การตอบสนองทางอิมมูนของกระต่ายต่อไลโปโซมของโปรตีนเมมเบรนชั้นนอกของเชื้อพาสเตอเรลลามัลโตซิดาสายพันธุ์ 8:A

นางสาวศศิวิมล เที่ยงแท้

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IMMMUNE RESPONSE OF RABBITS AGAINST LIPOSOME CONJUGATE OF OUTER MEMBRANE PROTEIN FROM *PASTEURELLA MULTOCIDA* SEROTYPE 8:A

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ศศิวิมล เที่ยงแท้ : การตอบสนองทางอิมมูนของกระต่ายต่อไลโปโซมของโปรตีนเมมเบรนชั้นนอกของเชื้อพาสเตอเรลลามัลโตซิดาสายพันธุ์ 8:A (IMMUNE RESPONSE OF RABBITS AGAINST LIPOSOME CONJUGATE OF OUTER MEMBRANE PROTEIN FROM *PASTEURELLA MULTOCIDA* SEROTYPE 8:A) อ. ที่ปรึกษา : รศ.ดร.วิมลมาศ ลิปิพันธ์, 152 หน้า. ISBN 974-3-0781-7

การศึกษาคุณสมบัติทางเคมีกายภาพ คุณสมบัติในการเป็นอิมมูโนเจนในการกระตุ้นการตอบสนองของภูมิคุ้มกันของไลโปโซมที่บรรจุโปรตีนเมมเบรนชั้นนอก (OMP) ของเชื้อพาสเตอเรลลามัลโตซิดา 8:A และการทดสอบการฆ่าเชื้อในหลอดทดลองของอิมมูนซีรัม โดยอนุภาคของไลโปโซมที่ได้มีขนาด 3.86, 3.90, 3.99 และ 3.42 ไมโครเมตร ส่วนเปอร์เซ็นต์ของโปรตีนที่อยู่ภายในไลโปโซมเท่ากับ 8.33, 81.5, 80.5, และ 78.6 เมื่อวิเคราะห์ที่เวลา 0, 4, 8, และ 12 สัปดาห์ตามลำดับ การวัดแอนติบอดีไตเตอร์ของอิมมูนซีรัมด้วยวิธีอีไลซาโดยทำปฏิกิริยากับเชื้อพาสเตอเรลลามัลโตซิดา 8:A ที่ทำให้ตายด้วยความร้อน และ OMP พบว่าซีรัมจากกระต่ายที่ฉีดไลโปโซม OMP และ OMP ที่ผสมกับฟรอยด์แอดจูแวนท์มีค่าแอนติบอดีไตเตอร์สูงกว่าซีรัมของกระต่ายที่ฉีดวัคซีนเชื้อพาสเตอเรลลามัลโตซิดาที่ทำให้ตายด้วยฟอร์มาลิน, อลัม OMP, และ OMP ที่ไม่มีแอดจูแวนท์ ($p<0.05$) โดยมีกระต่ายที่ได้รับไลโปโซมที่ไม่บรรจุโปรตีน และกระต่ายที่ได้รับ PBS เป็นกลุ่มควบคุม นอกจากนี้ผลการฆ่าเชื้อของอิมมูนซีรัมที่ถูกเหนี่ยวนำโดยไลโปโซม OMP และ OMP ที่ผสมกับฟรอยด์แอดจูแวนท์ยังให้ฤทธิ์ในการฆ่าเชื้อสูงกว่าอิมมูนซีรั่มของกระต่ายที่ถูกเหนี่ยวนำโดยวัคซีนชนิดอื่น ($p<0.05$) ดังนั้นไลโปโซมที่บรรจุ OMP และ OMP ที่ผสมกับฟรอยด์แอดจูแวนท์มีผลทำให้แอนติบอดีไตเตอร์และฤทธิ์ในการฆ่าเชื้อของอิมมูนซีรัมสูงที่สุด และพบว่ามีความสัมพันธ์ระหว่างแอนติบอดีไตเตอร์ที่จำเพาะกับแอนติเจนของแบคทีเรียทั้งที่เป็นเชื้อทั้งตัวและ OMP กับฤทธิ์การฆ่าเชื้อของอิมมูนซีรั่มต่อเชื้อพาสเตอเรลลามัลโตซิดา 8:A

การวิเคราะห์ ขนาดโปรตีนใน OMP ของเชื้อพาสเตอเรลลามัลโตซิดา 8:A โดยวิธี SDS-PAGE พบโปรตีนเด่นมีขนาด 37.5 kDa และโปรตีนอื่นๆ มีขนาด 23, 27.5, 45, และ 97.4 kDa และขนาดของโปรตีนใน OMP ที่เตรียมได้และ OMP ที่อยู่ในไลโปโซมมีรูปแบบเดียวกัน การศึกษาถึงความจำเพาะของแอนติบอดีของอิมมูนซีรัมจากกระต่ายที่ได้รับวัคซีนชนิดต่างๆ ต่อ OMP โดยวิธี western blot พบ strong reaction ของแอนติบอดีที่จำเพาะกับ OMP ที่ 37.5 kDa.

ภาควิชา จุลชีววิทยา	ลายมือชื่อนิสิต .....
สาขาวิชา จุลชีววิทยา	ลายมือชื่ออาจารย์ที่ปรึกษา .....
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KEY WORD : *Pasteurella multocida* / LIPOSOMES / OUTER MEMBRANE PROTEINS / BACTERICIDAL ACTIVITY / ADJUVANT / SDS-PAGE / WESTERN BLOT

SASIWIMON THEANGTAE: IMMUNE RESPONSE OF RABBITS AGAINST LIPOSOME CONJUGATE OF OUTER MEMBRANE PROTEIN FROM *PASTEURELLA MULTOCIDA* SEROTYPE 8:A. THESIS ADVISOR: ASSOCIATE PROFESSOR VIMOLMAS LIPIPUN, Ph.D. 152 pp. ISBN 974-03-0781-7

Liposome containing outer membrane protein (OMP) of *Pasteurella multocida* 8:A was studied on the physicochemical properties, immunogenicity to induce immune response and the *in vitro* bactericidal effect of immune sera. The particle size of liposome was 3.86, 3.90, 3.99, and 3.42 μm and the percent protein entrapment of liposome was 83.3, 81.5, 80.5, and 78.6 at week 0, 4, 8, and 12, respectively. The immunogenicity of a vaccine containing *P. multocida* OMP was investigated. Each group of rabbits was immunized with formalin killed whole cell, liposome OMP, OMP emulsified with Freund's adjuvant, alum OMP and OMP. The ghost liposome and PBS were used as the immunizing agents for control groups. The results indicated that liposome OMP and OMP with Freund's adjuvant significantly elicited the highest antibody titers ($p<0.05$), as shown by ELISA titers against whole cell and OMP of *P. multocida*. The liposome OMP and OMP emulsified with Freund's adjuvant also significantly produced higher bactericidal activity than other vaccines used ($p<0.05$). There was correlation between antibody titer against both whole cell and OMP and bactericidal activity of antisera against *P. multocida* 8:A.

The analysis by SDS-PAGE of OMP from *P. multocida* 8:A indicated the major band of 37.5 kDa and the minor bands of 23, 27.5, 45, and 97.4 kDa. The same protein bands were observed in both OMP from liposome vaccine and OMP freshly prepared. The study of specificity of rabbit immune sera by western blot showed that all immune sera strongly reacted to major protein band, 37.5 kDa.

Department Microbiology	Student's signature……...
Field of study Microbiology	Advisor's signature……..
Academic year 2001	Co-advisor's signature……-……

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CONTENTS

PAGE

LIST OF TABLES

Tables **Page**

LIST OF FIGURES

Figures **Page**

ABBREVIATIONS

APC	Antigen presenting cell
BSA	Bovine serum albumin
°C	Degree (s) celsius
CFU	Colony foming unit
CMC	Sodium carboxymethyl cellulose
DCM	Dichloromethane
ELISA	Enzyme-linked immunosorbent
FCA	Freund's complete adjuvant
FIA	Freund's incomplete adjuvant
g	gram
HDL	High density lipoprotein
IL	Interleukin
ISCOMs	Immunostimulatory complexes
kDa	Kilodalton
Kg	Kilogram
KSCN	Potassium thiocyanate
LPS	Lipopolysaccharides
M	Molar
mg	Milligram
MW	Molecular Weight
µg	Microgram
µl	Microliter
µm	Micrometers
µ	Micron
ml	Milliliter
mm	Millimeter
MPL	Monophosphoryl lipid A
N	Normal
nm	Nanometer
NSS	Normal saline solution
OD	Optical density

OMP	Outer membrane protein
PBS	Phosphate buffer solution
PBS-T	Phosphate buffer saline solution with tween-20
PC	Phosphatidylcholine
%	Percent
ppm	Part per million
PVA	Polyvinylalcohol
s.c.	Subcutaneous
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SEM	Scanning electron microscope
TBA	Tryptic soy blood agar
TEM	Transmission electron microscope
V	Volt

CHAPTER I

INTRODUCTION

Over a century has passed since the first attempt by Louis Pasteur at immunization against infection with Gram negative facultative bacterium, *Pasteurella multocida* the organism which bears his name (1). *P. multocida* is an important animal pathogen causing widespread infection such as snuffles in rabbits, pneumonia in cattle, sheep, and goats, atrophic rhinitis in swine, haemorrhagic septicaemia in cattle and buffaloes, and fowl cholera in chicken, bird and turkeys (2). Isolates may be grouped serologically into serogroups A, B, D, E, and F based on capsular antigen(3,4) or into 16 serotypes based on LPS (5). Serogroups A, D, and F are responsible for diseases in cattles, pigs, rabbits, and turkeys (4,6). Atrophic rhinitis is a worldwide disease of pigs. *P. multocida* serogroups A and D are often isolated from the nasal cavities of piglets from farms with atrophic rhinitis (7). *P. multocida* strains which cause fowl cholera generally belong to serogroup A, serotype 1, 3 or 4. Fowl cholera remains an important veterinary disease responsible for major losses in the poultry industry world wide. Prevention of pasteurellosis is at present carried out by vaccination with whole cell bacteria, which afford serotype-specific protection, or which confer protection against both homologous and heterologous serotypes. Unfortunately, these attenuated live vaccines have been implicated in recent outbreaks of fowl cholera in turkeys (8).

Because the current experimental vaccines do not provide a complete protection in animal, there is an interest in defining more specific and accurate responses of animal to *P. multocida* potential protective immunogens. Previous studies have demonstrated that a potassium thiocyanate (KSCN) extract of *P. multocida* and rabbit immune sera against a KSCN extract of *P. multocida* actively and passively protected rabbits against a homologous challenge, respectively (9,10,11). KSCN extracts of *P. multocida* contained outer membrane proteins (OMP), lipopolysaccharides (LPS), capsule, and nucleic acids (10) and that rabbits immunized with KSCN extracts produced antibodies against OMP and LPS of homologous *P.*

multocida and later they demonstrated that antibodies against OMP of *P. multocida* but not LPS inhibited pulmonary proliferation of *P. multocida* in mice (9,11).

Several studies have been reported to identify the potentially important OMP of *P. multocida* and to classify *P. multocida* strains based on the electrophoretic mobility of major OMP. Lu et al. (12) demonstrated that vaccination with OMP protected rabbits against homologous challenge. A monoclonal antibody (MAb) against a 37.5-kDa OMP protected both mice and rabbits against *P. multocida* infection (13). The OMP associated cross-protection factors have been identified in *in vivo*-grown *P. multocida* organisms. Ruffolo and Adler (14) identified an 87-kDa common OMP in all serotypes of *P. multocida* which elicited homologous protective immunity in mice. A 16-kDa common OMP of *P. multocida* with a high degree of similarity to the P6 protective OMP of *Haemophilus influenzae* was identified and considered to be immunogenic (15). A 50-kDa OMP of *P. multocida* has also been shown to have antiphagocytic activity (16). Chevalier et al. (17) identified a 37.5 kDa OMP (porin H) of *P. multocida* as a nonspecific, Gram-negative bacterial porin. Thus, the OMP of *P. multocida* were the antigens that have been considered effective vaccine candidates.

However, antigens may be limiting their ability to induce a strong protective immune response. While coadministering antigens with immunostimulating adjuvants, agent that could induce strong immunity to antigens (18). They appear to function by creating a depot site of injection and activating macrophages (19). Adjuvants suited for vaccine formulation should display universal properties of facilitating strong and sustained immune responses against varied immunogens as well as driving the response in appropriate direction to provide protective immunity, many adjuvants have undesirable side effects such as severe inflammatory. Liposome have been successfully used as drug carriers (20) and they have also been proposed as carriers of antigens and adjuvants to enhance the immune response to antigens. The theoretical basis focusing liposomes as carriers of vaccine has been extensively studied *in vitro* and in animal (21).

In this study, liposomes containing OMP from *P. multocida* were prepared by double emulsion techniques. The molar ratios of lecithin to cholesterol 1:1 and protein was dissolved in 0.5% polyvinylalcohol in phosphate buffer saline (PBS), used for preventing protein denaturation by organic solvent (22), and the carboxymethylcellulose in concentration 0.02% in PBS was used for stabilized

liposome (23). The physicochemical properties such as particle size, particle size distribution, percent protein entrapment, electronmicroscopic appearance were determined. Rabbits were administered vaccines by subcutaneous route with different adjuvants or without adjuvant, their sera were intended for measuring the protective capacity of vaccine against *P. multocida* by using enzyme-linked immunosorbent assay (ELISA), bactericidal activity, and immunoblot analysis.

The objectives of this research are

1. To determine the physicochemical properties of liposome containing OMP from *P. multocida*.
2. To determine and compare the immune response of rabbit that were administered liposome containing OMP from *P. multocida*, OMP emulsified with Freund's adjuvant, alum containing OMP, OMP without adjuvant, and formalin killed whole cell.

CHAPTER II

REVIEW OF LITERATURE

Pasteurella

Taxonomy

Pasteurella were first observed in the blood of bird with fowl cholera in 1877 (24). Recently, the taxonomy of the Genus *Pasteurella* was studied by DNA/DNA hybridization using the initial renaturation rate method of DeLey et al. (25) such as stringent reassociation conditions. Burgey's Manual of Systemic Bacteriology divided Genus *Pasteurella* among six species based on the beta hemolysin, growth on MacConkey agar, indole production, urease activity, gas from carbohydrates and acid production from lactose or mannitol as described in Table 1 (26). These species are *P. multocida, P. pneumotropica, P. haemolytica, P. ureae, P. aerogenes, and P. gallinarum.*

Morphology, growth characteristic and biochemical reaction

Pasteurella is Gram-negative anaerobe or facultative anaerobe bacteria, not acid-fast, cells usually coccobacillary or short rods in cultures from diseased tissues, strains from healthy animals are often pleomorphic with longer bacillary forms and occasional short filaments, their average size are 0.3 to 1.0 μ in diameter 1.0 to 2.0 μ in length (26). Bipolar staining is common, especially in preparation made from blood and infected animal tissue. Most of virulent strains of *P. multocida and P. haemolytica* produce capsules of varying size (27). The capsules are mostly composed of hyaluronic acid, which imparts a moist, mucoid character to the colonies (28). The majority of the species are non motile and spore forming. After repeated culture on agar, the bacteria trend to form longer rods and to become more pleomorphic, forming chains, filaments and rods of various sizes. When growing in carbohydrate media for prolonged periods, marked pleomorphicity is noted. It grow in temperature range between 22 and 44°C, most avian strains and a few others grow at 42°C, but the optimum temperature for growth is at 37°C. The maximum growth is

reached in 18 hours in fermenter or shaker condition. The pH growth range is from pH 6-8.8 with an optimum pH of 7.2-7.4. The use of digested protein media or protease peptone stimulates the growth of the organism. Bacteria could be grown in beef infusion media but better growth is obtained when blood or serum is added to the media. Some strains fail to grow in media not containing blood or serum. Increase CO_2 tension does not appreciably increase the growth or number of colonies. Three principle colony types are seen on clear screen agar (29): 1) a smooth or fluorescent colonies, virulent for rabbits, growing diffusely in broth, whitish, generally unstable, pathogenic and forming smooth, moderately opaque irridescent colonies on serum agar; 2) a rough or non-capsulated colonies (blue colonies), dewdrop-like and of a relatively low virulence. Giving a granular deposit in broth, and forming translucent bluish colonies; 3) a mucoid form of intermediate virulence. The highly virulent smooth form contains a type-specific polysaccharide capsular antigen; the mucoid form is rich in hyaluronic acid, and may or may not possess a polysaccharide capsular antigen in addition. The rough form has neither a capsular nor a mucoid antigen. Strains giving rise to blue colonies are most frequently recovered from infection. While acute infection usually yield strains giving rise to fluorescent colonies. Many group A strains produced mucoid variants on initial isolation on enriched media.

P. multocida produces acid, but not gas in glucose and sucrose; most strains ferment galactose, mannitol, mannose, sorbitol and xylose. Dextrose, dulcitol, lactose, maltose, and raffinose are occasionally acidified; inulin, inositol, rhamnose and salicin are usually not attacked. Strains from certain sources trend to possess particular biochemical characteristics (30). Strains of *P. multocida* are without action on litmus milk and gelatin. They produce indole, reduce nitrate and form a small quantity of H_2S as detected by lead acetate paper. The Methyl red and Voges-Proskauer reaction are both negative; the catalase and oxidase reaction are almost both positive, though rather weakly so. Methylene bule is reduced. Citrate cannot be used as the sole source of carbon. Other characteristics of the species of the Genus *Pasteurella* were shown in Table 2.

Table 1 Differential characteristics of the species of the genus *Pasteurella*[a]

Characteristics	*P. multocida*	*P. pneumo-tropica*	*P. haemo-lytica*	*P. ureae*	*P. aerogenes*	*P. gallinarum*
Hemolysis (β)	-	-	+	-	-	-
Growth on						
MacConkey's agar	-	-	+	-	+	-
Indole production	+	+	-	-	-	-
Urease activity	-	+	-	+	+	-
Gas from carbogydrates	-	-	-	-	+	-
Acid production from:						
Lactose	-	d	d	-	-	-
Mannitol	+[b]	-	+	+	-	-

[a] Data from Bergey's manual of systemic bacteriology. For symbols see standard definitions

[b] Strains from dogs and cats may be negative for mannitol

d Differs among strains

Table 2 Other characteristics of the species of the genus *Pasteurella* [a]

Characteristics	*P. multocida*	*P. pneumo-tropica*	*P. haemo-lytica*	*P. ureae*	*P. aerogenes*	*P. gallinarum*
Catalase test	+	+	+	+	+	+
Oxidase test (Kovac's)	[+]	+	+	+	+	+
Ornithine decarbosylase	[+]	+			+	[-]
Lysine decarboxylase	-	-			-	-
Arginine dihydrolase	-	-			-	-
H_2S production	+	[+]			+	[+]
Nitrate reduced to nitrile	[+]	+	+		+	+
Growth in KCN	[+]	d				
Methyl red and Voges-						
Proskauer test	-	-	-	-	-	-
Acid produciton from:						
Adonitol	[-]	[-]	-			-
Amygdalin	[-]	d				
Arabinose	d[b]	[-]			+	-
Cellobiose		[-]				
Dextrin	d		+			+
Dulcitol	d[b]	[-]	d		-	-
Erythritol	[-]	[-]	-	[-]		
Esculin						
Fructose	+			+		+
Galactose	+	[+]				+
Glucose	d[c]	[+]	+	+	+	+
Glycerol	[-]	[+]	d	[-]	+	-
Glycogen	[-]	[-]	+			
Inositol	[-]	d	+			-
Inulin	d		-			-
Maltose	+	[+]	+		+	+
Mannose		[+]				+

Table 2 Other characteristics of the species of the genus *Pasteurella*[a] (continued)

Characteristics	*P. multocida*	*P. pneumo-tropica*	*P. haemo-lytica*	*P. ureae*	*P. aerogenes*	*P. gallinarum*
Acid production from:						
Melizitose	[-]					
Melibiose	d		+			
Raffinose	d	d	+		-	d
Rhamnose	[-]	[-]	d		d	-
Salicin	[-]	[-]		[-]	-	
Sorbitol	[+][d]		+	+	-	d
Sorbose	[-]					
Starch		[+]	+			
Trehalose		[+]			-	+
Xylose	[+][d]	[-]		[-]	+	d

[a] Data from Bergey's manual of systemic bacteriology. Symbols: +, all strains positive; [+], most strains positive; d, differs among strains; [-], most strains negative; -, all strains negative.

[b] Arabinose and dulcitol feremetation is most common in strains form birds.

[c] Maltose fermentation is characteristic of strains from dogs and cats.

[d] Strains from cats and dogs may be negative for sorbitol and xylose.

Pasteurella multocida

Serotyping

P. multocida was first isolated in 1880 by Louis Pasteur. It has been given many names according to the host animal. In 1939 Rosenbanch and Merchant proposed the name of *P. multocida* in Bergey's manual of Systemic Bacteriology. The classification that gained widespread acceptance was based on serological properties of the organisms (31), capsular and cell wall compositions (32). At the present time, *P. multocida* have been identified as 5 capsular types designated A, B, D, E, and F based on capsular antigens (3, 4) and 16 somatic types based on somatic LPS antigens (5). The popular method of designating serotypes was combined both capsular and somatic types as described in Table 3 and 4.

The former serotyping system is identified by Arabic numbers 1 through 16 representing the somatic type followed by a capital letter representing the capsular type.

Maintenance procedures

P. multocida loses its viability in and on media when stored for more than 2-3 weeks in the refrigerator. All of the *Pasteurella* can be preserved for years in the lyophilized state by use of conventional suspending media for freeze-drying. Cells harvested from culture media maintain viability for long periods if suspended in defibrinated blood and stored at -40 to -70°C. Viability can be maintained indefinitely of the organisms are stored in liquid nitrogen (25).

Disease caused by *P. multocida*

P. multocida is an important veterinary and opportunistic human pathogen. The species is diverse and complex with respect to antigenic variation, host prediction and pathogenesis. Certain serological types are the etiologic agents of severe pasteurellosis, such as fowl cholera in domestic and wild bird, bovine haemorrhagic septicaemia and porcine atrophic rhinitis as described in Table 3 and 4.

Table 3 Designation of serotypes of *P. multocida* by the Carter-Heddleston method

Capsular type	Somatic type	Serotype	Disease
A	1, 3, 4	A:1	Fowl cholera
		A:3	
		A:4	
	5,6	A:5	Fowl cholera
		A:6	(Less common)
	7-10	A:7, A:8, A:9	
	12-15	A:10, A:12, A:13	
		A:14, A:15	
	16	A:16	Fowl cholera
B	2	B:2	Haemorrhagic septicaemia
D	11	D:11	Atrophic rhinitis
E	2	E:2	Haemorrhagic septicaemia

Table 4 Designation of serotypes of *P. multocida* by the Namioka-Carter method

Capsular type	Somatic type	Serotype	Disease
A	1, 3, 5,7,9	1:A	Pneumonia
		3:A	Pneumonia
		5:A	Fowl cholera
		7:A	Septicaemia
		8:A, 9:A	Fowl cholera
B	6, 11	6:B	Haemorrhagic septicaemia
		11:B	Would infection
D	1, 2, 3, 4, 10	1:D, 2:D, 10:D	Pneumonia
		3:D	Pneumonia
E	6	6:E	Haemorrhagic septicaemia

Fowl cholera

Fowl cholera is a major disease problem in poultry such as chickens, turkey, geese, ducks and many wild and zoologic birds. The important serotype which are the cause of fowl cholera belong to serogroup A, serotypes 1, 3, or 4. Sick birds with acute septicemia show anorexia, depression, nasal or oral discharge of mucus, white watery or green mucoid diarrhea. The course of illness is short and mortality often increase rapidly. The chronic cases often show swelling of a joint, wattle, footpad on tendon sheath. In chickens and turkeys, cholera is seen peracute, acute and chronic forms. The less spectacular, but still destructive chronic type disease is more frequently seen in some areas than the peracute form. Sporadic secondary infection of fowl are frequently found in the airsacs and sinuses. In addition to chickens, serious losses occur in domestic duck and geese (33).

Haemorrhagic septicaemia

Haemorrhagic septicaemia (HS) is caused by two specific serotypes of *P. multocida*. The Asian serotype is designated B:2, and the African serotype is E:2 by the Carter-Heddleston system, corresponding to 6:B and 6:E by the Namioka-Carter system. HS is considered economically to be the most important disease in Southeast Asia (34), including Indonesia, Phillipines, Thailand, Malaysia, Middle East, North East, central and south Africa. Cattle and buffalo are the usual hosts, although pigs, sheep, goats and camels are all susceptible. HS occurs in either the pectoral or edematous forms. The pectoral from involves the long and the plural cavity and is accompanied by petechial haemorrhages in those tissue. The edematous type appears as an extensive edema of the subcutaneous tissue and the organs and tissues of the peritoneal cavity (35).

Human infection

In humans, *P. multocida* causes several types of infections; the majority of these follow some sort of contact with domesticated animals. The first case reported was described in 1913 by Brugnatelli (24). Local wound infections in humans are associated with cat bites, cat scratches, or dog bites (36). These local wound infections are characterized by rapid development of pain, erythema, swelling, cellulitis, and purulent or serosanguineous drainage at the site of the wound. Systemic signs of infection may or may not be present (37). Local complications

include osetomyelitis of the bones near the bite wound site due to traumatic implantation of the organisms or extension of the cellulitis and septic arthritis. Since the wound is generally on the hand, bone and joint complications are usually seen at this site. Serious localized complications most frequently follow cat bites in which the wound may be deep, forceful, and traumatic to underlying tissues. Occasionally, this organism may be found in wounds that are not associated with animal bites or obvious animal exposure. The bacterium has been isolated from decubitus ulcers and from postsurgical abdominal, gynecologic, and orthopedic wound infections.

P. multocida may also be isolated from the respiratory tract, where it may exist as a commensal or as a cause of pneumonia, empyema, bronchitis, sinusitis, tonsillitis, and otitis media (38, 39). The organism may be isolated from respiratory tract secretions of persons with no respiratory tract disease or symptoms; in most cases, such persons have a history of occupational or recreational exposure to animals. Signs and symptoms of respiratory tract infection are present, and patients usually have some preexisting compromise of the lungs or upper airways such as chronic obstructive pulmonary disease, chronic bronchitis or sinusitis, lung carcinoma. These patients will also usually have some history of animal exposure. Central nervous system infections caused by *P. multocida* have included meningitis, subdural empyema, and brain abscess. Most reports have also documented that these patients had past or current exposure to animals, in most cases cats and/or dogs.

Antimicrobial susceptibility

Antimicrobial susceptibility testing and clinical response of infected patients indicated that *P. multocida* isolates are generally susceptible to a wide variety of antimicrobial agents (40). The organism is susceptible to penicillin, ampicillin, broad-spectrum penicillin, second-generation cephalosporins, third-generation cephalosporin, tetracycline, and chloramphenicol. Less activity has been noted for the first-generation cephalosporin and the semisynthetic penicillins such as methicillin and oxacillin.

Antigenic structure

Prevention of disease by using killed and live vaccine can induce protective immunity, but sometimes the results in mortality, morbidity and systemic infection are important problems. For these reasons, many researchers attempt to an improved

vaccine efficacy by the determination of the immunogenic antigens or subcellular materials. The membranes of each species of *Pasteurella* fall into certain antigenic patterns; even within each species, however, there are antigenic differences among strains, and different species appear interrelated by serologic tests. All *Pasteurella* possess somatic O antigens which are toxic for animals and which chemically are lipopolysaccharide-protein complexes. These have been subdivided by chemical fractionation into components of varying immunologic and serologic activity (41). The structure and cellular components of *P. multocida* may contribute to disease or stimulate host immune response. The major antigenic components are given below(42).

Potassium thiocyanate extract from whole cell

Potassium thiocyanate (KSCN) extract of *P. multocida* contains antigenic structure such as capsule, lipopolysaccharides (LPS), outer membrane protein (OMP), and nucleic acids (9), and rabbit which immunized with KSCN extracts produce antibodies against OMP and LPS of homologous *P. multocida* (10).

