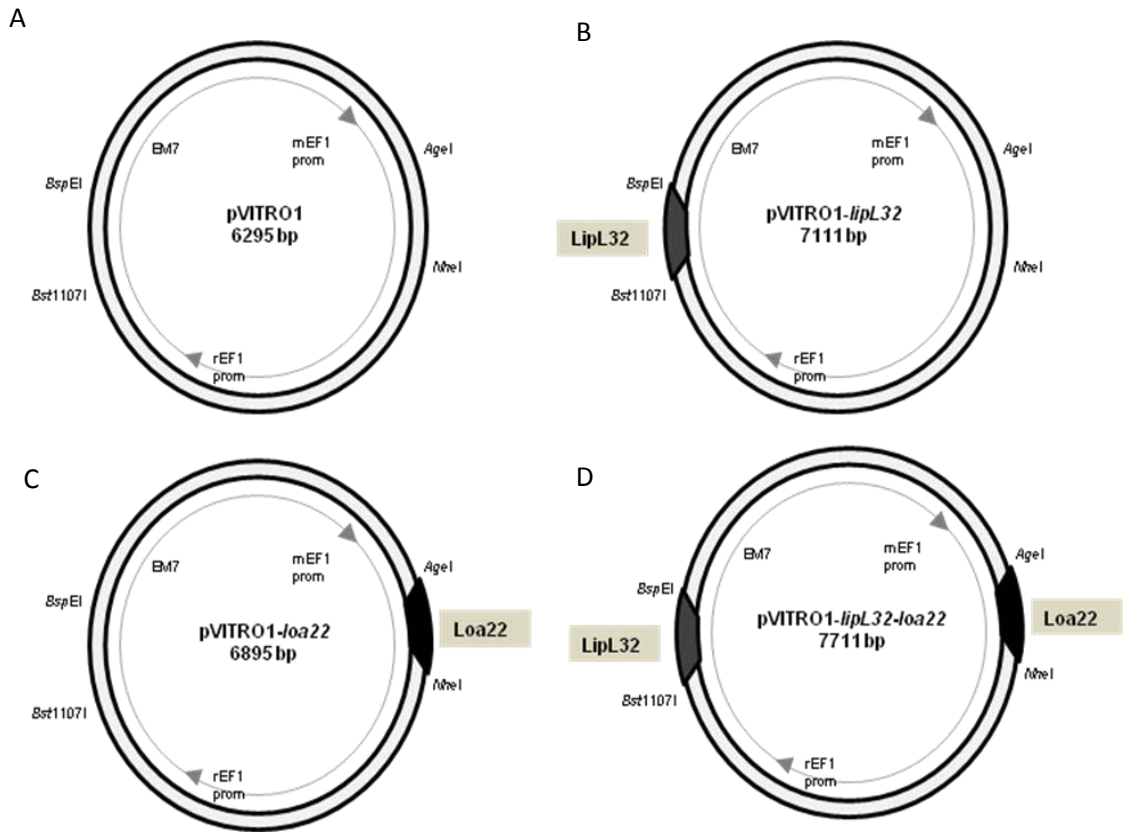


Figure 13. Plasmids used in this study. Restriction maps of plasmid pVITRO (A), pVITRO-*lipL32* (B), pVITRO-*loa22* (C), and pVITRO-*lipL32-loa22* are depicted. The direction of *lipL32* and *loa22* insertion is indicated.

Ligation

Ligation reaction was performed by T4 DNA ligase (NEB Biolab, New England) under the following conditions; 400 units of T4 ligase, digested *loa22* : digested pVITRO or pVITRO-*lipL32* at the molar ratio of 3:1 (0.075:0.025 pmole). The mixtures were incubated overnight at 16 ºC followed by heat inactivation at 65 ºC for 10 minutes. After incubation, ligation mixtures were 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 ºC water bath for 90 seconds and immediately incubated in ice for 2 minutes. One milliliter of LB medium was added to the solution, mixed, and incubated for an hour at 37 ºC under shaking condition. The transformants were plated onto LB agar containing 50 μg/ml of kanamycin, and incubated at 37 ºC overnight.

Colony PCR screening

Colonies of *E. coli* harboring the plasmid pVITRO-*loa22* or pVITRO-*lipL32-loa22* were screened by PCR reaction using specific primers for loa22 that were used for *loa22* amplification. The colonies from the master plate were picked and resuspended in PCR reaction mixture and the PCR reaction was carried out as describe above. The expected PCR products were observed by 1% agarose gel electrophoresis.

Endotoxin-free plasmid preparation

Large-scale extraction of plasmid (pVITRO, pVITRO-*lipL32*, pVITRO-*loa22*, and pVITRO-*lipL32-loa22*) ware performed with an endotoxin-free giga QIAGEN kit (Qiagen, Germany) according to the manufacturer's protocol. The extracted plasmids were used for an *in vitro* transfection studies and for vaccination studies in mice. The plasmid stocks were stored at -20 ºC until use.

DNA sequencing

Colonies containing indicated plasmids were cultured in LB broth containing 50 μg/ml of kanamycin at 37 ºC overnight and the 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 *loa22* sequence of *L. interrogans serovar Pomona* obtained from GenBank database (ACCESSION **No. EKN96269.1,** REGION: 195327-195914).

Transfection and Detection by Western Blot

To detect the expression of LipL32 and Loa22, HEK293T cell line was transfected with plasmid pVITRO, pVITRO-*lipL32*, pVITRO-*loa22*, or pVITRO-*lipL32-loa22* at the molar concentration of 0.2 pmole of each plasmid in a 24-well plate using X-treme gene trasfection reagent (Roche Applied Science) according to the manufacturer's protocol. After 48 hours of transfection, the culture supernatant was collected and the transfected cells were washed twice with ice-cold PBS and lysed in BufferA and Buffer B protein lysis buffers. Cell lysates were vortexed and the culture supernatants were centrifuged at 6,000xg at 4 ºC for 10 minutes. Proteins were evaluated by Western blot analysis using mouse anti-LipL32 and mouse anti-Loa22 polyclonal antibody.

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

Equal amount of total protein lysates or 20 μL of supernatants from transfected HEK293T cell line were boiled at 95 ºC for 10 minutes. Twenty microliters of samples (30 μg of total protein lysates) was loaded onto a 12% SDS-PAGE. Proteins were then transferred from SDS-PAGE onto polyvinyllidene fluoride membrane (PVDF, Millipore) by semi-dry transfer 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 in the 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 (1XPBS +0.1% Tween 20). The membrane was then incubated with polyclonal mouse anti-LipL32 mixed with polyclonal Loa22 antiserum (generated by immunization of female BALB/c mice with purified-6His tag-Loa22 protein) (1:10,000) in the blocking buffer) at room temperature for 1 hour at room temperature plus 4 ^{o}C overnight, followed by washing 3 times, 15 minutes each with washing buffer (1XPBS+ 0.1% Tween20). The membrane was incubated in horseradish peroxidase (HRP)-conjugated goat anti-mouse antiserum (1:5,000) in the blocking buffer at room temperature for 1 hour and then washed each 15 min for 3 times with washing buffer (1X PBS+0.1% Tween 20). The proteins were detected using enhanced chemiluminescence (ECL; 100 mM Tris-HCl pH. 8.5, luminal, coumaric acid, and hydrogen peroxide). The membrane was incubated with ECL reagents for 1 minute and then exposed to film in dark room.

Expression and Purification of recombinant LipL32 (rLipL32) and Loa22 (rLoa22)

Induction of rLipL32 and rLoa22 expression in *E. coli* by using IPTG

The obtained BL21(DE3) pLysS harboring plasmid pRSETC-*lipl32* with insert of signal sequence-deficient full-length *lipL32* and the one harboring plasmid p489A4-*loa22* with *loa22* insertion were cultivated in LB broth with 35 µg/ml choramphenicol and 100 µg/ml ampicillin by shaking at 250 rpm at 37 ºC. Next, the overnight culture was added to 25 ml of fresh media before incubation until the OD600 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 ºC 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 with 40% amplitude for 12 minutes on ice. The lysate was centrifuged at 16,000xg for 20 minutes at 4 ºC and the supernatant was transferred to a new tube. Keep LipL32 and Loa22 supernatant separately at -20^{o}C.

Purification using His-tag affinity chromatography

For protein purification by His-tag affinity column chromatography, the supernatant (rLipL32) ware directly loaded onto a Hi-Trap chelation column (Amersham Pharmacia Niotech) equilibrated with phosphate buffered saline (PBS). Proteins were eluted with stepwise gradient of 0, 50, 125, and 250 mM imidazole. The top of the peak of each fraction were collected to further check for the purity of protein by SDS-PAGE. Eluted fractions which provide the highest yield and purity were collected for further purification by hydrophobic-column chromatography. Purification of Loa22 was performed with the same gradient of immidazone as LipL32 protocol. Some proportion

of the purified rLipL32 and rLoa22 were dialyzed with 1XPBS at 4 ºC to remove residual immidazone. The others were collected for hydrophobic column chromatography.

Hydrophobic column chromatography

To further purify rLipL32 and rLoa22 by hydrophobic column chromatography, fractions with the highest amount of rLipL32 which obtained from the His-tag column were pooled and diluted with 4X concentrated ammonium sulfate buffer to 1X final concentration. Then, they were loaded onto a HiTrap butyl FF hydrophobic column (Amersham Bioscience) and using FPLC purification systems (GE Healthcare). Proteins were eluted with the stepwise gradient of 1000, 800, 600, 400, 200 mM ammonium sulfate and followed by double distilled water. The top of the peak of each fraction were collected to further check for the purity of the proteins by SDS-PAGE. Eluted fractions which provide the highest yield and purity were collected and pooled to use for immunization, ELISA, and in vitro re-stimulation assay. Purification of Loa22 was performed with the same gradient of ammonium sulfate as mentioned in the rLipL32 purification protocol. The best fraction was also collected for hydrophobic column chromatography.

After purifying at this step, all rLipL32 and rLoa22 fractions were determined for the purity of protein by SDS-PAGE and were subjected for measurement of protein concentration by Pierce BCA protein assay kit (Thermo Scientific). Proteins that would be used for immunization and cell stimulation were filtered via 0.2 μM membrane before measuring the concentration.

Optimization of amine to phosphate (N/P) ratio of chitosan and plasmid DNA

Preparation of Chitosan-DNA nanoparticle

Low molecular weight chitosan (CS; 22-24 kDa) was dissolved in 1% acetic acid and adjusted the pH to 5.5 with NaOH. Chitosan solutions were diluted with 5mM NaOAc until they reached the desired N/P ratio in the range of 1:1 to 20:1. DNA plasmid was diluted in 25 mM Na_2SO_4, and mixed with 100 µl of chitosan with equal volume of DNA. Nanoparticles were prepared by a complex coacervation technique described by Moa [40]. Briefly, after heating the DNA and chitoan solution at 55ºC for 5 minutes, an equal volume of the chitosan and the DNA solutions were mixed and immediately vortexed at maximum speed for 60 sec. The solution of nanoparticles was used immediately after preparation for transfection in HEK293T cell line for 5 days.

Gel retarding analysis

To monitor the encapsulation efficacy, naked pMax-GFP (Amaxza), a model plasmid, and various ratio of chitosan/pMax-GFP nanoparticles prepared at the N/P ratios of 1:1, 2:1, 4;1, 8:1, 10:1 and 20:1 were loaded onto 1% agarose gel in 1XTAE buffer. The calculation was done by fixing the amount of pMax-GFP at 1 µg of DNA per well and then changed the unit into molar ratio of N/P. Naked plasmid pMax-GFP was used as a control to compare the efficacy of chitosan in encapsulating DNA. The samples were run on the gel at 100 V for 40 minutes. The gel was stained with ethidium bromide and visualized under UV light.

Transfection Efficacy

After 5 days of transfection using CS/pMax-GFP at various ratio of N/P of 1:1, 2:1, 4:1, 8:1, 10:1 and, 20:1, GFP positive HEK293T cell line was evaluated under fluorescence microscopy.

Determination of transfection efficiency by CS/DNA plasmids by flow cytometry

To quantitatively measure the transfection efficiency at the different N/P ratios, CS/DNA nanoparticles at the N/P ratio of 1:1, 2:1, 4;1, 8:1, 10:1 and 20:1 were used to transfect into HEK293T cell line for 5 days. X-treme Gene Transfection Reagent (Roche, Applied Science) was used as positive controls. To quantify transfection efficiency, transfected cells were harvested and analyzled for GFP+ cells by flow cytometry (FC500, Beckman Coulter). The ratio that yields highest transfection efficiency was used for lipL32 and loa22 chitosan/DNA vaccine encapsulation.

Transfection and detection of CS encapsulation of lipL32 and loa22 plasmid *in vitro*

Since the result of N/P optimization indicated that the best ratio providing highest transfection efficiency was 20:1, this ratio was used to encapsulate *lipL32* and *loa22* DNA plasmid. Plasmids pVITRO, pVITRO-*lipL32*, pVITRO-*loa22*, and pVITRO-*lipL32-loa22* were encapsulated with chitosan nanoparticle by using the molar ratio of N/P ratio at 20:1. The molar concentration of each plasmid was fixed at 0.2 pmole. To detect the expression of LipL32 and Loa22, HEK293T $2.5x10^4$ cells/well were plated overnight on sterilized cover glass in 24-well plate. Cells were transfected with CS/pVITRO, CS/pVITRO-*lipL32*, CS/pVITRO-*loa22*, and CS/pVITRO-*lipL32-loa22* for 5 days. X-treme Gene Transfection Reagent was used as positive controls. Cells were stained by using immunofluorescent staining method to detect LipL32 and Loa22 expression. Briefly,

cells on the cover slide were carefully removed and fixed in cold acetone for 5 minutes, washed 3 times with 1XPBS. Cold methanol was added for 10 minutes to penetrate the cells. Cells were washed again in 1XPBS 3 times. Rabbit anti-LipL32 (1:00) and mouse anti-Loa22 (1:100) polyclonal antibodies which was prepared separately in PBS were added together. Cells were incubated at room temperature for 1 hour. This step is followed by washing with 0.1% PBST 3 times, each 5 minutes, and 1:200 of secondary antibodies of donkey anti-mouse Alexa Fluor488 $F(ab')_2$ fragments and 1:200 of anti-rabbit Alexa fluor555 $F(ab')_2$ fragments (Cell Signaling Technology) were added and incubated at room temperature for 30 minutes. Cells were washed with 0.1%PBST 5 times, each 5 minutes and directly observed under fluorescent microscope using anti-fade mounting media

SEM analysis of CS/*lipl32-loa22* DNA particles

The nanoparticles of CS, CS/pVITRO, CS/pVITRO-*lipL32*, CS/pVITRO-*loa22*, and CS/pVITRO-*lipL32-loa22*, prepared by encapsulation at the N/P ratio of 20:1 were directly observed using SEM to assess their size and morphology. Ten µl of nanoparticle suspension after encapsulation was placed on copper grids. The grids were air-dried and the samples were visualized at 15 kV 50,000X setting (Faculty of Science, Chulalongkorn University).

Cytotoxicity assay

HEK 293T cell line was plateed overnight at a density of 10,000 cells per well in 100 uL complete DMEM in 96-well plates. The medium was replaced by fresh serum-free media containing 0.04 pmole of DNA vaccine of CS/pVITRO, CS/pVITRO-*lipL32*, CS/pVITRO-*loa22*, or CS/pVITRO-*lipL32-loa22* nanoparticles. Untreated cells were used

as a negative control. After incubation for 96 hours, ten uL of 3-(4,5-diamethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) solution (5mg/mL in PBS) was added, and cells were incubated for additional 4 hours at 37ºC. After this incubation, 0.04 N HCl in isopropanol was added to dissolve the formazan. The plates were mildly shaken for 10 minutes to completely dissolve the formazan crystal. The absorbance was measured by using microplate reader at the wavelength of 450 nm. Three replicates were done for each sample. The mean value of the three replicates was used as the final result.

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

Evaluation of immunogenicity of CS/pVITRO-*lipL32-loa22* and coadministrtion of CS/pVITRO-*lipL32* and CS/pVITRO-*loa22*

Mice grouping

To evaluate the immunogenicity between administration of CS/pVITRO-*lipL32-loa22* and co-administration with CS/pVITRO-*lipL32* and CS/pVITRO-*loa22*, total of 15 female BALB/c mice at the age of eight weeks were used and randomly divided into following groups;

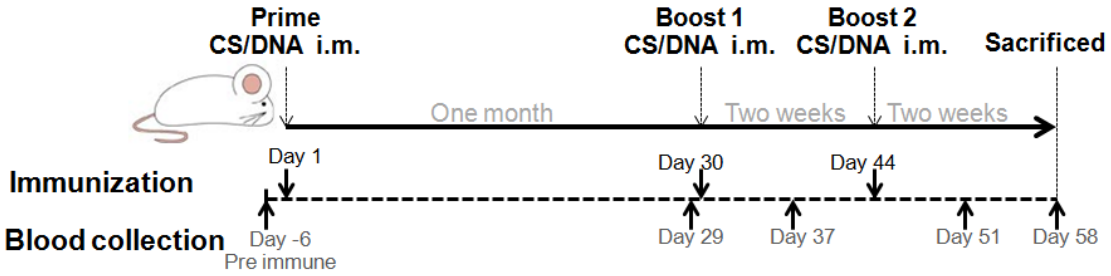
Group 1: CS/pVITRO (negative control group)

Group 2: CS/pVITRO-*lipL32-loa22* 10 pmole (two ORFs one plasmid)

Group 3: CS/pVITRO-*lipL32* 10 pmole + CS/pVITRO-*loa22* 10 pmole (two ORFs two plasmids)

Immunization protocol

Before the starting of immunization, pre-immune sera were collected by using retro-orbital breeding method after anesthesia with AERRANE (isoflurane, Baxter, USA). Mice were immunized three times by intramuscular (i.m.) injection with 10 pmole of DNA plasmid at day1, day30, and day 44 (Figure 14). Seven days after each immunization, sera were collected for determination of specific antibody titers.



Group	Prime	Boost1	Boost2
1 control	CS/pVITRO	CS/pVITRO	CS/pVITRO
2 One plasmid Two antigens	CS/pVITRO-*lipl32-loa22*	CS/pVITRO-*lipl32-loa22*	CS/pVITRO-*lipl32-loa22*
3 Two plasmids Two antigens	CS/pVITRO-*lipl32*+ CS/pVITRO-*loa22*	CS/pVITRO-*lipl32*+ CS/pVITRO-*loa22*	CS/pVITRO-*lipl32*+ CS/pVITRO-*loa22*

Figure 14. Scheme of vaccination to evaluate the immunogenicity in mice immunized with CS/pVITRO-*lipL32-loa22* and mice co-immunized with CS/pVITRO-*lipL32*+CS/pVITRO-*loa22* of lipL32-loa22 DNA vaccine.

Evaluation of immunogenicity of two antigens of LipL32-Loa22 and single antigen of LipL32 or Loa22 by heterologous prime-boost immunization

Mice grouping

To evaluate the immunogenicity between mice immunized with two antigens of LipL32-Loa22 and single antigen of LipL32 or Loa22 by heterologous prime-boost immunization, the total of 20 female BALB/c mice at the age of eight weeks were used and randomly divided into following groups;

Group 1: CS/pVITRO, PBS, PBS (negative control)

Group 2: CS/pVITRO-*lipL32*, rLipL32, rLipL32 (LipL32 only)

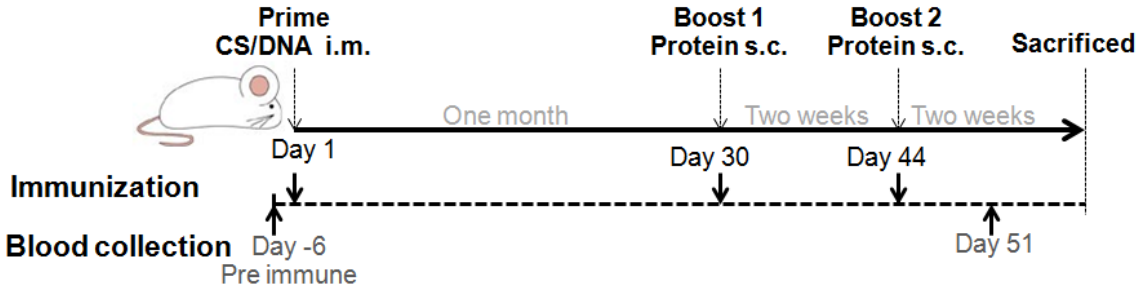
Group 3: CS/pVITRO-*loa22*, rLoa22, rLoa22 (Loa22 only)

Group 4: CS/pVITRO-*lipL32-loa22*, rLipL32+rLoa22, rLipL32+rLoa22

(LipL32 and Loa22)

Immunization protocol

Before beginning the experiments, pre-immune sera were collected by using retro-orbital breeding method after anesthesia with AERRANE (isoflurane, Baxter, USA). Mice were immunized by intramuscular (i.m.) with 10 pmole of DNA plasmid once at day1 (prime), and boost twice with the recombinant proteins by subcutaneous injection (s.c.) of rLipL32, rLoa22 or both at the concentration of each protein of 0.5 μM (11.63 μg of rLoa22, 15.63 μg of rLipL32) in AddaVax™ as an adjuvant at day30, and day 44 (Figure 15). Sera were collected 7 days after each immunization.



Group	Prime	Boost1	Boost2
1 control	CS/pVITRO	PBS	PBS
2 Single LipL32	CS/pVITRO-*lipl32*	rLipL32-AddaVax	rLipL32-AddaVax
3 Single Loa22	CS/pVITRO-*loa22*	rLoa22-AddaVax	rLoa22-AddaVax
4 Combine LipL32-Loa22	CS/pVITRO-*lipl32-loa22*	rLoa22+rLoa22-AddaVax	rLoa22+rLoa22-AddaVax

Figure 15. Vaccination scheme to evaluate the immunogenicity by comparing between immunization with a combination of two antigens of LipL32-Loa22 and with a single antigen of LipL32 or Loa22 by heterologous prime-boost regimen.

Blood sample collection

Blood samples were collected by retro-orbital puncture a week before immunization and were used as pre-immune sera. Other blood collections were performed one week after each immunization. To obtain the serum from the blood, blood was left at room temperature at least 30 minutes to clot. The clotted blood was centrifuge at 1000xg at 4ºC for 10 minute. Separated sera were collected and kept at -20ºC until use. The sera were used to measure the end point titer of antibody production after immunization by using ELISA.

Determination of total IgG, IgG1, and IgG2a by ELISA

To measure LipL32 and Loa22-specific total IgG1 and IgG2a titres, five hundred ng of rLIpL32 or rLoa22 were coated in each well in 96-well Immuno plate (SPL 96-wells immunoplate) with coating buffer (0.1 M Na2CO3, 0.1 M NaHCO3, pH 9.5) at 4ºC overnight. The plates were washed 5 times with washing buffer (1XPBS, 0.05% Tween20). The residual buffers were removed by absorbing on the paper towel. Then, the plates were blocked by using 5% skim milk in PBST. After blocking, diluted mice sera in blocking buffer were added to each well at 100 ml/well and the plates were incubated at room temperature for 1 hr. The plates were washed 5 times again with PBS with 1%Tween-20 (PBST) and horseradish peroxidase (HRPO)-conjugated rabbit anti-mouse IgG1 and rabbit anti-mouse IgG2a (Invitrogen) to separate wells in a 100 ml volume and incubated for 1 h at room temperature. The plates were washed 5 times in PBST, and 3, 39, 5, 59-tetramethylbenzidine (TMB) substrate (Sigma-Aldrich, Canada Ltd.) was added. The plates were incubated for 20 min at room temperature, followed by addition of 50 ml 2N sulfuric acid to stop the reaction. The assays were performed in duplicate with the mean values being reported for each biological replicate. Titers were reported as the end point titer when compared with the pre-immune group [174].

***In vitro* Re-stimulation Assay**

Lymphocyte proliferation assay

To measure cell proliferation in vitro upon re-stimulation, spleens from immunized mice were isolated and grinded. The cell suspension was centrifuged and red blood cells were lysed by RBC lysis buffer. The resulting cells were resuspended in culture medium (complete RPMI+2ME) at a final concentration of $1x10^6$ viable cells/ml and then CFSE proliferation assay were perform using CellTrace™ *CFSE* Cell Proliferation Kit (Molecular Probes/Invitrogen) as described by the manufacturer's

protocol. In the final step of washing, cell were plated into 24-well plate with the concentration of $1x10^6$ cells/well and were stimulated with 20 µg/ml of rLipL32 or rLoa22 or 5 µg/ml Concanavalin A (ConA) as a positive control. After 72 hours of stimulation, cells were collected, washed and stain for CD4+ T cell by using mouse anti-CD4-ECD (Beckman Coulter, Inc.) and were subjected to analyze by flow cytometry. The data were analyzed by FlowJo solfware (Tree star, Inc.).

Quantitative Analysis of the Released Cytokine by ELISA

To measure cytokine responses upon re-stimulation, $2x10^6$ splenocytes/mL in completed PPMI+2ME were seeded in a 12 well-plate and were stimulated with 20 µg/ml of rLipL32, rLoa22 or 5 µg ConA (positive control), or medium alone (negative control) for 72 hrs at 37ºC in CO_2 incubator. The culture supernatants were collected and were subjected to measured Th1 (IFN-γ, IL-2) and Th2 cytokines response (IL-4, IL-10) by using cytokine ELISA Max kit (Biolegend) according to the manufacturer's protocols.

