DEVELOPMENT OF RECOMBINANT VACCINES COMPOSED OF PLPE AND OMPH FROM *PASTEURELLA MULTOCIDA* A:3

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DEVELOPMENT OF RECOMBINANT VACCINES COMPOSED OF PLPE AND OMPH FROM *PASTEURELLA MULTOCIDA* A:3

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ABSTRACT

DEVELOPMENT OF RECOMBINANT VACCINES COMPOSED OF PLPE AND OMPH FROM *PASTEURELLA MULTOCIDA* A:3

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Pasteurella multocida serotype A:3 is a gram-negative bacterial pathogen which is one of the causative agents of shipping fever in cattle. In this study, *ompH* and two fragments of *plpE* gene (*plpE*N and *plpE*C) were cloned from the genomic DNA of *P. multocida* P-1062 (ATCC 15743, serotype A:3) and *plpE*N-*ompH* and *plpE*C-*ompH* fusions were constructed. In vitro expression of the genes was shown in HEK-293 cells. Later, full-length *plpE* gene was cloned and the recombinant proteins were expressed in *E. coli* and purified. Three DNA vaccine formulations, namely pCMV-ompH, pCMV-plpEN-ompH and pCMV-plpEC-ompH and five recombinant protein based vaccines, PlpEN-OmpH, PlpEC-OmpH, OmpH, PlpEC and PlpE were generated. Recombinant proteins were

formulated with at least one of the adjuvants: alum, CpG, alum-CpG, oil based and oil based-CpG. BALB/c mice were immunized with these vaccine formulations and their sera were used for the evaluation of antibody and serum IFN-γ titers. Protective capacities of the vaccines were also evaluated via challenge of mice with 10 LD_{50} of *P. multocida* A:3. DNA vaccines induced immune responses, but did not provide protection. All protein vaccine formulations increased antibody levels and CpG containing formulations enhanced serum IFN-γ titers. 100 μg of PlpEC-OmpH protein adsorbed on alum adjuvant conferred 40% protection while no protection was obtained with PlpEN-OmpH. Next, the effects of CpG, or its alum and oil based combinations as adjuvants were investigated on PlpEC-OmpH mediated protection. The vaccine formulation composed of PlpEC-OmpH and oil based-CpG adjuvant conferred 100% protection. Finally, the mice were vaccinated with recombinant OmpH, PlpEC and PlpE formulated with oil based-CpG adjuvant. OmpH, PlpEC and PlpE formulations provided 50%, 60% and 100% protection, respectively. These findings implicated that recombinant PlpE and PlpEC-OmpH fusion proteins when formulated with oil based-CpG adjuvant are potent acellular vaccine formulation candidates against shipping fever.

Keywords: *Pasteurella multocida*, OmpH, PlpE, recombinant vaccine

ÖZ

PASTEURELLA MULTOCIDA A:3'E AİT PLPE VE OMPH'DEN OLUŞAN REKOMBİNANT AŞILARIN GELİŞTİRİLMESİ

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Pasteurella multocida serotip A:3, sığırlardaki nakil humması hastalığı etmenlerinden biri olan gram-negatif bakteriyel bir patojendir. Bu çalışmada, *ompH* ve *plpE* geninin iki parçası (*plpE*N ve *plpE*C), *P. multocida* P-1062 (ATCC 15743, serotip A:3)'ün genomik DNA'sından kopyalanıp *plpE*N-*ompH* ve *plpE*C-*ompH* füzyonları oluşturuldu. Genlerin in vitro ekspresyonu HEK-293 hücrelerinde gösterildi. Daha sonra, *plpE* geninin bütünü kopyalandı ve rekombinant proteinler *E. coli*'de ekspres edilip saflaştırıldı. pCMV-ompH, pCMV-plpEN-ompH ve pCMV-plpEC-ompH olmak üzere 3 DNA aşısı, PlpEN-OmpH, PlpEC-OmpH, OmpH, PlpEC ve PlpE olmak üzere de 5 protein bazlı aşı üretildi. Rekombinant proteinler, alum, CpG, alum-CpG, yağ bazlı ve yağ bazlı-