A vaccine was prepared from potassium thiocyanate extract of *P. multocida* type A by an aqueous solution of 0.5 M KSCN in 0.08 M sodium chloride was found to be immunogenic in chicken against homologous and heterologous challenge (43). Subcellular fraction from 2.5% sodium chloride solution contained capsular antigen (44, 45) and complex substance with high molecular weight (40% protein and 15% carbohydrate) was immunogen. Truscott et al. reported that all of turkey inoculated with this antigen survived from challenge (16).

Antigen extracted from a virulent isolate of *P. multocida* (serotype 3, 12, 15:D) with potassium thiocyanate was prepared as vaccine by Ringler et al.,1985 (11). Pasteurella-free rabbits were vaccinated intranasally and intraconjuctivally twice with a 2-week interval and challenged intranasally with the homologous *P. multocida* 2 weeks after the second vaccination. The vaccinated rabbits produced serum IgG and nasal mucosal IgA against *P. multocida*. The vaccine protected the challenged rabbits from clinical disease and death, whereas rabbits immunized by the intramuscular route (43) produced persisting serum IgG and transient nasal IgA antibodies. The hyperimmune serum directed to the KSCN extract of *P. multocida* 3:A (10) provided significant protection against homologous challenge in rabbits.

Coadministration of *cholera* toxin with potassium thiocyanate extract enhanced protection immunity to *P. multocida* disease and infection in rabbits (46).

Capsules

The antigenic specificity of the capsular determines its serogroup (12). Capsular type A is composed of hyaluronic acid, which may act to mimic host antigens because hyaluronic acid naturally present in host tissue. Intimate associated with its other polysaccharides, proteins and lipids. The hyaluronic acid does not exert antiphagocytic activity, but saline-extractable capsule material contained a factor capable of inhibiting the function of bovine polymorphonuclear leucocyte. The capsule of avian strains provided production from the action of complement. Removal of the hyaluronic acid capsule increased both the adhesiveness of the organism to animal cell surfaces and its susceptibility to phagocytosis. The production of capsular material was affected by subminimal inhibitory concentration of antibiotics. Capsule was considered virulence factor because no encapsulated variants of pathogenic strains were less virulent than encapsulated forms. Capsular extract has been used to immunize cattle but it could not result in a practical vaccine. Oil adjuvant-type vaccine using capsular extracts obtained by solvent precipitation from the supernatant fluid of fermenter grown *P. multocida* type B has been used for immunization but did not yield encouraging results.

Lipopolysaccharides (LPS)

Examination of strains from a variety of animal sources confirmed that LPS from *P. multocida* was similar to semirough LPS of Enterobacteriaceae (16). The LPS contained lipid, 2-keto-3-deoxyoctanate (KDO), heptose, glucose and polysaccharide. The LPS of rabbit isolates contained either a non-serospecific antigen (R-LPS), a serospecific antigen (S-LPS) or both, from avian strains found to be R-LPS. The LPS responsible for antigenic specificity of somatic serotype, and when examined electrophoretically the molecular weight of LPS was low. *P. multocida* LPS has similar chemical and biological properties to the R-type LPS of the gram-negative bacteria. However, the level of antibody response following immunization depend on animal species, inoculated dose, LPS type, route and method of inoculation. The role of LPS as an immunogen in mammals remains controversial. Mice, cattle and rabbits have been readily protected against infection following immunization with LPS.

Toxins

Strains of *P. multocida* serogroup D which cause atrophic rhinitis produce a dermonecrotic toxin (PMT, for *P. multocida* toxin), which is the principle virulence factor in atrophic rhinitis. PMT induces localised osteolysis in the nasal turbinates, primarily through increased osteoclastic bone resorption. Recombinant toxin derivatives have been used as vaccine candidates (47, 48). Although toxin related sequences have occasionally been found in other serotypes, the synthesis of PMT is usually restricted to serogroup D. Treatment of mildly trypsinized toxin with dithiothreitol and sodium dodecyl sulphate produced 3 polypeptide chains of molecular weight 23, 67, and 74 kDa from which the whole toxin could be reconstructed (49). Crude toxin was inactivated by formaldehyde, heat and proteolytic enzyme suggesting it was a protein (26). Commercial vaccines therefore incorporate formaldehyde treated whole cell of toxigenic *P. multocida* or formaldehyde- detoxified crude bacterial extract of toxigenic organism (30). Strains from other hosts also produced the toxins including poultry, calves, cats, dogs, rabbits and human respiratory tract (50). Dermonecrotic type-A strain could produce turbinate atrophy similar to that caused by dermonecrotic type-D strains.

Peterson et al., demonstrated that immunized swine with purified *P. multocida* toxin can protect it against atrophic rhinitis (48). It was also reported that intranasal immunization with both *P. multocida* toxin and potassium thiocyanate extract (51) induced an effective response against homologous *P. multocida* challenge.

Plasmids

Several groups have undertaken studies to determine the presence of plasmids in numerous strains of *P. multocida* and to investigate the correlation between antibiotic resistance profiles, virulence attributes and the presence of plasmids. The rate of plasmid carriage has been shown to vary considerably between different *P. multocida* collections. Plasmid carriage in avian isolates from two studies varied from 24 (52) to 70.7% (53) of isolates tested. Studies with bovine and porcine strains by Schwarz et al. (54) found that 47% of strains carried plasmids. A similar figure of 51% of porcine serogroup D isolates carrying plasmids was found by Cote et al. (55). *P. multocida* isolated from rabbits demonstrated the highest plasmid carriage rate of 92% of the 28 isolates test (56).

P. multocida has been shown to harbour plasmids from 1.3 kb (57) to 100 kb (53) in size. However the majority of plasmid identified have been between 2 and 6 kb in size. Many phenotypically cryptic plasmids have been found in *P. multocida* isolated from avian (52, 53) and mammalian hosts (55, 56, 58).

Outer membrane protein

Outer membrane protein (OMP) is the specific protein that embedded in a phospholipid matrix of the outer membrane of gram-negative cell wall (59).

The wall of gram-negative bacteria is more complex than that of the gram-positive. It contains 3 component that lie outside the peptidoglycan layer: lipoprotein, outer membrane, and lipopolysaccharide as shown in Figure 1.

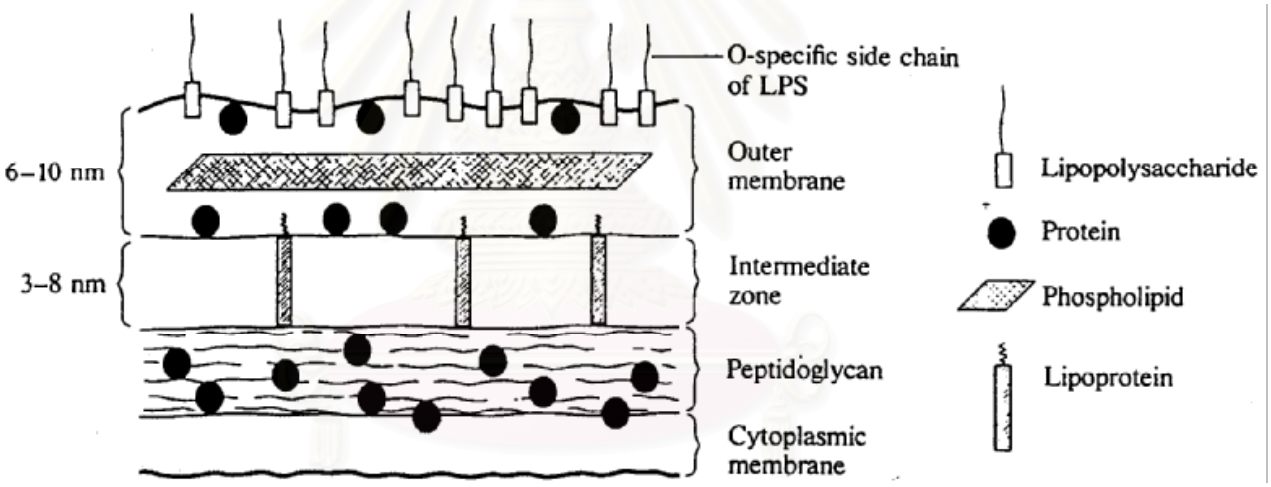


Figure 1 The model of gram negative cell wall (41).

The major proteins of the outer membrane, named according to the genes that code for them, have been placed into several functional categories on the basis of mutants in which they are lacking. The matrix porins, exemplified by OmpC, D, and F of *Escherichia coli* and *Salmonella typhimurium*, are trimeric proteins that penetrate both faces of the outer membrane. They form relatively nonspecific pores that permit the free diffusion of small (up to about 600 molecular weight) hydrophilic solute across the membrane. A second group of pore-forming proteins, exemplified by LamB and Tsx, show greater specificity: LamB, which is the receptor for lambda bacteriophage, is responsible for most of the trans-membrane diffusion of maltodextrins; Tsx, the receptor for T6 bacteriophage, is responsible for most of the transmembrane diffusion of nucleoside. LamB allows some passage of other solutes, however; its relative specificity may reflect weak interactions of solutes with configuration-specific sites within the channel.

A third group of major proteins are non-porins: they include OmpA, which participates noncovalently in the anchoring of the outer membrane to the peptidoglycan layer and is also the sex pilus receptor in F-mediated bacterial conjugation; lipoproteins, described above; and a protein designated “a” by one group and “3b” by another, which may be a protease and also functions as a regulator of capsular polysaccharide biosynthesis.

The outer membrane also contains a set of less abundant, so-called minor proteins, many of which are involved in the transport of specific small molecules such as vitamin B12 and the iron siderophores. They show high affinity for their substrates and probably function like the classic carrier transport systems of the inner (cytoplasmic) membrane.

In addition to their functions in transport and their functions as phage receptors and conjugation receptors, outer membrane proteins have been implicated in the control of DNA replication and cell division. The outer membrane also serves as a barrier to the diffusion of large molecules and as a protective envelope for hydrolytic enzymes and binding proteins that accumulate in the periplasmic space.

The topology of the major proteins of the outer membrane, based on cross-linking studies and analyses of functional relationships, as shown in Figure 2. These proteins are synthesized on ribosomes bound to the cytoplasmic surface of the inner membrane; how they are transferred to the outer membrane is still uncertain, but one

hypothesis suggests that transfer occurs at regions of adhesion between the inner and outer membranes, which regions are visible in the electron microscope.

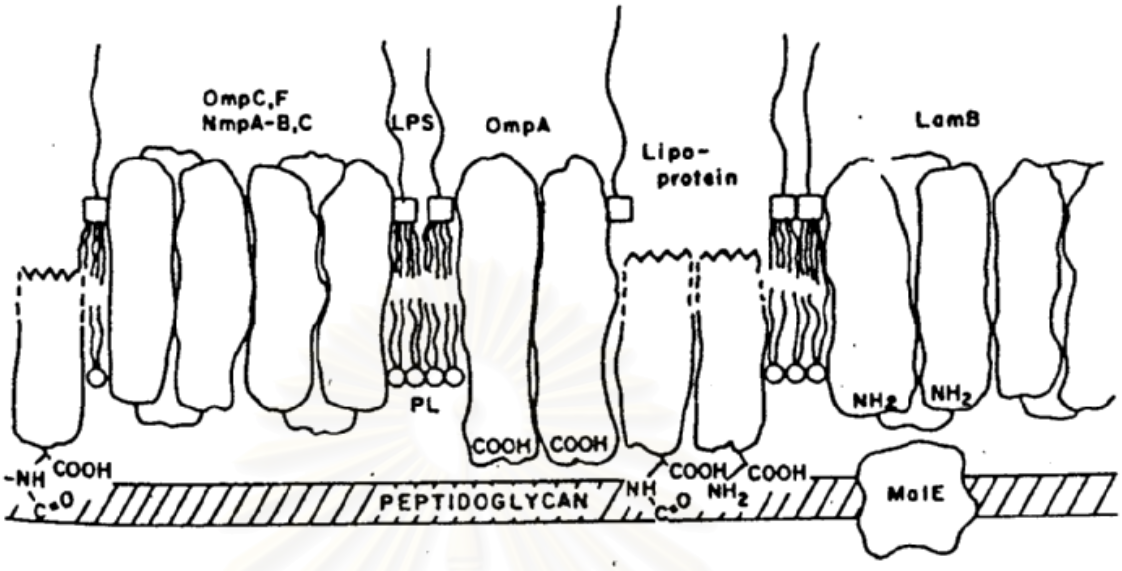


Figure 2 Schematic representation of the molecular organization of the major proteins in the outer membrane of gram negative bacteria (41).

LPS = lipopolysaccharide

PL = phospholipid

Omp, Lam, Nmp = major proteins of the outer membrane

MlaE = periplasmic binding protein for maltodextrins

OMP from *P. multocida* was extracted and its ability to immunize animals against *P. multocida* infection and enhancing the virulence in infected animals through the anti-phagocytic mechanism were investigated.

Abdullahi, et al. (60) studied the outer membrane proteins (OMP) profile of *P. multocida* type A and the related Taxon 13 strains isolated from bovine pneumonia to determine the heterogenicity of this group and to attempt to define a protective immunogen in a mouse septicemia model by investigation of the immunogenicity of the OMPs. They concluded that in the mouse model of pasteurellosis, the major OMPs were not protective antigens and therefore may be unlikely candidates for vaccines. However, their conclusions appear to contradict the findings of Lu, et al.(44) who showed that 31.5 kDa OMP (presumably outer protein) elicited protection against homologous challenge in a rabbit model, but in a mouse model, protection was afforded against heterologous challenge only if the challenge strain was shown by

probing with a monoclonal antibody, to express the 37.5-kDa antigen. Protection was not provided against strain lacking this antigen. They showed that OMP can be a protective immunogen in the strains that possessed it, but they concluded that antigen may be more important as vaccine candidates. Because only 24 % of their strain of *P. multocida* expressed this antigen. Out of ten major polypeptides of OMPs in the extract of *P. multocida* serotype B:2, immunoblotting showed that the polypeptide with the OMP vaccine or a commercial HS vaccine developed the highest mean $\log_{10}$ ELISA titers 21 days post-vaccination. The results suggested that OMP was protective and could be used in vaccines against HS (25).

The P6 OMP of *H. influenzae* has been shown to elicit protective imunity in animal models of infection. Kasten et al. cloned a gene encoding the *P. multocida* homologue of P6 and showed it to be present in all 16 somatic serotypes. However, immunization of turkeys with recombinant P6 failed to protect them against subsequent challenge (15)

The gene encoding the Oma87 OMP was cloned and characterised (14). Oma87 showed high similarity to the D15 protective OMP of *H. influenzae* (61) and rabbit antiserum against Oma87 was able to passively protect mice against infection.

OmpH is a homologue of the P2 porin of *H. influenzae* and a monoclonal antibody against OmpH could passively protect mice against infection (6). Luo et al.(62) cloned the *ompH* gene and showed experimentally that OmpH had porin activity. Immunisation of chickens with the recombinant mature length OmpH elicited immunity against homologous challenge, although heterologous protection was not investigated. Subsequent analysis of OmpH from different serotypes showed a high degree of conservation and predicted the presence of two large external loops. A cyclic synthetic peptide which mimicked the predicted structure of loop 2 was able to induce partial homologous protection in chickens (63).

OMP from other microorganism were reported as immunogenic in various animal such as, Kersten et al. found that liposome OMP of *Neisseria gonorrhoeae* induce immune response in mice (64) and Meenakshi et al. demonstrated that OMP of *Salmonella enteritides* gave a significantly higher antibody response than whole cell vaccine in chickens (65). Moreover, Jolley et al. demonstrated that immunized mice with recombinant Opc OMP from *Neisseria meningitidis* induced high levels of antibodies (66). This may indicated that the OMP contained the major immunodominant proteins to give rise to such high antibody titers.

Adjuvant

Practical development of modern vaccines has been greatly advanced by the availability of neutral and synthetic antigens, but progress has been hindered in some cases by poor immunogenicity of the antigens. The term adjuvant is widely used to describe a preparation that boosts the immune response to an antigen by increasing immunogenicity in a specific or nonspecific way. The term adjuvanticity strictly refers to the efficacy of the preparation but is sometimes used as an alternative to immunogenicity. The term vehicle describes the way in which the antigen is presented, as in mineral oil. The term carrier is used in the immunologic sense, as part of a hapten-carrier complex, and it is usually a protein. The final mode of presentation has been called the adjuvant formulation (67).

A variety of materials has been shown to display adjuvanticity. Probably the first adjuvant widely used and the only one still registered for general medical use is alum. It was first used nearly 60 years ago. Glenny and colleagues (68) noticed that an injected antigen gave only a short-lived antibody response. It was reasoned that this was because the deposited antigen rapidly left the injection site. By mixing with alum, an antigen depot was formed, resulting in a more prolonged immune response. The introduction by Freund (69) of the technique of emulsifying antigen in mineral oil with the addition of dead mycobacteria (Freund's complete adjuvant [FCA]) and a preparation not containing bacteria (Freund's incomplete adjuvant [FIA]) provided the means for greatly boosting an immune response. By using FCA, it was shown that strong delayed-type hypersensitivity (DTH) reactions to minute amounts of simple proteins could be obtained (70). For many years, FCA was regarded as the standard that new formulations must meet to be competitive. FCA was found to form granulomas at the injection site. This was once thought to be desirable, but an adjuvant with this type of reaction is no longer considered acceptable for medical use, nor is it used in many animal experiments.

Some of the properties and roles of adjuvants are listed in Table 5. As with vaccines, the safety of adjuvant formulations became a top priority and led to some guidelines about safety, specificity, feasibility, and formulations. It became clear over time that the final effector step of an adjuvant would be to enhance an already naturally occurring event, one that was most likely to involve specific interaction with

a receptor at the cell surface. The adjuvant should be easy and inexpensive to manufacture.

Table 5 Some desirable general properties of adjuvants.

Safety
It must not be carcinogenic, teratogenic, or abortogenic.
The formation of granulomas, local necrosis, hypersensitivity, fever, or autoimmune effects should be avoided.
Nonspecific effects on cell activation, caused by perturbation of cell membranes surfactants or oils, should be avoided.
It should be biodegradable and preferable gave a short half-life.
Specificity
Because most activation signals may be transduced by membrane phospholipase activation, the activity should be targeted to specific cells of the immune system. Such cells may already possess specific receptors, especially if the adjuvants derived from an infectious agent.
Having a known chemical structure is desirable.
Feasibility and formulation
The preparation should be stable, inexpensive, simple to (reproducibly) manufacture, and have a long storage life.
Presentation of the antigen with adjuvant as particles with multimeric arrays of antigen is advantageous.

A number of different adjuvant preparations have been developed are listed in Table 6. Most preparations also display some associated toxicity and, as a general rule.

Table 6 Overview of the adjuvant preparations that have been developed to date or are under investigation of aluminium. Based substances are the only adjuvants used to any significant degree in humans.

Mineral compounds	Aluminium phosphate ($AlPO_4$)
	Aluminium hydroxide ($Al(OH)_3$)
	Alum ($AlK(SO_4)_2.12H_2O$)
	Calcium phosphate ($Ca_3(PO_4)_2$)
Bacterial products	*Mycobacterial species*
	Mycobacterial components
	(e.g. trehalose dimycolate muramyl dipeptide)
	Corynebacterium species
	Bordetella pertussis
	Lipopolysaccharide (LPS)
Oil – based emulsions	Freund's complete adjuvants (FCA)
	Freund's incomplete adjuvants (FIA)
	Starch oil
Saponins	Quil A
Liposomes	
Immunostimulatory complexes (ISCOMs)	
Some cytokines	Interleukins 1 (IL-1)
	Interleukins 2 (IL-2)

The last decade has witnessed great activity in the field of adjuvant design, and a variety of studies point to a number of roles for adjuvants, some are listed in Table 7. There have been extensive reviews of candidate adjuvants (71-73). The complete issue of vaccine was reserved for such reports, and a group of candidate adjuvants was compared for activity and safety (74). One of the pioneers of the study of adjuvants, Chedid (75), proposed that the role of adjuvants could essentially be described by three concepts (which have been slightly modified):

1. The formation of a depot of antigen primarily at the site of application and from which the antigen was released over some period, which preferably could be predetermined.
2. The presentation (or delivery) of antigen to cells involved in immune responses, principally antigen-presenting cells (Acps).
3. The induction of secretion of enhancing factors, such as lymphokines, which would act principally on cells of the immune system, especially T and B lymphocytes.

With different preparations, there is some overlap between these stages. Nevertheless, this approach is followed in this presentation.

Table 7 Materials with adjuvant activity and possible sites of action.

Activities	Materials
Delay release of antigen	Depot formers, water in oil; oil in water emulsions; controlled release devices; inert carriers (e.g. Alum)
Mobilization on T-cell help	Proteins as carriers Polyclonal activators of T cells: PPD, Poly A : poly U
Modulate Ig receptor on B cells	B-cell mitogens, antigen-polymerizing factors
Localization of antigen in T-dependent areas	Hydrophobic antigens; addition of lipid tail to proteins
Stimulation of antigen-presenting cells	MDP and derivatives: LPS, lipid A
Facilitate cell-cell interaction	Surface-acting materials; saponin, lysolecithin, Quil A, liposomes, pluronic polymers
Focusing of antigen on leukocytes with Fc receptor	Alternate pathway of complement activators: inulin, zymosan, endotoxin

LPS, lipopolysaccharide; MDP, muramyl dipeptide; PPD, purified protein derivative

Mineral compounds

Alum-precipitated immunogen

The only adjuvant currently approved universally for use in humans is alum. The antigen solution usually is mixed with performed aluminium hydroxide, aluminium phosphate, or both compounds. A specific preparation, Alhydrogel, was chosen as a scientific standard in 1988 (73). Cells involved in immune responses, particularly eosinophils, may be attracted to the antigen depot (76), and complement may be activated, which could result in increased antigen localization of follicular dendritic cells and hence improved B-cell memory. When used intramuscularly it is claimed to induce plasma cell accumulation at the depot site. Its side effects, however, are minimal and it is universally used as adjuvants for diphtheria and tetanus toxoid vaccination, mainly because of the increase response to the toxoid. Immunogen precipitates can be injected subcutaneously or by the intraperitoneal route as well as into muscles. Its favoured use is in mice, often mixed with a *Bordetella pertussis* suspension and one of its major advantage is that it allows fairly rapid dissemination of immunogen without the risks attached to injection of molecules in solution into primed animals which can lead to rapid fatal anaphylaxis. A commonly used strategy is to divide alum precipitates into very small doses of a few μg and give these every few days over several weeks. Alum-precipitated toxiods and other immunogens are known to induce IgE antibodies in some species.

The only artificial adjuvants widely licensed are aluminium salts, often referred to as “alum”. Aluminum salts are very safe but they do have a number of disadvantages. These include:

1. variations in potency between different batches due to uncontrollable reactions between antigen and the aluminum salt
2. the requirement for refrigeration (complication delivery in developing countries) because they cannot be frozen or lyophilized
3. they occasionally produce abscesses or nodules
4. failure to work with certain antigens
5. at best their immunostimulating properties are limited, particulary with respect to the production of CMI.

In addition aluminium-based products display poor or no adjuvanticity when combined with some antigens (e.g. typhoid or *Haemophilus influenzae* type b capsular polysaccharides).

Bacterial products

Selected microorganisms have been identified which trigger particularly potent immunological responses. The immunostimulatory properties of these cells has generated interest in their potential application as, adjuvants. Examples include various microorganism such as *Mycobacterium* species, *Corynebacterium parvum*, *Corynebacterium granulosum* and *Bordetella pertussis*. Although some such microorganisms are used as antigens in vaccines, they are considered too toxic to be used solely in the role of adjuvant. Researchers thus have sought to identify the specific microbial biomolecules responsible for the observed immunostimulatory activity. It was hoped that these substances, when purified, might display lesser or no toxic side effects while retaining their immune stimulatory capacity.

Bordetella pertussis

The observed adjuvanticity of *Bordetella pertussis* is largely attributable to the presence of pertussis toxin and lipopolysaccharide (LPS). LPS constituent of the cell envelope of gram negative bacteria essentially consists of polysaccharide moieties to which lipid (lipid A) is covalently attached.

While purified LPS displays potent immunostimulatory properties. It also induces various toxic side effects listed in Table 8. The most prominent of which is pyrogenicity. These effects render application of LPS as an adjuvant unacceptable. Both its immunostimulatory and toxic properties are mainly associated with the lipid A portion of the molecule. Attempts have been made to chemically, or otherwise alter lipid A portion in order to ameliorate the observed toxicity.

Table 8 Some characteristic biological effects induced by lipopolysaccharide

Pyrogenicity
Generalized and severe toxicity
Adjuvanticity
Activation of macrophages and granulocytes
Activation of complement
Induction of synthesis of TNF, CSF, IL-1, IFN
Some antitumour activities

Succinylated or phthalylinated LPS displays significant reduction in toxicity (up to 100000 fold) while retain in its adjuvanticity. Acid treatment (0.1 M HCl) of LPS obtained from various *Salmonella* species resulted in the production of an LPS derivative termed monophosphoryl lipid A (MPL). This also displays adjuvanticity with title associated pyrogenicity or toxicity. This alteration of biological activity can also be achieved by removal of some of the fatty acids found in the LPS lipid A region. As LPS is effective in activation both cellular and humoral immune responses.

A suspension of killed *B. pertussis* injected jointly with immunogen is another good method of obtaining a potentiated antibody response, particularly in mice. The toxin is known to enhance both IgG and IgE antibody production.

Corynebacterium granulosum

Corynebacterium granulosum derived p40 particulate fraction, is composed of fragments of cell wall peptidoglycan and associated glycoproteins. Its administration to animals results in activation of various elements of immune function while displaying little or no toxic effects. In addition to activation of macrophages, p40 induces synthesis of a variety of cytokines, most notably IL-2, TNF, IFN and IFN not surprisingly, p40 was found to enhance both specific and non-specific resistance to a wide range of pathogens and also was shown to display antitumour activity. Clinical trials in humans appear to confirm many of these observations. P40, or derivatives, therefore may yet play a role in human or veterinary immunization programs.

Oil – based emulsion

Freund's Complete Adjuvant (FCA or CFA)

A water-in-oil emulsion was first used to boost an immune response about 80 year ago (73). This consists of a mixture of mineral oil, a suspension of heat killed *Mycobacterium butyrinum* or *Mycobacterium tuberculosis* and Arlacel A is a popular choice for an emulsifier such as squalene and a stabilizer is used to prepare the final product. Immunogens in solution are emulsified with these components to produce an antigen/water microdroplet phase within the oil phase. FCA greatly enhances and prolongs the antibody response as first described by Freund (77). The emulsion is stable and when injected intramuscularly forms a depot of immunogen which only slowly becomes available. The other major attribute is related to the activity of the mycobacteria, some of whose products act as powerful stimulants for cells of the immune system both at local lymph nodes and in the granuloma that forms around the depot. *M. tuberculosis* is the more potent of the two mycobacterial species used. Much antibody is synthesized within the granuloma and this activity continues so long as immunogen persists, which may be for many years. Because of granuloma formation and the tendency for FCA to render the body's own tissues autoimmunogenic it cannot be used in man, their toxic effect are listed in Table 9. It is however, by far the most effective and widely used adjuvant for routine antiserum production in animals to soluble antigens and antigens of emulsified cells. FCA induces an almost exclusive IgG antibody response in the rabbit, a predominant IgG, subclass response in sheep and IgG_2 antibodies in the guinea pig.

Table 9 Toxic effects noted when Freund's complete adjuvant (FCA) was administered to experimental animals

Inflammation or abcess formation at the side of injection
Pyrogenic effect (fever)
Severe pain
Possible organ damage
Possible induction of autoimmune disease
Hypersensitization
Induction of cancer in some animals under some conditions.