Detection of cytokine response from CD4+ T cells by intracellular cytokine staining (ICS)

To measure CD4+ T cell specific cytokine response, $2x10^6$ splenocytes/mL in completed PPMI+2ME were seeded in the 12 well-plate and were stimulated with 20 µg/ml rLipL32, rLoa22 or 5 ug/ml ConA (positive control), or medium alone (negative control) for 72 hrs at 37ºC in CO_2 incubator. Four hr before collecting the cells, 100 ng/ml PMA, 1 µg/ml ionomycin, and 1 µl of GolgiPlug™ protein transporter inhibitor (BD Bisciences) were added in to each well to boost cytokine production and trap the cytokines inside the cells. Cells were collected at 72 hours after the initial incubation and washed with PBS. Cells were further subjected to CD4+ cell surface marker staining at room temperature for 30 minutes by 1 µg/ml Alexa Fluor 488 anti-mouse CD4 (Biolegend). After the final washing, cells were stained using ICS using BD

Cytofix/Cytoperm™ (BD Bisciences) and stained with 1 μg/ml of PerCP/Cy5.5 anti-mouse IFN-γ (Biolegend), PE anti-mouse IL-2 (Biolegend), and 1 μg/ml biotinylated anti-mouse IL-4 (Biolegend) and 1 μg/ml streptavidin conjugated ECD (Beckman Coulter, Inc). After the final step of washing, cells were analyzed by flow cytometry (Beckman Couler FC500)

Stratistical analysis

All statistical analyses were performed using GraphPad Prism 5 software. The results are expressed as the mean and SD. One way ANOVA analysis of variance with Turkey-Kremer's post-hoc test was used to determine the statistical significance. All experiments were performed in duplicate and with five biological samples per group.

CHAPTER V

RESULTS

Construction of *lipL32* and *loa22* recombinant plasmids to use as a DNA vaccine

Optimization of PCR Reaction to Amplify Loa22 DNA Fragment

The genomic DNA of *L. interrogans* serovar Pomona was used as a template for PCR amplification of the full-length *loa22* with the primers, and PCR conditions as indicated in the materials and methods. The PCR products of 588 bpcorresponding to amino acid No.1 to No.195 of OmpA family lipoprotein (ACCESSION No. EKN96269.1, REGION: 195327-195914) was obtained at different amounts when amplified with different annealing temperatures (58, 60, 62ºC) and with different amounts of genomic DNA (100 or 200 ng) as a template. The highest amount of PCR products was observed without non-specific bands under the PCR condition where 100 ng of *L. interrogans* genomic DNA was used as the template with the annealing temperature of 58 ºC (Figure 16).

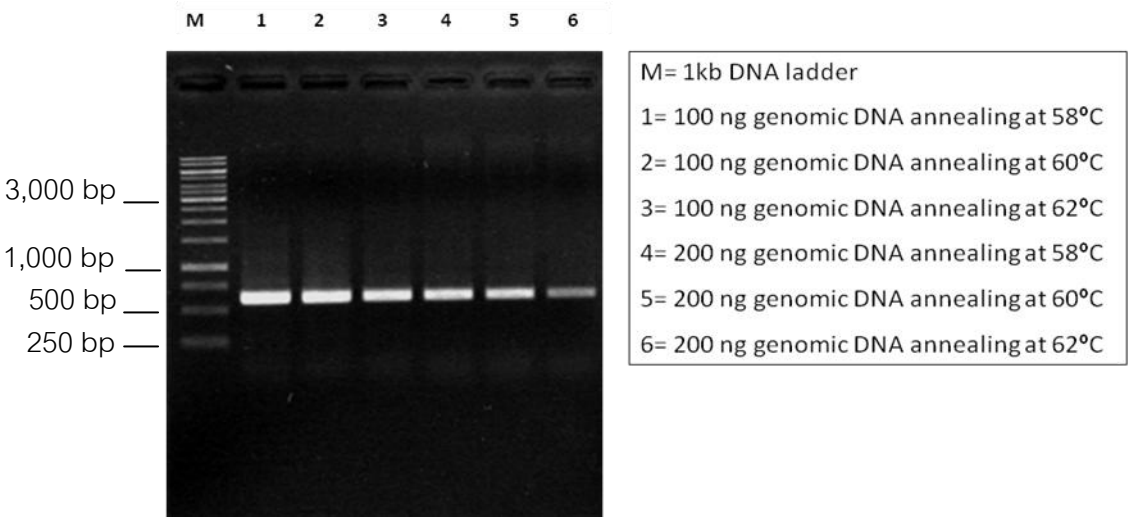


Figure 16. The PCR products of *loa22* after amplification using 100 or 200 ng of *L. interrogans* genomic DNA as the template under different annealing temperatures. Lane M, 1kB DNA ladder; Lane 1, 2, and 3, PCR product of *loa22* amplified with 100 ng DNA template at 58ºC, 60ºC and 62ºC respectively; Lane 4, 5, and 6, PCR product of loa22 amplified with 200 ng DNA template at 58ºC, 60ºC and 62ºC respectively.

Construction of recombinant plasmids containing *Loa22*

The PCR product of *loa22* was digested sequentially with restriction enzyme *Age*I and *Nhe*I. After digesting with *Age*I, the product was purified and subjected to *Nhe*I digestion. The resulting DNA fragment of *loa22* after cutting with both enzymes was analyzed for the size by agarose gel electrophoresis as shown in the Figure 17. This DNA was used for cloning in to multiple cloning site 1 (MCS1) of pVITRO or pVITRO-*lipL32*, respectively.

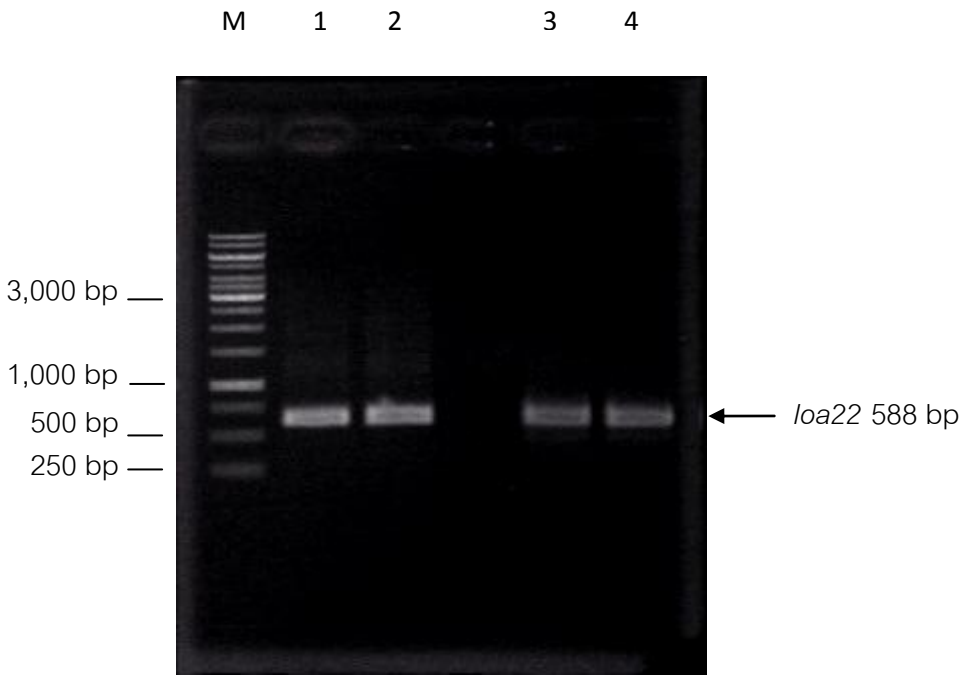


Figure 17. Digested product of *loa22*. The PCR product of *loa22* was sequentially cut with *Age*I and *Nhe*I . Lane M, 1 kB DNA ladder; Lane 1, *loa22* cut with *Age*I; Lane 2, *loa22* cut with *Nhe*I; Land 3 and 4, *loa22* completely cut with both enzymes.

The plasmid pVITRO1 (InvivoGen, USA) and pVITRO-*lipL32* are the mammalian expression vectors used in this study for the construction of DNA vaccine of *lipL32, loa22,* and *lipL32-loa22*. They were also subjected to digest with *Age*I and *Nhe*I. After digesting with *Age*I, the plasmids were purified and treated with *Nhe*I. Upon restriction enzyme treatement, the digested product of pVITRO and pVITRO-*lipL32* shifted the migration distance to the predicted size of 6,295 and 7,114 bp, respectively, on agarose gel electrophoresis, indicating that they were completely digested with restriction enzymes and were in the linear form (Figture 18). The *Age*I and *Nhe*I-digested plasmid pVITRO and pVITRO-*lipL32* were used to ligate with *loa22* obtained above.

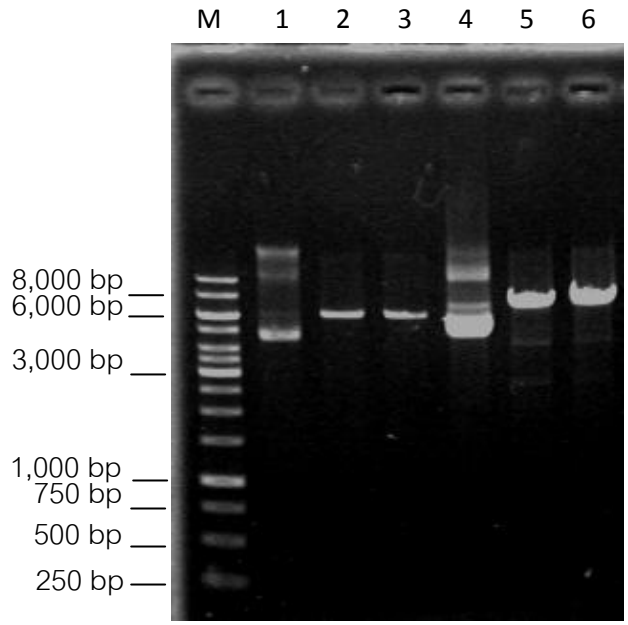


Figure 18. The pVITRO and pVITRO-*lipL32* plasmids digested with *AgeI* and *NheI* Lane M, 1 kB DNA ladder; Lane 1, uncut pVITRO; Lane 2 and 3, pVITRO cut with *Age*I and *Nhe*I; Lane 4, uncut pVITRO-*lipL32*; Lane 5 and 6, pVITRO-*lipL32* cut with *Age*I and *Nhe*I.

Transformation to obtain recombinant plasmids

After DNA ligantion, the products were used for transformation. The transformants were selected on kanamycin containing LB plates. All colonies were picked and screened for the insertion of *loa22* by using colony PCR method. For the cloning of *loa22* into pVITRO, there are 15 colonies appeared on the kanamycin containing LB plates. All 15 clones were screened using *loa22* specific primers. Only one colony from 15 colonies showed the 588 bp PCR product (colony number 5), indicating that it has the insertion of *loa22*. Other colonies did not have the insertion (Figure 19). Genomic DNA of *L. interrogans* serovar Pomona was used as a template for positive control

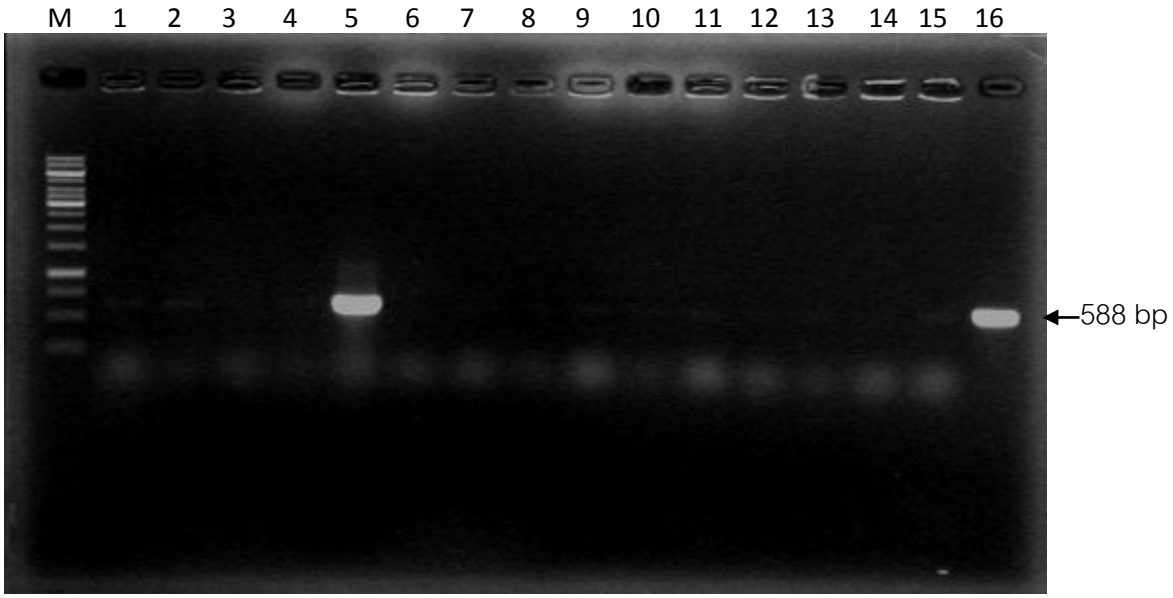


Figure 19. Colony PCR amplification to screen for *loa22* insertion in pVITRO. Lane M, 1 kB DNA ladder; Lane 1-15, PCR product when amplified from colonies number 1-15; Lane 16, positive control using genomic DNA of *L. interrogans* serovar Pomona as a DNA template for PCR amplification.

For cloning of *loa22* into pVITRO/*lipL32* to obtain pVITRO/*lipL32-lo22*, only one transformant colony appeared on the kanamycin containing LB plates. This colony has the *loa22* insertion after checking by colony PCR method (Figure 20).

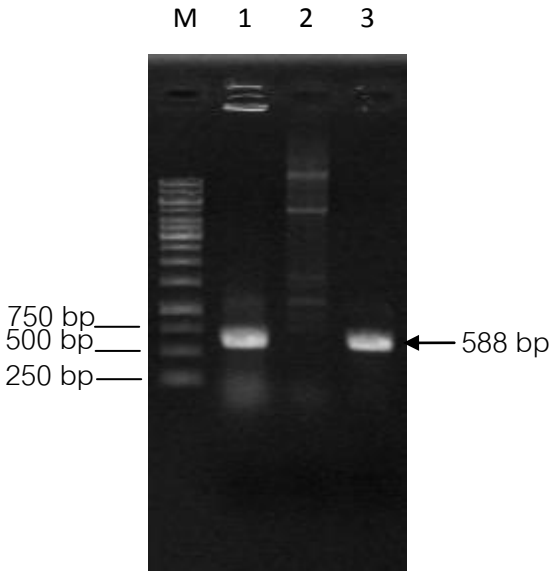


Figure 20. Colony PCR amplification to screen for *loa22* insertion in pVITRO-*lipL32*. Lane M, 1 kB DNA ladder; Lane 1, PCR product when amplified from a colony from kanamycin containing plate (colony number 1); Lane 2, negative control amplification using empty pVITRO as a template; Lane 3, positive control using genomic DNA of *L. interrogans* serovar Pomona as a DNA template for positive control.

DNA sequencing

pVITRO-*loa22* sequencing

Transformant of pVITRO-*loa22* (colony number 5 in Figure 4) was cultured in LB broth containing kanamycin and the plasmids were purified. Sequencing of *loa22* insertion in the plasmid was determined. There are 588 nucleotide encodes for full length Loa22 protein. The insertion of *loa22* in pVITRO-*loa22* plasmids has one mismatch at the nucleotide position 420 which changed nucleotide from G into A (Figure 21). Both GAG and GAA code for glutamic acid.

Sequence ID: lcl|30047 Length: 588 Number of Matches: 1

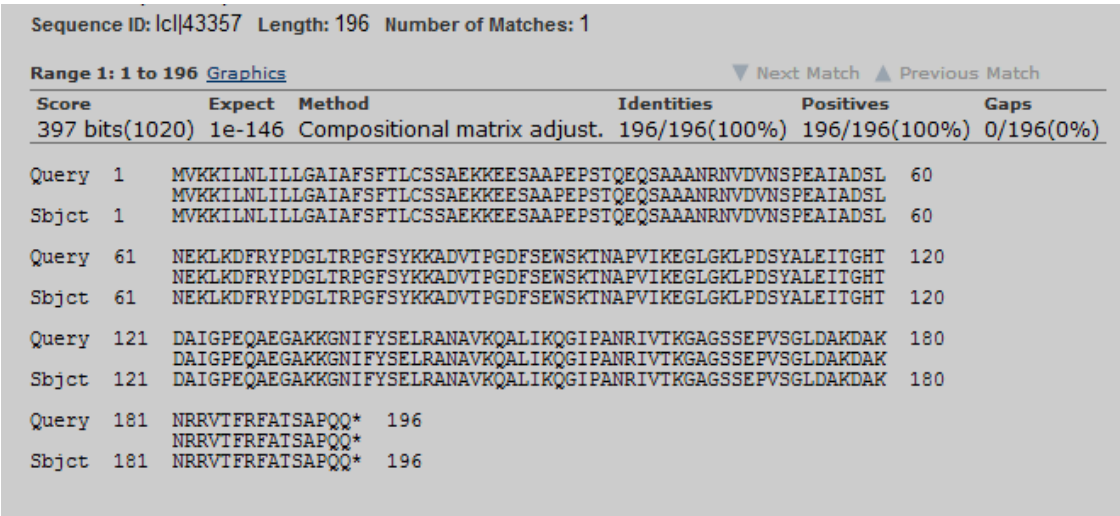
Range 1: 1 to 588 Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
1081 bits(585)	0.0	587/588(99%)	0/588(0%)	Plus/Plus

```
Query  1    ATGGTCAAAAAGATTTTGAATCTGATTCTGCTCGGTGCAATTGCATTTTCATTCACTCTC  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1    ATGGTCAAAAAGATTTTGAATCTGATTCTGCTCGGTGCAATTGCATTTTCATTCACTCTC  60

Query  61   TGCTCCTCTGCTGaaaaaaaaGAGGAATCCGCAGCTCCTGAGCCTTCAACGCAAGAGCAA  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61   TGCTCCTCTGCTGAAAAAAAAGAGGAATCCGCAGCTCCTGAGCCTTCAACGCAAGAGCAA  120

Query  121  TCCGCAGCTGCAAACAGAAACGTTGACGTCAATTCTCCGGAAGCGATCGCAGATTCTTTA  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  121  TCCGCAGCTGCAAACAGAAACGTTGACGTCAATTCTCCGGAAGCGATCGCAGATTCTTTA  180

Query  181  AACGAAAAACTAAAAGATTTCCGGTATCCAGACGGTTTAACTCGTCCTGGATTTAGTTAT  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  181  AACGAAAAACTAAAAGATTTCCGGTATCCAGACGGTTTAACTCGTCCTGGATTTAGTTAT  240

Query  241  AAAAAAGCGGATGTTACCCCTGGTGATTTCAGCGAGTGGTCTAAAACAAACGCTCCTGTA  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  241  AAAAAAGCGGATGTTACCCCTGGTGATTTCAGCGAGTGGTCTAAAACAAACGCTCCTGTA  300

Query  301  ATCAAAGAAGGTCTTGGAAAACTTCCAGATAGTTACGCTCTTGAAATTACAGGACACACC  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  301  ATCAAAGAAGGTCTTGGAAAACTTCCAGATAGTTACGCTCTTGAAATTACAGGACACACC  360

Query  361  GATGCGATCGGTCCCGAACAAGCAGAAGGTGCTAAAAAAGGAAATATTTTTTACTCTGAG  420
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  361  GATGCGATCGGTCCCGAACAAGCAGAAGGTGCTAAAAAAGGAAATATTTTTTACTCTGAA  420

Query  421  CTTCGTGCAAATGCAGTTAAACAAGCTTTAATCAAACAAGGGATTCCAGCAAATCGTATC  480
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  421  CTTCGTGCAAATGCAGTTAAACAAGCTTTAATCAAACAAGGGATTCCAGCAAATCGTATC  480

Query  481  GTTACTAAAGGTGCCGGTTCTTCCGAGCCAGTTTCTGGTCTTGATGCGAAAGATGCTAAA  540
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  481  GTTACTAAAGGTGCCGGTTCTTCCGAGCCAGTTTCTGGTCTTGATGCGAAAGATGCTAAA  540

Query  541  AATAGAAGAGTCACTTTCCGTTTTGCGACTTCCGCACCACAACAATAA  588
            ||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  541  AATAGAAGAGTCACTTTCCGTTTTGCGACTTCCGCACCACAACAATAA  588
```

Figure 21. DNA alignment of *loa22* sequence from genomic DNA of *L. interrogans* serovar Pomona (Query) and *loa22* from pVITRO-*loa22* clone number 5 (Sbjct). The nucleotide at the position 420 in the red box indicated the position with the silent mutation.

The predicted amino acid sequence of Loa22 in pVITRO-*loa22* was aligned with the full length of the amino acid sequence of Loa22 encoded from *L. interrogans* serovar Pomona (Gene bank AFLT02000039.1 Region: 87085 to 87672). The full length Loa22 contains 195 amino acids. The sequence of total amino acids of Loa22 from pVITRO-*loa22* plasmid is perfectly identical with Loa22 from *Leptospira* (Figure22). One point mutation in the nucleotide sequences did not change the amino acid sequence.



Sequence ID: lcl|43357 Length: 196 Number of Matches: 1

Range 1: 1 to 196 Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Method	Identities	Positives	Gaps
397 bits(1020)	1e-146	Compositional matrix adjust.	196/196(100%)	196/196(100%)	0/196(0%)

```
Query  1    MVKKILNLILLGAIAFSFTLCSSAEKKEESAAPEPSTQEQSAAANRNVDVNSPEAIADSL  60
            MVKKILNLILLGAIAFSFTLCSSAEKKEESAAPEPSTQEQSAAANRNVDVNSPEAIADSL
Sbjct  1    MVKKILNLILLGAIAFSFTLCSSAEKKEESAAPEPSTQEQSAAANRNVDVNSPEAIADSL  60

Query  61   NEKLKDFRYPDGLTRPGFSYKKADVIPGDFSEWSKTNAPVIKEGLGKLPDSYALEITGHT  120
            NEKLKDFRYPDGLTRPGFSYKKADVIPGDFSEWSKTNAPVIKEGLGKLPDSYALEITGHT
Sbjct  61   NEKLKDFRYPDGLTRPGFSYKKADVIPGDFSEWSKTNAPVIKEGLGKLPDSYALEITGHT  120

Query  121  DAIGPEQAEGAKKGNIFYSELRANAVKQALIKQGIPANRIVTKGAGSSEPVSGLDAKDAK  180
            DAIGPEQAEGAKKGNIFYSELRANAVKQALIKQGIPANRIVTKGAGSSEPVSGLDAKDAK
Sbjct  121  DAIGPEQAEGAKKGNIFYSELRANAVKQALIKQGIPANRIVTKGAGSSEPVSGLDAKDAK  180

Query  181  NRRVTFRFATSAPQQ*  196
            NRRVTFRFATSAPQQ*
Sbjct  181  NRRVTFRFATSAPQQ*  196
```

Figure 22. Alignment the amino acid sequence of Loa22 from genomic DNA of *L. interrogans* serovar Pomona (Query) and Loa22 from pVITRO/*loa22* clone number 5 (Sbjct). Percentage of amino acid similarity is 100%.

pVITRO-*lipL32-loa22* sequencing

Transformants of pVITRO/*lipL32-loa22* (colony number 1 in Figure 5) was cultured in the LB broth containing kanamycin and the plasmid was purified. of the nucleotide sequences of *loa22* insert in pVITRO-*lipL32* was determined. The insertion of *loa22* in pVitro/*lipL32* plasmids has one mismatch at the nucleotide position 420 similar to the mismatch of an insert of *loa22* in pVITRO (Figure 23). A single nucleotide changes from G into A (Figure 24) indicated the silent mutation since GAG and GAA both code for glutamic acid.