CpG adjuvanlarından en az biriyle formüle edildi. BALB/c fareleri bu aşı formülasyonlarıyla bağışıklandı ve serumları, antikor ve IFN-γ düzeylerinin belirlenmesinde kullanıldı. Aşıların koruyuculuk ları, farelerin 10 LD_{50} *P. multocida* A:3'e karşı hayatta kalma deneyi ile değerlendirildi. DNA aşıları, bağışıklık yanıtlarını tetiklemesine rağmen koruyuculuk sağlamadı. Protein aşı formülasyonları antikor düzeyinde artış sağladı ve serum IFN-γ titrelerindeki artış CpG içeren formülasyonlarla elde edildi. Alum adjuvanına adsorbe edilmiş 100 µg PlpEC-OmpH proteini %40 koruma sağlarken, PlpEN-OmpH proteiniyle hiç koruma elde edilemedi. Daha sonra, CpG ya da onun alum ve yağ bazlı kombinasyonlarının PlpEC-OmpH üzerine adjuvant etkisi araştırıldı. PlpEC-OmpH ve yağ bazlı-CpG adjuvanıyla hazırlanan aşı formülasyonu %100 koruma sağladı. Son olarak, fareler yağ bazlı-CpG adjuvanıyla formüle edilmiş rekombinant OmpH, PlpEC ve PlpE ile aşılandı. OmpH, PlpEC ve PlpE formülasyonları sırasıyla %50, %60 ve %100 koruma sağladı. Bu bulgular, yağ bazlı-CpG adjuvanıyla formüle edildiğinde rekombinant PlpE ve PlpEC-OmpH füzyon proteininin nakil humması hastalığına karşı hücresiz aşı formülasyonu adayı olduğunu gösterdi.

Anahtar kelimeler: *Pasteurella multocida*, OmpH, PlpE, rekombinant aşı

To My Family

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LIST OF ABBREVIATIONS

OmpH	Outer membrane protein H
PlpE	*Pasteurella* lipoprotein E
PlpEN	Amino terminal of PlpE
PlpEC	Carboxyl terminal of PlpE
GFP	Green fluorescent protein
bp(s)	Base pair(s)
HEK	Human embryonic kidney
BRD	Bovine respiratory disease
LPS	Lipopolysaccharide
LD	Lethal dose
IPTG	Isopropyl-β-D-thio-galactoside
ELISA	Enzyme-linked immunosorbent assay
i.m.	Intramuscular
i.p.	Intraperitoneal
s.c.	Subcutaneous
IgG	Immunoglobulin G
IFN-γ	Interferon gamma
ATCC	American Type Culture Collection

CHAPTER 1

INTRODUCTION

1.1. Bovine Respiratory Disease

1.1.1. Etiology

Bovine respiratory disease (BRD) has been one of the most serious problems in cattle industry causing high mortality (1.5-4.2%) and economic loss (almost $1 billion per year in US) in calves. BRD is related with stressful conditions in commingling and transportation of cattle like dust, dampness, fatigue, injury, hunger, dehydration, anxiety, irritant gases, and adverse weather conditions (heat, cold) coupled with bacterial and viral infections (Figure 1.1). Since transportation is the most universally accepted non-infectious risk factor for BRD, the disease is also known as shipping fever (Bagley, 1997; Welsh *et al*., 2004; Cho *et al*., 2008; Wildman *et al*., 2008; Angen *et al*., 2009; Taylor *et al*., 2010).

Bacterial pathogens associated with BRD complex are *Mannheimia haemolytica* (formerly *Pasteurella haemolytica*), *Pasteurella multocida*, *Histophilus somni* (formerly *Haemophilus somnus*), *Mycoplasma bovis* and *Arcanobacterium pyogenes* (formerly *Actinomyces pyogenes*) and most recently *Bibersteinia trehalosi* (formerly *Pasteurella trehalosi*) (Welsh *et al*., 2004; Cho *et al*., 2008; Confer, 2009).