Freund's Incomplete Adjuvant (FIA or IFA)

Water phase immunogen can be emulsified with mineral oil without mycobacteria to provide a depot immunization principle. This also provides as income insense long antibody response as there is only gradual uptake and loss of immunogen from the injection site. The primary effect of FIA is to serve as a depot and in some cases, oil droplets have been found in lymph nodes distant from the injection site (78). However, the response with FIA is commonly found to be less reliable and of lower order, this may be due to the fact that open active granulomas do not develop and persist at the FIA depot site. There may also be differences in the antibody isotype response using FIA exclusively. In guinea pigs the response to soluble proteins IgG_1. FIA is commonly used for booster immunizations in subcutaneous sites because of its reduced pathogenic effects.

Liposomes

Liposomes have concentric lipid membrane containing biodegradable lipids such as phospholipids in two layers that are separated by an aqueous compartment (73). They can also be bound by single bilayer membrane (unilamellar liposomes) or may be composed of multiple concentric membrane (multilamellar liposomes). Because phospholipids are amphiphilic substances, they form bilayer vesicles spontaneously when dispersed in an excess of aqueous solution, so that liposomes can entrap both hydrophilic and hydrophobic substances in their vesicles (49). Hydrophilic substances are entrapped within the aqueous compartment while hydrophobic substances are bound into the lipid membrane.

Liposome have been successfully used as drug carriers (20) and they have also been proposed as carriers of antigens and adjuvants to enhance the immune response to antigens. The theoretical basis for using liposomes as carriers of vaccines has been extensively studied in vitro and in animals (21). The present study describes the safety and immunogenicity of a liposomal vaccine injected into humans. Several factors, such as the composition, the charge, the number of layers, and the method of preparation, affect their adjuvanticity.

In 1965, the English scientist Alec D. Bangham made a chance discovery. Introducting water into an erlenmeyer flask containing a thin layer of phospholipid molecules makes these assume an ordinate structure now known as liposomes (49).

In the first time, liposomes had been studied as a biological membrane model (13, 26). In 1971, liposomes had been extensively studied as drug delivery system (30); one aspect of the studies involved the entrapment of enzymes which could then be administered intravenously to patients for the treatment of inherited storage diseases (50). Liposomes have an advantage as a drug delivery system because they were formed from natural molecules which can be metabolized in the body. Because of the versatility of liposomes structure and ability to incorporate almost any drug regardless of solubility, the use of liposomes as carrier vesicles has been extended to many hundred of drugs. They included chelating agent, antibiotics, drugs, which particular emphasis on anti-tumor drugs, peptide hormones, other proteins and genetic materials (79-86).

Interaction of liposome with the biological element

Since 1970 when liposomes were first proposed and tested in animals as a drug carrier, much of their behaviour within the biological element and ways to control such behaviour have become known (87). In this respect, relevant to most applications, including immunoadjuvant activity in vaccine, is the effect of biological fluids (with which liposomes come into contact on injection) on, firstly, the liposomal structural integrity and, secondly, the rate at which vesicles are cleared from the site of injection and distributed in tissues. With all these events, liposomal behaviour is determined by the structural characteristics of the vesicles. It is known (87), for instance, the plasma high density lipoprotein (HDL) remove phospholipid molecules from conventional liposomes (e.g. those made of egg phosphatidylcholine; PC) injected intravenously. Liposomes attacked by HDL become very leaky or dis-integrate and release their drug contents. By substituting PC with ‘high melting’ phospholipids such as distearoyl phosphatidylcholine(DSPC) or sphingomyelin, or supplementing phospholipids with excess cholesterol, vesicle bilayers become rigid at 37°C (the body’s temperature) or have their phospholipid molecules packed and thus resist phospholipids loss or HDL. Liposomal integrity is therefore preserved and entrapped solutes (e.g. antigens) remain with the carrier for longer periods of time.

It has been observed (87) that liposome stability (in terms of solute retention) in vivo is directly related to their rate of clearance from the blood circulation (i.e. the more stable the vesicles are the longer they circulate in the blood). The relationship between vesicle clearance and vesicle stability is abolished when a negative or a

positive surface charge is imposed on the bilayer surface (88, 89). For instance, even the most stable, negatively charged liposomes exhibit short half-lives. A similar reduction in the half-life of stable liposomes also occurs as their size increases (89). Recently, several groups (90-92) have shown that circulation time of liposomes can be prolonged significantly by rendering their bilayer surface highly hydrophilic, for instance, by the use of polyethyleneglycol covalently coupled to a liposomal phospholipid component. Not surprisingly, liposomes, which exhibit extended half-lives either because of their lipid composition or because of a highly hydrophilic surface are intercepted by the reticuloendothelial system (RES) much more slowly. Obviously, such long-lived liposomes are especially amenable to targeting to cells with which they do not normally interact in vivo. This is usually achieved by the anchoring of cell-specific ligands (e.g. antibodies, certain glycoproteins and glycolipids) onto the liposome surface. Regardless of the mechanism of interaction of liposomes (4) with cells, their uptake (when it occurs) proceeds though endocytosis, although fusion may be involved to some extent. Interestingly, recent work has shown that the lysosomotropic pathway of liposomal entry into cells can be interfered with. This is achieved (in vitro) by the use of liposomes which, owing to their lipid composition, can fuse with the lysosomes. Thus, it now appears possible to deliver antigens and drugs into more than one cell compartments.

Much of what is known of the fate of liposomes injected intravenously, also concerns preparation administratered by alternative parenteral routes (e.g. intraperitoneal, subcutaneous or intramuscular). For example, a proportion of liposomes, determined by their size, lipid composition and route of injection, enters the lymphatic and, subsequently, the blood circulation. Once there, and in terms of clearance rates, they behave as if given intravenously (93, 94). On the other hand, whereas liver, spleen and bone marrow intercept most of liposomes given by the intravenous route, they will account for only a small fraction of the dose administered by other routes. Much of the remainder (e.g. for liposomes injected subcutaneously or intramuscularly) is retained at the site of injection (94) or ends up in the lymph node draining the injected site. Uptake by lymph nodes is, in fact, much greater (over 100-fold in terms of percent uptake per gram tissue) than with any of the other RES tissues (93, 94).

The physiological basis of liposome adjuvanticity for proteins

The induction of a classical, T-dependent humoral response requires that the protein antigen be taken up via a phagocytotic or endocytotic mechanism by an antigen presenting cell (APC). The protein is then degraded in the lysosome to small fragments, some of which are subsequently presented on the surface of the cell in association with the MHC II glycoprotein complex. Recognition of this peptide-MHC II complex by T-helper lymphocytes (CD4) results in the secretion of a number of lymphokines by the T-lymphocyte. These lymphokines lead to proliferation of the T-lymphocyte recognizing the peptide and also to activation and proliferation of B-lymphocytes, leading ultimately to secretion of antibody recognizing the antigen that was originally taken up by the APC.

A number of cells are able to act as APCs, the most important of which are macrophages, B-lymphocyte and the dendritic and Langerhans cells. Of these, the macrophages have appeared as the most likely key components in the induction of an immune response towards liposomal antigen, since it is well known that liposomes are avidly phagocytosed by macrophages. A number of experiments *in vitro* (95, 96) and *in vivo* (97, 98) have now proven that macrophages are indeed the fulcrum of liposome adjuvanticity. Van Rooijen has demonstrated the key role played by macrophages *in vivo* by elimination the macrophages with toxic liposomes (99) and subsequently studying the immune response toward free and liposomal antigen. He showed that in the absence of macrophages, the response toward liposomal antigen is significantly reduced, yet the response against certain soluble antigens may be enhanced. Two mechanisms are therefore proposed (100):

1. Soluble antigen, when rendered particulate by encapsulation in, or binding to, liposomes is much more efficiently taken up and processed by macrophages than in the free from. This results in efficient recruitment of T-lymphocytes which are then able to stimulate B-lymphocytes recognizing the antigen as in Figure 3. It is also possible that the macrophages are able to transfer fragments of the antigen directly to B-lymphocytes or dendritic cells, which may in turn present the antigen to T-lymphocytes (101).
2. Liposome block macrophages by occupying their phagocytotic activity. These macrophages may otherwise suppress certain immune responses.

Once the macrophages are blocked, soluble antigen is free to interact with other cells of the immune system and induce an immune response

It is also possible that for certain liposome types the B-lymphocytes are able to act as APC, and there is also evidence that dendritic cells play an important role in presenting liposomal antigen (102). The adjuvanticity of liposomes may also arise in part from a slow-release effect. Liposome associated antigen has been shown to remain at the site of injection for a considerable time (103), during which time gradual leakage with result in continual stimulation of the immune system as shown in Figure 3.

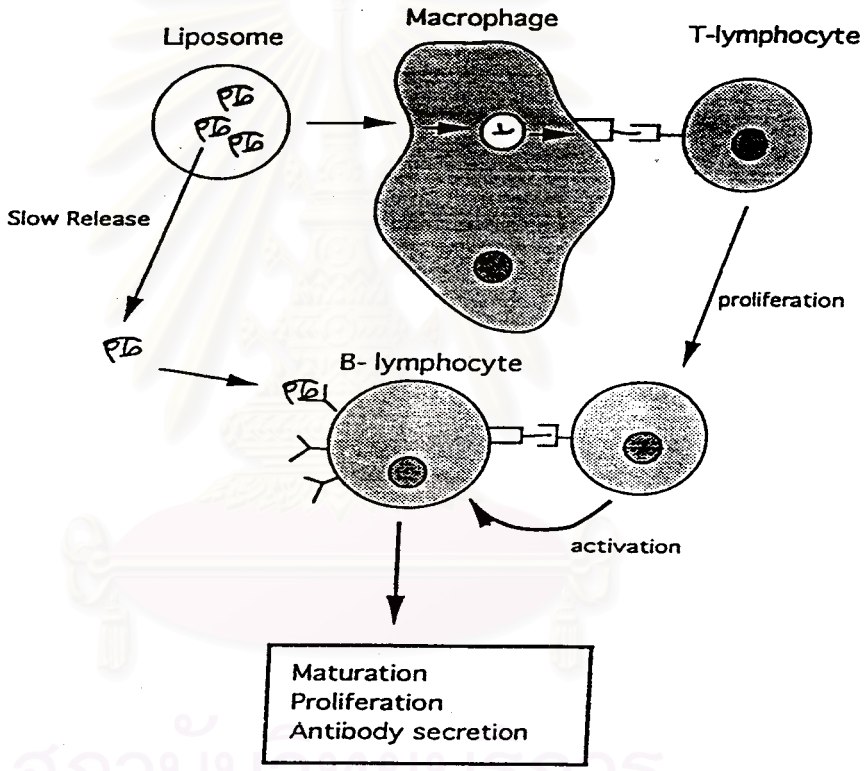


Figure 3 A simplified illustration of the proposed mechanism of liposomal adjuvanticity. Liposome encapsulated protein is phagocytosed by macrophages, leading to protein degradation in the lysosomes. Fragments of the protein are then presented on the surface of the macrophage associated with the MHC II complex, leading to specific T-helper cell stimulation. Lymphokine secretion, and direct interaction of the T-cell with B-cells which have bound and

endocytosed free antigen leads to specific B-cell stimulation and antibody secretion.

Induction of a humoral response

On the above evidence it appears that rendering the antigen particulate by simply associating it with liposomes is sufficient to enhance macrophage uptake and immune stimulation. A number of parameters, however, affect the adjuvanticity of the liposome. Parameters that have been investigated include, the size of the liposomes, (104) the lipid:protein ratio (105), the lipid transition temperature (106), and the lipid charge (106). While all of these factors play a crucial role on the intensity, it is difficult to draw any general conclusion regarding an optimal formulation. It is probably necessary to optimize these parameters for each different antigen, animal model, and site of injection, and also for the type of immune response desired, since what is deleterious for one antigen may be beneficial for another. One parameter that appears to play a role not on the intensity but rather on the type of immune response generated is the localization of antigen in or on the liposomes. While the intensity of the response is roughly the same for the same quantity of antigen encapsulated in or bound to the surface of liposomes, the ratio of IgG to IgM is significantly affected, with surface-bound antigen generating a high ratio of IgM to IgG (107, 108). This suggests that surface-bound antigen may, apart form inducing immunity via macrophages, interact directly with B-lymphocytes and activate B-lymphocytes via an alternative mechanism.

Induction of cellular immunity

When an antigen presenting cell takes up antigen via an endocytotic or phagocytotic mechanism, the antigen (or a fragment of the antigen) is presented on the surface associated with MHC II. This leads ultimately to the activation of B lymphocytes and production of antibodies. If, however, the antigen, instead of entering an endosomal route, is delivered into the cytoplasm, the antigen undergoes a different processing and is presented on the surface associated with the MHC I complex. This leads to the proliferation of a different group of T lymphocytes, cytotoxic T lymphocyte (CTL), specific for the antigen that was in the cytoplasm. CTL from another branch of the immune response called cell mediated immunity (CMI) which is often essential for immunity against viral pathogens, and hence it is of

prime importance that certain vaccines be capable of inducing CTL. Conventional adjuvants such as alum of Freund's adjuvant, generally speaking, are not capable of inducing significant CTL, yet liposomes, and in particular pH sensitive liposomes, appear able to inducing significant CTL for a wide variety of encapsulated antigens.

When liposomal antigen is taken up by cells (primarily macrophages) liposomes pass from the endosome to lysosome where degradation occurs, and antigen is then recycled to the endosomal compartment and associated with MHC II (109). It has, however, been shown *in vitro* that if the antigen is incorporated in pH sensitive liposomes, the liposome disrupts in the acidic endosomal compartment and fuses with the endosome, thus releasing part of the entrapped protein directly in to the cytoplasm. Once in the cytoplasm the antigen is processed and presented associated with the MHC I complex (110). While this effect is not observed with normal liposomes *in vitro*, *in vivo* it has been demonstrated that both types of liposome are able to induce a CTL response, with pH sensitive liposome possibly giving a slightly better response (111). The difference between the *in vitro* and *in vivo* results could be explained by a destabilization of pH dependent liposome *in vivo*, and possible also to the trafficking of antigen fragments form macrophages to dendritic cells *in vivo*. A number of other liposomal parameters affecting CMI induction have been investigated including using liposomes in which the surface charge varied (112), which has a negligible effect, or varying lipid transition temperature (113), which appears to have an important role, at least for a trans-membrane protein.

Immunological adjuvant in vaccines

Immunological adjuvants are agents that can provoke potent immune reponses to antigens. They include aluminium hydroxide (alum), saponins complexed to membrane protein antigens (immune stimulating complexes). Pluronic polymers with mineral oil, killed mycobacteria in mineral oil (Freund's complete adjuvant, FCA), bacterial products such as lipopolysaccharide (LPS) and muramyl dipeptide (MDP), and liposomes. Adjuvants appear to function by one or both of the following mechanisms.

The first mechanism involves creating a depot (as, for example, by alum and oil emulsions) at the site of injection, which prolongs the release and interaction of antigens with APC. The APC may also invade the depot area in the presence of local

inflammation, and adjuvant antigen complexes may migrate to area in the regional lymph nodes containing T cells. The second mechanism is activation of macrophages (for example LPS, MDP) which release interleukin 1 (lL-1). The combined action of IL-1 and antigen on T cells produces interleukin 2 (IL-2) and other mediators which activate effector T cells (CMI) or antibody-forming B cells (HI). Some of these factors also promote the retention of circulating lymphocytes in the lymphoid tissues adjacent to the site of injection. It has been suggested that strong and persistent immune responses may be elicited by targeting antigens to interdigitating cells and follicular dendritic cells which, unlike macrophages, constitutively express major MHC II antigens. As these specialize in presenting antigen to T and B lymphocytes respectively. CMI and HI could be favoured selectively. Of the adjuvants mentioned, only alum is licensed for use in humans. But it is far from idea: it is not always effective. It increases CMI only slightly if at all and, as it can not be lyophilized, it requires refrigerated storage. Many of the other adjuvants are toxic, inducing granulomas, acute and chronic inflammations (CFA), cytolysis (saponins, some Pluronic polymers) and pyrogenicity (LPS and MDP). However with some of the agents, toxicity can be curtailed without loss of adjuvanticity through changes in the adjuvant's structure (LPS, MDP) or through the choice and synthesis of appropriate adjuvant analogs (saponin, Pluronic polymers). Concerted efforts are now in progress to develop safe and effective adjuvants to meet the challenges of subunit and peptide vaccines and new insights into the ways in which immunity is produced. An ideal adjuvant should comply and certain criteria which, in addition to some practical ones (for example inexpensive raw materials, simplicity of manufacture, stability in storage (preferable in a freeze-dried form) would also include a biodegradable, nontoxic, and non-immunogenic nature, the ability to elicit both CMI and HI to antigens administered by a variety of routes, synergistic action with other adjuvants if needed, and a potential for selective interaction with populations of immunocompetent cells.

Adjusting the proportions of each polymer varies the rate of release of the entrapped antigens. Entrapped antigens can be released at predetermined rates or intervals after a single immunization and so may obviate the need for booster doses of a vaccine. The size of the microparticle is critical. Particles with a diameter of less than 10 μm are taken up by macrophages and transported to the draining lymph node and produce a more rapid rise in antibody levels compared with larger (>10 μm)

particles that remain at the injection site, comparable with the suitable in CFA emulsion for antigens is presented schematically.

Both the activation of T-cells and of B-cells is important for an effective imunogenic reaction, because, in a complex way, they interact with one another, either directly or through interleukins. The T-helper (T_H) cells are the "principal orchestors" of the immune response because they are needed for the activation of the major effector cells in the activation of the major effector cells in this response (i.e. cytotoxic T (T_c) cells and antibody-producing B cells). Antigen presentation is mediated by specialized macrophages (APC). After internalization of the antigen-loaded particle or free antigen, immunorelevant epitopes are presented on the surface of the APC, in combination with a major histocompatibility complex (MHC), the T_H cells are attracted and activated by two signals: binding of the T-cell antigen receptor to the MHC complex and production of interleukin-1 by the APC. The activated T_H cells trigger a complex cascade. They release lymphokine a) stimulating B lymphocytes to proliferate and produce specific antibodies after differentiation as well as memory cells, and b) activating granulocytes, macrophages, and natural killer cells. Cytotoxic T cells are activated directly. Moreover some long-lived T_H cells seem to provide a memory function in the T-cell compartment of the immune system.

Certain molecules appear to trigger B lymphocyte directly (thymus-independent antigens). This mechanism is still incompletely directly understood because the antibodies produced are mainly of the IgM class, and little or no immunological memory is affected.

The release of the antigen from microparticle is controlled by a variety of factors, such as microparticle morphology, microparticle size, and the polymer used for microencapsulation. In this selection the attempt to summarize some of *in vivo* results obtained with experimental vaccine-delivery system after parenteral application, although one has to bear in mind, that such a retrospective comparison is obscured by the unknown effect of experimental differences on the biological response.

For one, different species were used (e.g. mice, rats, and guinea pigs). Injection site and vehicles for microparticle administration were different, such as IP, IM, or SC. Therefore, it is not surprising reaction varied considerably from study to study. Second, there is no consensus on a generally accepted standard dose for the respective animal model used. Only two groups reported afforts in establishing an

adequate dose for tetanus toxoid in mice. A dose-response relation for microencapsulation antigens has been established to our knowledge; therefore, interspecies comparisons and *in vivo-in vitro* correlation of antigen release from animal studies are subjected of speculation. Third, and most importantly, there seems to be little agreement in the literature on a standard immunization protocol for *in vivo* studies. Positive and negative controls, time schedule for boostering, and time intervals for blood sampling differ considerably from group to group.

After subcutaneous primary and secondary immunization of mice with ovalbumin (OVA) in microparticles (5.3 μm). The antibody titers reached were comparable to those reached by immunization with OVA in FCA over 12-week period.

Microparticles (<10 μm) were taken up by macrophages and transported to the draining lymph nodes. The researchers also demonstrated that different sizes of microparticles could be administered in combination to provide a biphasic antibody response. In this review liposomes are discussed as attractive adjuvant candidates**.**

It is fairly clear that the adjuvant property of liposomes is a physical rather a chemical effect. When injected intravenous liposomes are taken up mainly by the liver and spleen. Interestingly, intravenous administration is a very poor method of immunizing mice with material in liposomes intraperitoneal and subcutaneous administration being routes with lead to a greater immune response. It seems likely that liposomes injected by these two routes will remain at the site of injection for a long period. Thus, liposomes may well exert their adjuvant effect by the same method as many other particulate adjuvant that is, by retaining a "depot" of antigen at the site of injection. The phagocytosis of liposomes by cells such as macrophages may also be an important factor. This suggested by the fact that the incorporation of more than 30 mol% cholesterol into liposomes markedly reduces the immune response to the antigen entrapped within them. This level of cholesterol has also been shown to reduce the rate of digestion of liposomes by macrophages.

The research group of Naito et al, 1996 (114) immunized adjuvant liposome and alum in rabbits. Antibody response after immunization with surface-coupled ovalbumin (OVA) of liposomes was investigated in mice. OVA was coupled to the surface of liposome via amino groups using glutaraldehyde. OVA-liposome conjugate induced a significant anti-OVA IgG antibody production in mice.

However, no IgE antibody production specific for OVA was observed. Immunization with OVA-liposome induced IgE-specific unresponsiveness even after the subsequent challenge with OVA adsorbed with aluminium hydroxide (OVA-alum), which induces a high level of IgE antibody production. Furthermore, following the primary immunization with OVA-alum. A secondary challenge with OVA-liposome boosted anti-OVA IgG but not anti-OVA IgE antibody production. These results show the potential of the antigen-liposome conjugate for the development of a vaccine with the least allergic reaction and also for the application of immunotherapy.

Since Allison and Gregoriadis 1974 (115) demonstrated that liposomes could enhance the antibody response to diphtheria toxoid. They have claimed that whilst liposomes enhance the immune response they also prevent any adverse hypersensitivity reaction to the antigen by shielding it from antibodies. They have so far demonstrated for diphtheria toxoid, that liposomal entrapment of antigen prevents arthus type hypersensitivity reactions when the material is injected into the footpad of a primed mouse, and also prevents the death of mice with high circulating antibody when injected intravenously, the role of liposomes as adjuvants in stimulating an immune response has received a great deal of attention. Since that time liposomes have been shown to be effective adjuvants for a large number of protein antigens.

Liposomes are being investigated in the design of subunit vaccines for viral diseases and are able to efficiently present the surface glycoproteins of many enveloped viruses for stimulation of a protective immune response. The viral glycoproteins are anchored in the liposomal bilayer via a trans-membrane segment and assume a conformation analogous to their native conformation in the viral envelope. Influenza virus surface proteins have also been shown by Almeioda et al. 1998 (116) to orientate themselves in liposomes in a manner which, under the electron microscope appears similar to their disposition in the intact influenza virus. Moreover the “virosomes” so formed can be agglutinated by anti-influenza antisera. The production of the virosome was prompted by the knowledge liposomes were adjuvants, and the need for a non-pyrogenic influenza vaccine. Whole influenza virus is a good immunogen, but is also pyrogenic. Influenza proteins are not pyrogenic, but also are not very good immunogen. It is hoped that the combination of non-pyrogenic lipid with the subunits should produce non-pyrogenic, but immunogenic, preparation.

CHAPTER III
MATERIALS AND METHODS

MATERIALS

1. Microrganisms

Lyophilized *P. multocida* vaccine strain serotype 8:A was obtained from Division of Biological Product, Department of Livestock, Ministry of Agriculture and Cooperation, Bangkok, Thailand.

2. Experimental Animals

Rabbits: The immune sera were produced in young adult New Zealand White rabbits weighing about 2 – 4 Kg each.

3. Media

Tryptic soy agar (Merck, Germany)

4. Blood

Sheep blood (Faculty of Veterinary Sciences, Chulalongkorn University)

5. Chemicals

Absolute ethanol (Merck, Germany)
Acetic acid (Merck, Germany)
Acetonitrile (Lab-scan analytical sciences, Irelane)
Acrylamide (Biorad, U.S.A)
Amido black (Fluka, Switzerland)
Ammonium persulfate (Biorad, U.S.A)
Barbital (BDH Chemicals LTD Poole, England)
Bis-acrylamide (Biorad, U.S.A)
Bovine serum albumin (Sigma, U.S.A)
Bromophenol blue (Biorad, U.S.A)
Cholesterol (Sigma, U.S.A)
4-Chloro-1-napthol (Sigma, U.S.A)

Citric acid (Mallinkrodt chemical works, England)

Coomassie brillant blue R-250 (Biorad, U.S.A)

Dichloromethane (Merck, Germany)

Disodium hydrogen phosphate (Biorad, U.S.A)

Fetal calf serum (Gibco, U.S.A)

Formaldehyde (May&Baker LTD Dagenham, England)

Freund's adjuvant complete (Difco, U.S.A)

Freund's adjuvant incomplete (Difco, U.S.A)

Glycerol (Merck, Germany)

Glycine (Promega, U.S.A)

Goat anti-rabbit IgG enzyme HRP conjugate (Zymed, U.S.A)

Hydrochloric acid (Merck, Germany)

Hydrogen peroxide solution, 35 % (Merck, Germany)

Lecithin (Sigma, U.S.A)

2- Mercaptoethanol (Biorad, U.S.A)

Methanol (BDH Chemicals LTD Poole, England)

N-laurylsakosine (Sigma, U.S.A.)

N,N-Diethyl-p-phenylene diamine sulfate salt (Biorad, U.S.A.)

N,N,'-methylene bis acrylamide (BRL, U.S.A)

Protein molecular weight standard (Biorad, U.S.A)

Polyvinyl alcohol (Merck, Germany)

Potassium aluminium sulfate (Fluka, Switzerland)

Potassium chloride (May&Baker LTD Dageham, England)

Potassium dihydrogen phosphate (May&Baker LTD Dagenham, England)

Potassium thiocyanate (Merck, Germany)

Skim milk (Difco, U.S.A)

Sodium barbital (BDH Chemicals LTD Poole, England)

Sodium bicarbonate (Wako pure chemical industries LTD, Japan)

Sodium carbonate (BDH Chemicals LTD Poole, England)

Sodium carboxymethylcellulose (Merck, Germany)

Sodium chloride (J.T.Baker, U.S.A.)

Sodium hydroxide pellets (Merck, Germany)

Sodium hydrogen phosphate (Merck, Germany)

Sulfuric acid 98 % (Mallinkrodt chemical works, England)

TEMED (Promega, U.S.A)

Thimerosol (Kecks, U.S.A)

Trizmabase (Sigma, U.S.A)

Tris-Hydrochloride (Sigma, U.S.A)

Triton X –100 (Amresco, U.S.A)

Tween 20 (Merck, Germany)

Xylene (BDH Chemicals LTD Poole, England)

6.Instrument

Analytical balance (Sartorious, U.S.A)

Autoclave (Hirayama, Japan)

Colony counter 500 (Suntex, Taiwan)

Critical point drier (Balzers, Liechtenstein)

Deep freezer -85 °C (Forma Scientific, U.S.A)

Freezer -20°C (Tropical Ariston, Italy)

Hamilton syringe (Helmilton, Switzerland)

Hot air oven (Precision, U.S.A)

Incubator (Memmert, Germany)

Light microscope (Olympus, Japan)

Larminar flow (ISSCO, Thailand)

Magnetic stirrer (Thermolyme, U.S.A)

Mastersizer S long bed (Malvern instruments Ltd., England)

Microplate Reader (Biorad, U.S.A)

Micropipette (Socorex, Switzerland)

Multichanel micropipette (Socorex, Switzerland)

pH meter (Beckman, U.S.A.)

Pipette aid (Accu-jet, Germany)

Power supply model 200/2.0

Refrigerator (Hitachi, Japan)

Refrigerated centrifuge (Sigma, U.S.A)

Scanning Electron Microscope (Jeol, Japan)

Sonicator (Heat System-Ultrasonic Inc., U.S.A)

Transmission Electron Microscope (Joel, Japan)

Trans-Blot SD Semi-Dry Transfer cell (Biorad, U.S.A.)

Ultracentrifuge (Beckman, U.S.A)

7. Glassware

Beaker (Pyrex, U.S.A)

Erlenmeyer flasks (Pyrex, U.S.A)

Funnel (Pyrex, U.S.A.)