Sequence ID: lcl|30047 Length: 588 Number of Matches: 1

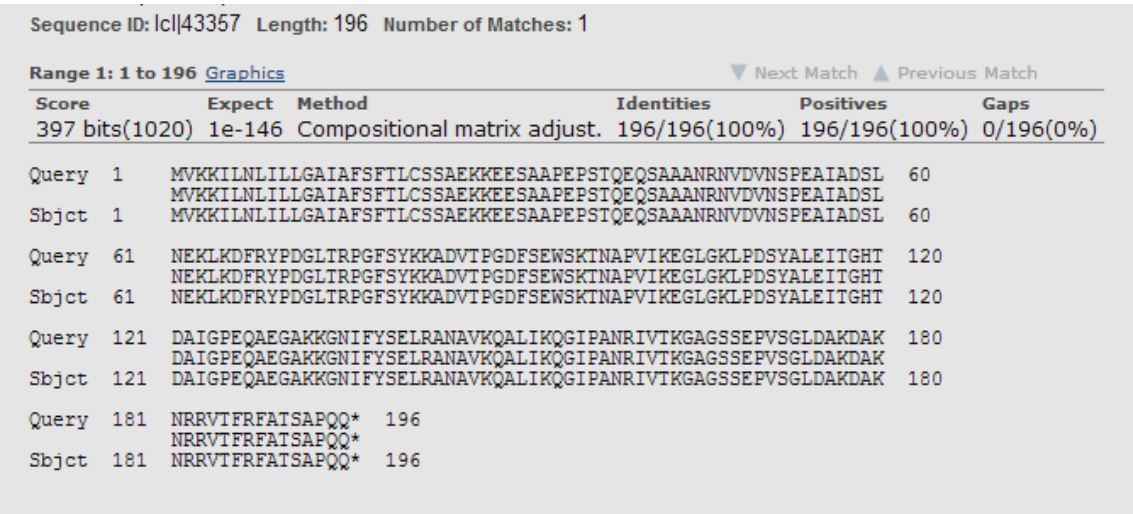
Range 1: 1 to 588 Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
1081 bits(585)	0.0	587/588(99%)	0/588(0%)	Plus/Plus

```
Query  1    ATGGTCAAAAAGATTTTGAATCTGATTCTGCTCGGTGCAATTGCATTTTCATTCACTCTC  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1    ATGGTCAAAAAGATTTTGAATCTGATTCTGCTCGGTGCAATTGCATTTTCATTCACTCTC  60

Query  61   TGCTCCTCTGCTGaaaaaaaaGAGGAATCCGCAGCTCCTGAGCCTTCAACGCAAGAGCAA  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61   TGCTCCTCTGCTGAAAAAAAAGAGGAATCCGCAGCTCCTGAGCCTTCAACGCAAGAGCAA  120

Query  121  TCCGCAGCTGCAAACAGAAACGTTGACGTCAATTCTCCGGAAGCGATCGCAGATTCTTTA  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  121  TCCGCAGCTGCAAACAGAAACGTTGACGTCAATTCTCCGGAAGCGATCGCAGATTCTTTA  180

Query  181  AACGAAAAACTAAAAGATTTCCGGTATCCAGACGGTTTAACTCGTCCTGGATTTAGTTAT  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  181  AACGAAAAACTAAAAGATTTCCGGTATCCAGACGGTTTAACTCGTCCTGGATTTAGTTAT  240

Query  241  AAAAAAGCGGATGTTACCCCTGGTGATTTCAGCGAGTGGTCTAAAACAAACGCTCCTGTA  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  241  AAAAAAGCGGATGTTACCCCTGGTGATTTCAGCGAGTGGTCTAAAACAAACGCTCCTGTA  300

Query  301  ATCAAAGAAGGTCTTGGAAAACTTCCAGATAGTTACGCTCTTGAAATTACAGGACACACC  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  301  ATCAAAGAAGGTCTTGGAAAACTTCCAGATAGTTACGCTCTTGAAATTACAGGACACACC  360

Query  361  GATGCGATCGGTCCCGAACAAGCAGAAGGTGCTAAAAAAGGAAATATTTTTTACTCTGAG  420
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  361  GATGCGATCGGTCCCGAACAAGCAGAAGGTGCTAAAAAAGGAAATATTTTTTACTCTGAA  420

Query  421  CTTCGTGCAAATGCAGTTAAACAAGCTTTAATCAAACAAGGGATTCCAGCAAATCGTATC  480
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  421  CTTCGTGCAAATGCAGTTAAACAAGCTTTAATCAAACAAGGGATTCCAGCAAATCGTATC  480

Query  481  GTTACTAAAGGTGCCGGTTCTTCCGAGCCAGTTTCTGGTCTTGATGCGAAAGATGCTAAA  540
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  481  GTTACTAAAGGTGCCGGTTCTTCCGAGCCAGTTTCTGGTCTTGATGCGAAAGATGCTAAA  540

Query  541  AATAGAAGAGTCACTTTCCGTTTTGCGACTTCCGCACCACAACAATAA  588
            ||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  541  AATAGAAGAGTCACTTTCCGTTTTGCGACTTCCGCACCACAACAATAA  588
```

Figure 23. DNA alignment of *loa22* sequence from genomic DNA of *L. interrogans* serovar Pomona (Query) and loa22 from pVITRO/*lipL32-loa22* clone number 1 (Sbjct). The nucleotide at the position 420 in the red box is the silent mutation.

The amino acid sequences of Loa22 from pVITRO/*lipL32-loa22* was aligned with the full length of amino acid sequences of Loa22 from *L. interrogans* serovar Pomona (Gene bank AFLT02000039.1 Range: 87085 to 87672). The full length Loa22 contains 195 amino acids. The amino acid sequence of Loa22 from pVITRO/*lipL32-loa22* plasmid is completely identical with Loa22 from *Leptospira* genome (Figure 24).



Sequence ID: lcl|43357 Length: 196 Number of Matches: 1

Range 1: 1 to 196 Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Method	Identities	Positives	Gaps
397 bits(1020)	1e-146	Compositional matrix adjust.	196/196(100%)	196/196(100%)	0/196(0%)

```
Query  1    MVKKILNLILLGAIAFSFTLCSSAEKKEESAAPEPSTQEQSAAANRNVDVNSPEAIADSL  60
            MVKKILNLILLGAIAFSFTLCSSAEKKEESAAPEPSTQEQSAAANRNVDVNSPEAIADSL
Sbjct  1    MVKKILNLILLGAIAFSFTLCSSAEKKEESAAPEPSTQEQSAAANRNVDVNSPEAIADSL  60

Query  61   NEKLKDFRYPDGLTRPGFSYKKADVTPGDFSEWSKTNAPVIKEGLGKLPDSYALEITGHT  120
            NEKLKDFRYPDGLTRPGFSYKKADVTPGDFSEWSKTNAPVIKEGLGKLPDSYALEITGHT
Sbjct  61   NEKLKDFRYPDGLTRPGFSYKKADVTPGDFSEWSKTNAPVIKEGLGKLPDSYALEITGHT  120

Query  121  DAIGPEQAEGAKKGNIFYSELRANAVKQALIKQGIPANRIVTKGAGSSEPVSGLDAKDAK  180
            DAIGPEQAEGAKKGNIFYSELRANAVKQALIKQGIPANRIVTKGAGSSEPVSGLDAKDAK
Sbjct  121  DAIGPEQAEGAKKGNIFYSELRANAVKQALIKQGIPANRIVTKGAGSSEPVSGLDAKDAK  180

Query  181  NRRVTFRFATSAPQQ*  196
            NRRVTFRFATSAPQQ*
Sbjct  181  NRRVTFRFATSAPQQ*  196
```

Figure 24. Amino acid alignment of Loa22 sequence encoded from genomic DNA of *L. interrogans* serovar Pomona (Query) with Loa22 encoded from pVITRO/*lipL32-loa22* clone number 1 (Sbjct). 100% of amino acid is identical. The transformant clone of pVITRO/*lipL32-loa22* has a perfect match of amino acid sequences with *Leptospira*.

Plasmid Preparation for Transfection and for Used a DNA Vaccine

Endotoxin free pVITRO, pVITRO-*lipL32*, pVITRO-*loa22*, and pVITRO-*lipL32-loa22* were obtained from *E.coli* DH5α as described in materials and methods. These plasmids were used for an *in vitro* transfection and for DNA vaccine immunization. The expected size of DNA of each plasmid was confirmed (Figure 25). The size of linearized pVITRO, pVITRO-*lipL32*, pVITRO-*loa22*, and pVITRO-*lipL32-loa22* are 6295, 7114, 6883, and 7702 bp. respectively.

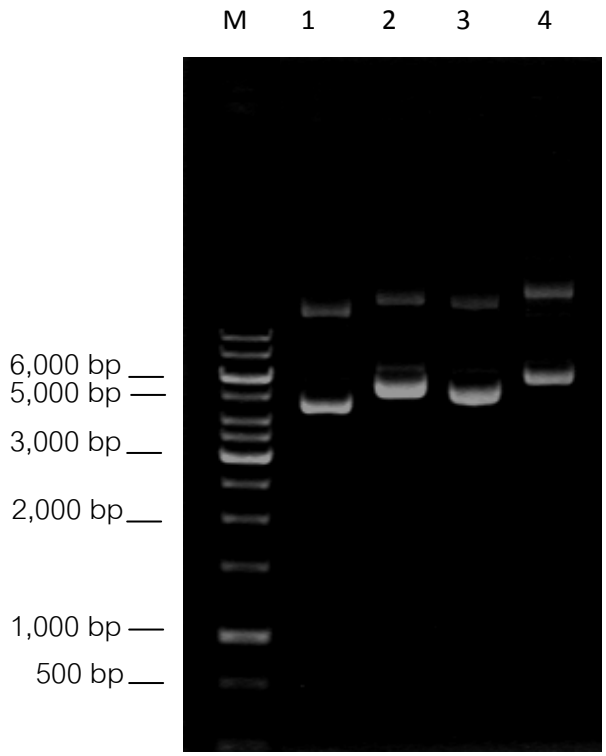


Figure 25. Endotoxin-free recombinant plasmid pVITRO, pVITRO-*lipL32*, pVITRO-*loa22*, and pVITRO-*lipL32-loa22* after purification. Land M, 1 kB DNA ladder, Lane 1, pVITRO; Lane 2, pVITRO-*lipL32*; Lane 3 pVITRO-*loa22*; Lane 4, pVITRO-*lipL32-loa22*

Transfection of Recombinant Plasmids in HEK293T Cell Line

HEK293T cell line was transfected with pVITRO, pVITRO-*lipL32*, pVITRO-*loa22*, or pVITRO-*lipL32-loa22* as described in materials and methods. After 48h of transfection, protein extracts from cell lysate and in culture supernatant were detected for LipL32 or Loa22 by Western blot. In both cell lysate and culture supernatant fractions of transfected HEK293T, LipL32 and Loa22 could be detected (Figure 26). In total cell lysate, a single major band corresponding to the predicted molecular weight of approximately 34 kDa of LipL32 was detected while those corresponding to Loa22 showed the molecular weight around 22 kDa. The control plasmid (pVITRO empty

vector) did not show any specific bands. In the culture supernatant, both LipL32 and Loa22 could be detected as well. The band corresponding to Loa22 has the same size as in the cell lysate. However, the secreted LipL32 has molecular weight of about 38 kDa which is higher than that observed in the cell lysate. Comparison of the expression level in pVITRO-*lipL32-loa22* transfection or pVITRO-*lipL32* and pVITRO-*loa22* co-transfection indicated that pVITRO-*lipL32-loa22* which carries both inserts in the same plasmid resulted in a higher expression of both proteins than the co-transfection method, especially for the level of Loa22 (Figure 26). The 43 kDa of β-actin which was used as an internal control could be detected only in the cell lysate fraction. Taken together, these results indicated that the cloned genes are correctly expressed both intracellularly, and as a secreted form. Using pVITRO-*lipL32-loa22* provided slightly higher expression of LipL32 and Loa22 than the co-transfection of each plasmid at equimolar concentration.

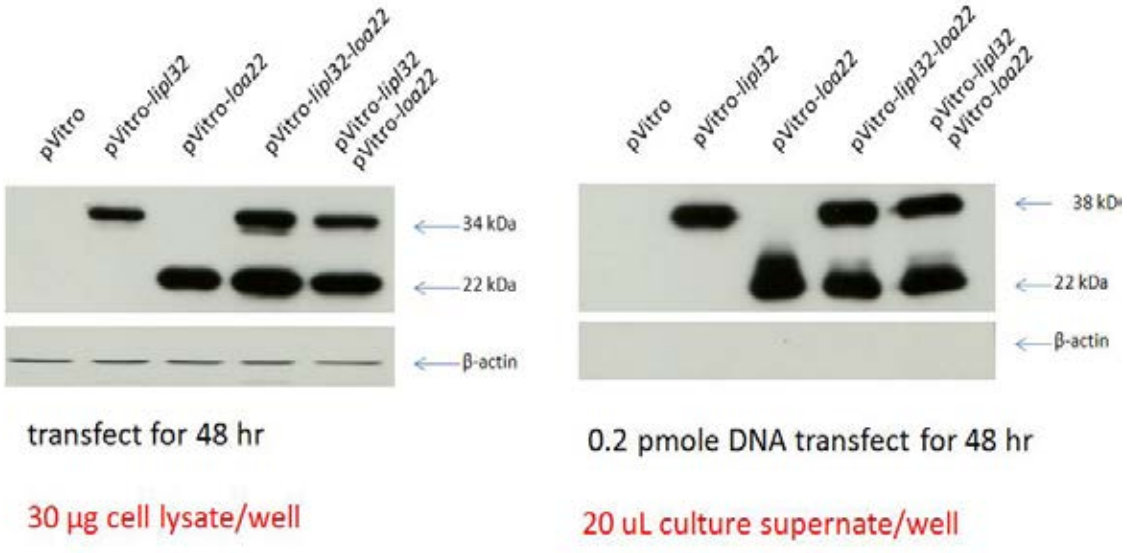


Figure 26. Detection of LipL32 and Loa22 in the cell lysate (left) and in the culture supernant (right) after transfection for 48 hrs in HEK293T. Lane 1-5 show HEK293 transfected with pVITRO, pVITRO-*lipL32*, pVITRO-*loa22*, pVITRO-*lipL32-loa22*, or pVITRO-*lipL32*+pVITRO-*loa22* co-transfection, respectively. LipL32 and Loa22 proteins were detected simultaneously with mouse polyclonal anti-LipL32 and mouse polyclonal anti-LipL32 from immunized mice.

Optimization of DNA Encapsulation by Chitosan

Transfection efficiency under fluorescent microscope

To develop a DNA vaccine for immunization, chitosan was used as DNA vaccine delivery system in this study. After demonstrating the correction of *loa22* sequence and the protein expression in cells transfected using pVITRO, pVITRO-*lipL32*, pVITRO-*loa22*, pVITRO-*lipL32-loa22* plasmids, the amount of molar ratio of chitosan and DNA was investigated for optimal encapsulation by determining the molar ratio of amine (N) of chitosan and phosphate (P) of DNA (N/P ratio). The objective is to determine the optimal

ratio that provides the highest transfection efficiency in a model cell line. In order to monitor the transfection efficiency, pMax-GFP was used as model plasmid for encapsulation. Ratios of N/P at 1:1, 2:1, 4:1, 8:1, 10:1 and 20:1 were tested. Encapsulation of CS/DNA at each ratio was performed by complex coacervation method [40]. Five days after transfection in HEK 293T cell line, GFP positive cells were monitored by fluorescent microscope. The transfection efficiency correlated well with an increasing ratio of N/P. The highest transfection efficiency was observed at the N/P ratio between 8:1 to 20:1 (Figure 27).

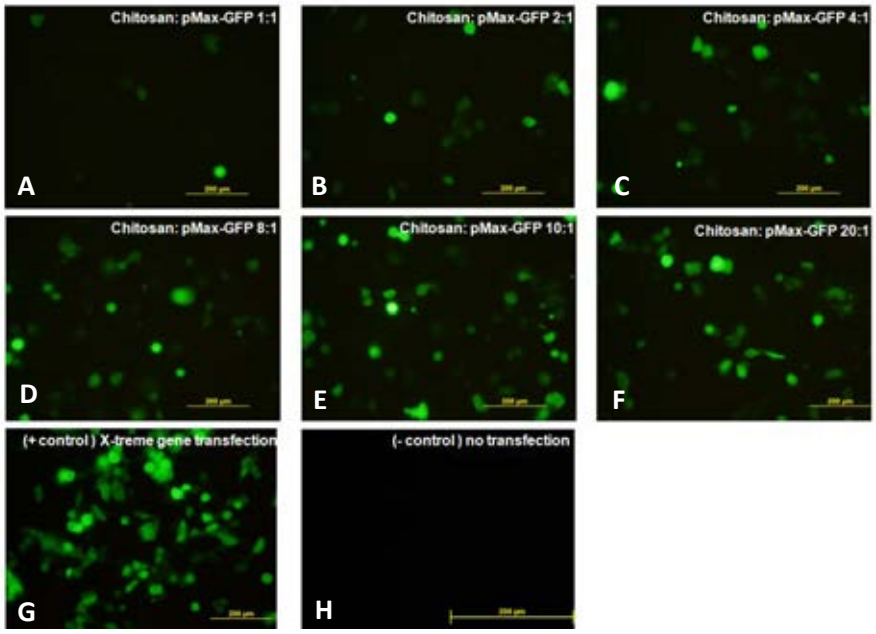


Figure 27. Expression of GFP in HEK 293 cells tranfected using CS/DNA transfection system. The plasmid pMax-GFP was used as a model plasmid. HEK293T cells were transfected with CS/pMax-GFP nanoparticles for 5 days at the N/P ratios of 1:1, 2:1, 4:1, 8:1, 10:1 and 20:1, and were shown in Figure 27A to 27F, respectively. Transfection efficiency was observed under fluorescent microscope. X-treme Gene transfection reagent was used as a positive control (Figure 27G). Untransfected cells were used for negative control (Figure 27H).

Encapsulation Efficacy of CS/DNA plasmid

The efficiency of DNA encapsulation by CS at different N/P ratios between 1:1 and 20:1 were determined by using agarose gel electrophoresis and the results were compared with the naked unencapsulated plasmid as a control. Immediately after encapsulation of CS/pMax-GFP at different ratios in the range of 1:1 to 20:1. encapsulated plasmid equivalent to 100 ng of DNA was directly loaded into 1% agarose gel electrophoresis. The naked DNA of pMax-GFP migrated at the expected size whereas the DNA encapsulated in chitosan nanoparticles remained in the loading wells when the N/P ratio was equal to or more than 8:1 (Figure 28).

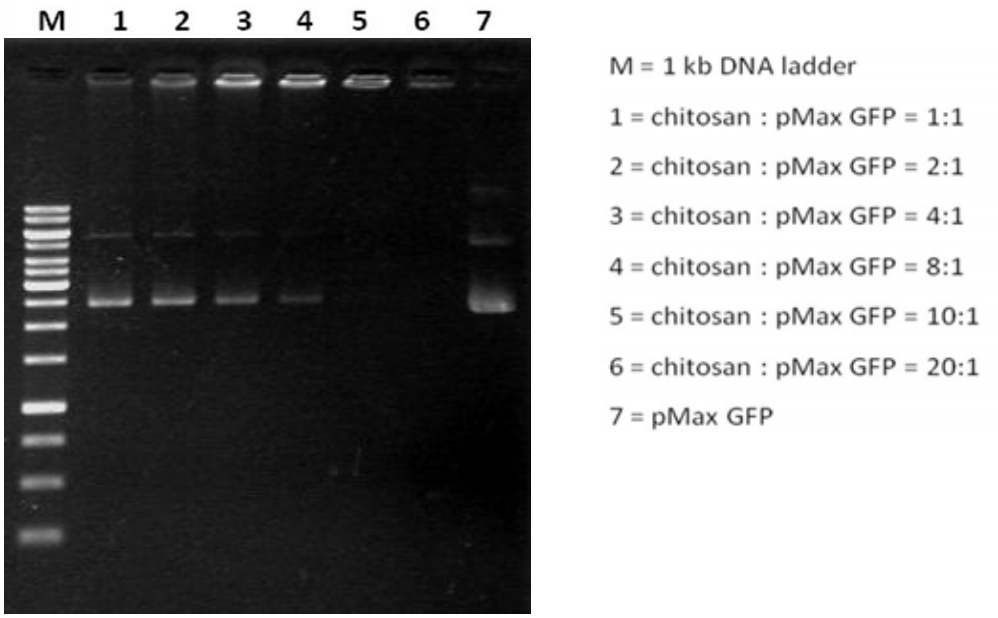


Figure 28. Encapsulation Efficacy of CS/DNA plasmid of CS/DNA partcles. Lane M marker. Lane 7 naked DNA. Lanes 1–6 nanoparticles prepared at N/P ratios of 1:1, 2:1, 4:1, 8:1, 10:1 and 20:1, respectively; Lane 7, pMax-GFP without encapsulation.

Quantitative Transfection Efficacy by Flow cytometry analysis

The transfection efficiency of CS/DNA particles of CS/pMax-GFP at the N/P ratio in the range of 1:1 and 20:1 were quantitatively measured by flow cytometer. The transfection efficiency of CS/DNA particles increased from 5.6% to 27.1% when the N/P ratio increased from 1:1 to 20:1(Figure 29). The transfection efficiency of commercial reagent was at 99.5% (data not show). Hence, the ratio of 20:1 was selected for further experiments in DNA vaccine preparation of *lipL32, loa22* vaccine

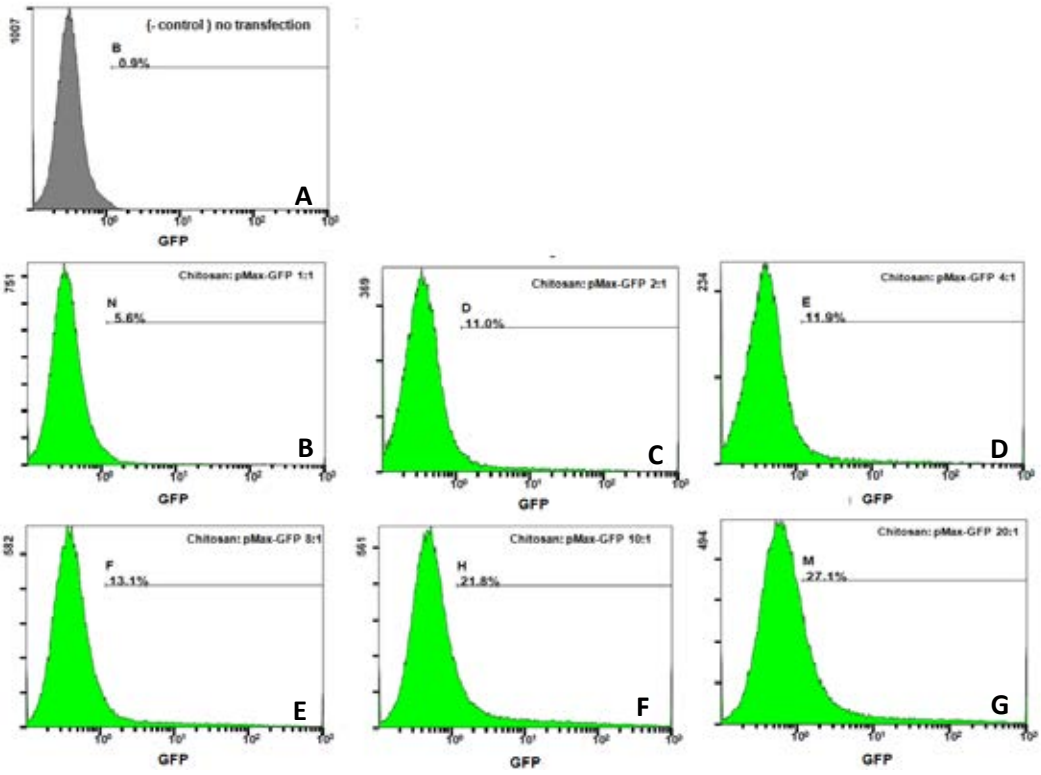


Fig at the N/P ratio of 1:1, 2:1, 4:1, 8:1, 10:1, or 20:1 observed by flow cytometry. The grey histrogram shows untransfected cell (Figure 29A). Other histograms (Figure 29B-29G) show the percent of transfection efficiency of CS/pMax-GFP at the ratio of 1:1, 2:1, 4:1, 8:1, 10:1 and 20:1, respectively.

Efficiency of chitosan as a *lipL32* and *loa22* DNA vaccine delivery system.

The efficacy of transfection using CS/DNA particles of pVITRO-*lipL32-loa22* at N/P ratio of 20:1 was determined by immunofluorescent staining in HEK 293T cell line. HEK293T cell line was transfected with CS/pVITRO-*lipL32-loa22* particles for five days and subjected to staining by rabbit anti-lipL32 and mouse anti-Loa22 polyclonal antibodies. The expression of LipL32 and Loa22 could be detected in the same cells when pVITRO-lipl32-loa22 was used (Figure 30). This result suggests that both proteins can be expressed in the same cells using CS as a delivery system.

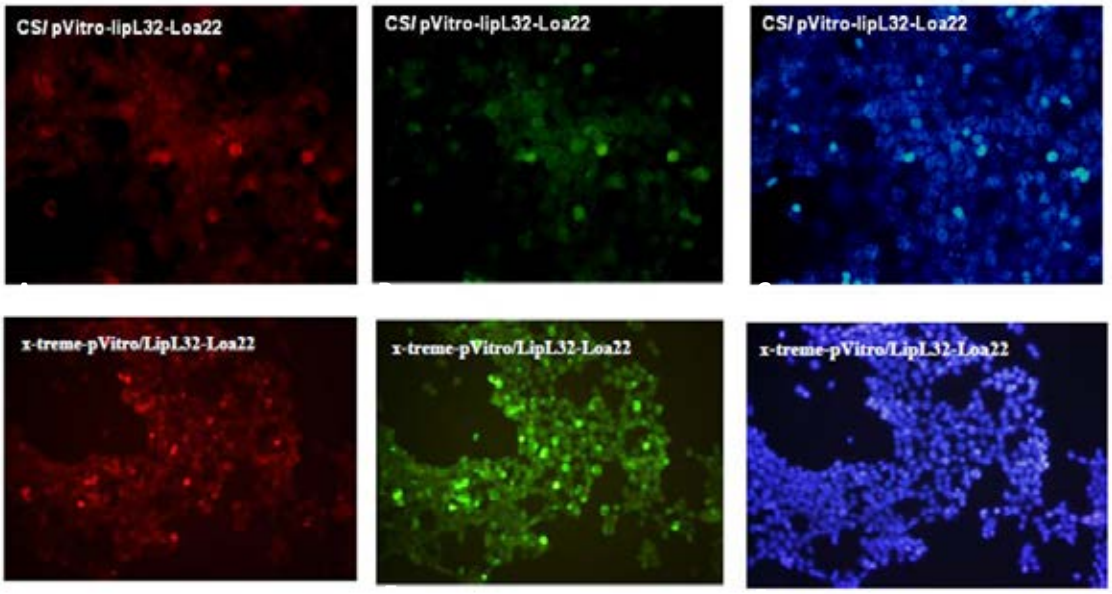


Figure 30. Immunofluorescent staining of Lipl32 and Loa22 in HEK293T cell line using CS/pVITRO-*lipl32-loa22*. LipL32 (red) and Loa22 (green) and DAPI (blue) are seen in cells after transfection with the N/P ratio of 20:1 using CS/pVITRO-*lipl32-loa22* particle for 5 days (Figure 30A-30C) compare with transfect with X-treme gene transfection reagent (Figure 30D-30F).