Glass bottle (Pyrex, Germany)

Glass plates for SDS-PAGE 7x10 cm, 8x10 cm (Biorad, U.S.A)

Glass slides (Clay Adam, U.S.A)

Measuring cylinders (Pyrex, U.S.A)

Measuring pipettes (HBG, Germany)

Pasteur pipettes (John poulten, England)

Petri dishes (Pyrex, U.S.A)

Syringes (Nipro, Thailand)

Test tubes (Pyrex, U.S.A)

Volumetric flask (Witeg Diffico, Germany)

Volumetric pipette (Brand, Germany)

8. Other

Aluminium foils (Diamond foil, U.S.A.)

Centrifuge tube plastic 50,500 ml (Nalgene, U.S.A)

Clip (Medi-clip, England)

Comb (Biorad, U.S.A)

Disposable gloves (Imperial, Thailand)

Hydrophilic polypropylene membrane filter 0.45 μm (Gelman, U.S.A)

Microtiter plate (Nunc, Denmark)

Microtube plastics (Treff Lab, Switzerland)

Nitrocellulose membrane 0.2 μm (Micron Separation Inc., U.S.A)

Reservoir 8 well (Labsystems, U.S.A)

Tip 100, 200 μl (Treff Lab, Switzerland)

Whatman filter paper No.1 (Whatman, England)

METHODS

1. Calibration curve of standard protein (assay by Lowry method)

The assay is based on the reaction of protein with an alkaline copper tartrate solution (Reagent A) and Folin reagent (Reagent B) (117). There are two steps which lead to color development: the reaction between protein and copper in alkaline medium, and the subsequent reduction of the Folin reagent by copper treated protein. Protein affects a reduction of the Folin reagent by loss of 1,2 or 3 oxygen atoms, thereby one or more of several possible reduced species which have a characteristic of blue color with maximum absorbance at 750 nm. and minimum absorbance at 605 nm.

The procedure could be explained as followed: the working reagent A was prepared by adding 20 μl of agents to each ml of reagent A. (This working reagent A was stable for 1 week even though a precipitate was formed after 1 day. The solution was warmed and vortexed when the precipitation was formed.)

Standard solution containing an accurate amount of bovine serum albumin (BSA) 1.35 mg/ml was diluted to 5 dilutions 50, 100, 200, 400, 800 μl/ml of BSA. Triplicate, 20 μl of each standard was pipetted into microtube. The 100 μl of reagent A was added and mixed immediately, subsequently 800 μl of reagent B was added into microtube and mixed again. The mixture was incubated in the dark for 15 minutes, absorbances could be read at 750 nm. The plot of the average absorbances versus known concentrations was made. The correlation between absorbances and concentrations was determined by linear regression.

2. Preparation of OMPs from *P. multocida*

2.1 Preparation of microorganisms

The cultures of *P. multocida* serotype 8:A were thawed from stock cultures, and streaked for isolation on TBA plates. Isolated colony was streaked on surface of TBA plates and incubated at 37^{o}C for 24 hr. Cells were harvested with NSS from cultures on agar surface and washed by centrifugation at 3,000×g for 10 minutes at 4 ^{o}C. The supernatant was removed, and the pellet was resuspended in NSS and counted by drop plate method.

2.2 Preparation of OMP and protein determination

The bacterial cell suspension was chilled on ice and sonicated by using sonicator (Heat System Ultrasonic Inc., U.S.A.) for 1 minutes 5 times. Unbroken cells were removed by centrifuging the suspension at 3,000×g for 10 minutes at 4°C and the supernatant was then centrifuged by using ultracentrifuge (Beckman, U.S.A.) at 100,000×g for 1 hour at 4°C to pellet the membrane components. The pellet containing total membrane was resuspended in 2% sodium lauryl sarcosinate (sakosyl) detergent in PBS pH 7.4 and incubated at 25°C for 20 minutes. After another centrifugation at 100,000×g for 1 hour at 4°C, the outer membrane was again treated with 2% sakosyl in PBS pH 7.4 and incubated at 25°C for 20 minutes and centrifuged at 100,000×g for 1 hour at 4°C to pellet the outer membrane sample. The supernatant was decanted, and the pellet was resuspended in distilled water (33). The total protein concentration of the outer membrane preparations was determined by the Lowry method. OMP samples were stored (1000 μl aliquots) at -20°C for further experiments.

3. Analysis of OMP of *P. multocida* by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

The protein patterns of OMP of *P. multocida* 8:A were analyzed by SDS-PAGE with discontinuous buffer according to the method as follows (118):

3.1 Preparation of vertical slab gels

3.1.1 Preparation of separating gels

Separation of the OMP was performed in an 8x10 cm vertical electrophoresis cell. The 12% separating gel was prepared in the lower part from 30% stock solution of acrylamide and N,N'-methylene-bis-acrylamide in the ratio of 30:0.8. The gels were prepared in 0.375 M tris-hydrochloride pH 8.8, and contained 0.1 % sodium dodecyl sulfate (SDS) and the gels were chemically polymerized by the addition of 0.2% of N,N,N',N'-tetramethylethylene diamine (TEMED) and 10 % ammonium persulfate, polymerizing agent. The gels were mixed by gently swirling and poured into the assembled gel sandwich about 5 cm, and were overlayed on the

top with distilled water. The gels were allowed to polymerize 1 hour at room temperature.

3.1.2 Preparation of stacking gels

The stacking gels were consisted of 4 % acrylamide in 0.125 M tris-hydrochloride pH 6.8, 0.2% SDS. The gels were chemically polymerized by the addition of TEMED and ammonium persulfate. The prepared gels were filled into assemblied gel sandwich with a comb of ten identical wells 0.5 cm length inserted in the upper part at a length of 2 cm and then the gels were allowed to polymerize at room temperature at least 1 hour.

3.2 Preparation of sample

Antigen extracts were prepared in the sample buffer of 0.6 M tris-hydrochloride pH 6.8 containing 2% SDS, 10% glycerol, 5% 2-mercaptoethanol and 0.5% bromophenol blue as the dye. Sample concentrations were 10 μg/well combined in equal volume of sample buffer and boiled at 95^{o}C for 5 minutes.

3.3 Molecular weight protein markers

The broad range reference marker protein standards (Biorad, U.S.A) used for indicating the molecular weights (MW) of the protein bands were aprotinin (MW 6,500), lysozyme (MW 14,500), trypsin inhibitor (MW 21,500), carbonic anhydrase (MW 31,000), ovalbumin (MW 45,000), serum albumin (MW 66,200), phosphorylase b (MW 97,400), β-galactosidase (MW 116,250) and myosin (MW 200,000) prepared 1 part in 19 part of sample buffer and boiled at 100^{o}C for 5 minutes.

3.4 Gel loading

The comb was slowly removed from the gel. Each well was rinsed with electrophoresis buffer. Sample was gently loaded beneath the buffer in each well. Every well was loaded with the same volume of each sample. The molecular weight protein markers were loaded in the first lane or among the sample lanes. If the well was not needed, the same volume of sample buffer was loaded instead.

3.5 Electrophoresis

The electrophoresis was carried out by using the mini-protein II vertical electrophoresis cells (Biorad, U.S.A). The electrophoresis buffer pH 8.3 containing 0.025 M tris, 0.192 M glycine and 0.1 % SDS. The buffer of 300 ml was put into the lower buffer tank and 100 ml into the upper buffer tank reservoir. The power (Biorad Power Supply model 200/2.0, U.S.A.) was set an initial voltage of 40 V for 30 minutes and a running voltage of 70 V for 2 hours or until the tracking dye was migrated about 1 cm from the bottom of separating gel.

3.6 Procedure for staining

The gels were fixed in the fixing solution containing 40% methanol with 10% acetic acid for 10-30 minutes and stained with 0.1% Coomassie brilliant blue R-250 in 40% methanol, 10% acetic acid for at least 30-60 minutes. The gels were then destained in the destaining solution until the protein bands were seen and the background were clear.

3.7 Determination of molecular weight

The relative mobility (Rf) of protein were determined and calculated from the formula

$$Rf = \frac{\text{distance of the protein migration}}{\text{distance of the tracking dye migration}}$$

The Rf were plotted against the known MW of protein markers in the semilog scale and the MW of the protein samples were estimated from standard calibration curve.

3.8 Storage of stained gels

After staining completed, gels were photographed on a bright light box (Spectroline, U.S.A.) and stored after drying on cellophane membrane. For drying a gel, two sheets of cellophane of the size bigger than the gel were soaked in water. One sheet of cellophane was placed onto a glass plate and stained gel was placed carefully avoiding air bubbles. The second sheet of cellophane was placed over the gel and air bubbles were removed. Cellophane was folded back to each edge of the

glass plate and was fixed with clips to prevent the cellophane moving when it dried. The gel was left to dry at room temperature or 37 ^{o}C for 24 hours.

4. Preparation of immunization antigens

4.1 Preparation of liposome containing OMP from *P. multocida* by double emulsion technique.

Liposome containing OMP from *P. multocida* was prepared by double emulsion technique (modified from the method of Kato, et al. (119), and Sukanya (23) as followed: total lipid mixture in the molar ratio of 1:1 of egg yolk lecithin to cholesterol was dissolved in 10 ml of dichloromethane at 50 mg/ml. OMP from *P. multocida* was dissolved in phosphate buffer solution (PBS), pH 7.4 and polyvinyl alcohol concentration at 0.5% (22) to obtain protein concentration at 2 mg/ml. To 10 ml of protein solution, an equal volume of the lipid solution was added, the mixture was vigorously agitated by vortex mixer for 10 minutes to give a w/o emulsion. The emulsion obtained was quickly added by stirring to 100 ml of 0.02 % sodium carboxymethylcellulose (CMC) in PBS pH 7.4 (23) to yield a w/o/w emulsion. After 10 minutes. stirring, another 100 ml of 0.02 % CMC in PBS pH 7.4 was added to complex emulsion and the stirring was further continued for 10 hours until the dichloromethane completely evaporated. In this way, liposome containing protein extract form *P. multocida* was obtained.

The aqueous dispersion of liposome was centrifuged at 20,000xg for 30 minutes. The pellet was washed three times with the PBS pH 7.4 by centrifugation and redispersed in 10 ml of the same medium for further analysis.

4.1.1 Percent protein entrapment

The volume of 1 ml of liposome suspension was pipetted into microcentrifuge tube. The encapsulated protein was separated from free protein by centrifugation at 20000 g for 30 minutes. The pellet containing entrapped protein was ruptured by adding 1 ml of 2 % w/v Triton X-100. After incubated at room temperature for 30 min, the clear solution was assayed by the modified Lowry method (Bio-rad DC protein assay). The absorbances were read at 750 nm and compared with the corresponding standard curve (all analysis were done in triplicate). The percent protein entrapment was calculated by following equation.

$$\text{Percent protein entrapment} = \frac{\text{Total protein content in liposome vesicle} \times 100}{\text{Initial protein content}}$$

4.1.2 Particle size analysis

Laser particle sizer (Mastersizers long bed Ver 2.11 with 300 mm range lens, 240 mm beam length, Malvern instruments Ltd., England) was used to determine particle size of liposome. Particle size distribution was analysed by the curve plotted between particle diameter versus percent volume of particles. Cumulative frequency curve was plotted between cumulative percent under size versus particle diameter, and the median diameters were determined.

4.1.3 Transmission electron microscopy (TEM)

In order to examine the characteristics of liposomes OMP, transmission electron microscopy (Jeol, Japan) was performed. Samples were stained with 1% phosphotungstic acid pH 7.0 on 300 mesh formyar film coated copper grids and air dried. The grids were examined under transmission electron micoscope and photographed.

4.1.4 Scanning electron microscopy (SEM)

Surface characteristics of liposomes were studied by using degydrate method and cryo-scanning electron microscope method.

A. Dehydrate method

The specimens were obtained by a specific fixation technique, which liposomes were adsorbed on the glass slide and were fixed with 2.5% glutaraldehyde in 0.1 M PBS pH 7.2 for 1 hour and subsequently fixed with 1% osmium tetraoxide in 0.1 M PBS pH 7.2, washed three times with 0.1 M PBS pH 7.2 for 5 minutes, dehydrated with a serial concentrations of 30%, 50%, 70%, 90% and 100% v/v acetone solution for 5 minutes in each step, dried with critical point drying method by Samtri 780 dryer (Balzers, Liechtenstein)., mounted, and coated with gold. The scanning electron micrographs were examined and photographed (Joel, Japan)

B. Cryo-SEM method

1-mm cube of liposome were fixed on the specimen stub by using an adhesive. Then the specimen stub was set on the specimen holder and frozen in liquid nitrogen. The specimen exchange rod was fit to the specimen holder. The holder was set in the cryo chamber though the airlock chamber. After that the specimen was cut with the knife. When the ice in the specimen was sublimated by using an etching heater, then transferred the specimen to the cooling stage. The observation was carried out by scanning electron micrographs (Joel, Japan).

4.1.5 Analysis of liposome OMP of *P. multocida* by SDS-PAGE

The protein patterns of OMP of *P. multocida* in liposome were analyzed by SDS-PAGE according to the method described in the part; analysis of OMP (118).

4.2 Preparation of OMP with Freund's adjuvant

An equal volume of OMP of *P. multocida* 1mg/ml was mixed with complete or incomplete Freund's adjuvant by vortex mixture for 5 minutes.

4.3 Preparation of alum OMP

OMP of *P. multocida* was dissolved and diluted in phosphate buffer solution (PBS), pH 7.4 to obtain protein concentration at 1000 μg/ml. The mixture containing 1 ml of protein, 500 μl of 1M sodium bicarbonate, and 1 ml of 0.2 M aluminium potassium sulfate was prepared and vigorously agitated by vortex mixer for 10 seconds (120).

The aqueous dispersion of alum was centrifuged at 13000 g for 5 minutes. The precipitates were washed three times with the PBS pH 7.4 by centrifugation and redispersed in 1 ml of the same medium.

4.4 Preparation of liposome without protein

Liposome was prepared as described in the part; liposome preparation but use PBS instead of solution of protein.

5. Experimental immunization design

Rabbits were divided into 5 groups (5 rabbits/group) for experiment and 2 groups (2 rabbits/group) for control, each was immunized 2 doses by subcutaneous route, 4 weeks apart:

Group 1 was immunized with formalin killed whole cell of *P. multocida* 8:A, 10^8 CFU per dose.

Group 2 was immunized with liposome OMP of *P. multocida* 8:A, 500 μg of protein per dose.

Group 3 was immunized with the OMP of *P. multocida* 8:A emulsified in Freund's adjuvant, 500 μg of protein per dose. The first dose was immunized OMP with complete Freund's adjuvant and the second dose was immunized OMP with incomplete Freund's adjuvant.

Group 4 was immunized with alum containing OMP of *P. mulocida* 8:A, 500 μg of protein per dose.

Group 5 was immunized with OMP of *P. multocida* 8:A, 500 μg of protein per dose.

Group control 1 was immunized with ghost liposome (liposome without protein), 1 ml.

Group control 2 was immunized with phosphate buffer pH 7.4, 1 ml.

Blood was collected from the lateral ear vein of each rabbit prior to immunization and every 2 weeks for 3 months. Serum was separated by centrifugation at 8000 g for 10 minutes, aliquoted in microtubes and frozen at -20°C until analyzed.

6. Determination of immune response

6.1 Determination of antibody titer by enzyme-linked immunosorbent assay (ELISA) (121)

Antibody against antigen determination was determined as followed: polystyrene 96-well plates were coated with 100 μl/well of OMP of *P. multocida* 10 μg per ml in coating buffer, and incubated overnight at 4°C. The antigen was removed. The wells were washed with PBS-T (pH 7.4) 3 times, blocked with 3 %

gelatin in PBS-T for 1 hour and were washed again as above. One hundred microliter of each serum dilution (ten-fold dilution) in 1% gelatin in PBS-T was added and incubated in triplicate OMP-coated wells for 1 hour, after the wells were washed with PBS-T, 100 μl of solution containing goat anti-rabbit-IgG-HRP conjugate was added to the wells and incubated for 1 hour at room temperature. After another PBS-T wash, 100 μl of the substrate (o-phenylenediamine) was added and incubated in dark for 15 minutes. The reaction was stopped by adding H_2SO_4, 50 μl per well. The optical density at 492 nm was read by microplate reader (Biorad, U.S.A) and the serum dilution showing OD of 0.5 was determined as antibody titer.

Antibody was also assayed by using the whole cell antigen, *P. multocida* 8:A as coating antigen. The ELISA process was performed by the similar protocol described above. The serial dilution of rabbit immune sera was reacted with homologous whole cell coated at about 10^8 CFU/well in microtiter plate and the antibody titers were monitored.

6.2 Determination of bactericidal activity of immune sera

Immune sera were tested for their bactericidal capacity. Triplicate of 20 μl of immune sera of each rabbit were incubated with 100 μl of suspension of *P. multocida* (about $2x10^3$ CFU/ml) in modified barbitone buffer for 10 minutes at room temperature in microtiter plates (122, 123). The plates were further incubated for 30 minutes at 37°C. From each well of triplicate, 20 μl sample was removed and plated on 5% sheep blood agar. The number of colonies at 30 minutes (T_{30}) was counted after incubation at 37°C, 24 hours. The initial bacterial inoculum was confirmed retrospectively by plate count and was calculated as 0.5 x the mean CFU/ml at T_0. The percentage of killing (K%) was then calculated by the formula:

$$\%K = \left[1 - \frac{\text{Mean cfu /ml at } T_{30}}{\text{Initial bacterial inoculum } (0.5xT_0)} \right] \times 100$$

Immune rabbit serum against the whole cell of *P. multocida* was incubated as positive serum control and modified barbitone buffer was included as negative control.

6.3 Western blot analysis of antigen extract of *P. multocida* with rabbit immune sera (121)

6.3.1 Western blot transfer

The OMP of *P. multocida* was separated by SDS-PAGE on 12% polyacrylamide gel, then transferred onto a 0.2 μm nitrocellulose membrane using semidry electrophoretic transfer cell (Biorad, U.S.A.). The concentration of antigen run on SDS-PAGE were about 0.05 μg. A comb of 2 wells was used, a large well for antigen loading and a small one for molecular weight protein markers. Following electrophoresis the gels were equilibrated in 48 mM tris-hydrochloride, 39 mM glycine, 20% methanol and 0.04% SDS transfer buffer pH 9.2 for 15 minutes to remove of electrophoresis buffer salts and detergents. Nitrocellulose membrane and filter papers wet by slowly aligning into transfer buffer, allowing to soak for 15-30 minutes. Three sheets of pre-soaked filter paper were placed onto the platinum anode, the pre-wetted nitrocellulose was then placed on top of the filter papers and the equilibrated gel was placed next on top of the nitrocellulose membrane, aligning the gel on the center of the membrane. The three sheets of pre-soaked filter papers were placed on top of the gel, for all between steps carefully removing air bubbles. The semidry electrophoresis transfer cell was assembled and run for 15 minutes at 15 V. After the transfer was finished, the gel was removed and stained with Coomassie brilliant blue for OMP to determine that the proteins were completely transferred. Efficient binding of antigen to the nitrocellulose membrane was determined by staining a portion of the membrane for sample proteins and molecular weight protein standard with 0.1% amido black in 40 % methanol with 10% acetic acid at least 1 hour.

6.3.2 Immuno-analysis of antigen with rabbit immune sera

The nitrocellulose was cut into strips for immunodetection. The strips were washed in tris buffered saline (TBS) pH 7.4 (50 mM tris-hydrochloride with 0.85% sodium chloride) and non specific binding sites were blocked with 5% skimmed milk in TBS pH7.4 (blocking buffer) for 2 hours at room temperature. Each two strips were probed with rabbit immune sera against whole cell, the sera against liposome OMP, OMP with Freund's adjuvant, alum OMP, and OMP. Immune sera were diluted 1:100 in blocking buffer, rabbit normal sera of each group were used as control.

Antigen and sera were incubated for 1 hour at room temperature. After washing with TBS pH 7.4 several times, the nitrocellulose strips were incubated with 1:1,000 diluted goat anti-rabbit immunoglobulin conjugated with horseradish peroxidase at room temperature for 1 hour and washed several times with TBS pH 7.4 to remove the unbound conjugate. The nitrocellulose strips were then incubated in the solution of 0.11 M 4-chloro-1-napthol and 0.06 M N,N diethyl-p-phenylene diamine in acetonitrile 200 μl with 1 μl of 37% hydrogen peroxide in 10 ml of citrate buffer pH 6.0 (substrate buffer) at room temperature for 5-20 minutes. The specific bands observed were dark blue in color. The enzymatic reaction was stopped by washing the strips in distilled water. The nitrocellulose membranes were air dried and photographed immediately after development. The storage was done by wrapping in aluminum foil to protect from light.

CHAPTER IV

RESULT AND DISCUSSION

1. Calibration curve of standard protein

In this study, Lowry method (117) was used to determine concentration of protein remaining in OMP from *P. multocida* 8:A that were treated with 2% sakosyl to solubilize differentially the inner membrane components and protein remaining in liposome vesicles were ruptured with Triton X-100 to release protein. Lowry method can be used for protein determination in the presence of detergent. The relationship between concentration of standard protein bovine serum albumin (BSA) in PBS 7.4 and absorbance at 750 nm was shown in Table 10 and standard curve was shown in Figure 4 with the correlation coefficient of 0.9990.

Table 10 Relationship between concentration of standard protein (BSA) in PBS pH 7.4 and absorbance at 750 nm. (Assay by Lowry method)

Concentration of BSA (µg/ml)	Absorbance[a]
50	0.075
100	0.092
200	0.112
400	0.160
800	0.250

$y = 0.0665 + 0.00023x$

$r^2 = 0.9990$

a, average of three determination

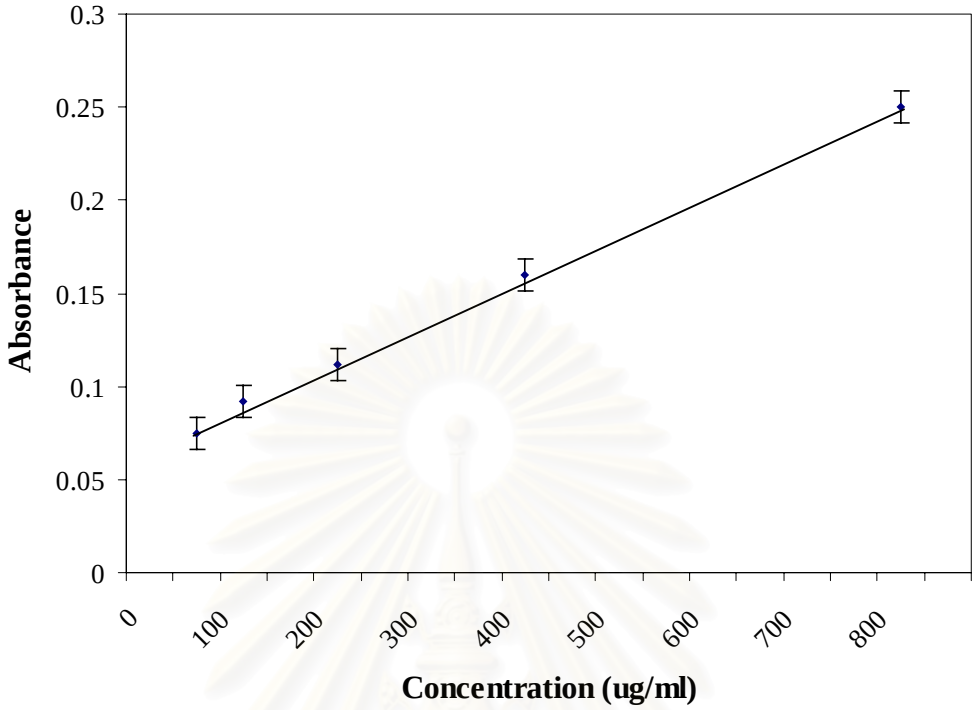


Figure 4 Calibration curve of standard protein in PBS 7.4 at 750 nm (Assay by Lowry method)

2. Preparation of OMP from *P. multocida*

2.1 Preparation of microorganism

The culture used in OMP extract was *P. multocida* 8:A, obtained from Division of Biological Product, Department of Livestock, Ministry of Agriculture and Cooperation, Bangkok, Thailand. This microorganism grew on tryptic soy blood agar (TBA) at 37°C about 16-18 hours gave white to yellow color colony. The cells were harvested with NSS from at lease of 10 plates, 6 ml per plate.

2.2 Preparation of OMP and protein determination

The OMP of *P. multocida* 8:A was prepared by using sakosyl detergent incubated with sonicated cell of *P. multocida* 8:A. The OMP is a part of the outer membrane of the cell wall exposed on the cell surface (59), antigenic determinants of OMP were reported as the target for protective antibodies (65). Protein content

determined by Lowry method was estimated from standard curve. The bovine serum albumin was used as standard protein at concentration 50, 100, 200, 400, and 800 μg/ml. The result of protein concentration in OMP extracts was displayed in Table 11.

Table 11 OMP concentrations of *P. multocida* 8:A

Lot. Protein	*P. multocida* 8:A		OMP	
	Concentration (cfu/ml)	Volume (ml)	Concentration (μg/ml)	Volume (ml)
A	$9.1X10^9$	60	400	5
B	$6.5X10^9$	120	1500	3
C	$2.6X10^{10}$	240	1800	6
D	$7.8X10^9$	300	1875	8
E	$9.75X10^9$	480	4500	5

3. Analysis of OMP of *P. multocida* by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

The electrophoretic patterns of OMP of *P. multocida* 8:A was analyzed on 12% polyacrylamide gels. The molecular weights of antigens were estimated from the calibration curve of protein standards. The molecular weight of standard protein were in the range of 6.5-200 kDa.

In this investigation, SDS-PAGE was performed to examine protein profile of OMP from *P. multocida* 8:A on 12% gels and the result was showed in Figure 2. Protein pattern of all lots of OMP preparation were the same and were shown the major band at the MW of 37.5 kDa and minor bands of 23, 27.5, 45, and 97.4 kDa.

There were the same intensities in major and minor protein pattern among OMP preparations from lot A to lot E. In 1989, Choi et al. using the same method for preparation of OMP from reference strain of the 16 serotypes of *P. multocida*, reported that the polypeptide profiles revealed 2 to 4 major OMP in all 16 strains and most strains had major 34.5 kDa and 38 kDa protein bands (124). By using the

immunogen by SDS-PAGE analysis, OMP is composed of proteins measuring 24.6, 27, 30.4, 37.5, 49.5, and 64.5 kDa (12). These OMP profiles were identical to immunogenic determinant of OMP determined in rabbits isolate. Lee, et al. (125) also reported that the OMP of avian strains migrated in the range of 28-40 kDa.

The interest in the potential use of OMP for serotyping is rapidly increasing (126-129). Lugtenberg et al. (130) analyzed cell membrane preparations form pig isolates of *P. multocida* of unknown capsular serotypes by SDS-PAGE. Three different cell envelope profiles were distinguished on the basis of the electrophoretic mobilities of the heavy and weak proteins. Johnson et al. (131) determined the protein profiles of capsular serotype B and E strains isolated from animals with haemorrhagic septicaemia and placed the isolates in two distinct groups on the basis of the molecular masses (32 to 37 kDa) of the major proteins. Ireland et al. (132) showed that the major difference among avian isolates of serotype A was the location of a major protein band in the 34- to 38-kDa region.

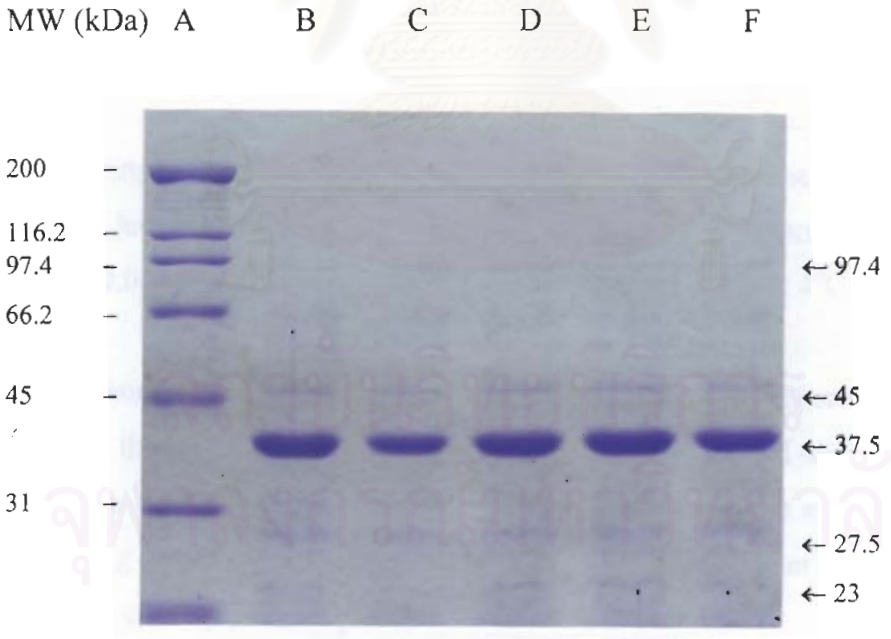


Figure 5 SDS-PAGE analysis of OMP extracts from *P. multocida* 8:A on 12% polyacrylamide slab gel stained with Coomassie blue. Lane A: Molecular weight protein markers, Lane B-F, OMP preparations from lot A-E, respectively.

4. Preparation of immunization antigen

4.1 Preparation of liposome containing OMP form *P. multocida* by double emulsion technique

White colloidal vesicles of liposome containing OMP from *P. multocida* was prepared by double emulsion technique with molar ratio of 1:1 of egg yolk lecithin and cholesterol that modified from the method of Kato et al. (119) and Sukanya (23). This technique was selected for preparing liposome containing protein extract from *P. multocida* because of some benefit factors. The first, it enables the polymer to become associated with surface of each bilayer in the multilamellar structure, resulted in a high stability. The second, because of its simplicity and reproducibility, the large scale production could be developed.

4.1.1 Percent protein entrapment

The percent protein entrapment of OMP from *P. multocida* 8:A in liposome was showed in Table 12. The percent protein entrapment of liposome OMP was 83.3,.81.5, 80.5 and 78.6 at 0, 4, 8, and 12 weeks after storage at 4°C, respectively. The result indicated that the liposome still contained the same percent protein entrapment during the time test ($p<0.05$). In 1996, Sukanya demonstrated that the 1:1 molar ratio of lecithin to cholesterol gave high entrapping efficiency and CMC in concentration 0.02% could remain the protein content over 90% at 4^{o}C for 3 months (23).

OMP from *P. multocida* is hydrophilic substance since entrapment was depended upon the volume of aqueous phase that was encapsulated during liposomes formation. The role of cholesterol on protein entrapment may be explained on the basis of swelling effect of the lipid membrane, that showed 10 water molecules were bound to each lecithin polar group (23).

Table 12 Percent protein entrapment of OMP in liposome prepared by double emulsion technique with 1:1 molar ratio of lecithin to cholesterol.

Duration of storage(weeks)	Percent protein entrapment
0	83.3±3.2
4	81.5±2.7
8	80.5±2.5
12	78.6±3.1

4.1.2 Particle size analysis

Table 13 showed the particle size of liposome containing OMP from *P. multocida* 8:A. The particle size was measured by Mastersizer S long bed (Malvern instruments Ltd., England), based on the principle of laser ensemble light scattering.

The protein concentration of freshly prepared liposome OMP of *P. multocida* 8:A at 1 mg/ml showed the diameter of 3.86, 3.90, 3.99, and 3.42 μm at 0, 4, 8, and 12 weeks after storage at 4^{o}C, respectively. The result indicated that the liposome still contained the same size during the time test: 12 weeks at 4^{o}C ($p<0.05$).

In previous study (120) the determination of dichloromethane (DCM) remaining in liposome during the primary emulsion step was done by using gas chromatography (GC). The result demonstrated that DCM concentration in liposome was not more than the maximum permissible concentration ($5x10^{4}$ ppm/ml in blood).

The effect of cholesterol on the liposome particle size could be explained on the basis of the influence of cholesterol uptake on the fluidity of the phosphatidyl choline bilayer (23), this result indicated that the larger liposome vesicle was formed when the cholesterol concentration was increased.

The particle size distribution of liposome and the cumulative undersize frequency curve of liposome OMP was shown in appendix I.

Table 13 Particle size of liposome prepared by double emulsion technique with 1:1 molar ratio of lecithin to cholesterol.

Duration of storage (weeks)	Average size (μm)
0	3.86±0.44
4	3.90±1.06
8	3.99±0.91
12	3.42±0.02

4.1.3 Liposome vesicles examined by transmission electron microscopy (TEM)

Figure 6 showed the negative staining of 1:1 molar ratio of egg yolk lecithin to cholesterol liposome containing OMP from *P. multocida* 8:A. They appeared to be round vesicles.

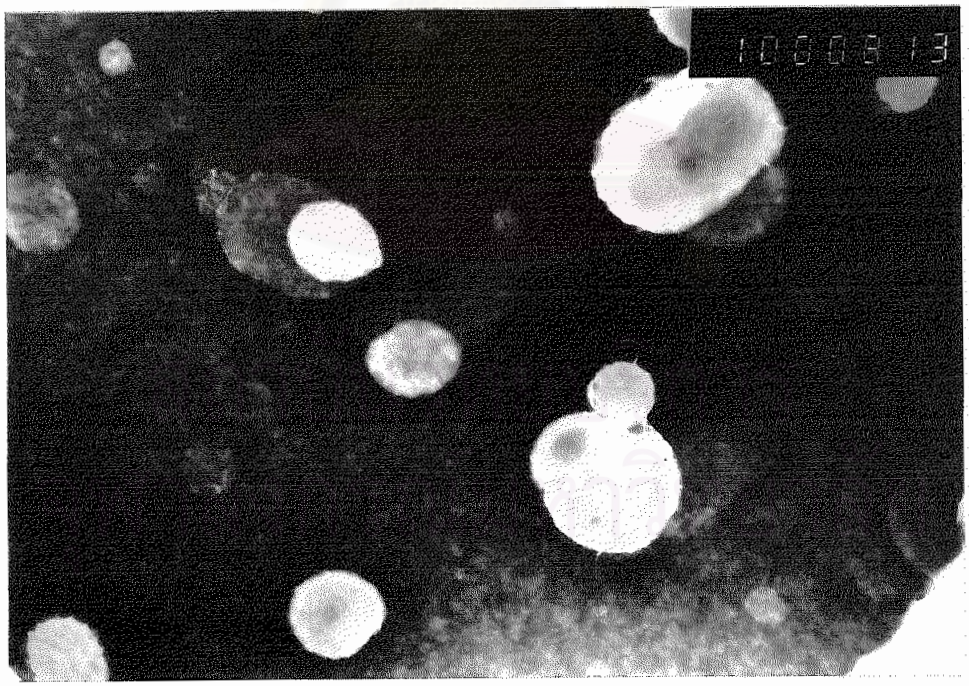


Figure 6 Transmission electron micrograph of liposome prepared by double emulsion technique with 1:1 molar ratio of lecithin to cholesterol, negative stained with phosphotungstic acid. (magnification 16,500x) (17mm = 1 μm)

4.1.4 Liposome vesicles examined by scanning electron microscopy (SEM)

Surface characteristics of liposomes were studied by using dehydrate method and cryo-SEM method.

A. Dehydrate method

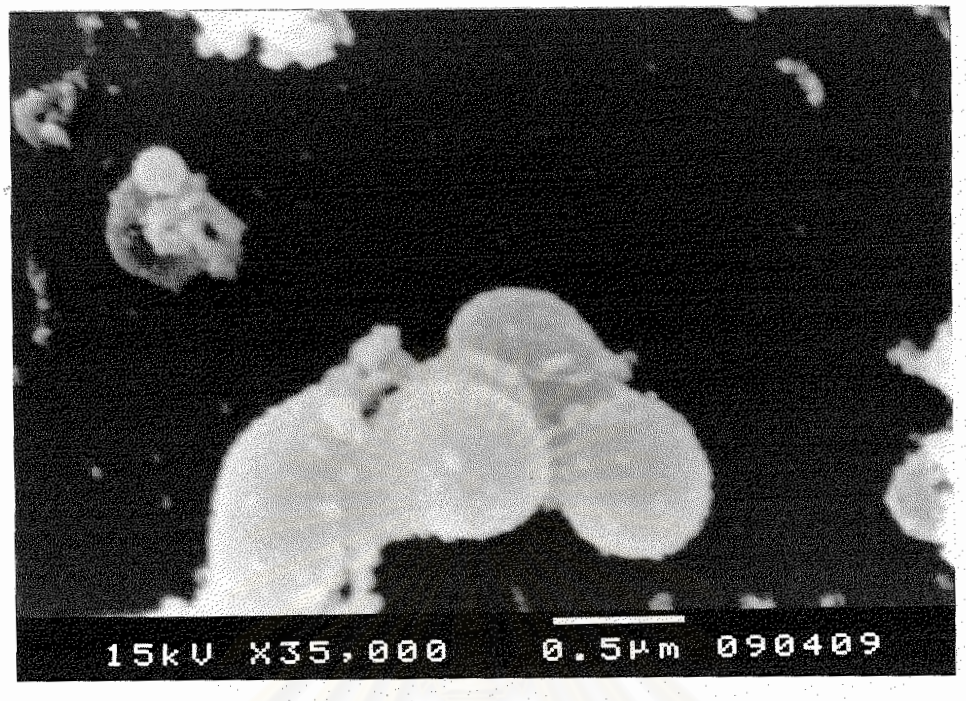
Figure 7a showed the shape, size, and aggregation of liposome OMP sample prepared by dehydrate method (Balzers, Liechtenstein).

B. Cryo-SEM method

Figure 7b showed the shape, size, and aggregation of liposome OMP sample prepared by cryo-SEM method (Joel, Japan).

They appeared to be round vesicles and smooth surface.

a.



b.

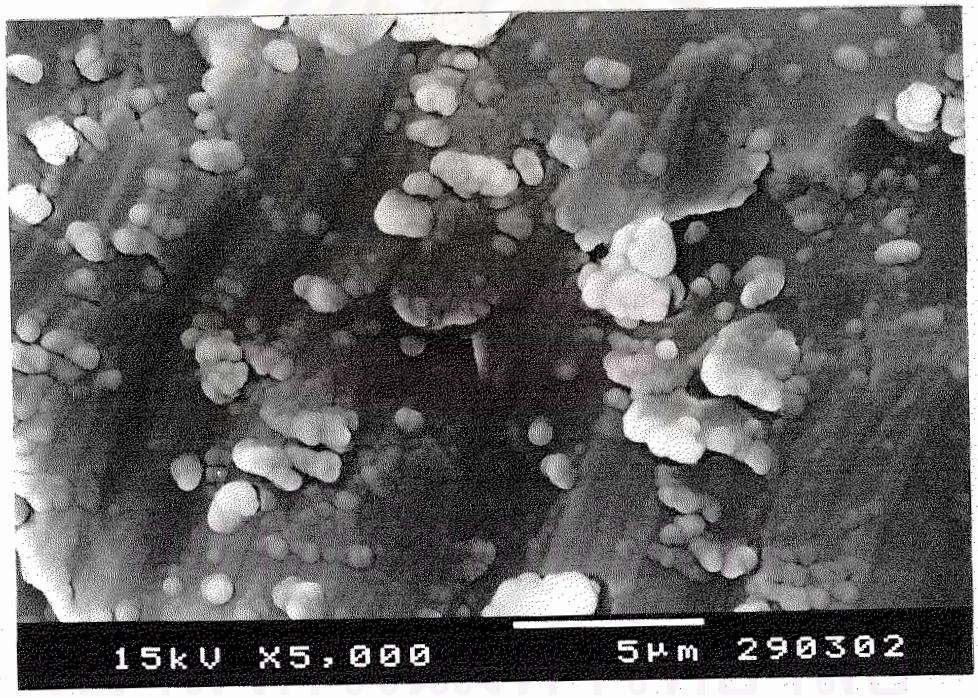


Figure 7 Scanning electron micrograph of liposome containing protein extract from *P. multocida* 8:A with 1:1 molar ratio of egg yolk lecithin to cholesterol; a., prepared by Dehydrate method (x35,000) ; b., prepared by Cryo-SEM method (x5,000)

4.1.5 Analysis of liposome OMP of *P. multocida* by SDS-PAGE

The protein patterns of OMP of *P. multocida* 8:A in liposome showed major band at the MW of 37.5 kDa and minor bands of 23, 27.5, 45, and 97.4 kDa, which was similar to protein patterns of OMP as shown in Figure 8. Thus, it was demonstrated liposome prepared by double emulsion technique showed the same characteristics as freshly prepared OMP.

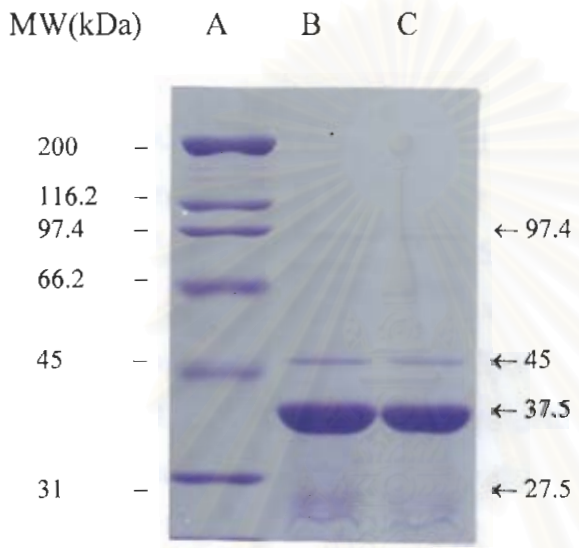


Figure 8 SDS-PAGE analysis of OMP and lioposome OMP from *P. multocida* 8:A on 12% polyacrylamide slab gels stained with Coomasie blue. Lane A: Molecular weight protein markers, Lane B: OMP preparation, Lane C: Liposomc OMP.

5. Determination of antibody titers of rabbit immune sera

Antibody titer of immune sera from rabbits immunized with formalin killed whole cell, liposome OMP, OMP with Freund's adjuvant, alum OMP and OMP without adjuvant were determined by enzyme-linked immunosorbent assay (ELISA). ELISA, a convenient, rapid and more sensitive method was used to determine the antibody titers of rabbit immune sera against whole cell and OMP of *P. multocida*. The immune response in rabbits were detected at day 0, 14, 28, 56, 72 and 85 after the first immunization. The absorbances of serial dilution of rabbit immune sera reacted with heat killed whole cell and OMP as coating antigen (10^7 cfu and 1 μg per well, respectively) in microtiter plate were demonstrated in Table 14-15 and Figure 9-10.

All of immune sera containing specific antibodies gave high antibody titer as shown in Figure 11-14. The difference in antibody response between group interaction was analyzed by Repeated Analysis (133) (Appendix II).

The average of antibody response of rabbits immunized with formalin killed whole cell, liposome OMP, OMP with Freund's adjuvant, alum OMP and OMP without adjuvant against whole cell (Figure 11-12) detected on day 42, 85, 85, 70 and 56 after the first immunization reached the highest titers of $1.44x10^5$, $6.11x10^8$, $1.41x10^8$, $1.38x10^6$, $2.94x10^5$, respectively. Alternatively, the average of antibody response of rabbits immunized with formalin killed whole cell, liposome OMP, OMP with Freund's adjuvant, alum OMP and OMP without adjuvant against OMP (Figure 13-14) detected on day 56, 70, 70, 70 and 70 after the first immunization reached the highest titers of $1.14x10^6$, $1.60x10^9$, $4.1x10^8$, $6.25x10^6$, $2.94x10^6$, respectively. The antibody responses induced by OMP with adjuvants were significantly different ($P< 0.05$) from OMP without adjuvant and formalin killed whole cell.

In the period of 12 weeks of immune sera collection, it was indicated that liposome OMP and Freund's adjuvant OMP induced the highest antibody titer compared to that of alum OMP, OMP and formalin killed whole cell.

Several researchers have demonstrated that protection of immunized mice against *P. multocida* infection was due to humoral immunity (HI) (134-136). Lu et al.(12) reported that IgG antibodies to major OMPs of *P. multocida* were abundantly present in the lung lavage sample of immunized rabbits. Both antibody-mediated killing by complement (137, 138) and antibody-mediated opsonophagocytosis (139-141) may be important mechanisms of HI against pasteurellosis. Marandi et al. (6) demonstrated that IgG2b monoclonal antibody to 32-kDa OMP were involved in the protection of mice against lethal challenge infection by means of opsonization and inhibition of proliferation of *P. multocida*, possibly as a result of an increased influx of PMNs into the secretion site.

The result from this study indicated that there were differences in lag times and durations of immune response elicited by different kind of antigen preparations. In addition, the extent of immune response may depend on route of administration, dose and frequency of infection. Further study should be investigated in other animal species especially chicken or ducks.

Table 14 Absorbance of rabbit immune sera against whole cell determined by ELISA.

Antigen	Reciprocal of serum dilution	Absorbance of preimmune sera	Absorbance of immune sera
Formalin killed whole cell	100	0.499	3
	1,000	0.156	3
	10,000	0.156	2.622
	100,000	0.136	0.874
	1,000,000	0.129	0.308
	10,000,000	0.105	0.231
	100,000,000	0.101	0.122
liposome OMP	1,000	1.128	3
	10,000	0.261	3
	100,000	0.108	3
	1,000,000	0.101	3
	10,000,000	0.101	2.513
	100,000,000	0.098	0.921
	1,000,000,000	0.089	0.125
Freund's adjuvant OMP	1,000	0.152	3
	10,000	0.146	3
	100,000	0.12	3
	1,000,000	0.113	3
	10,000,000	0.112	2.193
	100,000,000	0.107	0.438
	1,000,000,000	0.102	0.111
Alum OMP	100	1.711	3
	1,000	0.305	3
	10,000	0.283	3
	100,000	0.216	3
	1,000,000	0.155	0.892
	10,000,000	0.12	0.279
	100,000,000	0.112	0.169

Table 14 Absorbance of rabbit immune sera against whole cell determined by ELISA. (continued)

Antigen	Reciprocal of serum dilution	Absorbance of preimmune sera	Absorbance of immune sera
OMP	100	1.131	3
	1,000	0.203	3
	10,000	0.207	3
	100,000	0.156	1.545
	1,000,000	0.115	0.243
	10,000,000	0.107	0.092
	100,000,000	0.099	0.088
Liposome without OMP	100	1.116	1.759
	1,000	0.371	0.732
	10,000	0.163	0.240
	100,000	0.094	0.133
	1,000,000	0.103	0.119
	10,000,000	0.088	0.095
	100,000,000	0.081	0.082
PBS	100	2.176	1.968
	1,000	0.913	0.845
	10,000	0.551	0.526
	100,000	0.324	0.304
	1,000,000	0.24	0.207
	10,000,000	0.154	0.156
	100,000,000	0.115	0.127

Table 15 Absorbance of rabbit immune sera against OMP determined by ELISA.

Antigen	Reciprocal of serum dilution	Absorbance of preimmune sera	Absorbance of immune sera
Formalin killed whole cell	100	1.437	3
	1,000	0.298	3
	10,000	0.12	3
	100,000	0.117	3
	1,000,000	0.108	1.155
	10,000,000	0.098	0.359
	100,000,000	0.098	0.155
liposome OMP	100	1.892	3
	1,000	0.376	3
	10,000	0.124	3
	100,000	0.121	3
	1,000,000	0.123	3
	10,000,000	0.115	2.957
	100,000,000	0.102	1.861
Freund's adjuvant OMP	100	2.982	3
	1,000	0.787	3
	10,000	0.21	3
	100,000	0.107	3
	1,000,000	0.102	3
	10,000,000	0.098	1.019
	100,000,000	0.097	0.168
Alum OMP	100	1.259	3
	1,000	0.404	3
	10,000	0.184	3
	100,000	0.165	3
	1,000,000	0.12	1.465
	10,000,000	0.106	0.358
	100,000,000	0.104	0.128

Table 15 Absorbance of rabbit immune sera against OMP determined by ELISA. (continued)

Antigen	Reciprocal of serum dilution	Absorbance of preimmune sera	Absorbance of immune sera
OMP	100	1.908	3
	1,000	0.364	3
	10,000	0.136	3
	100,000	0.115	3
	1,000,000	0.085	1.135
	10,000,000	0.078	0.205
	100,000,000	0.072	0.108
Liposome without OMP	100	0.897	1.802
	1,000	0.303	0.594
	10,000	0.124	0.234
	100,000	0.096	0.130
	1,000,000	0.092	0.116
	10,000,000	0.080	0.091
	100,000,000	0.081	0.083
PBS	100	2.873	2.788
	1,000	1.185	1.282
	10,000	0.742	0.761
	100,000	0.464	0.476
	1,000,000	0.323	0.341
	10,000,000	0.289	0.216
	100,000,000	0.111	0.114

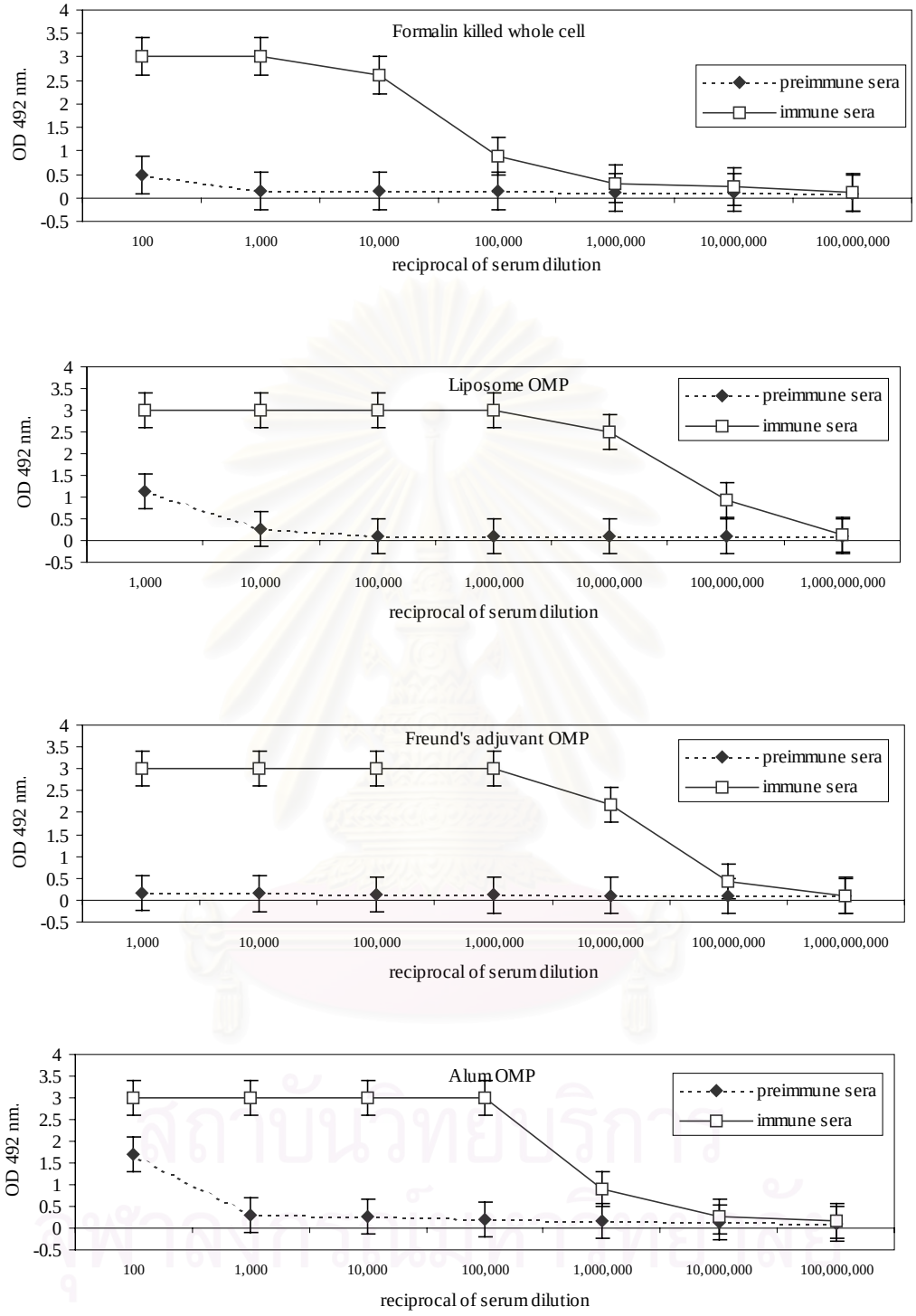


Figure 9 Absorbance of serial dilution of rabbit immune sera against whole cell determined by ELISA were compared to those from normal rabbit sera (n=5). Points indicated mean value, and bars represent standard deviation.