Size and morphology of CS/pVITRO-lipL32, CS/pVITRO-*loa22*, CS/pVITRO-*lipL32-loa22* particles

The morphology of particles of CS, CS/pVITRO, CS/pVITRO-*lipL32*, CS/pVITRO-*loa22*, CS/pVITRO-*lipL32-loa22*, or CS/pVITRO-*lipL32*+CS/pVITRO-*loa22* was analyzed by scanning electron microscope (SEM). After encapsulating the plasmid with CS, sizes of the particles were still in the range of nanometers (approximately 100 nm). Different shapes and sizes were observed but most particles have the spherical morphology (Figure 31). Therefore, the CS/DNA plasmid formulated in this study is referred to as a nanoparticle in this study.

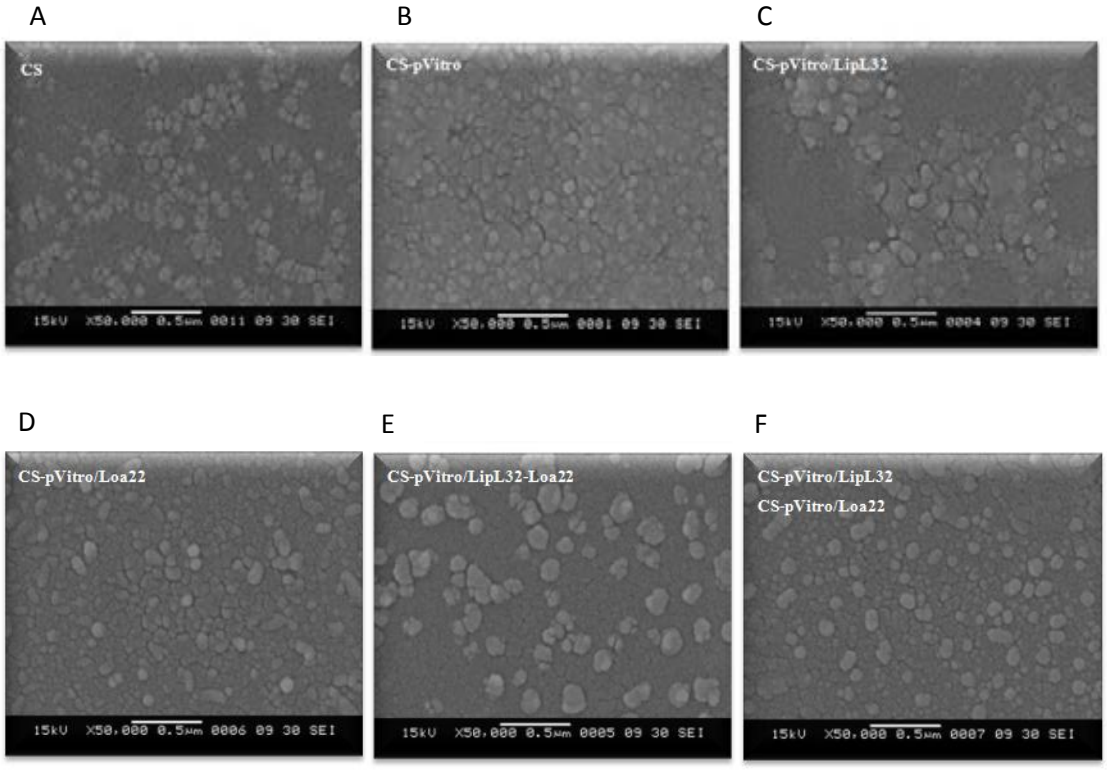


Figure 31. Scanning electron micrograph of the CS/DNA nanopartcile. The encapsulation was carried out at the N/P ratio of 20:1. A, CS only; B, CS/pVITRO; C, CS/pVITRO-*lipL32*; D, CS/pVITRO-*loa22*; E, CS/pVITRO-*lipL32-loa22* and F, CS/pVITRO-*lipL32*+pVITRO/*loa22* co-encapsulation.

To quantitatively measure the nanoparticle sizes of CS, CS/pVITRO, CS/pVITRO-*lipL32*, CS/pVITRO-*loa22*, CS/pVITRO-*lipL32-loa22*, or CS/pVITRO-*lipL32*+CS/pVITRO-*loa22*, the images of particles obtained from scanning electron microscope (SEM) were analyzed by ImageJ program. Total 50 particles in each sample were measured for the diameter and calculated for mean of diameter. After encapsulating DNA plasmid with CS, sizes of CS/DNA in all conditions were approximately 100-125 nm. A marginal different size between each CS/DNA was observed (Figure 32).

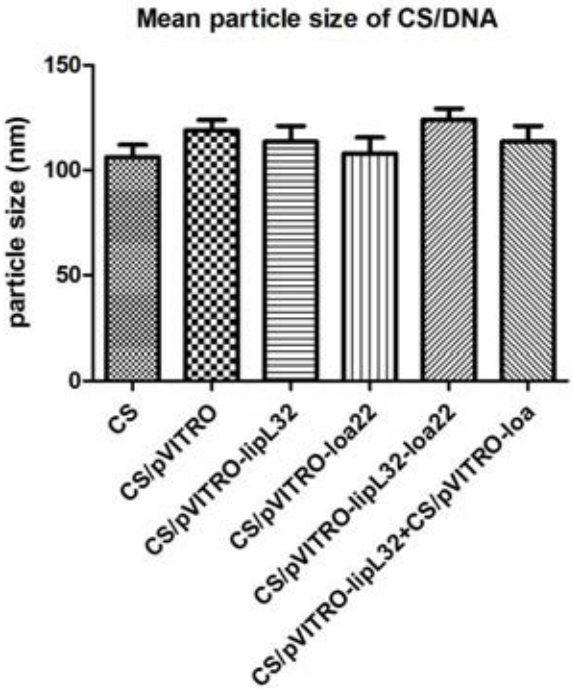


Figure 32. The mean diameter of CS, CS/pVITRO, CS/pVITRO-*lipL32*, CS/pVITRO-*loa22*, CS/pVITRO-*lipL32-loa22*, or CS/pVITRO-*lipL32*+CS/pVITRO-*loa22* particles was analyzed by ImageJ program from the data obtained by scanning electron microscope (SEM).

Cytotoxicity of CS/pVITRO, CS/pVITRO-*lipL32*, CS/pVITRO-*loa22*, CS/pVITRO-*lipL32-loa22* nanoparticles

After encapsulation of CS/DNA of pVITRO, pVITRO-*lipL32,* pVITRO-loa22, and pVITRO-*lipL32-loa22*, they were tested for the cytotoxicity against HEK293T cell line by MTT assay. All types of CS/DNA nanoparticles resulted in little to no toxicity effect against HEK293T cell line. This result was observed at all N/P ratios of all types of CS/DNA nanoparticle (Figure 33).

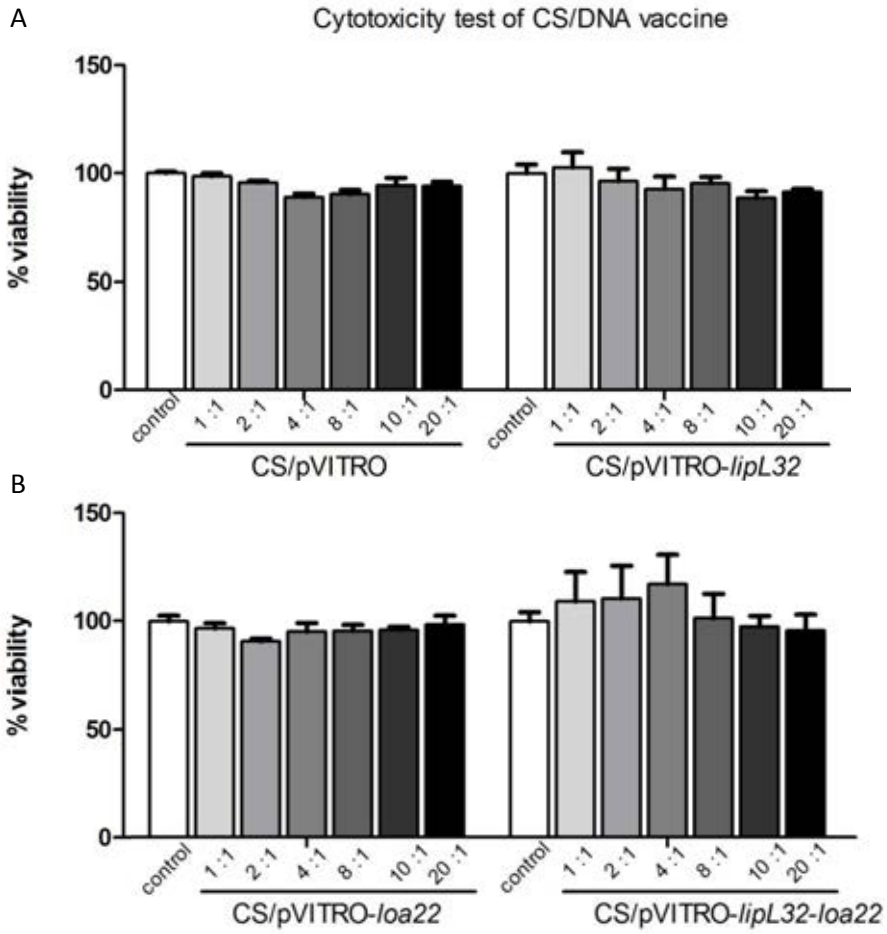


Figure 33. Cytotoxicity of (A) CS/pVITRO and CS/pVITRO-*lipL32*, (B) CS/pVITRO-*loa22* and CS/pVITRO-*lipL32-loa22* in HEK293T cell line (n=3, error bars represent standard deviation). The result is representative of the two independent experiments with similar trends.

Preparation of recombinant LipL32

Induction of expression of recombinant LipL32 (rLipL32) in *E. coli* strain BL21 (DE3) pLysS was carried out by 0.5 mM IPTG at 37ºC for 3 hrs. The expression of rLipL32 was clearly observed at the size around 34 kDa in the soluble part (supernatant) of extracted protein (Figure 34A). To purify rLipL32 in large scale (four liters of *E. coli* culture), his-tag affinity column chromatography and hydrophobic column chromatography were applied for two step protein purification. At the first step of purification by using his-tag affinity column chromatography, large amount of rLIpL32 was obtained in the 250 mM and 500 mM of imidazole fraction (Figure 34B). These two fractions were collected and purified with the hydrophobic column chromatography. With this step of purification, the purity of rLipL32 increased and could be observed in 600, 400, and 200 mM ammonium sulfate to DDW fractions (Figure 34C). All three fractions were pooled and used for immunization, ELISA, and *in vitro* splenocyte re-stimulation.

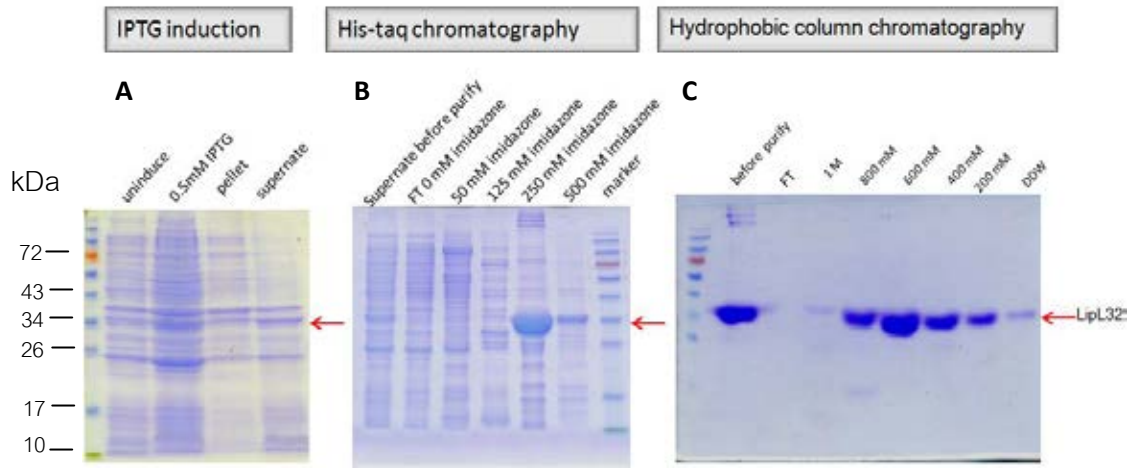


Figure34. SDS-PAGE of rLipL32. A, induction of rLipL32 by 0.5 mM IPTG; B, purification of soluble part of rLipL32 after induction by His-tag column chromatography; C, purification of rLipL32 with hydrophobic column chromatography after purifying by His-tag column chromatography each lane represent each fraction as indicated above.

Preparation of recombinant Loa22 protein

Induction of recombinant Loa22 (rLoa22) in *E. coli* strain BL21 (DE3) pLysS were conducted by 0.1 mM IPTG at 37ºC for 2 hrs. The expression of rLoa22 could be clearly observed at the size around 22 kDa in the soluble part (supernatant) of extracted protein (Figure 35A). To purify rLoa22 in large scale (four liters of *E. coli* culture), his-tag affinity column chromatography and hydrophobic column chromatography were used. At the first step of purification by His-tag affinity column chromatography, large amount of rLoa22 was obtained in the 125 mM and 250 mM of imidazole fractions (Figure 35B). These two fractions were collected and purified with the hydrophobic column chromatography. With this step of purification, the purity of rLoa22 was observed in 800 mM to 400 mM ammonium sulfate fractions (Figure 35C). Both fractions were pooled and used for immunization and cell stimulation. Other fractions were used for ELISA plate coating.

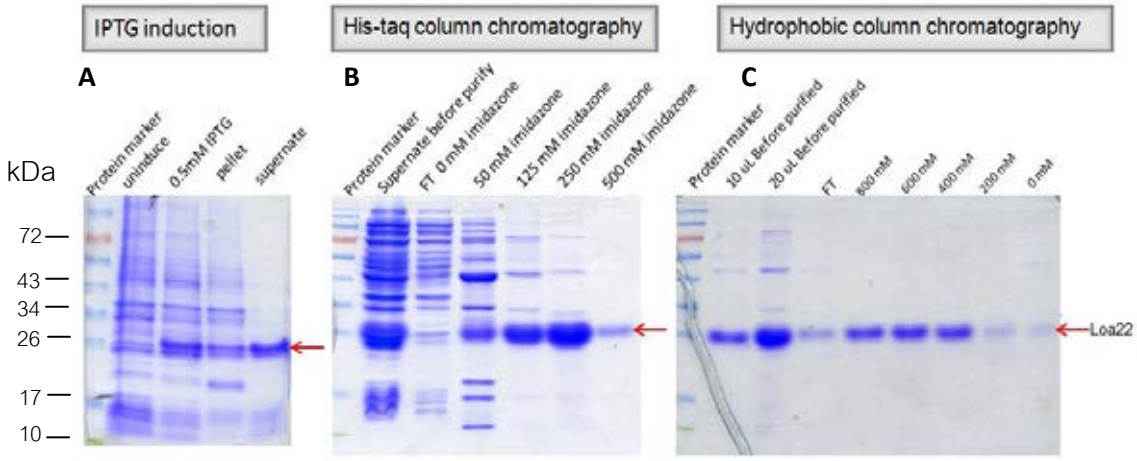


Figure 35. SDS-PAGE of rLoa22. A, induction of rLoa22 by 0.1 mM IPTG; B, purification of soluble part of rLipL32 by His-tag column chromatography; C, purification of rLoa22 with hydrophobic column chromatography after purifying by His-tag column chromatography each lane represent each fraction as indicated above.

Humoral immune response induced by vaccination using CS/pVITRO-*lipL32-loa22* vs CS/pVITRO-*lipL32*+CS/pVITRO-*loa22* co-administration

To use plasmids containing *lipL32* and *loa22* as a DNA vaccine, two formulas of lipL32 and loa22 DNA vaccine were compared. One formula was CS/pVITRO-*lipL32-loa22* which contained *lipL32* and *loa22* genes on the same plasmid (two ORFs in one plasmid); another was CS/pVITRO-*lipL32*+CS/pVITRO-*loa22* co-administration (two ORFs on two different plasmids). Plasmids used for immunization were 10 pmole each. These two formulas were used to compare the immunogenicity in stimulating antibody production in mice. Plasmids were encapsulated using the optimal conditions described above. Sera of BALB/c mice immunized with different formulation were measured for total IgG responses. Figure 36 shows the level of total IgG antibody specific for LipL32 and Loa22 after intramuscular immunization 3 times (Priming immunization, boost immunization 1, and boost immunization 2 on day1, day31, and day45, respectively) with CS/pVITRO-*lipL32-loa22* or CS/pVITRO-*lipL32*+CS/pVITRO-*loa22*.

The level of total IgG specific for LipL32 and/or Loa22 increased after repeated administration of vaccines which could be detected after the first boost and the second boost. Specific total IgG response against LipL32 is not significantly different between the two formulations of vaccine (Figure 36A). However, total IgG response against Loa22 in mice immunized with CS/pVITRO-*lipL32-loa22* (two ORFs in one plasmid) was higher than those immunized with CS/pVITRO-*lipL32*+CS/pVITRO-*loa22* (Figure 36B). The *in vivo* immune responses correlated well with the amount of Loa22 and LipL32 expression *in vitro* (Figure 26). The expression level of Loa22 from pVITRO-*lipL32-loa22* transfected cell is higher than that co-transfected with CS/pVITRO-*lipL32*+CS/pVITRO-*loa22*. Therefore, CS/pVITRO-*lipL32-loa22* plasmid was selected to use for further experiments.

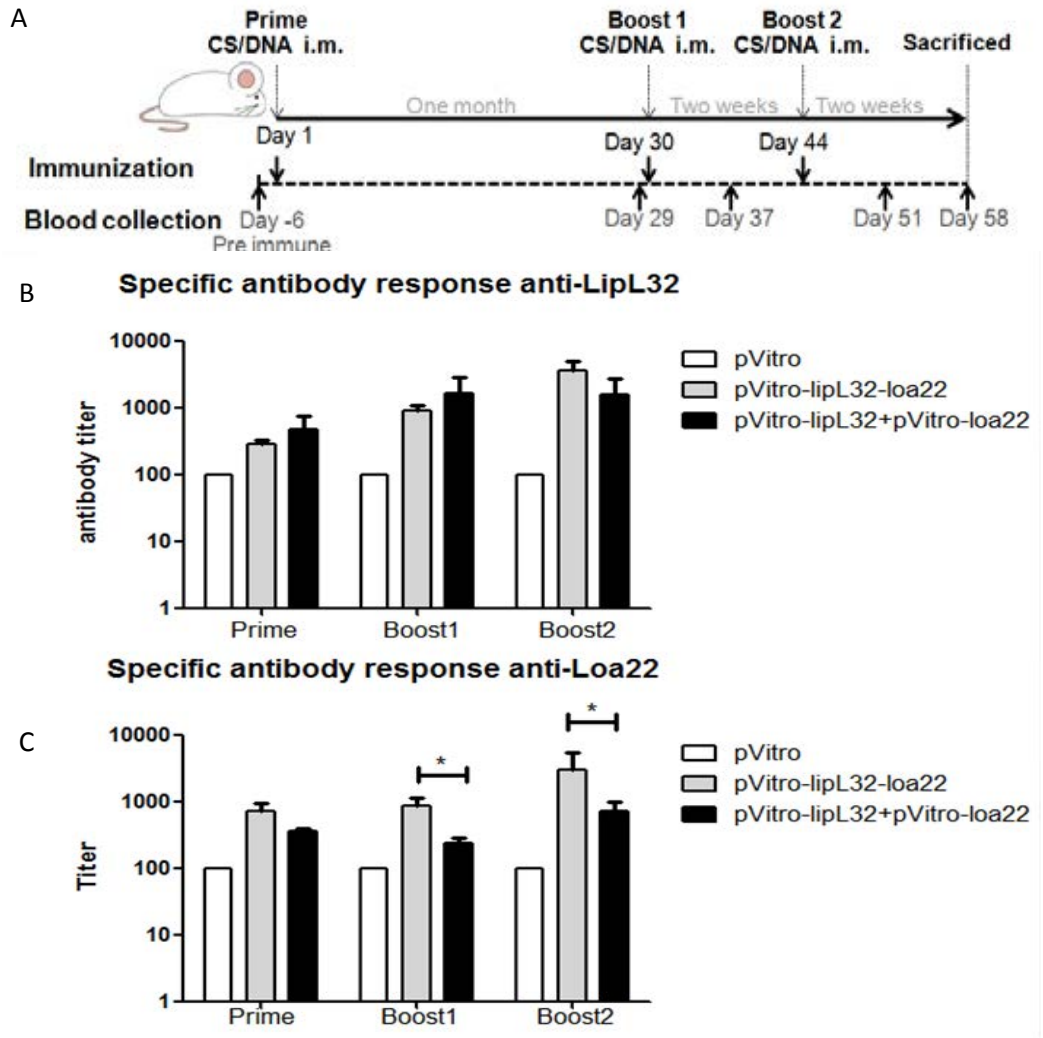


Figure 36. Schematic of immunization and blood collection (A). The endpoint antibody titers of sera collected on day 29 (four weeks after prime immunization), day 37 (one week after boost1 vaccination), and day 51 (one week after boost2 immunization). The titers of specific total IgG against LipL32 (B) and Loa22 (C) were shown. Mice were divided into three groups and immunized with the following vaccines; CS/pVITRO (white bar), CS/pVITRO-*lipL32-loa22* (grey bar), and CS/pVITRO-*lipL32*+CS/pVITRO-*loa22*. Five mice per group were used. Each group was immunized three times with the similar doses of 10 pmole of DNA plasmids. Data are reported as geometric means ± SD. *P < 0.05 indicated statistical significance when compared with the CS/pVITRO control group.

Humoral immune responses to LipL32, Loa22, and LipL32-Loa22 in heterologous prime-boost immunization

Heterologous prime-boost regimen was used for this study to test whether two antigens (LipL32 and Loa22) induce better humoral responses than single antigen (LipL32 or Loa22). Immunization begins with an i.m. priming with CS/DNA (CS/pVITRO-*lipL32* or CS/pVITRO-*loa22* or CS/pVITRO-*lipL32-loa22*), and subsequently followed by s.c. boost immunization twice with the recombinant protein in commercial adjuvants AddaVax™. The humoral immune responses of immunized mice were observed by the level of total specific IgG, IgG1, and IgG2a. Figure 22 showed the ELISA results of total IgG, IgG1, and IgG2a against LipL32 and Loa22.

All experimental groups showed significant increases in antibody titer compared with the CS/pVITRO-PBS-PBS control group. For the antibody production against LipL32, mice vaccinated with two antigens provided significantly higher level of IgG1 than mice receiving only LipL32 single antigen. However, the level of anti-LipL32 specific total IgG and IgG2a level are not statistically different between the two groups (Figure 37A).

For antibody production against Loa22, mice vaccinated with two antigens provided no significant increase in the level of anti-Loa22 specific total IgG, IgG1, or IgG2a antibodies when compared with those that received only Loa22 (Figure 37B).

The proclivity of immune response seem to be Th2 biased, since the level of IgG1 (Th2) is higher than the level of IgG2a (Th1), especially in mice receiving two antigens (Figure 37A and 37B).