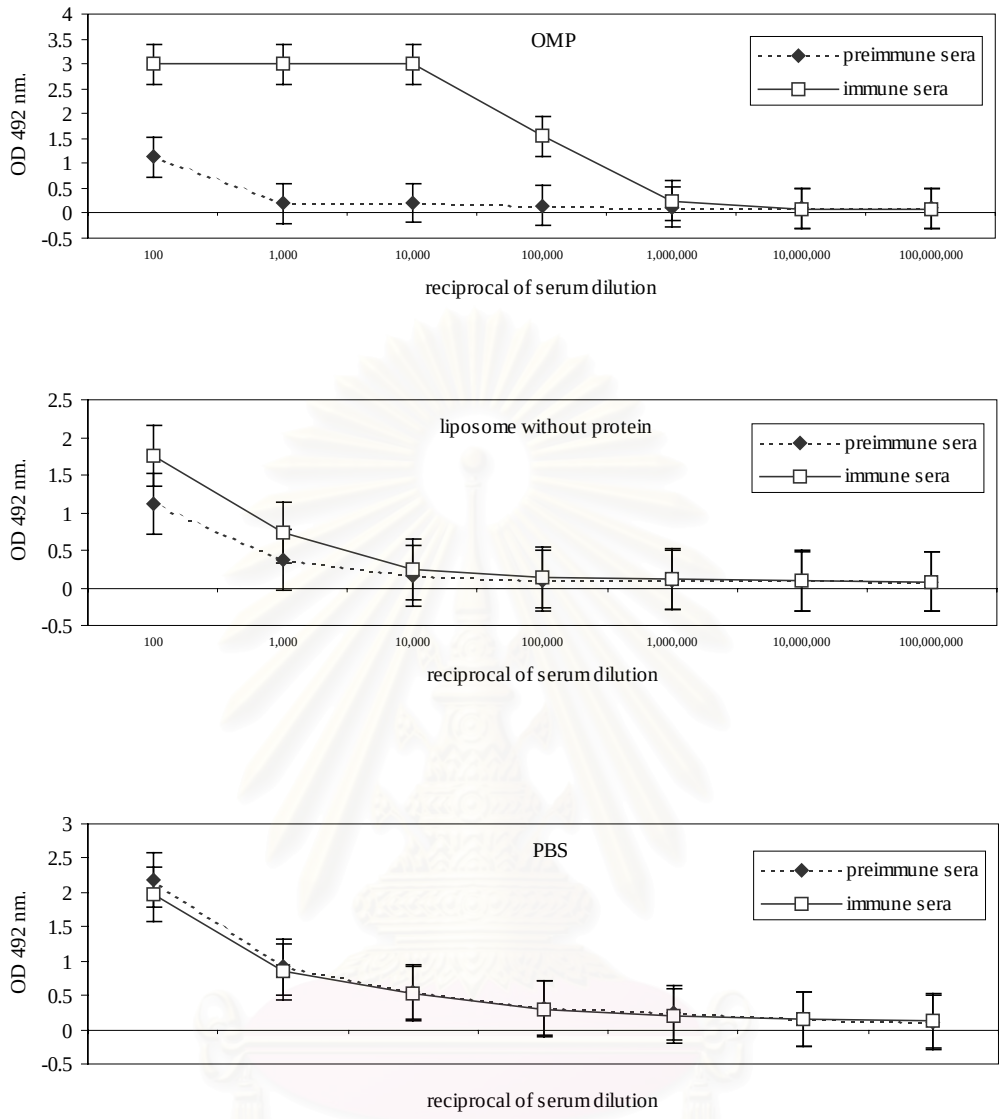


Figure 9 Absorbance of serial dilution of rabbit immune sera against whole cell determined by ELISA were compared to those from normal rabbit sera (n=5). Points indicated mean value, and bars represent standard deviation. (continued)

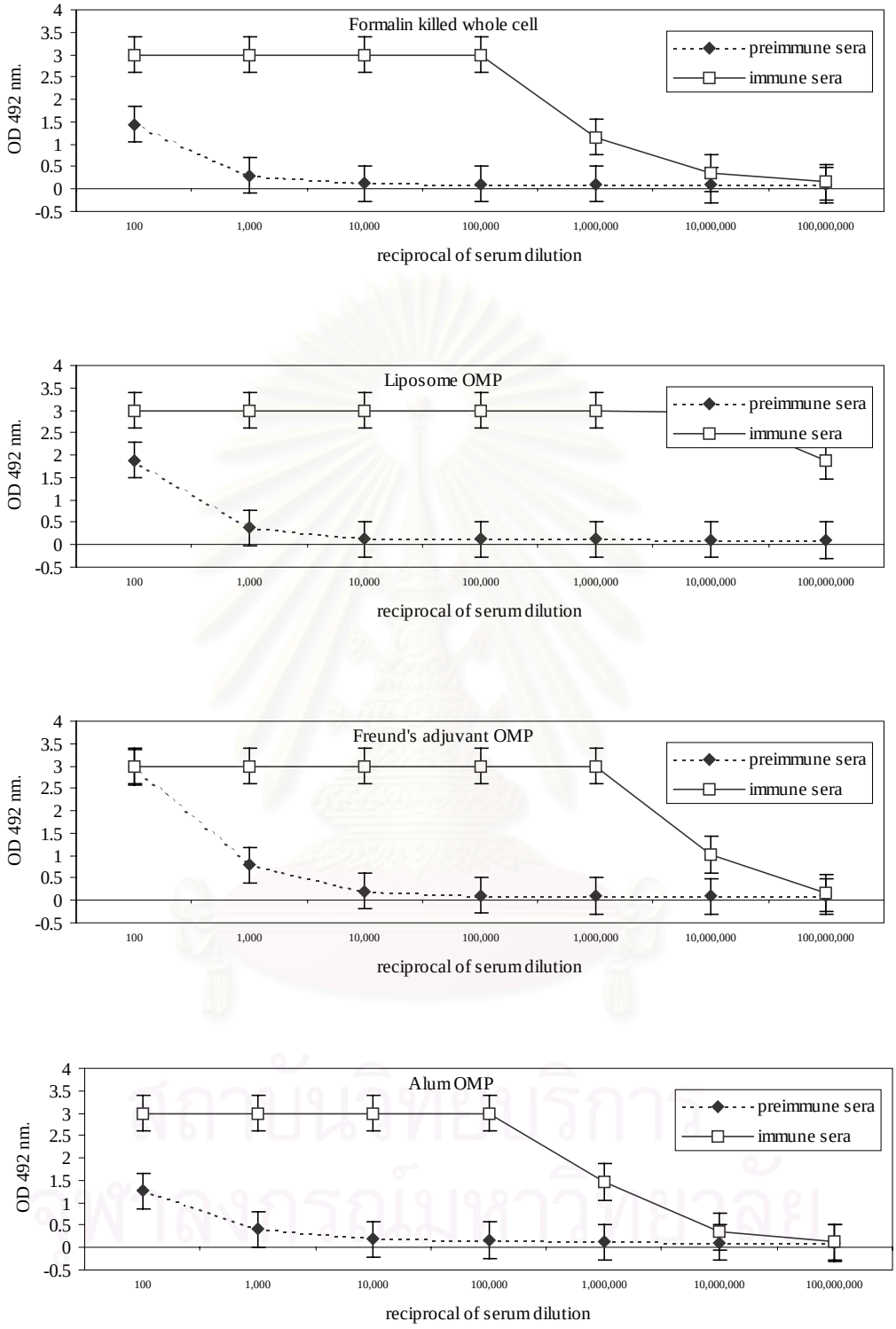


Figure 10 Absorbance of serial dilution of rabbit immune sera against OMP determined by ELISA were compared to those from normal rabbit sera (n=5). Points indicated mean value, and bars represent standard deviation.

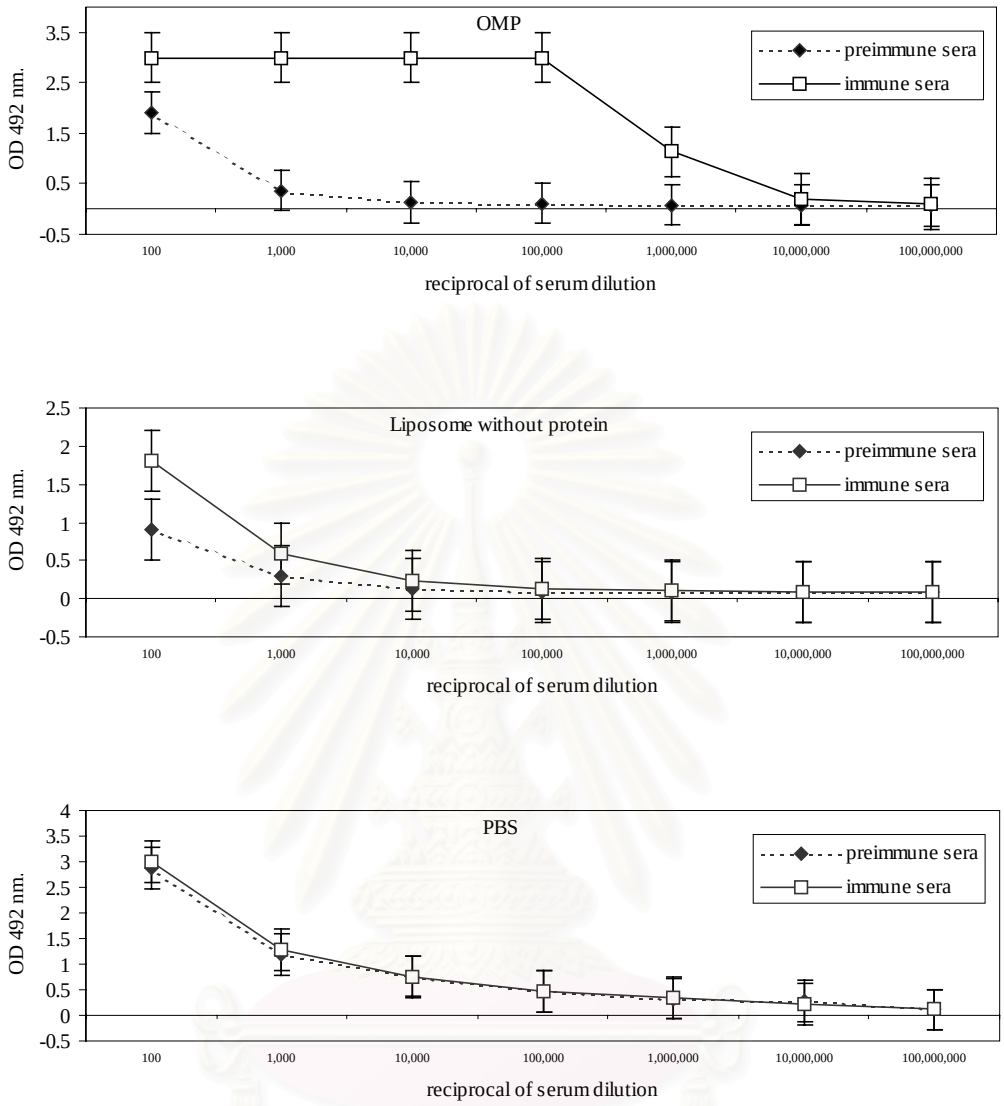


Figure 10 Absorbance of serial dilution of rabbit immune sera against OMP determined by ELISA were compared to those from normal rabbit sera (n=5). Points indicated mean value, and bars represent standard deviation. (continued)

Day	Antibody titers						
	Whole cell	Liposome OMP	Freund's adjuvant OMP	Alum OMP	OMP	Ghost liposome	PBS
0	240	220	240	300	340	270	430
14	4500	19300	15520	7000	9770	330	350
28	24900	107520	48320	7633	9800	590	550
42	144400	531560	594000	37033	208000	550	250
56	104080	8740000	3535000	1052500	294640	720	340
70	65860	74340000	108370000	1375000	267400	400	650
85	49880	611200000	141650000	820000	71200	600	800

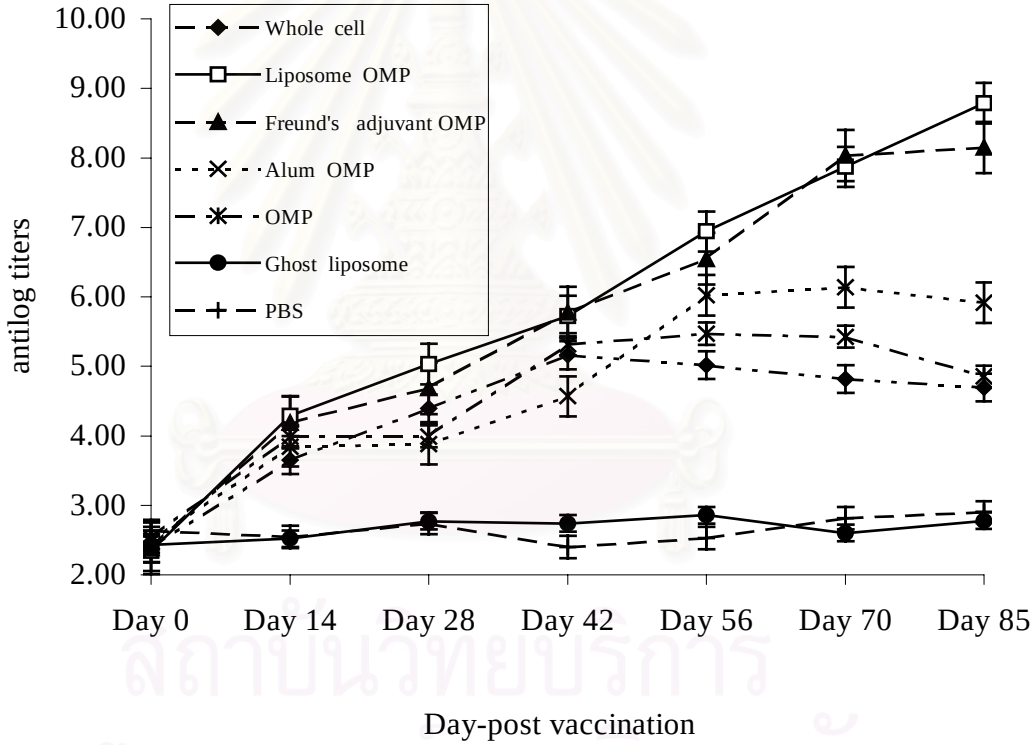


Figure 11 Antibody titer determined by ELISA of antisera from rabbits (n=5) immunized subcutaneously on day 0 and 28 with whole cell, liposome OMP, Freund's adjuvant OMP, alum OMP, OMP, ghost liposome, and PBS against whole cell.

Day	Antibody titers						
	Whole cell	Liposome OMP	Freund's adjuvant OMP	Alum OMP	OMP	Ghost liposome	PBS
0	240	220	240	300	340	270	430
14	4500	19300	15520	7000	9770	330	350
28	24900	107520	48320	7633	9800	590	550
42	144400	531560	594000	37033	208000	550	250
56	104080	8740000	3535000	1052500	294640	720	340
70	65860	74340000	108370000	1375000	267400	400	650
85	49880	611200000	141650000	820000	71200	600	800

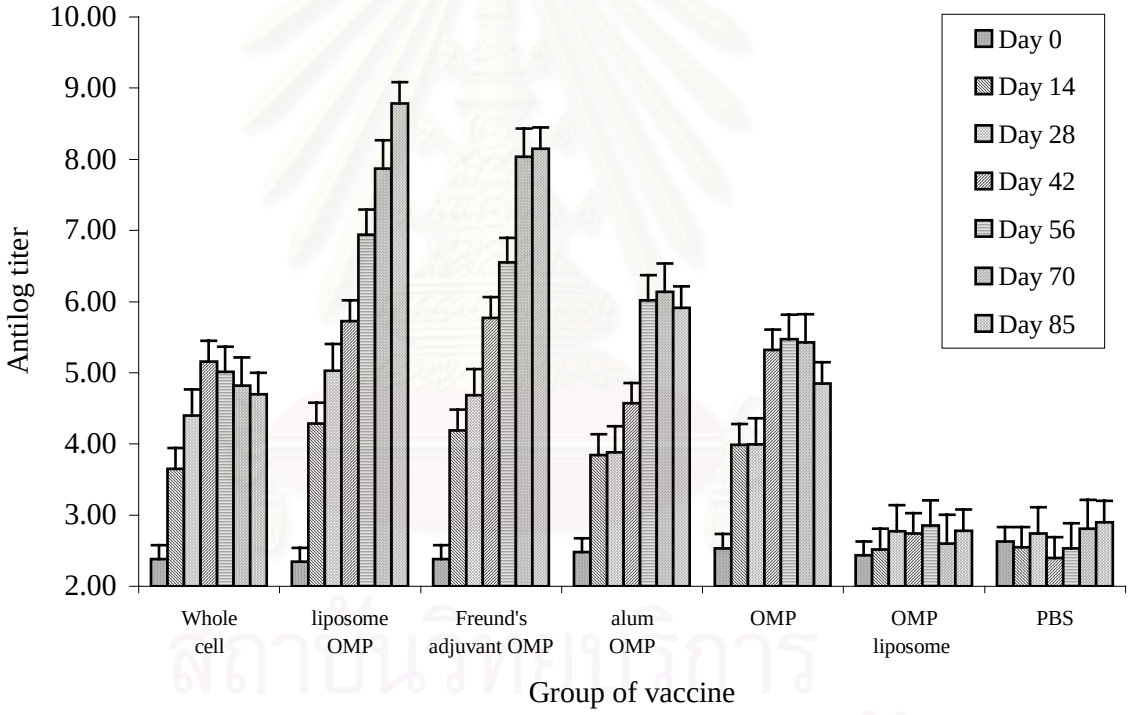


Figure 12 Comparison of antibody titers of immune sera (n=5) against whole cell by ELISA on day 0, 14, 28, 42, 56, 70, and 85.

	Antibody titers

Day	Whole cell	Liposome OMP	Freund's adjuvant OMP	Alum OMP	OMP	Ghost liposome	PBS
0	540	440	560	500	500	350	450
14	40300	38920	47280	9200	20992	550	440
28	35720	275520	157280	46933	21150	840	650
42	385010	3137400	2698140	351200	2422000	620	430
56	1138000	343000000	2073000	4690000	668010	850	680
70	219250	1600000000	410000000	6250000	876500	600	880
85	293140	1570000000	366000000	5878667	435210	750	950

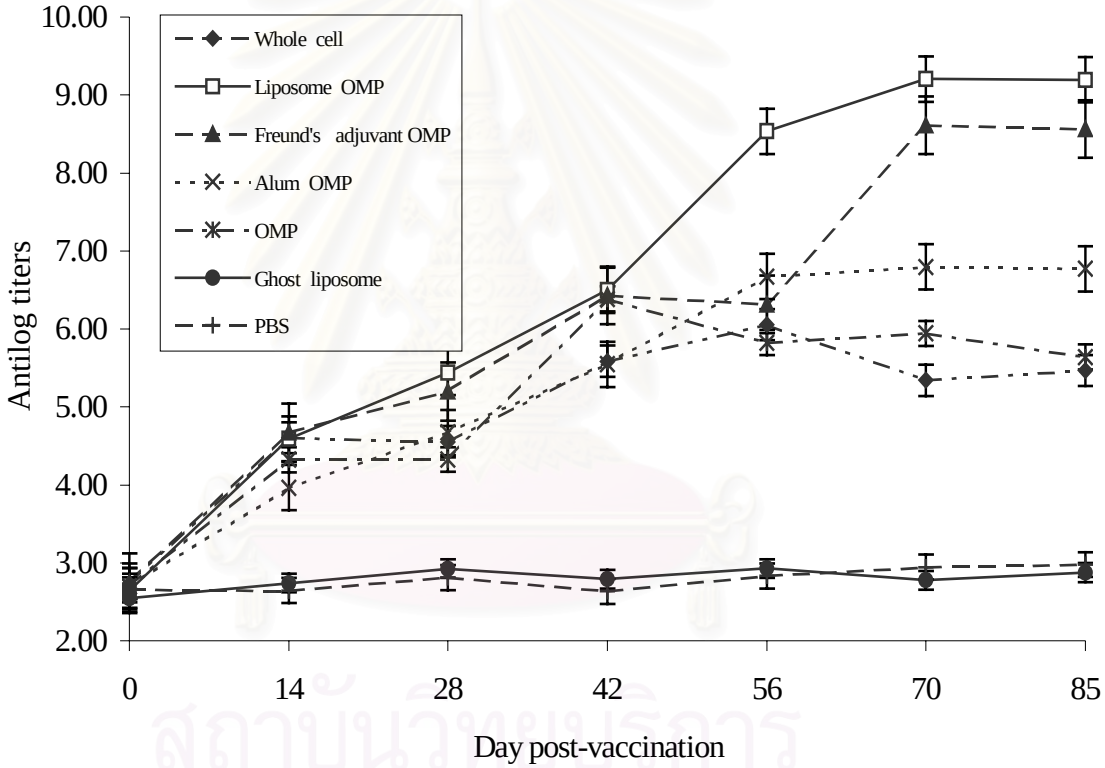


Figure 13 Antibody titer determined by ELISA of antisera from rabbits (n=5) immunized subcutaneously on day 0 and 28 with whole cell, liposome OMP, Freund's adjuvant OMP, alum OMP, OMP, ghost liposome, and PBS against OMP.

	Antibody titers

Day	Whole cell	Liposome OMP	Freund's adjuvant OMP	Alum OMP	OMP	Ghost liposome	PBS
0	540	440	560	500	500	350	450
14	40300	38920	47280	9200	20992	550	440
28	35720	275520	157280	46933	21150	840	650
42	385010	3137400	2698140	351200	2422000	620	430
56	1138000	343000000	2073000	4690000	668010	850	680
70	219250	1600000000	410000000	6250000	876500	600	880
85	293140	1570000000	366000000	5878667	435210	750	950

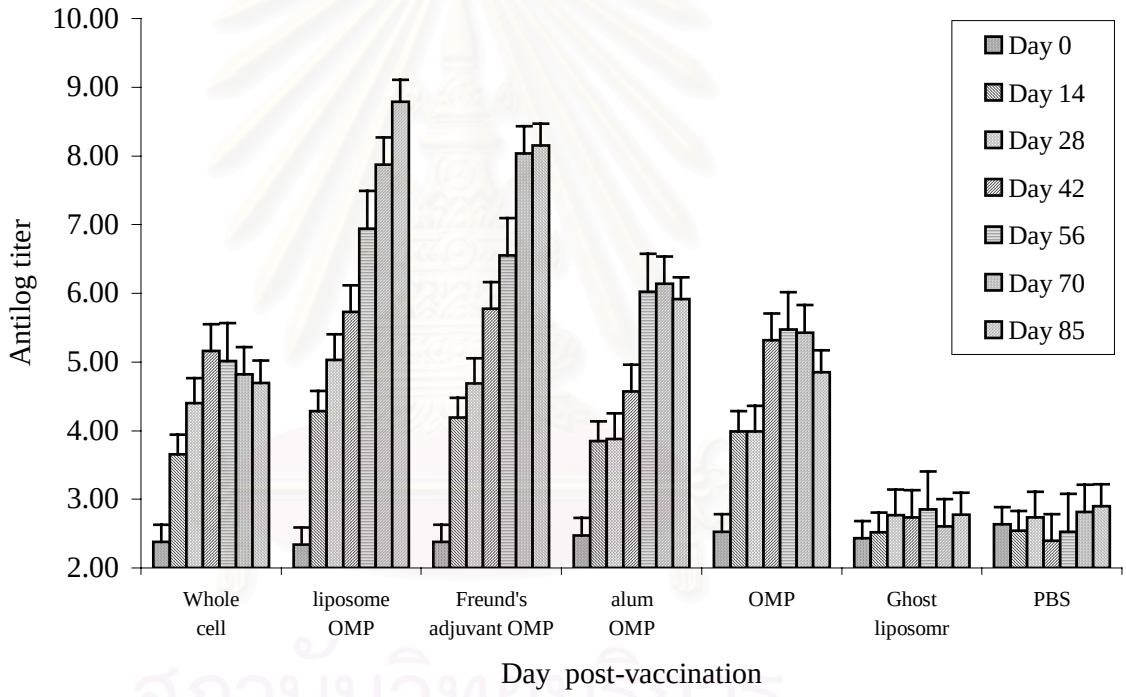


Figure 14 Comparison of antibody titers of immune sera (n=5) against OMP by ELISA on day 0, 14, 28, 42, 56, 70, and 85.

6. Determination of bactericidal activity of immune sera

Bactericidal activities of immune sera from rabbits were showed in Figure 15. The comparisons between bactericidal activities and antibody titers of immune sera immunized with formalin killed whole cell, liposome OMP, Freund's adjuvant OMP, alum OMP, and OMP, against whole cell and OMP determined by ELISA were exhibited in Figure 16-20. The bactericidal activity of rabbit immunized with formalin killed whole cell, liposome OMP, alum OMP and OMP reached the maximum on day 42 (64.53$\pm$1.68), 85 (94.12$\pm$4.16), 70 (65.05$\pm$1.70) and 56 (77.68$\pm$1.84) respectively which were the same as antibody titers against whole cell. While the bactericidal activity of rabbit immunized with Freund's adjuvant reached the maximum on day 70 (89.38$\pm$2.77) which were the same as antibody titers against OMP. The immune sera obtained from rabbits immunized with liposome OMP and Freund's adjuvant OMP elicited higher bactericidal capacity than immune sera from alum OMP, OMP and formalin killed whole cell ($P < 0.05$).

Hyperimmune rabbit sera against *P. multocida* 8:A were included as positive control. The bactericidal capacity of positive control were in range of 95.5-99.5%. Complement and barbital buffer that included as negative control showed zero percent bactericidal capacity. The bactericidal data reported in this study clearly showed that serum form rabbits immunized with all adjuvant could kill *P. multocida* 8:A under the experimental test conditions. As the bactericidal reaction is important for protection, the finding of killing activity provides some theoretical basis for understanding how antibody might be efficient in animal protection against infection.

The bactericidal assay was rapid method that gave little variation amongst replicates and was comparable on a day-to-day basis (122). MacDonale et al. (141) showed *that P. haemolytica* was not killed in the absence of a functional classical complement pathway. Also, the standard positive serum was not bactericidal unless in combination with complement, indicating that bactericidal activity was due to an antibody-complement complex.

During the optimization of the bactericidal assay both complement and specific antibodies were found to be essential components indicating that this activity was operating via the classical pathway of complement activation and that in the absence of antibody the alternative pathway was not activated. Antibody-mediated killing by the classical pathway of complement therefore appears to be an important

mechanism of immunity against *P. multocida* and can be stimulated by vaccination(125). Previous studies have shown that IgM is more active than IgG in bactericidal assays (142-144), although the activity of IgG has been found in rabbit serum to increase during sequential immunization (145).

The investigation of the bactericidal activities of sera suggested that a cell-surface antigen was associated with this mechanism of immunity, because the bactericidal capacity of sera was closely correlated with whole-cell ELISA titers. Although the normal sera showed no bactericidal activities, the immune sera showed higher degree of killing.

Day	Bactericidal activity (%)						
	Whole cell	Liposome OMP	Freund's adjuvant OMP	Alum OMP	OMP	Ghost liposome	PBS
0	0.79±2.70	1.60±3.70	0.56±2.00	0.28±1.40	0.11±2.50	1.10±1.50	0.00±1.35
14	4.50±2.39	18.54±3.03	12.14±3.03	14.10±1.72	16.56±1.35	0.50±0.88	0.80±1.11
28	28.21±2.61	38.17±3.09	30.43±2.50	22.27±1.48	15.92±2.70	0.00±1.42	1.50±1.50
42	64.53±1.68	77.84±2.68	78.44±1.72	50.07±2.16	71.19±2.90	0.80±1.50	0.00±0.88
56	61.90±1.86	83.99±2.80	82.14±1.67	59.92±1.70	77.68±1.84	1.60±0.45	1.10±0.93
70	62.01±2.90	93.02±1.34	89.38±2.77	65.05±1.70	76.9±2.81	0.00±0.87	0.70±1.20
85	53.43±3.53	94.12±1.46	88.08±2.05	61.69±1.88	50.45±4.27	1.20±1.47	0.00±1.12

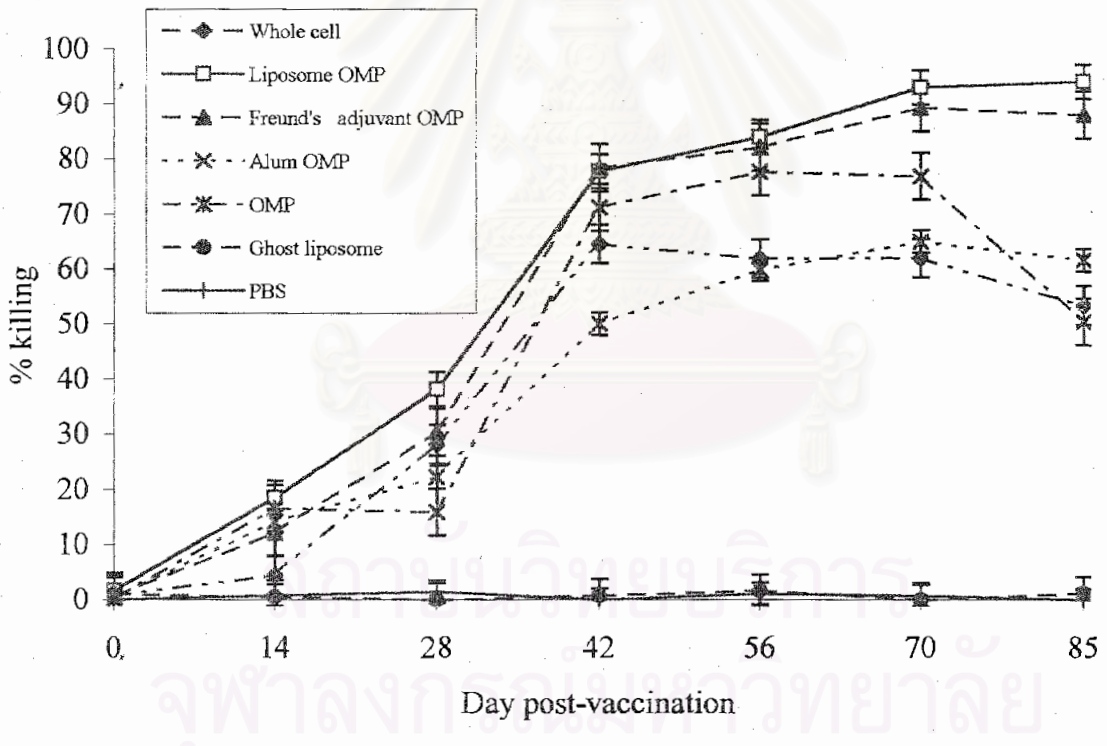


Figure 15 Bactericidal activity of rabbits immune sera (n=5) immunized with formalin killed whole cell, liposome OMP, Freund's adjuvant OMP, alum OMP, OMP, ghost liposome, and PBS .