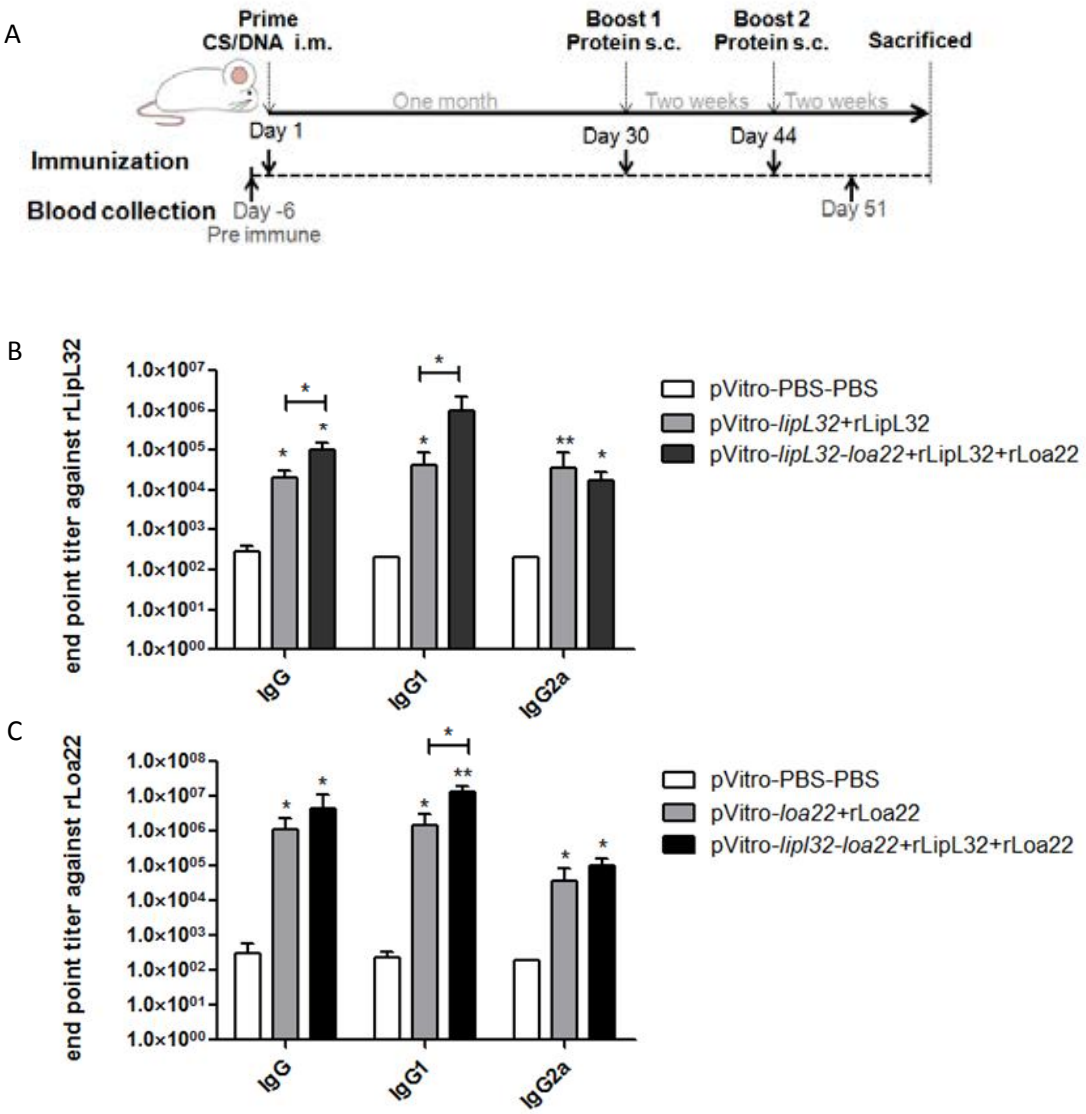


Figure 37. Schematic of immunization and blood collection (A) Serum titers of specific total IgG, IgG1, and IgG2a response against LipL32 (B) and Loa22 (C). Mice were immunized using heterologous prime-boost regimen with the following vaccines; CS/pVITRO+PBS+PBS (white bar), CS/pVITRO-*lipL32*+rLipL32+rLipL32 (grey bar), or CS/pVITRO-*lipL32-loa22*+rLipL32-rLoa22+rLipL32-rLoa22 (black bar). The results show antibody titer of sera collected seven days after bosst2. Each groups contained five mice. Mice were immunized with 10 pmole of DNA plasmids and 0.5 μmole of rLipL32, rLoa22 or both. Data are shown as geometric means ± SD. *P < 0.05, **P < 0.01 indicated statistical significance when compared with the CS/pVITRO control group.

The propensity of immune response generated by LipL32 or Loa22 single antigen with the combination of LipL32 and Loa22 in a heterologous prime boost regimen were analyzed by comparing the ratio of IgG1 antibody titer which is represent for Th2 response to IgG2a antibody titer which indicates Th1 response (IgG1/IgG2a). Figure 38 showed that immunization with a single antigen of LipL32 or Loa22 or with a combination of two antigens mainly drove Th2 response. This tendency is evidently observed in mice vaccinated with two antigens which showed higher ratio of IgG1/IgG2a than mice immunized with a single antigen of LipL32 or Loa22 antigen (Figure 38).

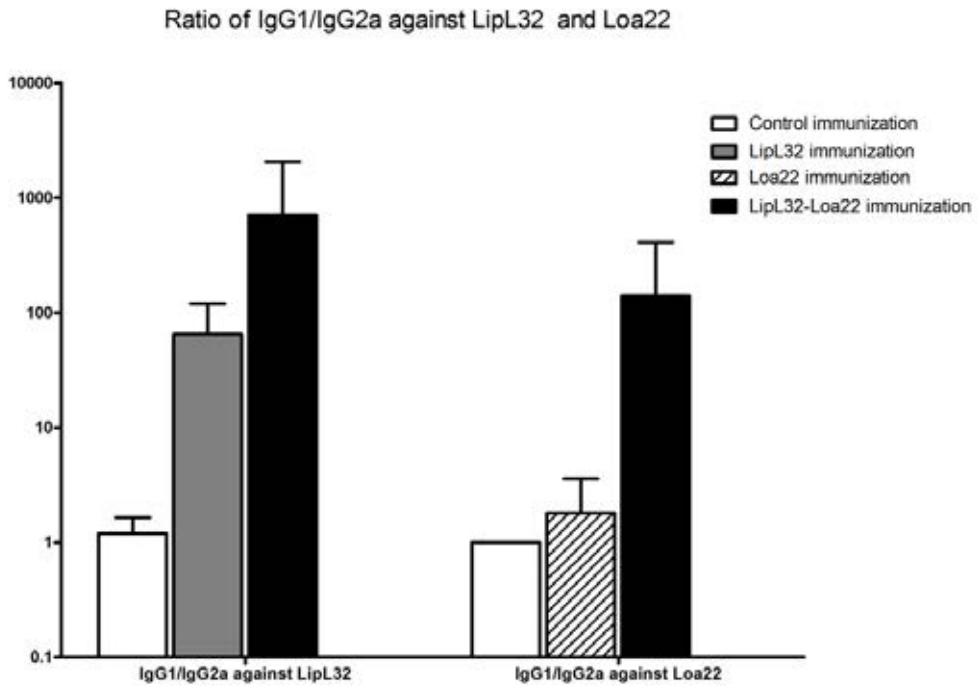


Figure 38. The ratio of IgG1/IgG2a antibody titer against LipL32 and Loa22. Mice were immunized using heterologous prime-boost regimen with the following vaccine; CS/pVITRO+PBS+PBS (open bar), CS/pVITRO-*lipL32*+rLipL32+rLipL32 (grey bar), CS/pVITRO-*oa22*+rLoa22+rLoa22 (hatched bar) or CS/pVITRO-*lipL32-loa22*+rLipL32-rLoa22+rLipL32-rLoa22 (closed bar). The antibody titers were from sera collected seven days after boost 2. Each group contained five mice.

T cells response to LipL32, Loa22, and LipL32-Loa22 in an *in vitro* restimulation.

Proliferation of CD4+ T cells

To compare whether using two antigens help to improve immunogenicity by stimulating better T cells responses than when using LipL32 or Loa22 single antigen alone, mice were immunized with a single antigen of LipL32, Loa22, or LipL32 and Loa22. Immunization was performed using the heterologous prime-boost strategy. CFSE was used for monitoring cell proliferation after restimulation with recombinant antigens and CD4+ T cells was analyzed for cell proliferation by flow cytometry.

Mice immunized with two antigens of LipL32-Loa22 or vaccinated with a single antigen of LipL32 and Loa22 provided no significant differences in CD4+ T cell proliferation upon restimulation with **20μg/ml of** LipL32 or Loa22 (Figure 40C). However, immunized mice indicated specific response against LipL32 or Loa22 since only little proliferation was observed in control vaccinated mice. Higher proliferation was observed when stimulated with mitogen ConA (Figure 40A and 40B) which was used as a positive control for cell stimulation. Mice in the control group (pVITRO-PBS-PBS) exhibited less T cell proliferation when it was stimulated with LipL32 or Loa22 (Figure 39A and 39E).

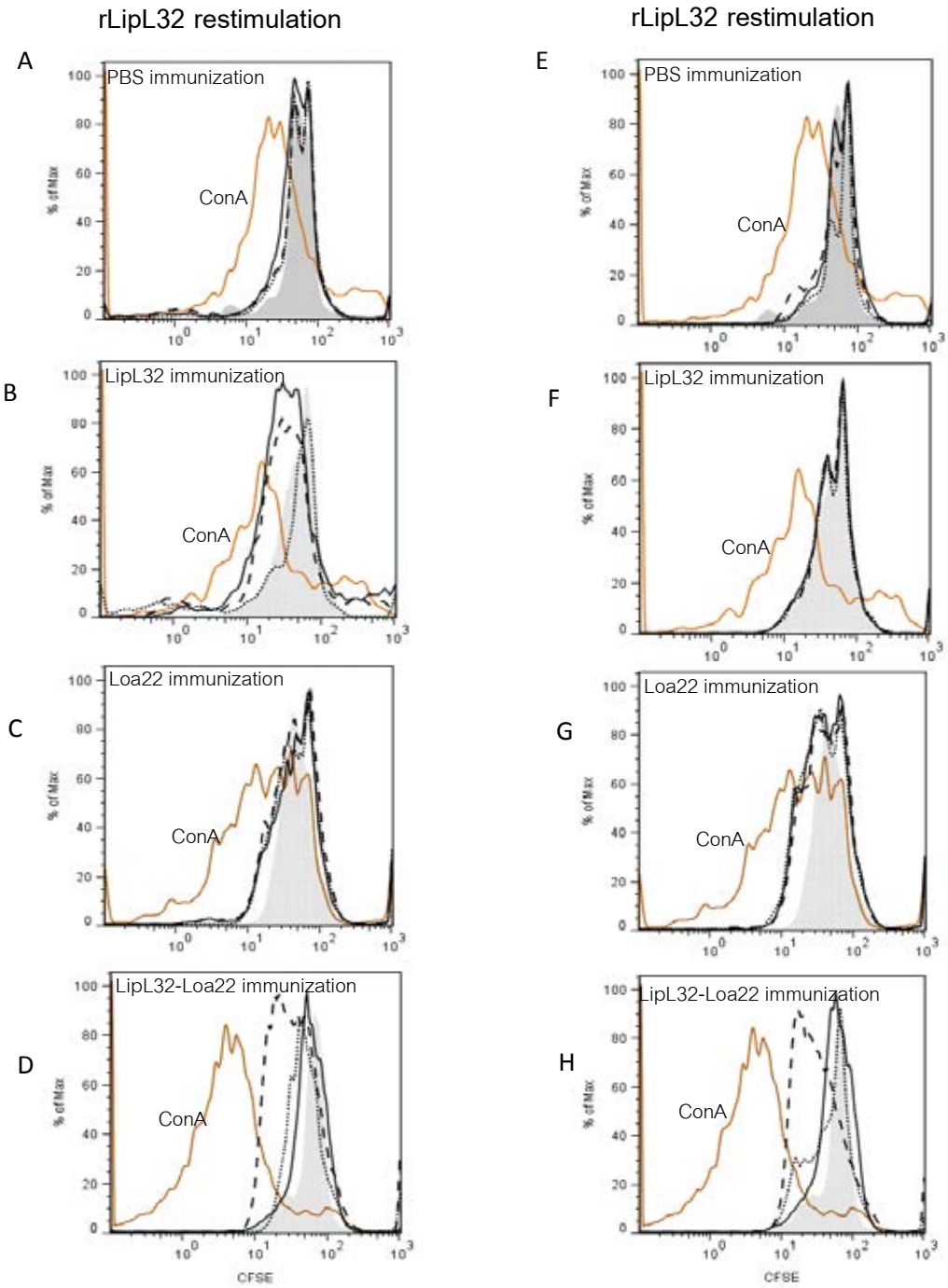


Figure 39. Proliferation of CD4+ T cells from all mice after restimulating with 20 μg/mL of rLipL32 (A-D) or rLoa22 (E-H) or 5 μg/mL of conA (brown line). Mice were immunized with pVITRO-PBS-PBS control (A, E), or single antigen of LipL32 (B, F) or single antigen of Loa22 (C, G) or both of LipL32 and Loa22 antigens (D, H). Three mice per group were represented by solid, dotted, or dashed black lines. The grey histrograms represent unstimulated cells.

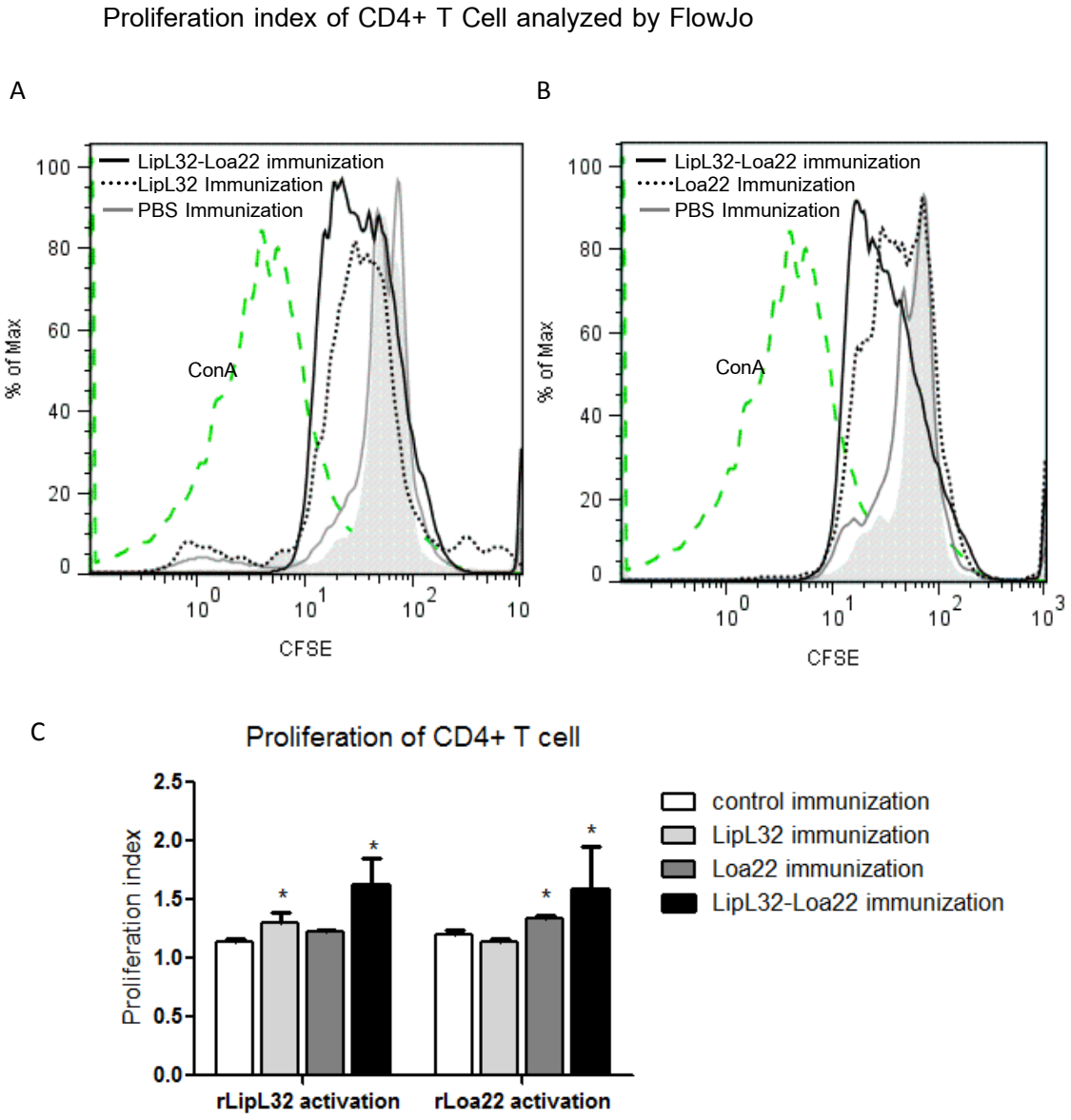


Figure 40. Proliferation of CD4+ T cells using CFSE staing. 20 µg/mL recombinant protein of LipL32 or Loa22 or 5 µg/mL of conA were used to cell stimulation. After 48 h of reactivation, splenocytes were stained for CD4+ T cell and measured by flow cytometry. The data were analyzed by FlowJo. The proliferation overlay (A and B) and the proliferation index (C) of CD4+ T cells obtained from LipL32 or Loa22 or LipL32-Loa22 vaccinated mice. The stimulation index is defined as the number of divisions that took place divided by the number of cells of the original population that went into division. Data are reported as means ± SD for three animals per group. *P < 0.05 compared to the pVITRO-BBS-PBS control immunization group.

Cytokine productions in response to *in vitro* restimulation by recombinant antigens

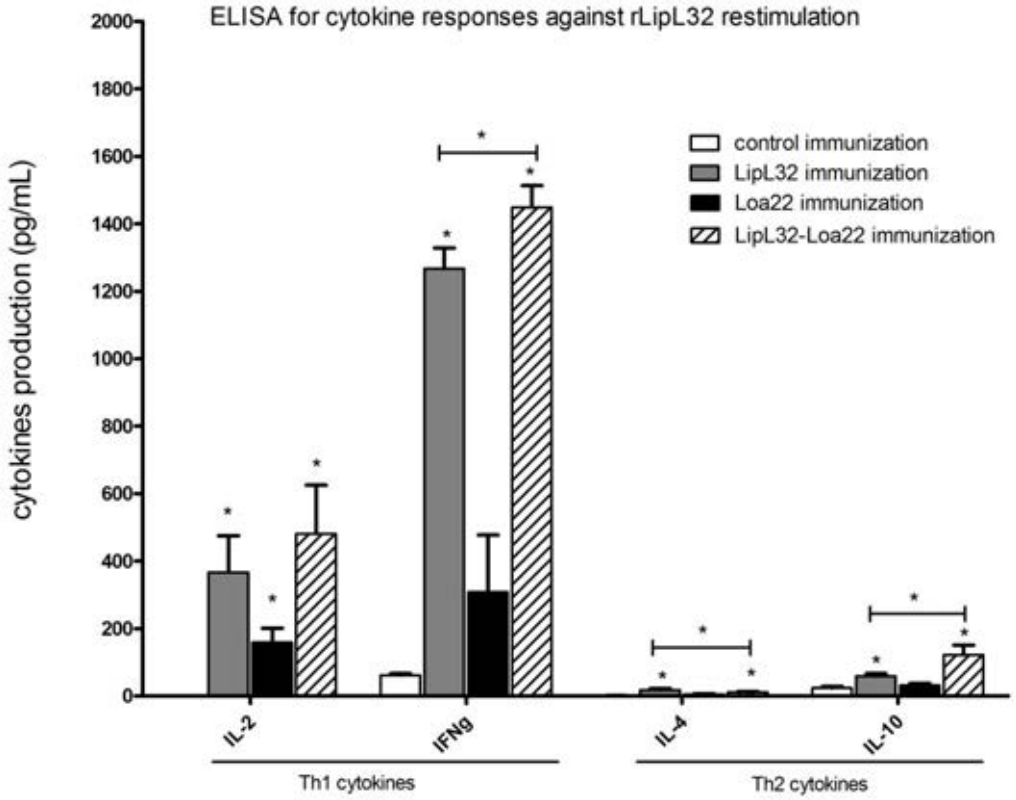
To compare cytokine profiles in response to restimulation with LipL32 or Loa22 splenocytes from mice in each group were restimulated with recombinant antigens as indicated and the cytokine response including Th1 (IFN-γ and IL-2) and Th2 (IL-4 and IL-10) of immunized mice were measured. The levels of cytokines IFN-γ, IL-2, IL-4, and IL-10 were measured by ELISA.

All vaccinated groups showed significant increases in all cytokines detected in this study when compared with CS/pVITRO-PBS-PBS control groups. However, IL-4 and IL-10 were produced at only low level.

The level of IFN-γ (typical Th1 cytokines) in mice immunized with two antigens are significantly higher than those received only one antigen of LipL32 (Figure 41A). However, the level of IL-2 is not different between LipL32 and LipL32-Loa22 immunized mice. For Th2 cytokines, the level of both IL-4 and IL-10 are extremely low. Splenocytes from mice receiving two antigens showed higher IL-10 level but lower IL-4 production when compared to those from mice receiving only LipL32 vaccine (Figure 41A)

In term of Th1 response against Loa22, vaccination with two antigens resulted in stronger Th1 responses (IFN-γ and IL-2) than when immunized only Loa22 antigen (Figure 41B). Nevertheless, no significant difference of Th2 cytokines response (IL-4, IL-10) between group sreceived two antigens or one antigen.

A


ELISA for cytokine responses against rLipL32 restimulation
cytokines production (pg/mL)
2000
1800
1600
1400
1200
1000
800
600
400
200
0
control immunization
LipL32 immunization
Loa22 immunization
LipL32-Loa22 immunization
IL-2
IFNg
IL-4
IL-10
Th1 cytokines
Th2 cytokines

B

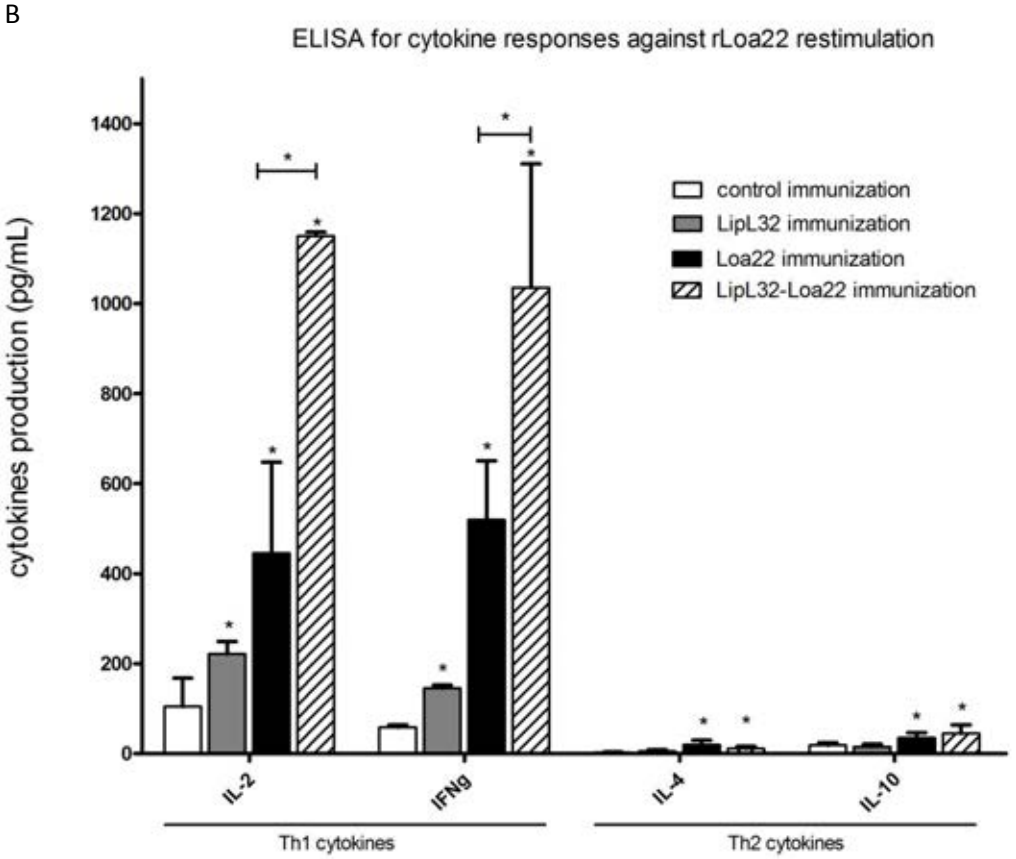

ELISA for cytokine responses against rLoa22 restimulation
cytokines production (pg/mL)
1400
1200
1000
800
600
400
200
0
control immunization
LipL32 immunization
Loa22 immunization
LipL32-Loa22 immunization
IL-2
IFNg
IL-4
IL-10
Th1 cytokines
Th2 cytokines

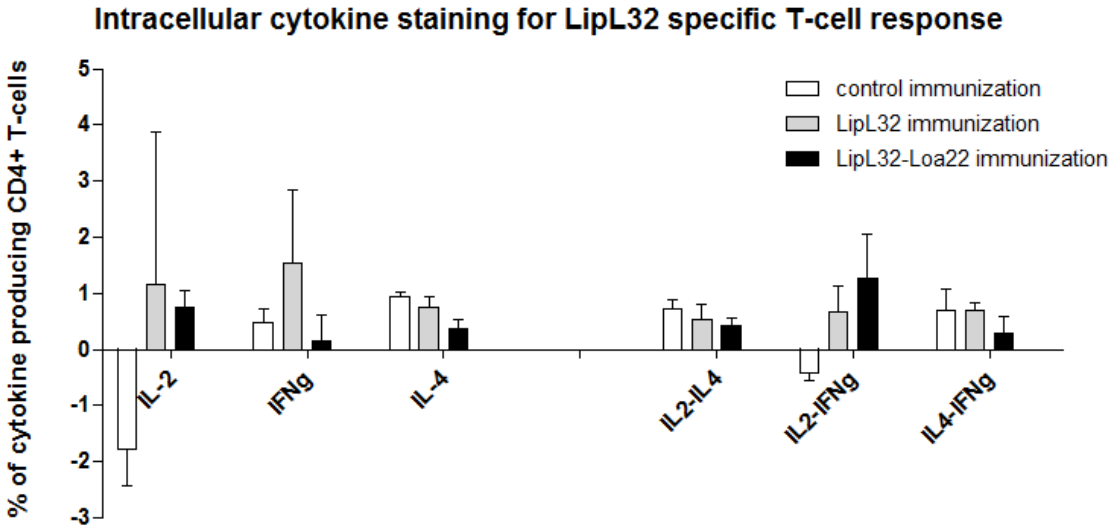
Figure 41. Cytokine responses of splenocytes from mice immunized with two antigens (LipL32-Loa22) or single antigen of LipL32 or Loa22. A, Th1 and Th2 cytokines response against rLipL32; B, Th1 and Th2 cytokines response against rLoa22 restimulation. The experiment was performed duplicate. The results represent from three mice per group. The statistical significant is $p<0.05$.

Intracellular cytokines staining for specific CD4+ T cell cytokine response

To compare the cytokine responses generated by CD4+ T cells at a single cell level, heterologous prime-boost regimen was used for immunization strategy. The cytokine response including Th1 (IFN-γ and IL-2) and Th2 (IL-4) of immunized mice were observed after restimulation of splenocytes with 20 μg/ml of rLipL32 or rLoa22. Cytokine response from CD4+ T cells were subjected to intracellular cytokine staining and measured by flow cytometry.

The level of Th1 or Th2 cytokines produced from CD4+ T cells in response to LipL32 or Loa22 showed no significant difference between mice receiving two antigens and mice receiving a single antigen (Figure 42A and 42B). Furthermore, there is no difference in the frequency of poly-functional T cells which can produce more than one cytokines simultaneously.

A



B

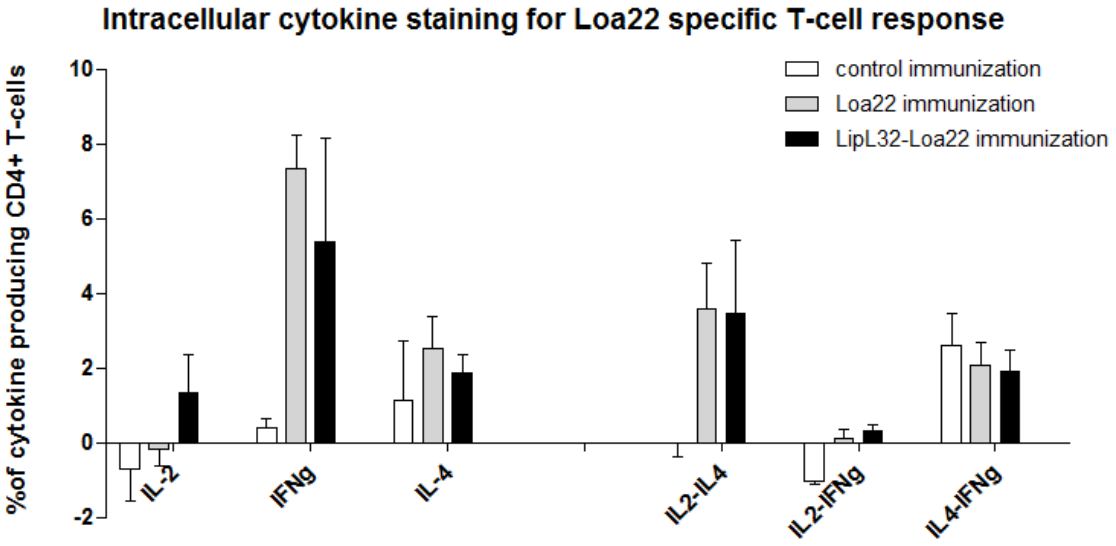


Figure 42. Intracellular cytokine production from $CD4^+$ T cells were analyzed by flow cytometry. BALB/c mice were immunized with a single antigen of LipL32 or Loa22 or both the combination of two antigens by heterologous prime-boost regimen. The splenocytes were stimulated with 20 μg/ml of recombinant LipL32 (A) or Loa22 (B). The results are the average from three mice in each group. The data shown were the percentages of cells which were subtracteded by background of percentages of CD4+ T cells with positive staining in unstimulated splenocytes.

CHAPTER VI

DISCUSSIONS

Subunit vaccines for leptospirosis have been developed to overcome the serovar-specific immunity generated by the whole cell-killed vaccine. Leptospiral outer membrane proteins have many advantages over current whole cell-based leptospiral vaccines. Subunit antigens which are shared by pathogenic *Leptospira* provide important characteristics in generating cross-protection among different serovars [109]. Immunological responses required for protection against leptospiral infection is thought to mainly rely on humoral immune response [2]. Cellular immune response, however, seems to be partially indispensible for protection of cattle from *Leptospira* infection [7]. Thus, with the available knowledge on immune response to infection, good vaccine candidates for leptospirosis should induce both humoral and cell mediated immune responses and should also provide protection across pathogenic serovars with negligible adverse effect.

Previous investigations showed that LipL32 is a conserved outer membrane protein among pathogenic serovars [110]. It was to be expressed during infection [22]. LipL32 was used as a subunit vaccine provided partial protection against pathogenic leptospiral challenge with different formulations [25-27, 100]. The suitability of LipL32 as a good vaccine candidate is still controversial because of its reported protective efficacy is not consistent in the literatures [117]. Therefore, using single antigen of LipL32 might not be sufficed to achieve a complete protection. The objective of this study is to test whether combining LipL32 with another antigen could promote better immune response in term of humoral and cell-mediated immune responses than when using only LipL32 or another antigen alone.

Another antigen used to combine with LipL32 in this study is Loa22, the protein in the OmpA family lipoprotein [28]. Loa22 possess many characteristics to be a good

vaccine candidates; *i.e.* 1) Loa22 is a surface exposed protein [29], 2) Loa22 is confirmed by mutagenesis study to be the only known virulence factor [31], 3) Loa22 is expressed during infection [22], 4) Loa22 can be recognized by patient sera [30] and 4) Loa22 provides partial protection in challenged hamsters [19]. Moreover, overcoming the narrow serovar specific immunity of leptospirosis vaccine is critical in the field of leptospirosis vaccine development. The characteristics of LipL32 and Loa22 could fulfill those aims since both of them are conserved among pathogenic serovars of leptospiras. Therefore, the immune responses generated against LipL32 and Loa22 antigens may provide heterologous protection. With the combination of these two antigens, not only the efficacy in stimulation of humoral and cellular immune response, but also the ability to provent the infection by heterlogous pathogenic serovars may be improved. The study to explore the combination of LipL32 and Loa22 as a vaccine candidate has never been undertaken.

DNA vaccines have been described to be effective against tumors [122] and several intracellular and extracellular pathogens, including HIV [123], SARS [124], and leptospires [25, 27, and 98]. However, in order to use *lipL32* and *loa22* as a DNA vaccine or LipL32 and Loa22 as recombinant subunit vaccines to elicit both humoral and cellular immune responses, many factors contributing to the success of the vaccine need to be considered such as vaccine delivery system, route of administration, dose, and vaccination strategy. Therefore, in this study, combination of LipL32 and Loa22 was investigated as a candidate vaccine by using biopolymer chitosan as a delivery system for *lipL32* and *loa22* DNA vaccine and AddaVax™ as an adjuvant for subunit recombinant protein vaccine, and the heterologous prime-boost immunization regimen for immunization.

Upon transfection, DNA plasmids containing full-length *lipL32* and *loa22* were able to produce both LipL32 and Loa22 proteins intracellularly and also secreted outside the cells. The releasing of both proteins is unexpected because full-length

lipL32 and *loa22* containing bacterial signal sequences were transfected into mammalian host. The secretion of both proteins outside the cells indicates that the signal peptides of either *lipL32* or *loa22* from *L. interrogans* can be recognized in higher eukaryotes such as mammalian cells. The signal peptide from bacteria can recognizes the mitochondrial receptors and functions as a mitochondrial leader sequence in mammalian host cell line HeLa [179]. This result correlates with the secretion of bacterial endoglucanase (endoglucanase E) from stably transfected Chinese Hamster Ovary cells (CHO). Either prokaryote or eukaryote signal peptides fusion with endoglucanase gene resulted in functional endoglucanase E because expressed proteins of both leader origins were post-translationally modified before secretion [178]. In our study, secreted LipL32 has higher molecular weight than that of LipL32 produced intracellularly, indicating that the post translational modification occurs and leptospiral leader sequence is functional in HEK293T mammalian cell line. Interestingly, LipL32 were predicted to contain potential N-glycosylation sites [185]. Taken together, the secretion of bacterial proteins by eukaryotic machinery may be a regular phenomenon and it may not have restriction of the origin of the signal sequences.

To construct a recombinant DNA plasmid of *lipL32* and *loa22*, we therefore decided to use the mammalian expression vector pVITRO1 which possesses two multiple cloning sites. This plasmid makes it possible to insert both *lipL32* and *loa22* in the same plasmid. DNA fragment of *lipL32* was inserted into the MCS2 and that of *loa22* was inserted in the MCS1 of pVITRO1. *lipL32* and *loa22* were expressed under the control of different promoters. *lipL32* was controlled by rEF1 promoter and *loa22* was controlled by mEF1 promoter. According to the manufacturer, both promoters display strong activity that yields similar levels of expression. Therefore, the difference in the level of expression of both antigens was not due to difference in promoter activity in our study. Encapsulation of DNA vaccine by chitosan showed the optimal N/P ratio of 20:1 and the efficacy of chitosan in delivering of DNA vaccine of *lipL32* and *loa22* was consistent with several studies which demonstrated the ability of chitosan in promoting

DNA uptake by cells [40, 137] and inducing immune response against DNA vaccine [138, 146].

Because the combination of *lipL32* and *loa22* as a vaccine was our objective, two formulations of vaccine were compared in term of the expression *in vitro* and the efficacy in inducing humoral immune responses *in vivo*. One formulation is CS/pVITRO-*lipL32-loa22* (two ORFs in one plasmid) and another is CS/pVITRO-lipL32+CS/pIVTRO-loa22 co-administration (two ORFs in two plasmids). There is no significant difference in the expression level of LipL32 protein *in vitro* after transfection between the two formulations. This result is in contrast with the level of Loa22 expression, where transfection of CS/pVITRO-*lipL32-loa22* (two ORFs in one plasmid) resulted in higher level of Loa22 expression in cell lysate and in the culture supernatant than that co-transfection method. This result was consistent with the results of an *in vivo* immunization with the same molar concentration of DNA vaccine because the level of total IgG response specific for Loa22 was higher in mice immunized with CS/pVITRO-*lipL32-loa22* than co-immunization by the two plasmids. The higher level of antibody against lipL32 and loa22 in single plasmid injection of our study may be supported by the study of Sedegah in 2004 which demonstrated the significant suppression or complete abrogation of immune response when the plasmids were pooled in nine-plasmid cocktail encoding candidate malaria vaccine antigens [177]. The antibody and IFN-γ responses to each antigen induced by the mixture were suppressed relative to the response induced by immunization with single plasmids of each antigen. Moreover, removal of single gene from the mixture frequently reduced the observed suppression [177]. Nevertheless, our result is in contrast with the study by Grifantini which demonstrated that there was no difference in the level of antibody responses in mice co-immunized with a combination of four plasmids encoding four malarial antigens (*Plasmodium falciparum* circumsporozoite protein, thrombospondin-related anonymous protein, major merozoite surface protein MSP1 and Pfs25) with those obtained with single-plasmid injections. The antibody response against MSP1 from single plasmid administration strongly potentiated by the presence of additional plasmids which

probably caused by the adjuvant effect from other plasmid [176]. In our study, several mechanisms might be responsible for the observed diminished effect of two plasmids formulation. Two plasmids might compete for uptake by host cells; the probability of uptaking one plasmid should be higher when compared with uptaking two plasmids at the same time. CS/pVITRO-*lipL32-loa22* carries two ORFs, after plasmid uptake, LipL32 and Loa22 can be expressed simultaneously in the same cell. For two plasmid co-immunization, however, once the plasmid of *lipL32* or *loa22* was uptaken by one cell, another may not be taken up by the same cell. Therefore, transfection with CS/pVITRO-*lipL32*-loa22 yielded higher level of Loa22 expression *in vitro* and immunization with this plasmid generated higher total IgG response against Loa22 *in vivo*. Using CS/pVITRO-*lipL32-loa22* not only results in the higher expression and higher ability in stimulating antibody response, but this formulation is also practical in lowering the cost and reducing time needed for preparation because one plasmid can carry two ORFs. Therefore we prepared only one plasmid and use it for both *lipL32* and *loa22*. In contrast to the co-administration method, this required two plasmids for lipL32 and loa22.

In other vaccine studies [119, 120], the immune responses induced by recombinant or multiple-component vaccines are better than those induced by a single-component vaccine. In our study, we compared whether using LipL32 and Loa22 in combination provides better immune response than when using single antigen of LipL32 or Loa22. To test this hypothesis, we applied heterologous prime-boost immunization regimen in order to promote both humoral and cellular immune responses. Many vaccine studies in other infectious diseases such as HIV [125, 126], tuberculosis [127], influenza [128], and leptospirosis [39] have demonstrated that heterologous prime-boost immunization is a promising effective strategy to stimulate both humoral and cellular immune responses than either vaccine formulation alone.

With the results we obtained from heterologous prime-boost immunization; i.m. priming with CS/DNA once and s.c. boosting with recombinant protein in AddaVax™

twice, all the antibodie titers elicited in the protein-boosted groups showed higher levels than the results obtained by immunization with CS/pVITRO-*lipL32-loa22* DNA vaccine. This result indicates that a protein-boost strategy can improve the immunogenicity of DNA vaccines against both LipL32 and Loa22. Priming immunization with CS/DNA vaccine and boosting two times with recombinant LipL32 and Loa22 proteins in AddaVax™ drives immune response into Th2 type. The level of IgG1 and total IgG are higher than the level of IgG2a. Addavax™ is an adjuvant described by the manufacturer as a balanced inducer of Th1 and Th2 responses better than alum which usually provides Th2 biased response. However, in this study the humoral immune response still leans toward Th2 with slightly higher IgG2a (Th1). Thus, this vaccination strategy could be valuable for inducing both Th1 and Th2 responses against *lipL32* and *loa22* DNA vaccines.

Comparing the immune responses between mice receiving one antigen of LipL32 or Loa22 and mice receiving both LipL32 and Loa22, the antibody response including total IgG, IgG1, and IgG2a against LipL32 and Loa22 were not different between these two groups. To measure CD4+ T cell responses, we used a splenocyte proliferation assay to monitor cellular immune responses. The results showed that splenocytes from all test groups proliferated more vigorously that those from the pVITRO control group. The splenocyte proliferation in the LipL32-Loa22 vaccinated group was the same as those of single LipL32 or Loa22 group, indicating that no enhanced CD4+ T cell proliferation response was elicited in the LipL32-Loa22 vaccinated groups.

It is known that subsets of Th cells can be distinguished by the pattern of cytokines that they produce. Th1 cells mainly produce IL-2 and IFN-γ and play a critical role in directing cell-mediated immune responses, which are important for clearance of intracellular pathogens. Th2 cells produce IL-4 and IL-10, which are important for eliciting responses against parasitic infection [121]. In our cytokine assays for IL-4 and IL-10, all vaccinated groups showed significantly increased levels of these cytokines,

compared with the control groups. However, the level of Th2 cytokines is extremely low. The possible explanation for this phenomenal is the antagonistic effect of Th1 and Th2 cytokines. With respect to IL-2 and IFN-γ, higher level of IL-2 and IFN-γ expression may suppress the expression of IL-4 and IL-10 cytokines. Th1 cytokines (IFN-γ and IL-2) and Th2 cytokine (IL-4) responses from CD4+ T cells observed by intracellular cytokine staining also provide no significant different between mice receiving one and two antigens.

Combining LipL32 and Loa22 antigens did not provide synergistic effect in stimulating humoral and cellular immune responses against LipL32 or Loa22 and the combination of both antigens also did not act antagonistically against each other. These results are in contrast with various studies indicating that combining two antigens, for example, OmpL1 and LipL41 DNA vaccine provide synergistic effect [95]. Immune response generated by one antigen can promote response of each other when compared with using a single antigen [119, 120]. In the study of leptospirosis vaccine, the LipL32-41-OmpL1 vaccinated groups showed better responses than the single-gene groups (LipL32, LipL41 or OmpL1). In addition, another study of vaccine against *Mycobacterium tuberculosis* indicates that there is a synergistic effect of three antigens combination [129]. Nevertheless, the result of no synergistic effect in our study may be explained by the difference in the recombinant LipL32 and Loa22 protein used in our study. LipL32 and Loa22 recombinant subunit vaccine were co-administered into mice as a separate protein in our study but all those studies used fusion proteins which combined two or three antigens in one peptide. Antibody and T-cell response may react against the overlapping region of each antigen in the peptide. Therefore, this may promote cross reaction and provide synergistic effect response. In our study, however, *lipL32* and *loa22* DNA plasmid was not constructed as a fusion gene and LipL32 and Loa22 recombinant proteins are also not the fusion protein. Hence, it is possible that no synergistic effect was seen in our study. Even though the synergistic effect between LipL32 and Loa22 may not be observed, combining the two antigens might promote

better protective efficacy than when using single LipL32 or Loa22 vaccine. The protective efficacy of LipL32 and Loa22 in combination need to be studied further in pathogen challenged animal model. Our study has some limitations because BALB/c mice were used to evaluate the immune response of *Leptospira* vaccine candidates but they are not susceptible to the pathogenic challenge. To determine the precise synergistic protective effects of LipL32-Loa22 vaccine candidates and protective efficiency, hamster or guinea pig models should be further tested.

CHAPTER VII

SUMMARY

In vitro studies

1. DNA vaccine and the ability of CS as a vaccine delivery system

Both *lipL32* and *loa22* can be expressed in HEK293 cell line in both intracellular and secreted forms. Construction of DNA plasmid of *lipL32* and *loa22* was achieved by inserting both *lipL32* and *loa22* into the same plasmid. CS effectively encapsulated DNA plasmids and the resulting particles exhibited the size of nanoparticles without significant toxicity against teste cell line. At the N/P ratio of 20:1, CS promoted uptake of *lipL32* and *loa22* into HEK293T cell line and the expression of both proteins was detected.

In vivo studies

1. Single plasmid containing two antigens promoted better antibody response than co-immunization of two plasmids of either *lipL32* or *loa22*.

2. Administrating two antigens of LipL32 and Loa22 via heterologous prime-boost immunization did not provide significantly higher overall humoral and cellular immune responses against LipL32 or Loa22 than a single antigen formulation, nor did it provide antagonistic effect against each LipL32 or Loa22.

The combination of LipL32-Loa22 and heterologous vaccination strategy should be further studied for the protective efficacy in animal models of leptospirosis.

REFERENCES

[1] Bharti, A. R., *et al*. Leptospirosis: a zoonotic disease of global importance. Lancet Infect Dis 3, no. 12 (2003): 757-771.

[2] Faine, S. *et al*. *Leptospira* and leptospirosis, 2nd (ed.), MediSci, pp. 1-272. Melbourne, Australia, 1999.

[3] Ko, A. I., Goarant, C., and Picardeau, M. *Leptospira*: The dawn of the molecular genetics era for an emerging zoonotic pathogen. Nat Rev Microbiol 7, no. 10 (2009): 736-747.

[4] Adler, B., and de la Pena, A. Moctezuma. *Leptospira* and leptospirosis. Vet Microbiol 140, no. 3-4: 287-296.

[5] McBride, A. J., Athanazio, D. A., Reis, M. G., and Ko, A. I. Leptospirosis. *Curr Opin Infect Dis* 18, no. 5 (2005): 376-386.

[6] Jost, B. H., Adler, B., Vinh, T., and Faine, S. A monoclonal antibody reacting with a determinant on leptospiral lipopolysaccharide protects guinea pigs against leptospirosis. J Med Microbiol 22, no. 3 (1986): 269-275.

[7] Bolin, C. A., Cassells, J. A., Zuerner, R. L., and Trueba, G. Effect of Vaccination with a monovalent *Leptospira Interrogans* serovar hardjo type Hardjo-Bovis vaccine on type Hardjo-Bovis infection of cattle. Am J Vet Res 52, no. 10 (1991): 1639-1643.

[8] Bolin, C. A., Zuerner, R. L., and Trueba, G. Effect of vaccination with a

pentavalent leptospiral vaccine containing *Leptospira Interrogans* serovar hardjo type hardjo-bovis on type hardjo-bovis infection of cattle. Am J Vet Res 50, no. 12 (1989): 2004-2008.

[9] Bolin, C. A., and Alt, D. P. Use of a monovalent leptospiral vaccine to prevent renal colonization and urinary shedding in cattle exposed to *Leptospira Borgpetersenii* serovar hardjo. Am J Vet Res 62, no. 7 (2001): 995-1000.

[10] Bolin, C. A., and Alt, D. P. Protection of cattle from renal and genital tract colonization with *Leptospira borgpetersenii* serovar hardjo. Proceedings of the 21st World Buiatrics Congress. Punte del Este, Uruguay. 2000.

[11] Challa, S., Nally, J. E., Jones, C., and Sheoran, A. S. Passive immunization with *Leptospira* lps-specific agglutinating but not non-agglutinating monoclonal antibodies protect guinea pigs from fatal pulmonary hemorrhages induced by serovar copenhageni challenge. Vaccine 29, no. 27: 4431-4434.

[12] Herrmann, J. L. Genomic techniques for identification of *Leptospira* strains. Pathol Biol (Paris) 41, no. 10 (1993): 943-950.

[13] Herrmann, J. L., *et al*. A new serovar in the grippotyphosa serogroup comprising leptospiral isolates from different regions. Int J Syst Bacteriol 44, no. 2 (1994): 362-364.

[14] Herrmann, J. L., Baril, C., Bellenger, E., Perolat, P., Baranton, G., and Saint Girons, I. Genome conservation in isolates of *Leptospira Interrogans*. J Bacteriol 173, no. 23 (1991): 7582-7588.

[15] Faisal, S. M., Yan, W., McDonough, S. P., and Chang, Y. F. *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, no. 3 (2009): 378-387.

[16] Ren, S. X., *et al.* Unique Physiological and pathogenic features of *Leptospira Interrogans* revealed by whole-genome sequencing. Nature 422, no. 6934 (2003): 888-893.

[17] Nascimento, A. L., *et al*. Genome features of *Leptospira Interrogans* serovar copenhageni. Braz J Med Biol Res 37, no. 4 (2004): 459-477.

[18] Haake, D. A., Walker, E. M., Blanco, D. R., Bolin, C. A., Miller, M. N., and Lovett, M. A. Changes in the surface of *Leptospira Interrogans* serovar grippotyphosa during in vitro cultivation. Infect Immun 59, no. 3 (1991): 1131-1140.

[19] Yan, W., *et al*. Identification and characterization of ompa-Like proteins as novel vaccine candidates for leptospirosis. Vaccine 28, no. 11: 2277-2283.

[20] Gamberini, M., *et al*. Whole-genome analysis of *Leptospira Interrogans* to identify potential vaccine candidates against leptospirosis. FEMS Microbiol Lett 244, no. 2 (2005): 305-313.

[21] Cullen, P. A., Cordwell, S. J., Bulach, D. M., Haake, D. A., and Adler, B. Global analysis of outer membrane proteins from *Leptospira Interrogans* serovar Lai. Infect Immun 70, no. 5 (2002): 2311-2318.

[22] Nally, J. E., Whitelegge, J. P., Bassilian, S., Blanco, D. R., and Lovett, M. A. Characterization of the outer membrane proteome of *Leptospira Interrogans* expressed during acute lethal infection. Infect Immun 75, no. 2 (2007): 766-773.

[23] Haake, D. A., *et al*. The leptospiral major outer membrane protein LipI32 is a lipoprotein expressed during mammalian infection. Infect Immun 68, no. 4 (2000): 2276-2285.

[24] Guerreiro, H., *et al*. Leptospiral proteins recognized during the humoral immune response to leptospirosis in humans. Infect Immun 69, no. 8 (2001): 4958-4968.

[25] Branger, C., *et al*. Protection against *Leptospira Interrogans* sensu lato challenge by DNA immunization with the gene encoding hemolysin-associated protein 1. Infect Immun 73, no. 7 (2005): 4062-4069.

[26] Seixas, F. K., *et al*. Recombinant *Mycobacterium bovis* bcg expressing the LipI32 antigen of *Leptospira Interrogans* protects hamsters from challenge. Vaccine 26, no. 1 (2007): 88-95.

[27] Branger, C., *et al*. Identification of the hemolysis-associated protein 1 as a cross-protective immunogen of *Leptospira Interrogans* by adenovirus-mediated vaccination. Infect Immun 69, no. 11 (2001): 6831-6838.

[28] Koizumi, N., and Watanabe, H. Molecular cloning and characterization of a novel leptospiral lipoprotein with ompa domain. FEMS Microbiol Lett 226, no. 2 (2003): 215-219.

[29] Picardeau, M., *et al*. Genome sequence of the saprophyte *Leptospira Biflexa* provides insights into the evolution of *Leptospira* and the pathogenesis of leptospirosis. PLoS One 3, no. 2 (2008): e1607.