Day	Immune sera of rabbit immunized with formalin killed whole cell		
	ELISA titers		% killing
	against whole cell	against OMP	
0	240	540	0.79
14	4500	40300	4.5
28	24900	35720	28.21
42	144400	385010	64.53
56	104080	1138000	61.9
70	65860	219250	62.01
85	49880	293140	53.43

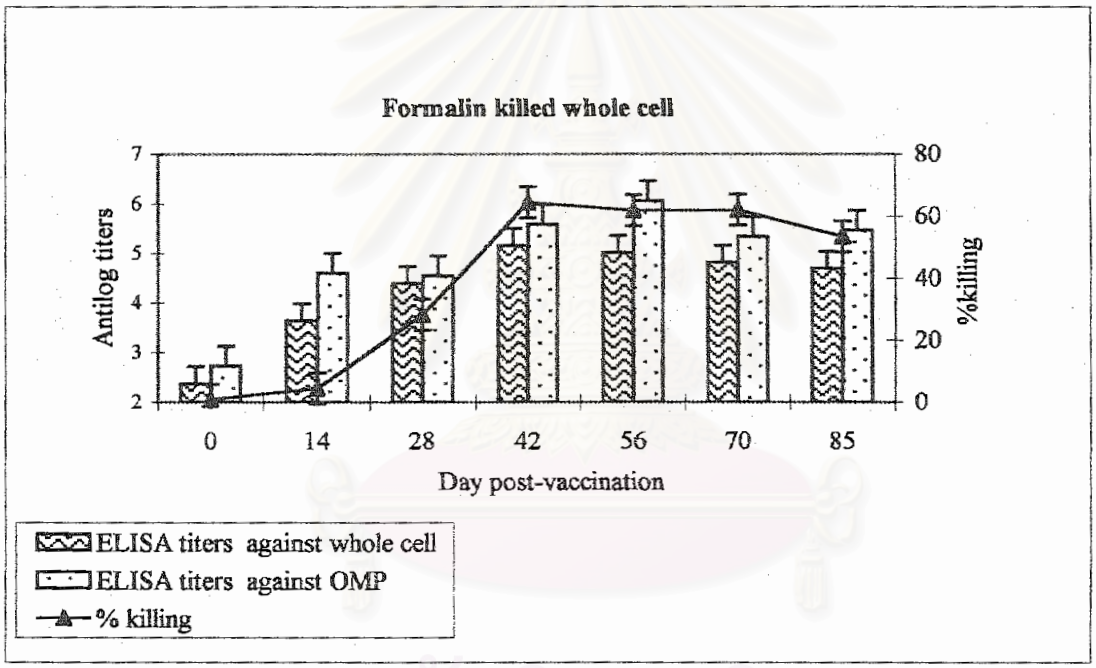


Figure 16 Comparison between percent killing of bactericidal activity and antibody titers of formalin killed whole cell against whole cell and OMP.

Day	Immune sera of rabbit immunized with liposome OMP		
	ELISA titers		% killing
	against whole cell	against OMP	
0	220	440	1.6
14	19300	38920	18.54
28	107520	275520	38.17
42	531560	3137400	77.84
56	8740000	343000000	83.99
70	74340000	1600000000	93.02
85	611200000	1570000000	94.12

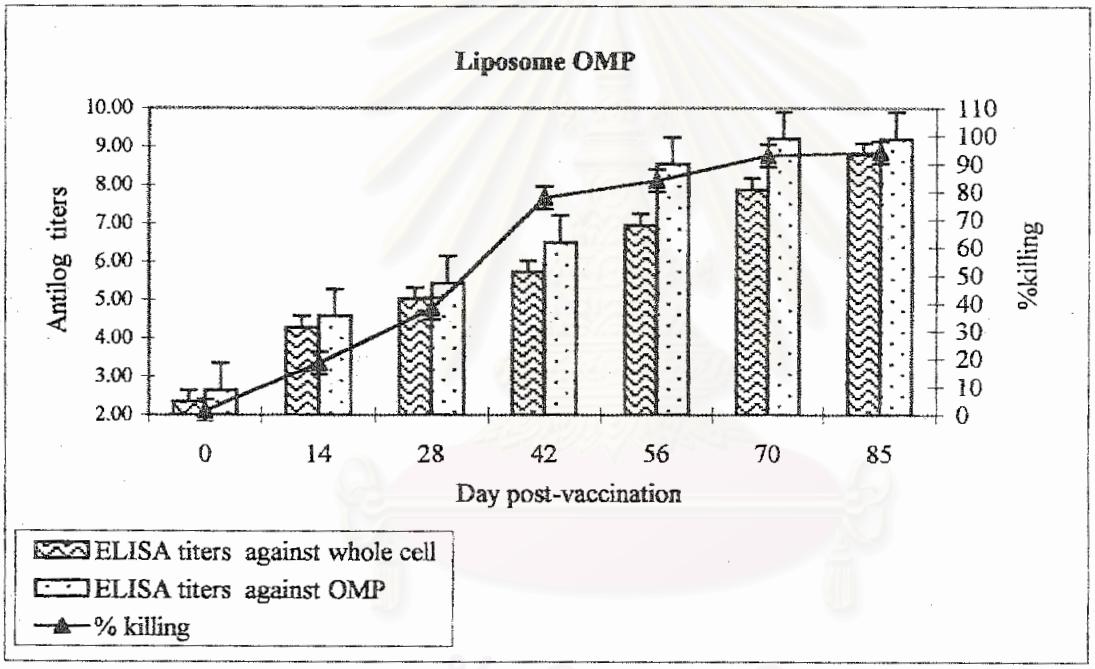


Figure 17 Comparison between percent killing of bactericidal activity and antibody titers of liposome OMP against whole cell and OMP.

Day	Immune sera of rabbit immunized with Freund's adjuvant OMP		
	ELISA titers		% killing
	against whole cell	against OMP	
0	240	560	0.56
14	15520	47280	12.14
28	48320	157280	30.43
42	594000	2698140	78.44
56	3535000	2073000	82.14
70	108370000	410000000	89.38
85	141650000	366000000	88.08

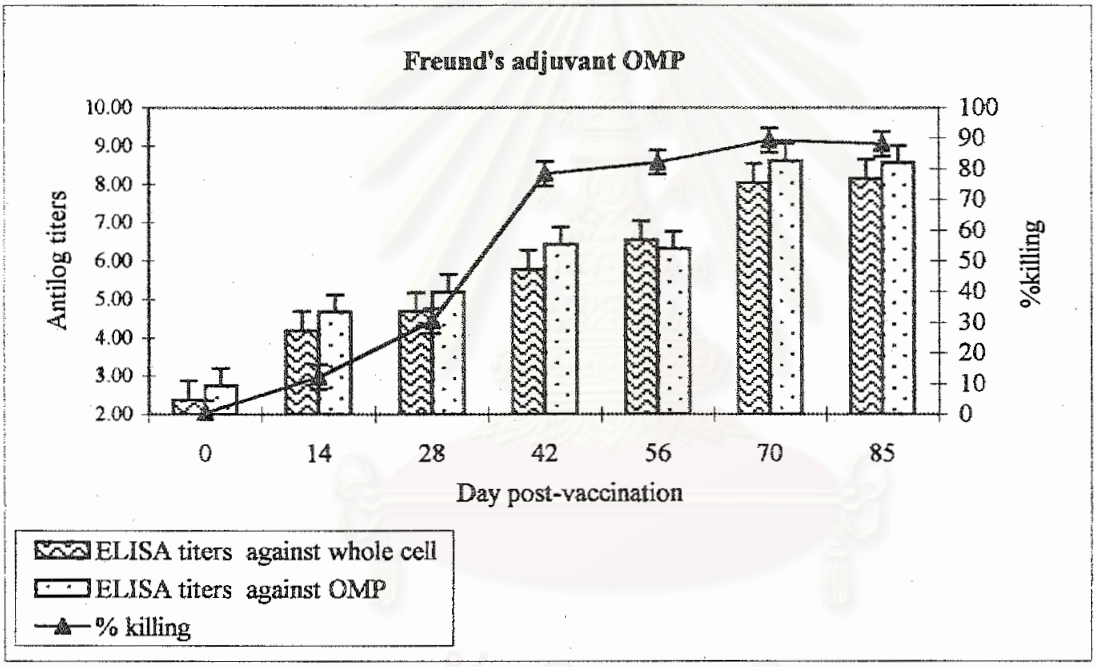


Figure 18 Comparison between percent killing of bactericidal activity and antibody titers of Freund's adjuvant OMP against whole cell and OMP.

Day	Immune sera of rabbit immunized with alum OMP		
	ELISA titers		% killing
	against whole cell	against OMP	
0	300	500	0.28
14	7000	9200	14.1
28	7633	46933	22.27
42	37033	351200	50.07
56	1052500	4690000	59.92
70	1375000	6250000	65.05
85	820000	5878667	61.69

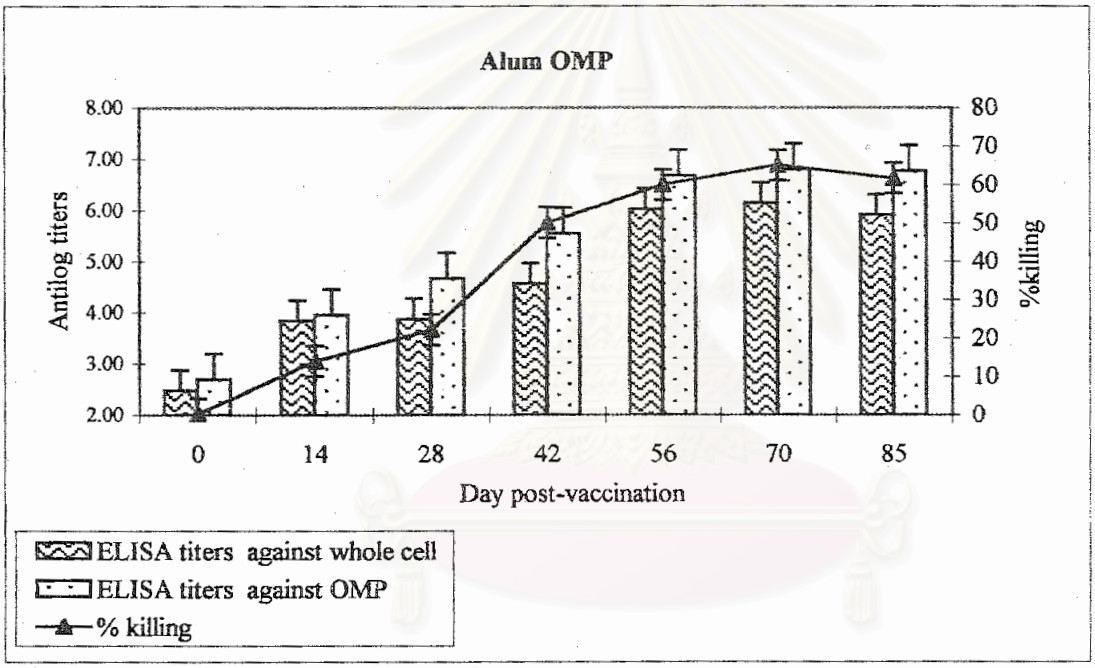


Figure 19 Comparison between percent killing of bactericidal activity antibody titers of alum OMP against whole cell and OMP.

Day	Immune sera of rabbit immunized with OMP		
	ELISA titers		% killing
	against whole cell	against OMP	
0	340	500	0.11
14	9770	20992	16.56
28	9800	21150	15.92
42	208000	2422000	71.19
56	294640	668010	77.68
70	267400	876500	76.9
85	71200	435210	50.45

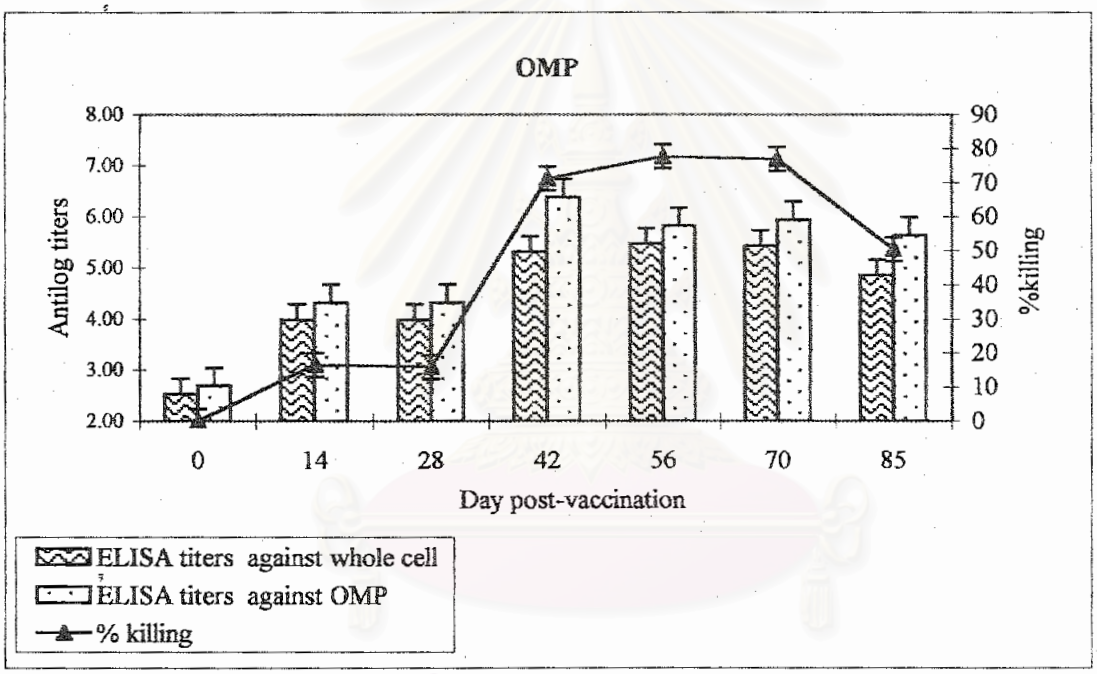


Figure 20 Comparison between percent killing of bactericidal activity and antibody titers of OMP against whole cell and OMP.

7. Western blot analysis of antigen from *P. multocida* 8:A with rabbits immune sera

In western blot analysis, OMP from *P. multocida* 8:A was transferred from polyacrylamide gels to nitrocellulose membrane, cut into strips and probed with rabbits immune sera against formalin killed whole cell, liposome OMP, Freund's adjuvant OMP, alum OMP, OMP, and preimmune sera of all antigen (used as control). The result was showed in Figure 21 and demonstrated that major band of 37.5 kDa was recognized by all immune sera.

Western blot analysis of KSCN antigen extract of *P. multocida* with all immune sera was shown in Figure 22. The result revealed that the proteins recognized by immune sera were between 31 and 97.4 kDa. Strong reactions were shown against 37.5 kDa protiens with all rabbit immune sera, protein 55 kDa showed weak reaction with immune sera against whole cell, liposome OMP, Freund's adjuvant OMP and OMP, protein 26 and 31 kDa reacted with immune sera against OMP.

It was reported in the western blot analysis of OMP that homologous immune sera against antigen extract recognized major proteins at 27, 37.5, 49.5, 58.7, and 64.4 kDa (12). The result indicated that OMP immunogen was exposed on the cell surface and accessibled by antibodies, since adsorption of immune sera with intact *P. multocida* resulted in a significantly reduction of antibody activities, especially the 37.5 kDa and the 37.5 kDa appeared to be the major protein of OMP. In 1991, Lu et al. (13) demonstrated that monoclonal antibody 1608 (146) directed against 37.5 kDa could recognize protective protein immunogen on the OMP of 24% clinical isolates of *P. multocida*. This monoclonal antibody protected mice, rabbits with passive immunization against afforded to be vaccine candidate in many reports (13).

The OMP and KSCN extract of *P. multocida* 8:A were recognized by most immune sera with strong reaction on 37.5 kDa and weak reaction on various protein components, normal rabbit sera showed no reaction with both antigens. Some factors such as heat during transfer of antigen to nitrocellulose and the method of OMP preparation with various adjuvants, such as preparation of liposome OMP, protein must be exposed to organic solvent, preparation of alum OMP and Freund's adjuvant OMP, the components must be mixed vigorously, that may be the cause of

denaturation and change in antigenic determinants of protein, and the weak reaction with immune sera were observed.

Figure 21 Western blot analysis of OMP from *P. multocida* 8:A probed with rabbits immune sera against antigens. Lane A: Molecular weight protein markers, Lane B, D, F, H, and J : Rabbit preimmune sera against formalin killed whole cell, liposome OMP, Freund's adjuvant OMP, alum OMP and OMP respectively: Lane C, E, G, I, and K: Rabbit immune sera against formalin killed whole cell, liposome OMP, Freund's adjuvant OMP, alum OMP, and OMP, respectively.

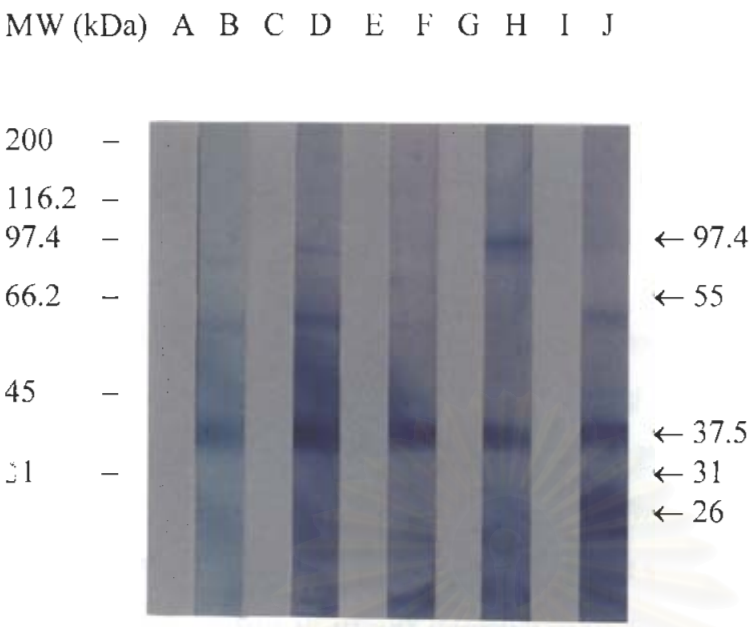


Figure 22 Western blot analysis of KSCN from *P. multocida* 8:A probed with rabbits immune sera against antigens. Lane A, C, E, G, and I : Rabbit preimmune sera against formalin killed whole cell, liposome OMP, Freund's adjuvant OMP, alum OMP and OMP respectively: Lane B, D, F, H, and J: Rabbit immune sera against formalin killed whole cell, liposome OMP, Freund's adjuvant OMP, alum OMP, and OMP, respectively.

CHAPTER V

CONCLUSION

The particle size of liposome containing OMP from *P. multocida* 8:A prepared by double emulsion technique was 3.86, 3.90, 3.99, and 3.42 μm in diameter and the percent protein entrapment of liposome was 83.3, 81.5, 80.5, and 78.6 at week 0, 4, 8, and 12, respectively, indicated that there was no significant difference between time of storage and particle size of liposome OMP when kept at 4^{o}C for 12 weeks.

Surface characteristic of freshly prepared liposome OMP was studied by TEM and SEM indicated that liposome OMP appeared round vesicle and smooth surface.

The immunogenicity of liposome vaccine containing OMP of *P. multocida* 8:A and OMP emulsified with Freund's adjuvant that investigated in rabbits elicited significantly higher antibody titer than in rabbits vaccinated with formalin killed whole cell, alum OMP, and OMP without adjuvant ($p<0.05$) as indicated by ELISA titer against heat killed whole cell and OMP of *P. multocida*. This may indicate that the OMP contains the major immunodominant protein and the concentration of OMP was not adequate to give rise to such high antibody titers. Bouzoubaa et al and Pati et al (147, 148) showed that OMP confererd better protection than whole bacteria. The use of adjuvant has been well documented as enhancing the immunogenicity of OMP vaccine (149, 150). Multiple mechanisms probably underlie the adjuvant effect of liposome (21). Liposome presumably provide depot effect and the removal of antigen from the injection site is primarily accomplished by macrophage, which avidly phagocytose liposomes. After ingesting the antigen-bearing liposomes, the macrophage assumes the role of antigen presenting cell. Aving et al (21) indicated that liposomal antigen that is taken up by macrophages is processed and present to helper T lymphocytes. This result indicated the liposome was not only processed by macrophages, but it also directly affect B and T lymphocytes. The magnitude of this effect also depends on bilayer composition (18). However, the main impediments to the use of conventional liposome as vaccine adjuvants have been the need to formulate complex mixtures with other immunoadjuvant, the consequent undertainty of safety in human, and the lack of cost-effectiveness in obtaining purified lipids

(151). The result of this study provide preliminary that liposome OMP vaccine may be useful for the control of *P. multocida* in pasteurellosis.

The investigation of the bactericidal activities of sera suggested that the liposome OMP and OMP emulsified with Freund's adjuvant produced higher bactericidal activity than other vaccines used in this study ($p<0.05$). The maximum killing activities were 94.12 ± 1.46 at day 85 and 89.38 ± 2.77% at day 70 for anti-liposome OMP and anti-Freund's adjuvant OMP, respectively.

The result of western blot analysis of OMP and KSCN were reported that all rabbit immune sera against vaccines in this study recognized major proteins at 37.5 kDa, which appeared to be the major protein of OMP.

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APPENDIX I

Particle size distribution data
of liposome outer membrane protein from *Pasteurella multocida* 8:A

Particle size distribution data of freshly prepared outer membrane protein liposome from *Pasteurella multocida* 8:A.

Size low (um)	Frequency %	Size high (um)	Cumulative under %
0.05	0.03	0.06	0.03
0.06	0.06	0.07	0.08
0.07	0.09	0.08	0.17
0.08	0.13	0.09	0.30
0.09	0.18	0.11	0.48
0.11	0.25	0.13	0.73
0.13	0.35	0.15	1.08
0.15	0.49	0.17	1.56
0.17	0.70	0.20	2.26
0.20	0.99	0.23	3.25
0.23	1.35	0.27	4.60
0.27	1.66	0.31	6.26
0.31	1.83	0.36	8.09
0.36	1.90	0.42	9.99
0.42	2.03	0.49	12.02
0.49	2.19	0.58	14.21
0.58	2.24	0.67	16.45
0.67	2.32	0.78	18.77
0.78	2.31	0.91	21.08
0.91	2.36	1.06	23.44
1.06	2.44	1.24	25.88
1.24	2.61	1.44	28.49
1.44	2.91	1.68	31.40
1.68	3.40	1.95	34.8
1.95	4.12	2.28	38.92
2.28	5.01	2.65	43.93
2.65	5.99	3.09	49.91
3.09	6.90	3.60	56.81
3.60	7.65	4.19	64.46

Particle size distribution data of freshly prepared outer membrane protein liposome from *Pasteurella multocida* 8:A.

Size low (um)	Frequency %	Size high (um)	Cumulative under %
4.19	8.24	4.88	72.7
4.88	7.66	5.69	80.36
5.69	6.46	6.63	86.82
6.63	4.89	7.72	91.71
7.72	3.25	9.00	94.96
9.00	1.82	10.48	96.79
10.48	0.77	12.21	97.56
12.21	0.15	14.22	97.7
14.22	0.00	16.57	97.7
16.57	0.00	19.31	97.7
19.31	0.16	22.49	97.87
22.49	0.39	26.20	98.26
26.2	0.50	30.53	98.76
30.53	0.47	35.56	99.23
35.56	0.36	41.43	99.6
41.43	0.23	48.27	99.83
48.27	0.12	56.23	99.95
56.23	0.05	65.51	100
65.51	0.00	76.32	100
76.32	0.00	88.91	100
88.91	0.00	103.58	100
103.58	0.00	120.67	100
120.67	0.00	140.58	100
140.58	0.00	163.77	100
163.77	0.00	190.8	100
190.8	0.00	222.28	100
222.28	0.00	258.95	100
258.95	0.00	301.68	100

Particle size distribution data of freshly prepared outer membrane protein liposome from *Pasteurella multocida* 8:A.

Size low (um)	Frequency %	Size high (um)	cumulative under %
301.68	0.00	351.46	100
351.46	0.00	409.45	100
409.45	0.00	477.01	100
477.01	0.00	555.71	100
555.71	0.00	647.41	100
647.41	0.00	754.23	100
754.23	0.00	878.67	100

Particle size distribution data of outer membrane protein liposome from *Pasteurella multocida* 8:A after 4 weeks storage.

Size low (um)	Frequency %	Size high (um)	Cumulative under %
0.05	0.00	0.06	0.00
0.06	0.00	0.07	0.00
0.07	0.00	0.08	0.00
0.08	0.00	0.09	0.01
0.09	0.01	0.11	0.02
0.11	0.02	0.13	0.03
0.13	0.03	0.15	0.07
0.15	0.08	0.17	0.14
0.17	0.19	0.20	0.33
0.20	0.48	0.23	0.82
0.23	1.08	0.27	1.90
0.27	1.80	0.31	3.69
0.31	2.10	0.36	5.79
0.36	2.07	0.42	7.87
0.42	2.25	0.49	10.11
0.49	2.57	0.58	12.68
0.58	2.52	0.67	15.2
0.67	2.62	0.78	17.82
0.78	2.24	0.91	20.06
0.91	2.23	1.06	22.29
1.06	2.29	1.24	24.58
1.24	2.43	1.44	27.01
1.44	2.71	1.68	29.72
1.68	3.22	1.95	32.94
1.95	4.02	2.28	36.96
2.28	5.03	2.65	41.98
2.65	6.18	3.09	48.16
3.09	7.14	3.60	55.3
3.60	7.64	4.19	62.94

Particle size distribution data of outer membrane protein liposome from *Pasteurella multocida* 8:A after 4 weeks storage.

Size low (um)	Frequency %	Size high (um)	Cumulative under %
4.19	7.49	4.88	70.43
4.88	6.76	5.69	77.19
5.69	5.58	6.63	82.76
6.63	4.21	7.72	86.98
7.72	2.94	9.00	89.92
9.00	1.90	10.48	91.82
10.48	1.15	12.21	92.97
12.21	0.69	14.22	93.66
14.22	0.48	16.57	94.14
16.57	0.78	19.31	94.92
19.31	0.94	22.49	95.86
22.49	1.02	26.20	96.88
26.2	0.98	30.53	97.86
30.53	0.82	35.56	98.67
35.56	0.59	41.43	99.26
41.43	0.36	48.27	99.63
48.27	0.20	56.23	99.82
56.23	0.09	65.51	99.91
65.51	0.03	76.32	99.95
76.32	0.00	88.91	99.96
88.91	0.00	103.58	99.96
103.58	0.00	120.67	99.96
120.67	0.00	140.58	99.96
140.58	0.00	163.77	99.96
163.77	0.00	190.8	99.96
190.8	0.00	222.28	99.96
222.28	0.00	258.95	99.96
258.95	0.00	301.68	99.96

Particle size distribution data of outer membrane protein liposome from *Pasteurella multocida* 8:A after 4 weeks storage.

Size low (um)	Frequency %	Size high (um)	cumulative under %
301.68	0.01	351.46	99.97
351.46	0.03	409.45	100
409.45	0.00	477.01	100
477.01	0.00	555.71	100
555.71	0.01	647.41	100
647.41	0.02	754.23	100
754.23	0.03	878.67	100

Particle size distribution data of outer membrane protein liposome from *Pasteurella multocida* 8:A after 8 weeks storage.

Size low (um)	Frequency %	Size high (um)	Cumulative under %
0.05	0.00	0.06	0.00
0.06	0.00	0.07	0.01
0.07	0.01	0.08	0.01
0.08	0.01	0.09	0.03
0.09	0.02	0.11	0.05
0.11	0.04	0.13	0.08
0.13	0.07	0.15	0.15
0.15	0.13	0.17	0.28
0.17	0.26	0.20	0.53
0.20	0.52	0.23	1.05
0.23	0.97	0.27	2.02
0.27	1.44	0.31	3.46
0.31	1.64	0.36	5.1
0.36	1.63	0.42	6.73
0.42	1.73	0.49	8.47
0.49	1.89	0.58	10.36
0.58	1.83	0.67	12.19
0.67	1.83	0.78	14.02
0.78	1.80	0.91	15.82
0.91	1.88	1.06	17.69
1.06	2.05	1.24	19.75
1.24	2.42	1.44	22.17
1.44	3.04	1.68	25.21
1.68	4.00	1.95	29.21
1.95	5.25	2.28	34.46
2.28	6.61	2.65	41.07
2.65	7.89	3.09	48.96
3.09	8.72	3.60	57.68
3.60	9.09	4.19	75.84

Particle size distribution data of outer membrane protein liposome from *Pasteurella multocida* 8:A after 8 weeks storage.