[30] Monahan, A. M., Callanan, J. J., and Nally, J. E. Proteomic analysis of *Leptospira Interrogans* shed in urine of chronically infected hosts. Infect Immun 76, no. 11 (2008): 4952-4958.

[31] Ristow, P., *et al*. The Ompa-like protein Loa22 is essential for leptospiral virulence. PLoS Pathog 3, no. 7 (2007): e97.

[33] Srikram, A., *et al*. Cross-protective immunity against leptospirosis elicited by a live, attenuated lipopolysaccharide mutant. J Infect Dis 203, no. 6: 870-879.

[34] Wolff, J. A., *et al*. Direct gene transfer into mouse muscle in vivo. Science 247, no. 4949 Pt 1 (1990): 1465-1468.

[36] Alpar, H. O., and Bramwell, V. W. Current status of DNA vaccines and their route of administration. Crit Rev Ther Drug Carrier Syst 19, no. 4-5 (2002): 307-383.

[37] Estcourt, M. J., Ramsay, A. J., Brooks, A., Thomson, S. A., Medveckzy, C. J., and Ramshaw, I. A. Prime-boost immunization generates a high frequency, high-avidity CD8(+) cytotoxic T lymphocyte population. Int Immunol 14, no. 1 (2002): 31-37.

[38] McShane, H. Prime-boost immunization strategies for infectious diseases. Curr Opin Mol Ther 4, no. 1 (2002): 23-27.

[39] Feng, C. Y., Li, Q. T., Zhang, X. Y., Dong, K., Hu, B. Y., and Guo, X. K. Immune strategies using single-component Lipl32 and multi-component recombinant Lipl32-41-Ompl1 vaccines against *Leptospira*. Braz J Med Biol Res 42, no. 9 (2009): 796-803.

[40] Mao, H. Q., *et al*. Chitosan-DNA nanoparticles as gene carriers: synthesis, characterization and transfection efficiency. J Control Release 70, no. 3 (2001): 399-421.

[41] Paster, B. J., *et al*. Phylogenetic analysis of the spirochetes. J Bacteriol 173, no. 19 (1991): 6101-6109.

[42] Li, C., Motaleb, A., Sal, M., Goldstein, S. F., and Charon, N. W. Spirochete periplasmic flagella and motility. J Mol Microbiol Biotechnol 2, no. 4 (2000): 345-354.

[43] Ellis, W. A., and Michno, S. W. Bovine leptospirosis: a serological and clinical study. Vet Rec 99, no. 20 (1976): 387-391.

[44] Cullen, P. A., *et al*. Surfaceome of *Leptospira* spp. Infect Immun 73, no. 8 (2005): 4853-4863.

[45] Shang, E. S., *et al*. The rare outer membrane protein, Ompl1, of pathogenic *Leptospira* species is a heat-modifiable porin. Infect Immun 63, no. 8 (1995): 3174-3181.

[45] Reyes, E. A., *et al*. A. Expression in *Escherichia coli* of the gspd(L) gene of the type Ii secretion system in *Leptospira Borgpetersenii* serovariety hardjo. Rev Cubana Med Trop 57, no. 1 (2005): 45-46.

[46] Ellinghausen, H. C., Jr., and McCullough, W. G. Nutrition of *Leptospira Pomona* and growth of 13 other serotypes: a serum-free medium employing oleic albumin complex. Am J Vet Res 26 (1965): 39-44.

[47] Johnson, R. C., and Harris, V. G. Differentiation of pathogenic and saprophytic letospires. I. growth at low temperatures. J Bacteriol 94, (1967): 27-31.

[48] Bulach, D. M., *et al*. Genome reduction in *Leptospira Borgpetersenii* reflects limited transmission potential. Proc Natl Acad Sci U S A 103, no. 39 (2006): 14560-14565.

[49] Chulalongkorn University, Metalurgy and Material Science Research institute building, Information corner [Online]. Available from: http://www.material.chula.ac.th/CCB/enCCB/en_Info_Corner.htm [2013, Jan 21]

[50] Hartwig, D. D., *et al*. High yield expression of leptospirosis vaccine candidates LigA and LipI32 in the methylotrophic yeast *Pichia pastoris*. Microb Cell Fact 9: 98.

[51] Newman, M. J., and Powell, M. F. Immunological and formulation design considerations for subunit vaccines. Pharm Biotechnol 6 (1995): 1-42.

[52] Cinco, M. New insights into the pathogenicity of leptospires: evasion of host defences. New Microbiol 33, no. 4: 283-292.

[53] Ristow, P., *et al*. Biofilm formation by saprophytic and pathogenic leptospires. Microbiology 154, no. Pt 5 (2008): 1309-1317.

[54] Bovet, P., Yersin, C., Merien, F., Davis, C. E., and Perolat, P. Factors

associated with clinical leptospirosis: a population-based case-control study in the seychelles (Indian Ocean). Int J Epidemiol 28, no. 3 (1999): 583-590.

[55] Levett, P. N. Leptospirosis. Clin Microbiol Rev 14, no. 2 (2001): 296-326.

[56] Waitkins, S. A. Leptospirosis as an occupational disease. Br J Ind Med 43, no. 11 (1986): 721-725.

[57] Campagnolo, E. R., *et al*. Analysis of the 1998 outbreak of leptospirosis in Missouri in humans exposed to infected swine. J Am Vet Med Assoc 216, no. 5 (2000): 676-682.

[58] Terry, J., Trent, M., and Bartlett, M. A Cluster of leptospirosis among abattoir workers. Commun Dis Intell 24, no. 6 (2000): 158-160.

[59] Demers, R. Y., Frank, R., Demers, P., and Clay, M. Leptospiral exposure in Detroit rodent control workers. Am J Public Health 75, no. 9 (1985): 1090-1091.

[60] Ko, A. I., Galvao Reis, M., Ribeiro Dourado, C. M., Johnson, W. D., Jr., and Riley, L. W. Urban epidemic of severe leptospirosis in Brazil. Salvador leptospirosis study group. Lancet 354, no. 9181 (1999): 820-825.

[61] Merien, F., and Perolat, P. Public health importance of human leptospirosis in the south pacific: a five-year study in New Caledonia. Am J Trop Med Hyg 55, no. 2 (1996): 174-178.

[62] Yersin, C., Bovet, P., Merien, F., Wong, T., Panowsky, J., and Perolat, P.

Human leptospirosis in the Seychelles (Indian Ocean): a population-based study. Am J Trop Med Hyg 59, no. 6 (1998): 933-940.

[63] Bureau of Epidemiology, Annual Epidemiological Surveillance Report, Ministry of Public Health, Thailand (2007)

[64] Tangkanakul, W., Smits, H. L., Jatanasen, S., and Ashford, D. A. Leptospirosis: an emerging health problem in Thailand. Southeast Asian J Trop Med Public Health 36, no. 2 (2005): 281-288.

[65] Wuthiekanun, V., *et al*. Clinical diagnosis and geographic distribution of leptospirosis, Thailand. Emerg Infect Di*s* 13, no. 1 (2007): 124-126.

[66] Kline, K. A., S. Falker, S. Dahlberg, S. Normark, and B. Henriques-Normark. Bacterial adhesins in host-microbe interactions. Cell Host Microbe 5, no. 6 (2009): 580-592.

[67] Farrelly, H. E., Adler, B., and Faine, S. Opsonic monoclonal antibodies against lipopolysaccharide antigens of *Leptospira Interrogans* serovar Hardjo. J Med Microbiol 23, no. 1 (1987): 1-7.

[68] Masuzawa, T., *et al*. Immunological reactivity and passive protective activity of monoclonal antibodies against protective antigen (PAG) of *Leptospira Interrogans* serovar Lai. Zentralbl Bakteriol 272, no. 3 (1990): 328-336.

[69] Schoone, G. J., *et al*. An Immunoprotective monoclonal antibody directed against *Leptospira Interrogans* serovar Copenhageni. J Gen Microbiol 135, no. 1 (1989): 73-78.

[70] Bernheimer, A. W., and Bey, R. F. Copurification of *Leptospira Interrogans*

serovar Pomona hemolysin and sphingomyelinase C. Infect Immun 54, no. 1 (1986): 262-264.

[71] Lee, S. H., Kim, S., Park, S. C., and Kim, M. J. Cytotoxic activities of *Leptospira Interrogans* hemolysin sphh as a pore-forming protein on mammalian cells. Infect Immun 70, no. 1 (2002): 315-322.

[72] Lo, M., *et al.* Effects of temperature on gene expression patterns in *Leptospira Interrogans* serovar Lai as assessed by whole-genome microarrays. Infect Immun 74, no. 10 (2006): 5848-5859.

[73] Martinez, R., *et al*. Efficacy and safety of a vaccine against human leptospirosis in Cuba. Rev Panam Salud Publica 15, no. 4 (2004): 249-255.

[74] Barbosa, A. S., *et al*. A newly identified leptospiral adhesin mediates attachment to laminin. Infect Immun 74, no. 11 (2006): 6356-6364.

[75] Hauk, P., *et al*. In LipI32, the major leptospiral lipoprotein, the C terminus is the primary immunogenic domain and mediates interaction with collagen Iv and plasma fibronectin. Infect Immun 76, no. 6 (2008): 2642-2650.

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

[77] Figueira, C. P., *et al*. Heterologous expression of pathogen-specific genes LigA

and LigB in the saprophyte *Leptospira Biflexa* confers enhanced adhesion to cultured cells and fibronectin. BMC Microbiol 11, (2011): 129.

[78] Lin, Y. P., and Chang, Y. F. A domain of the *Leptospira* LigB contributes to high affinity binding of fibronectin. Biochem Biophys Res Commun 362, no. 2 (2007): 443-448.

[79] Murray, G. L., *et al*. Major surface protein LipI32 is not required for either acute or chronic infection with *Leptospira Interrogans*. Infect Immun 77, no. 3 (2009): 952-958.

[80] Koizumi, N., and Watanabe, H. Leptospiral immunoglobulin-like proteins elicit protective immunity. Vaccine 22, no. 11-12 (2004): 1545-1552.

[81] Matsunaga, J., *et al*. Pathogenic *Leptospira* species express surface-exposed proteins belonging to the bacterial immunoglobulin superfamily. Mol Microbiol 49, no. 4 (2003): 929-945.

[82] Matsunaga, J., Lo, M., Bulach, D. M., Zuerner, R. L., Adler, B., and Haake, D. A. Response of *Leptospira Interrogans* to physiologic osmolarity: relevance in signaling the environment-to-host transition. Infect Immun 75, no. 6 (2007): 2864-2874.

[83] Croda, J., *et al*. Targeted mutagenesis in pathogenic *Leptospira* species: disruption of the LigB gene does not affect virulence in animal models of leptospirosis. Infect Immun 76, no. 12 (2008): 5826-5833.

[84] Choy, H. A., Kelley, M. M., Chen, T. L., Moller, A. K., Matsunaga, J., and

Haake, D. A. Physiological osmotic induction of *Leptospira Interrogans* adhesion: LigA and LigB bind extracellular matrix proteins and fibrinogen. Infect Immun 75, no. 5 (2007): 2441-2450.

[85] Qin, J. H., *et al.* Genome-wide transcriptional analysis of temperature shift in *L. Interrogans* serovar Lai strain 56601. BMC Microbiol 6 (2006): 51.

[86] Adler, B., and Faine, S. Host immunological mechanisms in the resistance of mice to leptospiral infections. Infect Immun 17, no. 1 (1977): 67-72.

[87] Adler, B., and Faine, S. The antibodies involved in the human immune response to leptospiral infection. J Med Microbiol 11, no. 4 (1978): 387-400.

[88] Adler, B., and Faine, S. Pomona serogroup-specific, agglutinating antigen in *Leptospira*, identified by monoclonal antibodies. Pathology 15, no. 3 (1983): 247-250.

[89] Naiman, B. M., Alt, D., Bolin, C. A., Zuerner, R., and Baldwin, C. L. Protective killed *Leptospira Borgpetersenii* vaccine induces potent Th1 immunity comprising responses by CD4 and gammadelta T lymphocytes. Infect Immun 69, no. 12 (2001): 7550-7558.

[90] Brown, R. A., Blumerman, S., Gay, C., Bolin, C., Duby, R., and Baldwin, C. L. Comparison of three different leptospiral vaccines for induction of a type 1 immune response to *Leptospira Borgpetersenii* serovar Hardjo. Vaccine 21, no. 27-30 (2003): 4448-4458.

[91] Naiman, B. M., *et al*. Evaluation of type 1 immune response in naive and

vaccinated animals following challenge with *Leptospira Borgpetersenii* serovar Hardjo: involvement of wc1(+) gammadelta and CD4 T cells. Infect Immun 70, no. 11 (2002): 6147-6157.

[92] Ido, Y., Hoki, R., Ito, H., and Wani, H. The prophylaxis of weil's disease (spirochaetosis icterohaemorrhagica). J Exp Med 24, no. 5 (1916): 471-483.

[93] Verma, R., Khanna, P., and Chawla, S. Whole-cell inactivated leptospirosis vaccine: future prospects. Hum Vaccin Immunother 9, no. 4. (2013): 1-3.

[94] Gonzalez, A., *et al*. Immunogenicity and protective capacity of leptospiral whole-cell monovalent serogroup ballum vaccines in hamsters. Rev Argent Microbiol 37, no. 4 (2005): 169-175.

[95] Haake, D. A., *et al*. Leptospiral outer membrane proteins OmpL1 and LipL41 exhibit synergistic immunoprotection. Infect Immun 67, no. 12 (1999): 6572-6582.

[96] Palaniappan, R. U., *et al*. Immunoprotection of recombinant leptospiral immunoglobulin-like protein a against *Leptospira interrogans* serovar pomona infection. Infect Immun 74, no. 3 (2006): 1745-1750.

[97] Silva, E. F., *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, no. 33 (2007): 6277-6286.

[98] Faisal, S. M., Yan, W., Chen, C. S., Palaniappan, R. U., McDonough, S. P., and

Chang, Y. F. Evaluation of protective immunity of *leptospira* immunoglobulin like protein A (LigA) DNA vaccine against challenge in hamsters. Vaccine 26, no. 2 (2008): 277-287.

[99] Yan, W., *et al*. Immunogenicity and protective efficacy of recombinant *Leptospira* immunoglobulin-like protein B (rLigB) in a hamster challenge model. Microbes Infect 11, no. 2 (2009): 230-237.

[100] Grassmann, A. A., *et al*. Protection against lethal leptospirosis after vaccination with LipL32 coupled or coadministered with the b subunit of *Escherichia coli* heat-labile enterotoxin. Clin Vaccine Immunol 19, no. 5: 740-745.

[101] Lindstedt, S., and Samuelsson, B. Bile Acids and Steroids. Lxxxiii. On the inter-conversion of cholic and deoxycholic acid in the rat. J Biol Chem 234, no. 8 (1959): 2026-2030.

[102] Coutinho, M. L., *et al*. A LigA three-domain region protects hamsters from lethal infection by *Leptospira Interrogans*. PLoS Negl Trop Dis 5, no. 12: e1422.

[103] Chen, T. Development and present status of a leptospiral vaccine and the technology of vaccine production in China. Nihon Saikingaku Zasshi 40, no. 4 (1985): 755-762.

[104] Martinez, R., *et al*. Efficacy and safety of a vaccine against human leptospirosis in Cuba. Rev Panam Salud Publica 15, no. 4 (2004): 249-255.

[105] Kitaoka, M., and Inoue, S. Standard procedures of Weil's disease vaccine and Weil's disease and Akiyami combined vaccine. Jpn Med J 1478, (1952): 2845-2847

[106] Levett, P. N. Leptospirosis. Clin Microbiol Rev 14, no. 2 (2001): 296-326.

[107] Rathinam, S. R. Ocular leptospirosis. Curr Opin Ophthalmol 13, no. 6 (2002): 381-386.

[108] Yan, Y., *et al*. An evaluation of the serological and epidemiological effects of the outer envelope vaccine to *Leptospira*. J Chin Med Assoc 66, no. 4 (2003): 224-230.

[109] Sonrier, C., Branger, C., Michel, V., Ruvoen-Clouet, N., Ganiere, J. P., and Andre-Fontaine, G. Evidence of cross-protection within *Leptospira* interrogans in an experimental model. Vaccine 19, no. 1 (2000): 86-94.

[110] Haake, D. A., Suchard, M. A., Kelley, M. M., Dundoo, M., Alt, D. P., and Zuerner, R. L. Molecular evolution and mosaicism of leptospiral outer membrane proteins involves horizontal DNA transfer. J Bacteriol 186, no. 9 (2004): 2818-2828.

[111] Hoke, D. E., Egan, S., Cullen, P. A., and Adler, B. LipL32 is an extracellular matrix-interacting protein of *Leptospira* spp. and *Pseudoalteromonas tunicata*. Infect Immun 76, no. 5 (2008): 2063-2069.

[112] Vivian, J. P., *et al*. Crystal structure of LipL32, the most abundant surface protein of pathogenic *Leptospira* spp. J Mol Biol 387, no. 5 (2009): 1229-1238.

[113] Flannery, B., *et al*. Evaluation of recombinant *Leptospira* antigen-based enzyme-linked immunosorbent assays for the serodiagnosis of leptospirosis. J Clin Microbiol 39, no. 9 (2001): 3303-3310.

[114] Lee, S. H., Kim, K. A., Park, Y. G., Seong, I. W., Kim, M. J., and Lee, Y. J. Identification and partial characterization of a novel hemolysin from *Leptospira Interrogans* serovar Lai. Gene 254, no. 1-2 (2000): 19-28.

[115] Yang, C. W., Wu, M. S., Pan, M. J., Hsieh, W. J., Vandewalle, A., and Huang, C. C. The *Leptospira* outer membrane protein lipl32 induces tubulointerstitial nephritis-mediated gene expression in mouse proximal tubule cells. J Am Soc Nephrol 13, no. 8 (2002): 2037-2045.

[116] Pinne, M., and Haake, D. A. Lipl32 is a subsurface lipoprotein of *Leptospira interrogans*: presentation of new data and reevaluation of previous studies. PLoS One 8, no. 1: e51025.

[117] Murray, G. L. The lipoprotein Lipl32, an enigma of leptospiral biology. Vet Microbiol 162, no. 2-4: 305-314.

[118] Koebnik, R. Proposal for a peptidoglycan-associating alpha-helical motif in the C-terminal regions of some bacterial cell-surface proteins. Mol Microbiol 16, no. 6 (1995): 1269-1270.

[119] Doria-Rose, N. A., and Haigwood, N. L. DNA vaccine strategies: candidates for immune modulation and immunization regimens. Methods 31, no. 3 (2003): 207-216.

[120] Williamson, E. D., Bennett, A. M., Perkins, S. D., Beedham, R. J., Miller, J., and

Baillie, L. W. Co-immunisation with a plasmid dna cocktail primes mice against anthrax and plague. Vaccine 20, no. 23-24 (2002): 2933-2941.

[121] Gor, D. O., Rose, N. R., and Greenspan, N. S. Th1-Th2: a procrustean paradigm. Nat Immunol 4, no. 6 (2003): 503-505.

[122] Stevenson, F. K., *et al*. DNA vaccines to attack cancer. Proc Natl Acad Sci U S A 101 Suppl 2 (2004): 14646-14652.

[123] Barouch, D. H., *et al*. Augmentation and suppression of immune responses to an HIV-1 DNA vaccine by plasmid cytokine/ig administration. J Immunol 161, no. 4 (1998): 1875-1882.

[124] Zhao, P., *et al*. Immune responses against sars-coronavirus nucleocapsid protein induced by DNA vaccine. Virology 331, no. 1 (2005): 128-135.

[125] Harari, A., *et al*. An Hiv-1 Clade C DNA Prime, Nyvac Boost Vaccine Regimen Induces Reliable, Polyfunctional, and Long-Lasting T Cell Responses. J Exp Med 205, no. 1 (2008): 63-77.

[126] Wang, S., *et al*. Polyvalent HIV-1 Env vaccine formulations delivered by the dna priming plus protein boosting approach are effective in generating neutralizing antibodies against primary human immunodeficiency virus type 1 isolates from subtypes A, B, C, D and E. Virology 350, no. 1 (2006): 34-47.

[127] Magalhaes, I., *et al*. RBCG induces strong antigen-specific t cell responses in Rhesus macaques in a prime-boost setting with an adenovirus 35 tuberculosis vaccine vector. PLoS One 3, no. 11 (2008): e3790.

[128] Wang, S., Parker, C., Taaffe, J., Solorzano, A., Garcia-Sastre, A., and Lu, S. Heterologous HA DNA vaccine prime--inactivated influenza vaccine boost is more effective than using DNA or inactivated vaccine alone in eliciting antibody responses against H1 or H3 serotype influenza viruses. Vaccine 26, no. 29-30 (2008): 3626-3633.

[129] Aagaard, C., *et al*. A multistage tuberculosis vaccine that confers efficient protection before and after exposure. Nat Med 17, no. 2: 189-194.

[130] Kutzler, M. A., and Weiner, D. B. DNA vaccines: ready for prime time? Nat Rev Genet 9, no. 10 (2008): 776-788.

[131] Gurunathan, S., Klinman, D. M., and Seder, R. A. DNA vaccines: immunology, application, and optimization. Annu Rev Immunol 18 (2000): 927-974.

[132] Hubbert, W. T., and Miller, J. N. Studies on immunity in experimental leptospirosis: the immunogenicity of *Leptospira Icterohemorrhagiae* attenuated by gamma-irradiation. J Immunol 95, no. 4 (1965): 759-764.

[133] Babudieri, B., Castelli, M., and Pisoni, F. Comparative tests with formolized and irradiated vaccines against leptospirosis. Bull World Health Orga*n* 48, no. 5 (1973): 587-590.

[134] Matsuo, K., Isogai, E., and Araki, Y. Control of immunologically crossreactive leptospiral infection by administration of lipopolysaccharides from a nonpathogenic strain of *Leptospira Biflexa*. Microbiol Immunol 44, no. 11 (2000): 887-890.

[135] Morein, B., Sundquist, B., Hoglund, S., Dalsgaard, K., and Osterhaus, A. Iscom,

a novel structure for antigenic presentation of membrane proteins from enveloped viruses. Nature 308, no. 5958 (1984): 457-460.

[136] Pearse, M. J., and Drane, D. Iscomatrix adjuvant for antigen delivery. Adv Drug Deliv Rev 57, no. 3 (2005): 465-474.

[137] Sailaja, A.K., Amareshwar, P., and Chakravarty, P. Chitosan nanoparticles as a drug delivery system. Res J Pharm Biol Chem Sci 1, (2010): 474–484

[138] van der Lubben, I. M., Verhoef, J. C., Borchard, G., and Junginger, H. E. Chitosan and its derivatives in mucosal drug and vaccine delivery. Eur J Pharm Sci 14, no. 3 (2001): 201-207.

[139] Bachmann, M. F., and Jennings, G. T. Vaccine delivery: a matter of size, geometry, kinetics and molecular patterns. Nat Rev Immunol 10, no. 11: 787-796.

[140] Saranya, N., Moorthi, A., Saravanan, S., Devi, M. P., and Selvamurugan, N. Chitosan and its derivatives for gene delivery. Int J Biol Macromol 48, no. 2: 234-238.

[141] Knorr, D. Use of chitinous polymers in foodda challenge for food research and development. Food Technol 38, (1984): 85–97.

[142] Kurita, K. Chemistry and application of chitin and chitosan, Polym Degrad Stab 59, (1998): 117–120

[143] Razdan, A., and Pettersson, D. Effect of chitin and chitosan on nutrient

digestibility and plasma lipid concentrations in Broiler chickens. Br J Nutr 72, no. 2 (1994): 277-288.

[144] van der Lubben, I. M., Verhoef, J. C., van Aelst, A. C., Borchard, G., and Junginger, H. E. Chitosan microparticles for oral vaccination: preparation, characterization and preliminary in vivo uptake studies in murine Peyer's patches. Biomaterials 22, no. 7 (2001): 687-694.

[145] De Campos, A. M., Sanchez, A., and Alonso, M. J. Chitosan nanoparticles: a new vehicle for the improvement of the delivery of drugs to the ocular surface. application to cyclosporin A. Int J Pharm 224, no. 1-2 (2001): 159-168.

[146] Illum, L., Jabbal-Gill, I., Hinchcliffe, M., Fisher, A. N., and Davis, S. S. Chitosan as a novel nasal delivery system for vaccines. Adv Drug Deliv Rev 51, no. 1-3 (2001): 81-96.

[147] Zhu, Y., Wang, J., Zhang, Y., Wang, Q., Xu, Y., and Wang, H. Chitosan microspheres enhance the immunogenicity of an ag85b-based fusion protein containing multiple T-cell epitopes of *Mycobacterium tuberculosis*. Eur J Pharm Biopharm 66, no. 3 (2007): 318-326.

[148] Nishimura, K., Nishimura, S., Nishi, N., Saiki, I., Tokura, S., and Azuma, I. Immunological activity of chitin and its derivatives. Vaccine 2, no. 1 (1984): 93-99.

[149] Seferian, P. G., and Martinez, M. L. Immune stimulating activity of two new chitosan containing adjuvant formulations. Vaccine 19, no. 6 (2000): 661-668.

[150] Woodland, D. L. Jump-starting the immune system: prime-boosting comes of age. Trends Immunol 25, no. 2 (2004): 98-104.

[151] Bansal, A., *et al.* Multifunctional T-cell characteristics induced by a polyvalent dna prime/protein boost human immunodeficiency virus type 1 vaccine regimen given to healthy adults are dependent on the route and dose of administration. J Virol 82, no. 13 (2008): 6458-6469.

[152] Lu, S. Heterologous prime-boost vaccination. Curr Opin Immunol 21, no. 3 (2009): 346-351.

[153] Biswas, S., Reddy, G. S., Srinivasan, V. A., and Rangarajan, P. N. Preexposure efficacy of a novel combination dna and inactivated rabies virus vaccine. Hum Gene Ther 12, no. 15 (2001): 1917-1922.

[154] Amara, R. R., *et al*. Control of a mucosal challenge and prevention of aids by a multiprotein dna/mva vaccine. Science 292, no. 5514 (2001): 69-74.

[155] Hanke, T., *et al*. Effective induction of simian immunodeficiency virus-specific cytotoxic T lymphocytes in macaques by using a multiepitope gene and dna prime-modified vaccinia virus ankara boost vaccination regimen. J Virol 73, no. 9 (1999): 7524-7532.

[156] Kent, S. J., *et al*. Enhanced T-cell immunogenicity and protective efficacy of a human immunodeficiency virus type 1 vaccine regimen consisting of consecutive priming with DNA and boosting with recombinant Fowlpox Virus. J Virol 72, no. 12 (1998): 10180-10188.

[157] Shiver, J. W., *et al*. Replication-incompetent adenoviral vaccine vector elicits

effective anti-immunodeficiency-virus immunity. Nature 415, no. 6869 (2002): 331-335.

[158] Egan, M. A., *et al*. Priming with plasmid DNAs expressing interleukin-12 and simian immunodeficiency virus gag enhances the immunogenicity and efficacy of an experimental aids vaccine based on recombinant vesicular stomatitis virus. AIDS Res Hum Retroviruses 21, no. 7 (2005): 629-643.

[159] Skinner, M. A., *et al*. The order of prime-boost vaccination of neonatal calves with *Mycobacterium bovis* BCG and a DNA vaccine encoding mycobacterial proteins HSP65, HSP70, and APA is not critical for enhancing protection against bovine tuberculosis. Infect Immun 73, no. 7 (2005): 4441-4444.

[160] Hu, S. L., *et al*. Protection of macaques against siv infection by subunit vaccines of siv envelope glycoprotein gp160. Science 255, no. 5043 (1992): 456-459.

[161] Dunachie, S. J., *et al*. A DNA prime-modified vaccinia virus ankara boost vaccine encoding thrombospondin-related adhesion protein but not circumsporozoite protein partially protects healthy malaria-naive adults against *Plasmodium falciparum* sporozoite challenge. Infect Immun 74, no. 10 (2006): 5933-5942.

[162] Park, S. H., Yang, S. H., Lee, C. G., Youn, J. W., Chang, J., and Sung, Y. C. Efficient induction of T helper 1 CD4+ T-cell responses to hepatitis c virus core and E2 by a DNA prime-adenovirus boost. Vaccine 21, no. 31 (2003): 4555-64.

[163] Sin, J. I., Bagarazzi, M., Pachuk, C., and Weiner, D. B. DNA priming-protein boosting enhances both antigen-specific antibody and th1-type cellular immune responses in a murine herpes simplex virus-2 gd vaccine model. DNA Cell Biol 18, no. 10 (1999): 771-779.

[164] Xiao-wen, H., *et al*. Augmented humoral and cellular immune responses of a hepatitis B DNA vaccine encoding HBSAG by protein boosting. Vaccine 23, no. 14 (2005): 1649-1656.

[165] D. T. O'Hagan New generation vaccine adjuvants, ENCYCLOPEDIA OF LIFE SCIENCES, John Wiley & Sons, Ltd., 1-7 (2007).

[166] Freund, J., Casals, J., and Hosmer, E. Sensitization and antibody formation after injection of tubercle bacili and paraffin oil. Proc Soc Exp Biol Med 37, (1937): 509–513

[167] Pascual, D. M., Morales, R. D., Gil, E. D., Munoz, L. M., Lopez, J. E., and Casanueva, O. L. Adjuvants: present regulatory challenges. Vaccine 24 Suppl 2 (2006): S2-88-89.

[168] HogenEsch, H. Mechanisms of stimulation of the immune response by aluminum adjuvants. Vaccine 20 Suppl 3 (2002): S34-S39.

[169] Schirmbeck, R., Melber, K., Kuhrober, A., Janowicz, Z. A., and Reimann, J. Immunization with soluble hepatitis B virus surface protein elicits murine H-2 class I-restricted CD8+ cytotoxic T lymphocyte responses in vivo. J Immunol 152, no. 3 (1994): 1110-1119.

[170] Podda, A. The adjuvanted influenza vaccines with novel adjuvants: experience with the MF59-adjuvanted vaccine. Vaccine 19, no. 17-19 (2001): 2673-2680.

[171] O'Hagan, D. T. MF59 is a safe and potent vaccine adjuvant that enhances protection against influenza virus infection. Expert Rev Vaccines 6, no. 5 (2007): 699-710.

[172] Coffman, R. L., Sher, A., and Seder, R. A. Vaccine adjuvants: putting innate immunity to work. Immunity 33, no. 4: 492-503.

[173] Mbow, M. L., De Gregorio, E., Valiante, N. M., and Rappuoli, R. New adjuvants for human vaccines. Curr Opin Immunol 22, no. 3: 411-6.

[174] Frey, A., Di Canzio, J., and Zurakowski, D. A Statistically defined endpoint titer determination method for immunoassays. J Immunol Methods 221, no. 1-2 (1998): 35-41.

[175] Grifantini, R., *et al*. Multi-plasmid dna vaccination avoids antigenic competition and enhances immunogenicity of a poorly immunogenic plasmid. Eur J Immunol 28, no. 4 (1998): 1225-1232.

[176] Sedegah, M., *et al*. Reduced immunogenicity of DNA vaccine plasmids in mixtures. Gene Ther 11, no. 5 (2004): 448-456.

[177] Hall, J., Hazlewood, G. P., Surani, M. A., Hirst, B. H., and Gilbert, H. J. Eukaryotic and prokaryotic signal peptides direct secretion of a bacterial endoglucanase by mammalian cells. J Biol Chem 265, no. 32 (1990): 19996-19999.

[178] Mukhopadhyay, A., Ni, L., Yang, C. S., and Weiner, H. Bacterial signal peptide recognizes HeLa cell mitochondrial import receptors and functions as a mitochondrial leader sequence. Cell Mol Life Sci 62, no. 16 (2005): 1890-1899.

APPENDICES

APPENDIX A

BUFFER AND REAGENT

Reagent for DNA extraction

1. 0.5 M EDTA pH8.0

Disodium ethylenediamine tetraacetate	18.66	g

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

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 minutes.

3. 10% Sodium lauruyl sarcosine (SDS)

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

Mix the solution and store at room temperature.

4. 5M NaCl (100 mL)

NaCl	14.61	g

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

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

Saturated phenol	50	ml
Chloroform	48	ml
Isoamyl alcohol	2	ml

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

Reagent for agarose gel electrophoresis

1. 50X Tris-Acetate buffer (TAE)

Tris base	420	g
Glacial acetic acid	57.1	ml
0.5 M EDTA pH8.0	100	ml

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

2. Running buffer for agarose gel electrophoresis

50XTAE was distilled 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 is present.

4. 10 mg/ml Ethidium bromide

Ethidium bromide	1.0	g
Distilled water	100	ml

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

Reagent for cloning

1. 1M glucose (10 ml)

Glucose	1.8	g

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

2. 2M $MgCl_2$ (10ml)

$MgCl_2$	1.9	g

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

3. SOB (100 ml)

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

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

4. SOC (10 ml)

SOB	10	ml
2 M MgCl2	50	µl
1 M Glucose	200	µl

Mix the solution and store at 4º

5. Ampicillin stock (100 mg/ml)

Ampicillin	1	g

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

6. Choramphinicol stock (35 mg/ml)

Choramphinicol	140	mg

Dissolve in 10 ml of absolute ethanol. Store at -20ºC.

7. Kanamycin stock (50 mg/ml)

Kanamycin	0.5	g

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

8. Lauria-Bertani (LB) Medium

Bacto tryptone	10	g
Yeast tryptone	5	g
NaCl	10	g

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

9. LB-Ampicillin 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 121ºC for 15 minutes. After autoclaved, allowed media cool down, added 1 ml ampicillin stock, poured and stored plates at 4ºC

10. LB-Ampicillin-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 121ºC for 15 minutes. After autoclaved, allowed media cool down, added 1 ml Ampicilin stock, poured and stored plates at 4ºC

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 1000 ml. Sterilize the solution by autoclaving at 121ºC for 15 minutes. After autoclaved, allowed media cool down, added 1 mL Kanamycin stock, poured and stored plates at 4ºC

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

1. 1M Tris-HCl pH 8.8

Tris base	12.11	g

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

2. 0.5 M Tris-HCl pH 6.8

Tris base	6.055	g

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

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

1M Tris-HCl pH 6.8	1	ml (final conc. 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 -20ºC.

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

Tris base	18.21	g
SDS	0.4	g

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

5. Running buffer

Tris 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 -20ºC

8. 10% sodium laury sacosine (SDS)

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

Mix the solution and steor at -20ºC

9. 30% Acrymide/80% 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. 12% SDS PAGE

Separating gel (8 ml)

Distilled water	3.436	ml
1.5 M Tris-HCl pH 8.8	2.4	ml
40% Acrylamide and Bis-acrylamide solution	2	ml
10% SDS	80	µl
10% APS	80	µl
TEMED	4	µl

Stacking gel (2 ml)

Distilled water	1.204	ml
1.5 M Tris-HCl pH 8.8	0.504	ml
40% Acrylamide and Bis-acrylamide solution	0.25	ml
10% SDS	20	µl
10% APS	20	µl
TEMED	2	µl

Reagent for Western blot

1. TBS	20	ml
1M 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 121ºC 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 4ºC

5. 5X Running buffer

Tris-base	15.2	g

Glycine	94	g
SDS	5	g
Deionize water	1000	ml

Mix the solution and store at room temperature.

6. Transfer buffer for Western blot

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

7. ECL substrate for HRP

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

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

<u>Solution A</u>

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% H2O2	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_4-2H2O	0.356	g
20 mM NaH_2PO_4-H2O	0.276	g

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

2. Buffer B (Vary concentration of imidazole)

60 mM imidazone

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 imidazone

0.5 M NaCl	2.922	g
60 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 imidazone

0.5 M NaCl	2.922	g
60 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 imidazone

0.5 M NaCl	2.922	g

60 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 -20ºC

Reagent for chitosan

1. 25 mM Na2SO4 pH 5.5

Na_2So_4	0.35	g

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

2. 5 mM sodium acetate pH 5.5

Sodium acedate	0.04	g

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

Reagent for cell culture

1. Complete RPMI 1640 100 mL

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

2. Complete DMEM 100 ml

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

3. Freezing media 10 ml

Complete media	90	ml
DMSO	10	ml

4. FBS inactivation

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

5. RIPA buffer for protein extraction 10 mL

50 mM 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

Adjust volume to 10 ml using deionized water

6. 1XPBS pH 7.4

NaCl	8	g
KCl	0.2	g
Na2HPO4	1.44	g
KH2PO4	0.24	g
Deionized water	1000	ml

Autoclaved at 121ºC for 15 minutes.

Reagent for indirect immunofluorescent

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 65ºC in a chemical hood. Then, the solution was cooled to room temperature and adjusted to pH 7.4

Reagent for MTT assay

1. MTT 5 mg/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 4ºC

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, IgG1, IgG2a)

1. Coating buffer

NaHCO3	7.13	g
Na2CO3	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 4ºC.

3. Washing buffer

1XPBS	100	ml
Tween 20	50	µl
BSA	1	g

Mix the solution and store at room temperature.

4. Stop reaction solution

0.5 M	H_2SO_4	2.67	ml
DDW		97.33	ml

Mix the solution and store at room temperature.

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 ^{o}C storage.

7. Washing buffer

1X PBS	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
DDW	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. RPMI +2ME

RPMI	100	ml
2ME	35	µl

Mix the solution and store at 4ºC.

2. 1XPBS

NaCl	8	g
KCl	0.2	g
Na2HPO4	1.44	g
KH2PO4	0.24	g
Deionized water	1000	ml

Autoclave at 121ºC and pressure for 15 minutes.

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, filtered by 0.02 µM membrane, and store at 4ºC

APPENDIX B

1. > *L.interrogans* serovar serovar Pomona OmpA family lipoprotein Loa22 (ACCESSION No. EKN96269.1, REGION: 195327-195914) and primers used in the study.

loa22 with natural reader sequence

Amplify *loa22* from genomic DNA of *L. interrogans* serovar Pomona

For_signalLoa22_*AgeI*	GCGCCACCGGTATGGTCAAAAAGATTTTG *AgeI*
Rev_signalLoa22_*NheI*	CGGCTAGCTTATTGTTGTGGTGCGGAAGT *NheI*

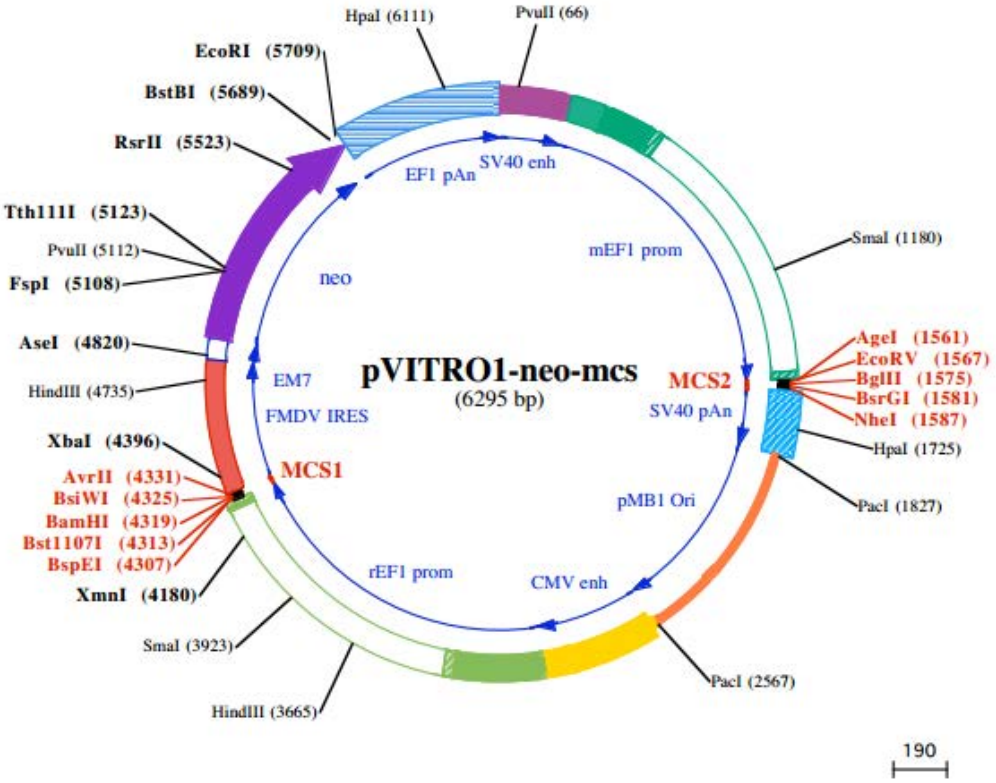
loa22 sequence from genomic DNA of *L. interrogans* serovar Pomona

>Pomona/1/3803786/3803199 OmpA-family lipoprotein
ATGGTCAAAAAGATTTTGAATCTGATTCTGCTCGGTGCAATTGCATTTTCATTCACTCTCTGCTCCTCTGCTGAAA
AAAAAGAGGAATCCGCAGCTCCTGAGCCTTCAACGCAAGAGCAATCCGCAGCTGCAAACAGAAACGTTGACGTC
AATTCTCCGGAAGCGATCGCAGATTCTTTAAACGAAAAACTAAAAGATTTCCGGTATCCAGACGGTTTAACTCGTC
CTGGATTTAGTTATAAAAAAGCGGATGTTACCCCTGGTGATTTCAGCGAGTGGTCTAAAACAAACGCTCCTGTAATC
AAAGAAGGTCTTGGAAAACTTCCAGATAGTTACGCTCTTGAAATTACAGGACACACCGATGCGATCGGTCCCGAA
CAAGCAGAAGGTGCTAAAAAAGGAAATATTTTTTACTCTGAGCTTCGTGCAAATGCAGTTAAACAAGCTTTAATCA
AACAAGGGATTCCAGCAAATCGTATCGTTACTAAAGGTGCCGGTTCTTCCGAGCCAGTTTCTGGTCTTGATGCGAA
AGATGCTAAAAATAGAAGAGTCACTTTCCGTTTTGCGACTTCCGCACCACAACAATAA

Amino acid sequence of OmpA family lipoprotein Loa22 protein ID: EJO76777.1 from Gene bank database

MVKKILNLILLGAIAFSFTLCSSAEKKEESAAPEPSTQEQSAAANRNVDVNSPEAIADSL
NEKLKDFRYPDGLTRPGFSYKKADVTPGDFSEWSKTNAPVIKEGLGKLPDSYALEITGHT
DAIGPEQAEGAKKGNIFYSELRANAVKQALIKQGIPANRIVTKGAGSSEPVSGLDAKDAK
NRRVTFRFATSAPQQ-

2. pVITRO-neo1-MCS



Feature of pVITRO1-neo-mcs

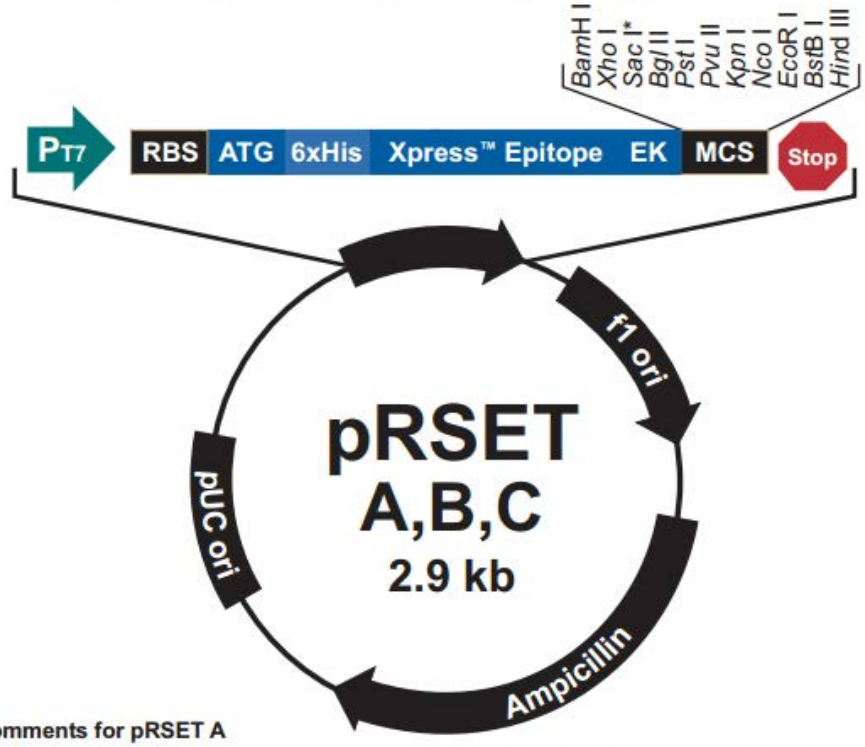
1. rEF1 and mEF1 prom: pVITRO1-neo-mcs plasmid carries two elongation factor 1 alpha (EF-1α) promoters, from rat and mouse origins. Similarly to their human counterpart, both promoters display a strong activity that yield similar levels of expression. EF-1α promoters are expressed at high levels in all cell cycles and lower levels during G0 phase. EF-1α promoters are also non-tissue specific; they are highly expressed in all cell types.
2. SV40 enhancer which is comprised of a 72-base-pair repeat allows the enhancement of gene expression in a large host range. The enhancement varies from 2-fold in non-permissive cells to 20-fold in permissive cells.

3. CMV enhancer: The major immediate early enhancer of the human cytomegalovirus (HCMV), located between nucleotides -118 and - 524, is composed of unique and repeated sequence motifs. The HCMV enhancer can substitute for the 72-bp repeats of SV40 and is severalfold more active than the SV40 enhancer.
4. SV40 pAn: the Simian Virus 40 late polyadenylation signal enables efficient cleavage and polyadenylation reactions resulting in high levels of steady-state mRNA.
5. pMB1 ori: a minimal E. coli origin of replication to limit vector size, but with the same activity as the longer Ori. FMDV IRES: The internal ribosome entry site of the Foot and Mouth Disease Virus enables the translation of two open reading frames from one mRNA with high levels of expression
6. EM7 is a bacterial promoter that enables the constitutive expression of the antibiotic resistance gene in E. coli.
7. Neo: The neo gene from Tn5 confers resistance to Kanamycin in *E. coli* and G418 in mammalian cells. In bacteria, neo is expressed from the constitutive E. coli EM7 promoter. In mammalian cells, neo is transcribed from the rat EF-1α promoter as a polycistronic mRNA and translated via the FMDV IRES.
8. EF1 pAn is a strong polyadenylation signal. InvivoGen uses a sequence starting after the stop codon of the EF1 cDNA and finishing after a bent structure rich in GT.
9. MCS1 and MCS2: Each multiple cloning site contains several restriction sites that are compatible with many other enzymes, thus facilitating cloning.

3. pRSET A, B, C used for LipL32 and Loa22 recombinant protein productions.

Map of pRSET A, B, and C

pRSET A, B, and C The map below shows the features of pRSET A, B, and C. **The complete sequence of the vector is available for downloading from our website at www.invitrogen.com or from Technical Support (see page 18).**



Comments for pRSET A
2897 nucleotides

*Version C does not contain *Sac* I

T7 promoter: bases 20-39
6xHis tag: bases 112-129
T7 gene 10 leader: bases 133-162
Xpress™ epitope: bases 169-192
Multiple cloning site: bases 202-248
T7 reverse priming site: bases 295-314
T7 transcription terminator: bases 256-385
f1 origin: bases 456-911
bla promoter: bases 943-1047
Ampicillin (*bla*) resistance gene (ORF): bases 1042-1902
pUC origin: bases 2047-2720 (C)

Features of pRSET A, B, and C

Features

The important elements of pRSET A, B, and C are described in the table below. All features have been functionally tested.

Feature	Benefit
T7 promoter	Provides tight, dose-dependent regulation of heterologous gene expression. Provides a binding site for most T7 promoter primers for sequencing into the insert.
Ribosome binding site	Optimally spaced from the multiple cloning site for efficient translation of the gene of interest.
Initiation ATG	Provides a translational initiation site for the fusion protein.
N-terminal 6×His tag	Permits purification of recombinant fusion protein on metal-chelating resins (i.e. ProBond™). In addition, it allows detection of the recombinant protein with the Anti-HisG Antibody (R940-25) or the Anti-HisG-HRP Antibody (Cat. no. R941-25)
T7 gene 10 sequence	Provides protein stability
N-terminal Xpress™ epitope tag	Allows detection of the fusion protein by the Xpress™ Antibody (Cat. no. R910-25) or the Xpress™-HRP Antibody (Cat. no. R911-25)
Enterokinase cleavage site	Provides a site for efficient removal of the fusion tag.
Multiple cloning site	Allows insertion of your gene of interest and facilitates in cloning in frame with the N-terminal epitope tag.
T7 reverse priming site	Allows sequencing of the insert.
T7 terminator	Permits efficient transcription termination.
f1 origin	Allows single strand rescue of DNA
bla promoter	Allows expression of the ampicillin resistance gene.
Ampicillin resistance gene (β-lactamase)	Allows selection of the plasmid in *E. coli*.
pUC origin	High copy replication and growth in *E. coli*.

BIOGRAPHY

NAME: Supawadee Umthong

DATE OF BIRTH: 15th April 1988

PLACE OF BIRTH: Ang-Thong, Thailand

BACKGROUND OF EDUCATION: B.Sc. in Biology (1st Class Honors with GPA 3.69), 2006 - 2009 Chulalongkorn University, Thailand

SCHOLARSHIPS:

- DPST scholarship for undergraduate and graduate (master level) programs, 2006 – 2013
- Undergraduate Training Scholarship under Young Scientist and Technologist Program (YSTP) from National Center for Genetic Engineering and Biotechnology (BIOTEC), NSTDA Thailand, March – May, 2009

PUBLICATIONS

Umthong, S., Phuwapraisirisan, P., Puthong, S., & Chanchao, C., In vitro antiproliferative activity of partially purified *Trigona laeviceps* propolis from Thailand on human cancer cell lines. *BMC Complement Altern Med* 11, 37 (2011).

Umthong, S., Puthong, S., & Chanchao, C., *Trigona laeviceps* propolis from Thailand: antimicrobial, antiproliferative and cytotoxic activities. *Am J Chin Med* 37(5), 855-865 (2009).

Umthong, S., Patarakul, K., Wanichwecharungruang, S., & Palaga, T. (2012), Chitosan nanoparticle for delivery of *lipl32-loa22* as DNA vaccine for leptospirosis. *Proceeding in 1st Asian plus three graduate research congress*, Chiang Mai, Thailand. p.212 (March 1-2, 2012)