Size low (um)	Frequency %	Size high (um)	Cumulative under %
4.19	9.06	4.88	83.45
4.88	7.61	5.69	89.22
5.69	5.77	6.63	93.07
6.63	3.85	7.72	95.23
7.72	2.17	9.00	96.12
9.00	0.89	10.48	96.19
10.48	0.06	12.21	96.19
12.21	0.00	14.22	96.19
14.22	0.00	16.57	96.19
16.57	0.00	19.31	96.19
19.31	0.00	22.49	96.38
22.49	0.20	26.20	96.77
26.2	0.38	30.53	97.26
30.53	0.49	35.56	97.8
35.56	0.54	41.43	98.33
41.43	0.53	48.27	98.81
48.27	0.48	56.23	99.22
56.23	0.41	65.51	99.54
65.51	0.33	76.32	99.79
76.32	0.24	88.91	99.94
88.91	0.15	103.58	100
103.58	0.06	120.67	100
120.67	0.00	140.58	100
140.58	0.00	163.77	100
163.77	0.00	190.8	100
190.8	0.00	222.28	100
222.28	0.00	258.95	100
258.95	0.00	301.68	100

Particle size distribution data of outer membrane protein liposome from *Pasteurella multocida* 8:A after 8 weeks storage.

Size low (um)	Frequency %	Size high (um)	cumulative under %
301.68	0.00	351.46	100
351.46	0.00	409.45	100
409.45	0.00	477.01	100
477.01	0.00	555.71	100
555.71	0.00	647.41	100
647.41	0.00	754.23	100
754.23	0.00	878.67	100

Particle size distribution data of outer membrane protein liposome from *Pasteurella multocida* 8:A after 12 weeks storage.

Size low (um)	Frequency %	Size high (um)	Cumulative under %
0.05	0.06	0.06	0.06
0.06	0.12	0.07	0.18
0.07	0.19	0.08	0.37
0.08	0.26	0.09	0.63
0.09	0.34	0.11	0.97
0.11	0.44	0.13	1.41
0.13	0.56	0.15	1.97
0.15	0.70	0.17	2.67
0.17	0.88	0.20	3.54
0.2	1.09	0.23	4.63
0.23	1.30	0.27	5.93
0.27	1.45	0.31	7.38
0.31	1.50	0.36	8.88
0.36	1.47	0.42	10.36
0.42	1.44	0.49	11.79
0.49	1.37	0.58	13.16
0.58	1.22	0.67	14.38
0.67	1.05	0.78	15.43
0.78	0.69	0.91	16.11
0.91	0.71	1.06	16.83
1.06	0.91	1.24	17.74
1.24	1.37	1.44	19.11
1.44	2.17	1.68	21.29
1.68	3.42	1.95	24.71
1.95	5.10	2.28	29.80
2.28	6.97	2.65	36.77
2.65	8.72	3.09	45.49
3.09	9.99	3.60	55.48
3.60	10.69	4.19	66.17

Particle size distribution data of outer membrane protein liposome from *Pasteurella multocida* 8:A after 12 weeks storage.

Size low (um)	Frequency %	Size high (um)	Cumulative under %
4.19	10.96	4.88	77.14
4.88	9.08	5.69	86.22
5.69	6.65	6.63	92.87
6.63	4.19	7.72	97.06
7.72	2.14	9.00	99.20
9.00	0.75	10.48	99.95
10.48	0.05	12.21	100
12.21	0.00	14.22	100
14.22	0.00	16.57	100
16.57	0.00	19.31	100
19.31	0.00	22.49	100
22.49	0.00	26.20	100
26.20	0.00	30.53	100
30.53	0.00	35.56	100
35.56	0.00	41.43	100
41.43	0.00	48.27	100
48.27	0.00	56.23	100
56.23	0.00	65.51	100
65.51	0.00	76.32	100
76.32	0.00	88.91	100
88.91	0.00	103.58	100
103.58	0.00	120.67	100
120.67	0.00	140.58	100
140.58	0.00	163.77	100
163.77	0.00	190.80	100
190.80	0.00	222.28	100
222.28	0.00	258.95	100
258.95	0.00	301.68	100

Particle size distribution data of outer membrane protein liposome from *Pasteurella multocida* 8:A after 12 weeks storage.

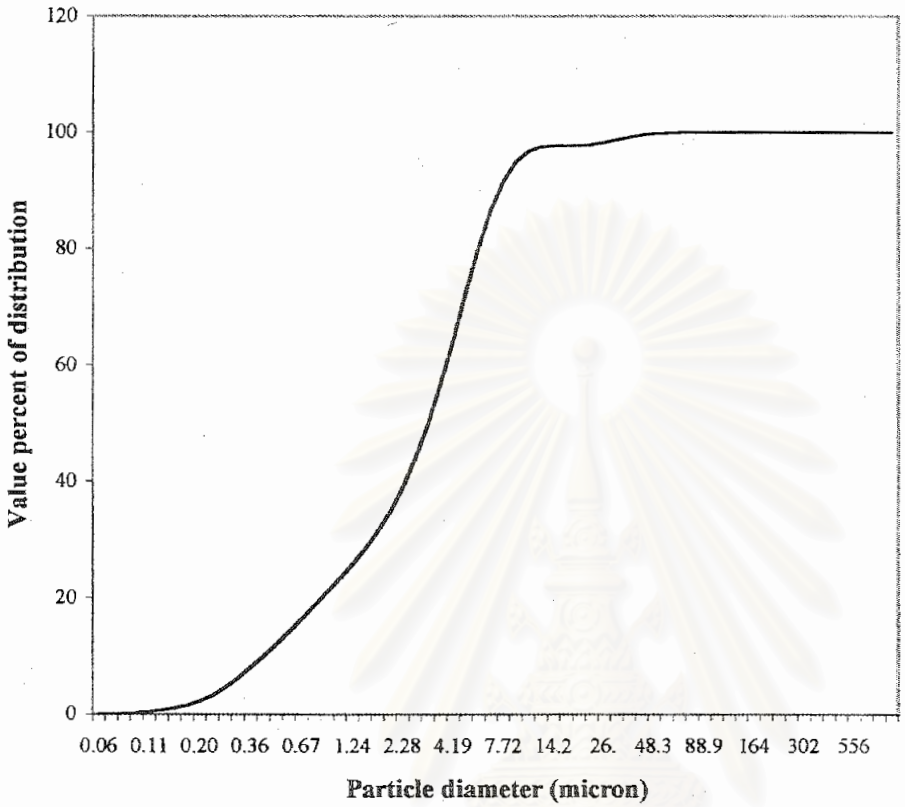
Size low(um)	Frequency%	Size high(um)	cumulative under%
301.68	0.00	351.46	100
351.46	0.00	409.45	100
409.45	0.00	477.01	100
477.01	0.00	555.71	100
555.71	0.00	647.41	100
647.41	0.00	754.23	100
754.23	0.00	878.67	100

APPENDIX II

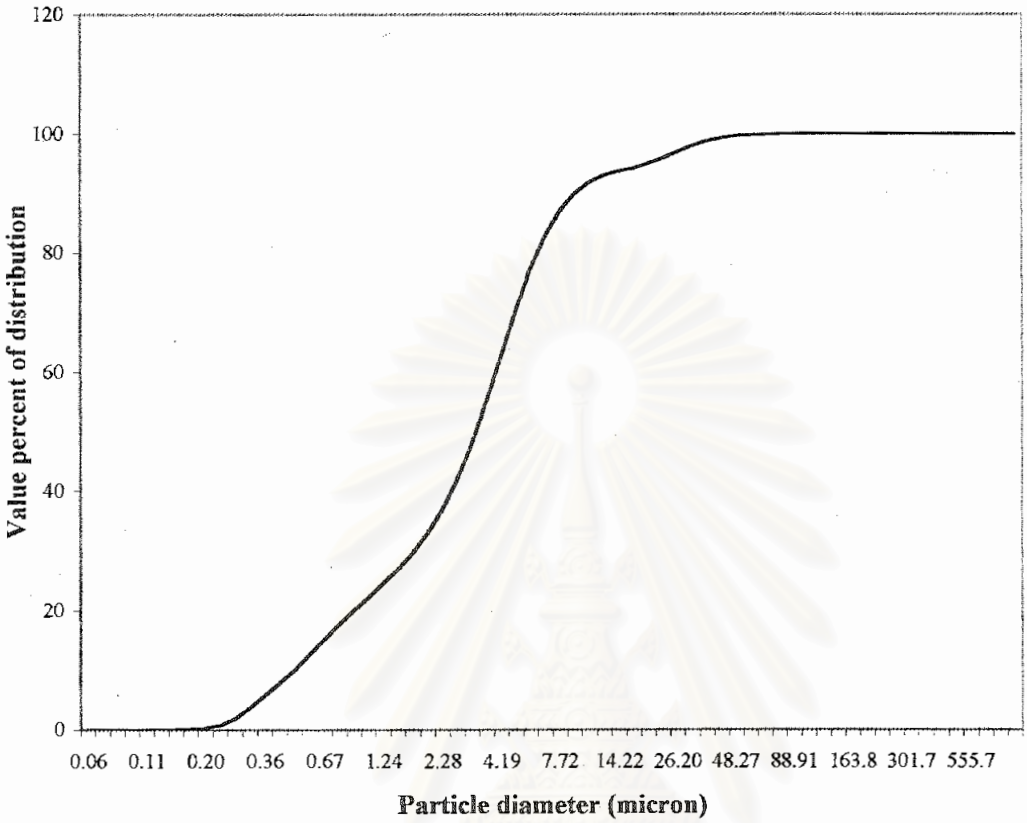
Cumulative undersize frequency curve

of liposome outer membrane protein from *Pasteurella multocida* 8:A

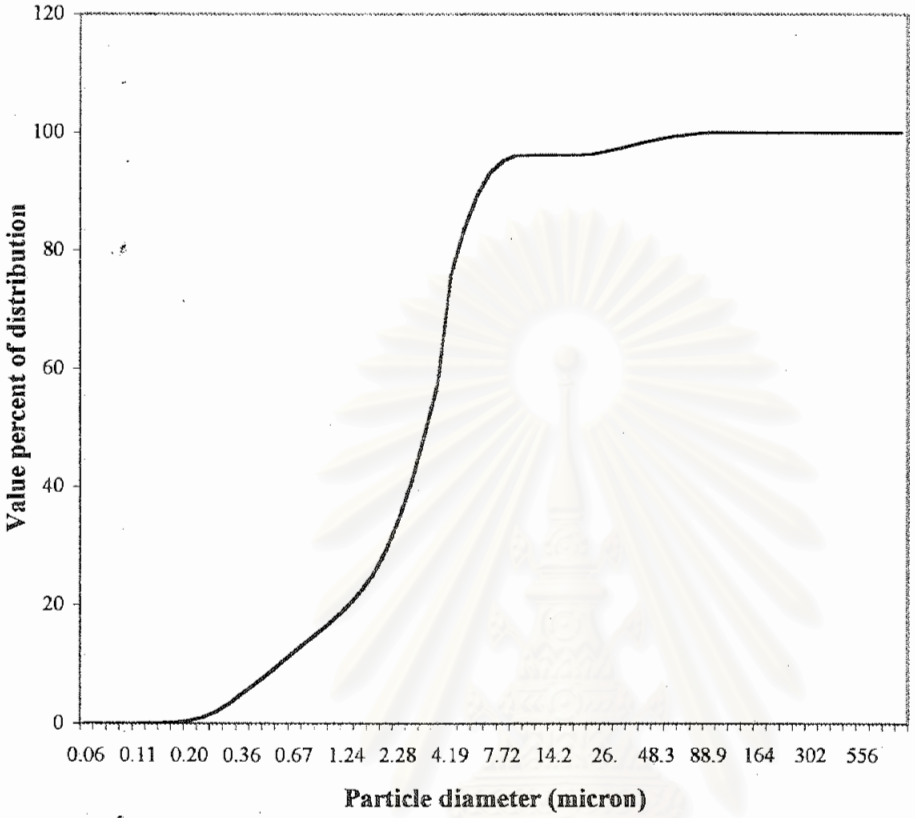
Week 0



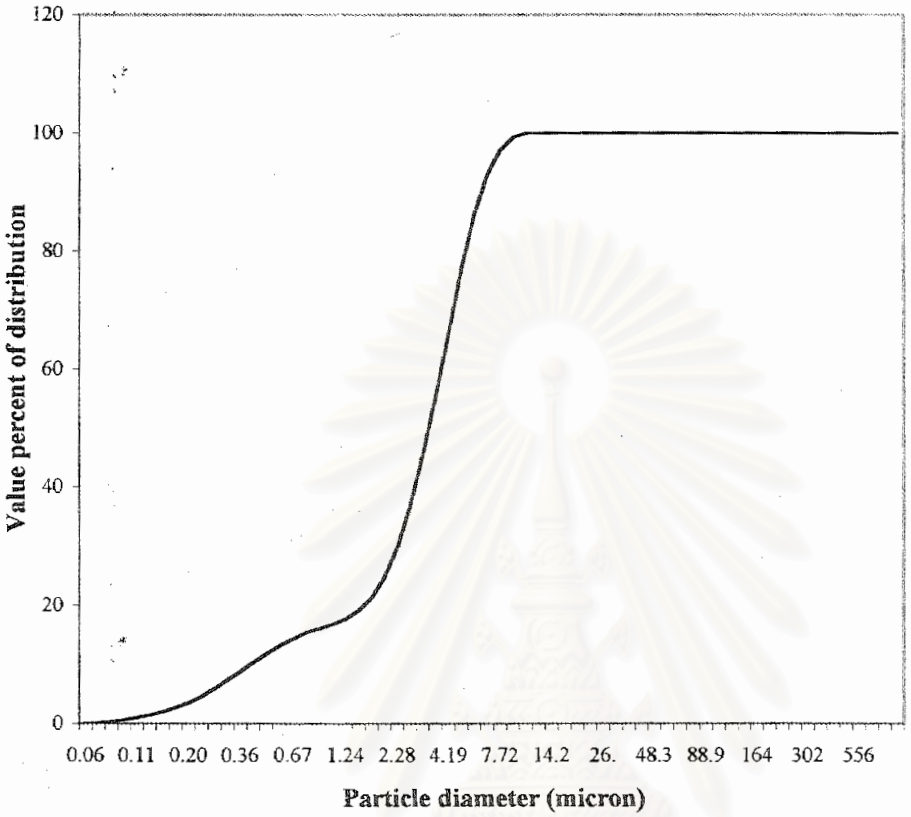
Week 4



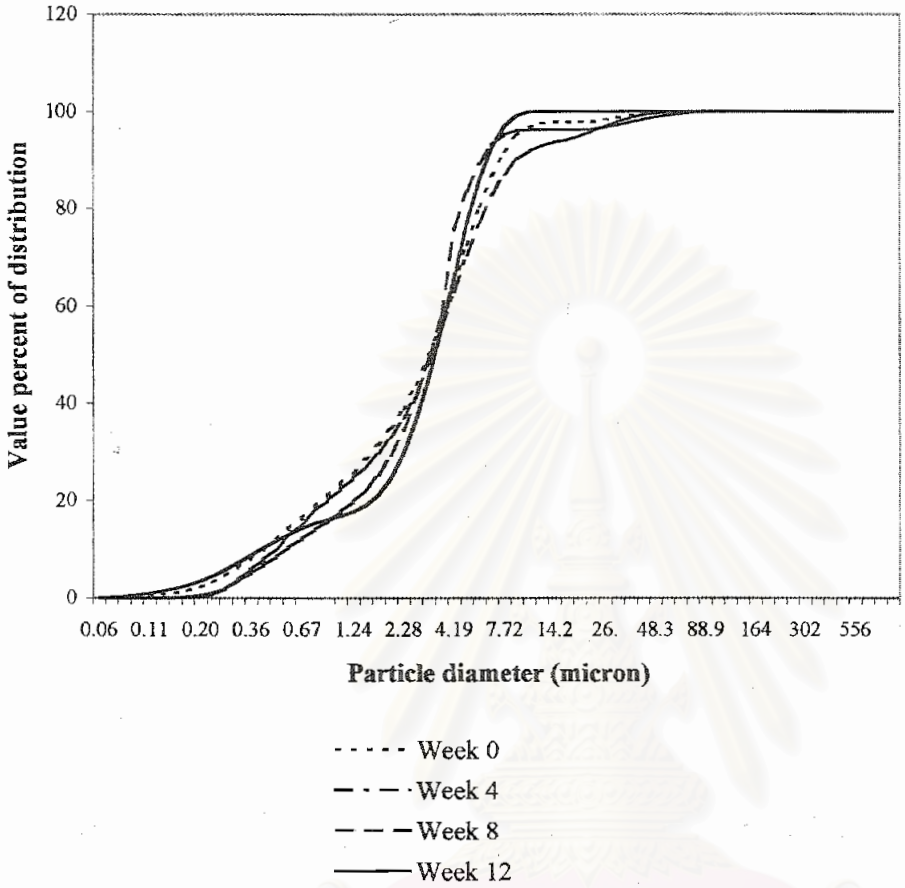
Week 8



Week 12



Week 0-12



APPENDIX III

Statistic significant difference between treatment groups of vaccine

Statistical significant difference between vaccine preparation, sera were collected in period of 12 weeks after vaccination. (Analysed by Repeated Analysis)

Treatment group	Statistical significant (P) difference				
	FKWC	OMPL	OMPF	OMPA	OMP
FKWC	-	0.000	0.000	0.009	
OMPL	0.000	-		0.000	0.000
OMPF	0.000		-	0.000	0.000
OMPA	0.000	0.000	0.000	-	
OMP		0.009	0.000		-

There were significantly difference in antibody titers against whole cell between groups, with a P value < 0.05.

FKWC = immune sera of rabbits vaccination with formalin killed whole cell

OMPL = immune sera of rabbits vaccination with liposome OMP

OMPF = immune sera of rabbits vaccination with OMP with Freund's adjuvant

OMPA = immune sera of rabbits vaccination with alum OMP

OMP = immune sera of rabbits vaccination with OMP

Statistical significant difference between vaccine preparation, sera were collected in period of 12 weeks after vaccination. (Analysed by Repeated Analysis)

Treatment group	Statistical significant (P) difference				
	FKWC	OMPL	OMPF	OMPA	OMP
FKWC	-	0.000	0.000	0.000	0.000
OMPL	0.000	-		0.000	0.000
OMPF	0.000		-	0.000	0.000
OMPA	0.000	0.000	0.000	-	0.000
OMP	0.000	0.011	0.011	0.000	-

There were significantly difference in antibody titers against OMP between groups, with a P value < 0.05.

FKWC = immune sera of rabbits vaccination with formalin killed whole cell

OMPL = immune sera of rabbits vaccination with liposome OMP

OMPF = immune sera of rabbits vaccination with OMP with Freund's adjuvant

OMPA = immune sera of rabbits vaccination with alum OMP

OMP = immune sera of rabbits vaccination with OMP

Statistical significant difference between vaccine preparation, sera were collected in period of 12 weeks after vaccination. (Analysed by Repeated Analysis)

Treatment group	Statistical significant (P) difference				
	FKWC	OMPL	OMPF	OMPA	OMP
FKWC	-	0.000	0.000	0.009	
OMPL	0.000	-		0.001	0.000
OMPF	0.000		-	0.006	0.006
OMPA		0.001	0.006	-	
OMP		0.009	0.006		-

There were significantly difference in % bactericidal activity between groups, with a P value < 0.05.

FKWC = immune sera of rabbits vaccination with formalin killed whole cell

OMPL = immune sera of rabbits vaccination with liposome OMP

OMPF = immune sera of rabbits vaccination with OMP with Freund's adjuvant

OMPA = immune sera of rabbits vaccination with alum OMP

OMP = immune sera of rabbits vaccination with OMP

APPENDIX IV

Antilog ELISA titers and standard deviation between treatment groups of vaccine

Antilog ELISA titers (plates were coated with whole cell of *P. multocida*)

Day	Antibody titers						
	Whole cell	Liposome OMP	Freund's adjuvant OMP	Alum OMP	OMP	Ghost liposome	PBS
0	2.38±0.24	2.34±0.34	2.38±0.36	2.48±0.34	2.53±0.36	2.43±0.13	2.63±0.14
14	3.65±0.16	4.29±0.13	4.19±0.22	3.85±0.13	3.99±0.03	2.52±0.08	2.54±0.12
28	4.40±0.24	5.03±0.25	4.68±0.19	3.88±0.25	3.99±0.08	2.77±0.16	2.74±0.16
42	5.16±0.24	5.73±0.37	5.77±0.23	4.57±0.37	5.32±0.13	2.74±0.21	2.4±0.19
56	5.02±0.23	6.94±0.19	6.55±0.45	6.02±0.19	5.47±0.23	2.86±0.17	2.53±0.04
70	4.82±0.15	7.87±0.27	8.03±0.57	6.14±0.27	5.43±0.25	2.6±0.09	2.81±0.05
85	4.70±0.16	8.79±0.48	8.15±0.53	5.91±0.48	4.85±0.07	2.78±0.14	2.9±0.21

Antilog ELISA titers (plates were coated with OMP of *P. multocida*)

Day	Antibody titers						
	Whole cell	Liposome OMP	Freund's adjuvant OMP	Alum OMP	OMP	Ghost liposome	PBS
0	2.73±0.37	2.64±0.38	2.75±0.38	2.70±0.31	2.70±0.25	2.54±0.15	2.65±0.06
14	4.61±0.41	4.59±0.24	4.67±0.34	3.96±0.04	4.32±0.18	2.74±0.12	2.64±0.21
28	4.55±0.20	5.44±0.21	5.20±0.24	4.67±0.43	4.33±0.35	2.92±0.05	2.81±0.08
42	5.59±0.40	6.50±0.41	6.43±0.39	5.55±0.22	6.38±0.34	2.79±0.09	2.63±0.14
56	6.06±0.60	8.54±0.98	6.32±0.55	6.67±0.61	5.82±0.41	2.93±0.10	2.83±0.11
70	5.34±0.38	9.20±0.96	8.61±0.46	6.80±0.10	5.94±0.21	2.78±0.20	2.94±0.10
85	5.47±0.48	9.20±0.84	8.56±0.62	6.77±0.07	5.64±0.36	2.88±0.18	2.98±0.16

APPENDIX V

Preparation of media for stock microorganism and reagent solution

Media for stock microorganism

Skim milk

Skim milk	100	g
Distilled water to	1000	ml

Sterile at 121-124°C, 10 minutes.

5% blood agar

Tryptic soy agar	40	g
Sheep blood	50	ml
Distilled water	950	ml

Sterile at 121-124 °C, 15 minutes before adding sheep blood.

0.9% Normal saline

Sodium chloride	9	g
Distilled water	1000	ml

0.1 M Tris – HCl

Tris – Hydrochloride	15.76	g
Distilled water	1000	ml

Reagent solution for SDS-PAGE

30% Acrylamide-0.8% Bis-acrylamide

Acrylamide	30	g
Bis-acrylamide	0.8	g
Distilled water to	100	ml

Filter and store up to 3 months at 4^{o}C in the dark

5x Running buffer pH 8.3 (0.025 M tris, 0.102 M glycine, 0.1% SDS)

Trizmabase	15	g
Glycine	72	g
SDS	5	g
Distilled water to	1000	ml

Adjust pH to 8.3 before bring to volume

Stracking gel buffer pH 6.8 (0.5 M Tris-hydrochloride)

Trizmabase	6	g
Distilled water to	100	ml

Adjust pH to 6.8 with 6 N hydrochloric acid before being to volume

Separating gel buffer pH 8.8 (1.5 M Tris-hydrochloride)

Trizmabase	18.2	g
Distilled water to	100	ml

Adjust pH to 8.8 with 6 N hydrochloric acid before being to volume

10% Sodium dodecyl sulfate (SDS)

SDS	10	g
Distilled water to	100	ml

Store at room temperature

10% Ammonium persulfate

Ammonium persulfate	0.1	g
Distilled water to	1.0	ml
use fresh, do not store		

Sample buffer (0.66 M Tris-hydrochloride pH 6.8, 10% Glycerol, 2% SDS, 5% Mercaptoethanol, 0.025% Bromophenol blue)

0.5 M Tris-hydrochloride pH 6.8	1.2	ml
Glycerol	1.0	ml
10% SDS	2.0	ml
2-Mercaptoethanol	0.5	ml
Distilled water	2.0	ml

Straining solution (0.1 % Coomassie brilliant blue, 40% Methanol, 10% Acetic acid)

Coomassie brilliant blue R-250	1	g
Methanol	400	ml
Acetic acid	100	ml
Distilled water	500	ml
filter before use		

De-straining solution 40% Methol, 10% Acetic acid)

Methanol	400	ml
Acetic acid	100	ml
Distilled water to	500	ml

Reagent solution for Alum preparation

1.0M Sodium bicarbonate ($NaHCO_3$)

Sodium bicarbonate	84.01	g
Distilled water	1000	ml

0.2 M Potassium aluminium sulfate ($ALK(SO_4)_3$)

Potassium aluminium sulfate	51.68	g
Distilled water	1000	ml

Reagent solution for ELISA

0.05 M Carbonate bicarbonate buffer pH 9.6 (coating buffer)

Sodium carbonate	0.8	g
Sodium hydrogen carbonate	1.5	g
Distilled water to	500	ml

Adjust pH to 9.6 before bring to volume

Citrate-phosphate buffer pH 5.0 (substrate buffer)

Citric acid	9.3	g
Sodium hydrogen phosphate	18.3	g
Thimerosol	0.1	g
Distilled water to	1000	ml

Adjust pH to 5.0 before bringing to volume

Phosphate buffer saline pH 7.4 with Tween 20 (washing buffer)

Sodium chloride	8.0	g
Potassium dihydrogen phosphate	0.2	g
Sodium hydrogen phosphate	2.9	g
Potassium chloride	0.2	g
Thimerosol	0.1	g
Tween 20	0.5	ml

Adjust pH 7.4 before bringing to volume by distilled water to 1000 ml.

1% Bovine serum albumin in PBS –Tween 20 (diluent)

Bovine serum albumin	1	g
PBS-T	100	ml

3% Bovine serum albumin in PBS-Tween 20 (blocking solution)

Bovine serum albumin	3	g
PBS-T	100	ml

4N Sulfuric acid

98% Sulfuric acid	54.4	ml
Distilled water to	500	ml

Reagent solution for Western Blot

Blotting buffer pH 9.2 (48 mM Tris, 39 mM Glycine, 20% Methanol, 0.0375% SDS)

Trizmabase	5.8	g
Glycine	2.9	g
SDS	0.04	g
Methanol	200	ml
Distilled water to	1000	ml
do not adjust pH		

Tris buffer saline pH 7.4 (50 mM Tris-hydrochloride, 0.85% NaCl)

Tris-hydrochloride	7.9	g
Sodium chloride	8.5	g
Thimersol	0.1	g
Distilled water to	1000	ml

Tris buffer saline pH 9.5 (0.1 M Trizmabase, 0.85% NaCl)

Trizmabase	12.1	g
Sodium chloride	8.5	g
Distilled water to	1000	ml

Substrate

N,N-diethyl-p-phenylene diamine sulfate	0.06	M
4-Chloro-1-napthol	0.11	M
Acetonitrile	200	µl
0.1 M sodium citrate pH 6	10	ml
30% hydrogen peroxide	1	µl

0.1% Amido black

Amido black	0.1	g
Methanol	40	ml
Acetic acid	10.	ml
Distilled water to	100	ml

VITA

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