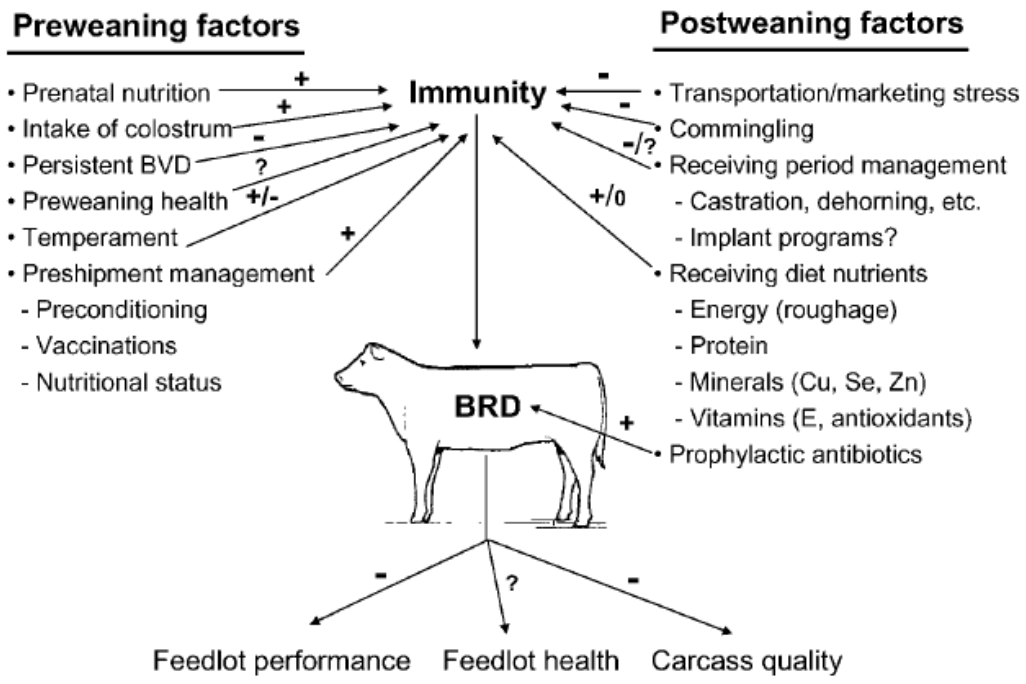

Figure 1.1. The factors affecting BRD in cattle and the results of the disease. (+): factors decreasing the incidence; (−): factors increasing the incidence; (?): factors not fully understood according to the available data. BVD: bovine viral diarrhea virus (Duff and Galyean, 2007).

1.1.1.1. *Mannheimia haemolytica*

Gram negative bacterium *M. haemolytica* is a pathogen of both ovine and bovine (Michael *et al*., 2011). Among the bacteria associated with BRD, it is the most important and commonly isolated pathogen from fatal cases of shipping fever pneumonia in cattle (Confer *et al*., 2006). *M. haemolytica* is often found in the upper respiratory tract of healthy cattle. After a viral infection and stress factors, it migrates to the lungs and multiplies rapidly. Major virulence factors are outer membrane proteins (adhesins), lipopolysaccharide (LPS), leukotoxin (Lkt), and iron-binding proteins. The immunosuppressive effects of *M. haemolytica* occur via secretion of the pro-inflammatory cytokines (IL-8, IL-1β and TNF-α) by alveolar macrophages after LPS stimulation (Srikumaran *et al*., 2007; Confer,

2009). Serotype specificity mostly comes from capsular lipopolysaccharides (CPS) but LPS related antigenic determinants were also observed (Michael *et al*., 2011). Of the *M. haemolytica* serotypes isolated from bovine, 65% belongs to S1 (serotype 1) and approximately 35% are S2 and S6. Majority of ovine isolates belongs to S2. Although a number of commercial vaccines for *M. haemolytica* S1 are in the market, their efficacy was shown to be approximately 50% in field studies. A combination that increases antibodies both to Lkt and a cell surface target would be the most effective vaccine against *M. haemolytica* (Ayalew *et al*., 2008; Michael *et al*., 2011). Ayalew *et al*. (2008) obtained a chimeric protein composed of immunogenic regions of Lkt A and an outer membrane protein PlpE (*Pasteurella* lipoprotein E) from *M. haemolytica*. The immune responses were elevated in the mice vaccinated with these fusion proteins and the hyperimmune sera were able to kill the pathogen.

1.1.1.2. *Pasteurella multocida*

P. multocida is the etiological agent of a wide range of animal diseases like pneumonia in cattle and sheep, atrophic rhinitis in swine, hemorrhagic septicemia in buffalo and cattle, and fowl cholera in chicken (Chung *et al*., 2005; Atashpaz *et al*., 2009). It has been also isolated both from healthy animals as a member of the oropharyngeal flora of calves and does not cause a serious disease but, in stress conditions, it is one of the bacterial pathogens associated with BRD complex (Ishiguro *et al*., 2005; Boyce *et al*., 2010). *P. multocida* strains are classified in five capsular serogroups (A, B, D, E and F) and 16 somatic LPS serotypes (1-16). Serogroup A, predominantly serotype A:3, causes severe pneumonia in young feedlot calves (Dabo *et al*., 2008b). Detailed information about *P. multocida* will be given in Section 1.2.

1.1.1.3. *Histophilus somni*

H. somni is the causative agent of pneumonia, septicemia, myocarditis, arthritis, thrombotic meningoencephalitis and reproductive failure in cattle and other ruminants (Geertsema *et al*., 2011). *H. somni* infections result in cranioventral fibrinous pleuropneumonia with hemorrhage and coagulation necrosis, and the gross lesions are similar to those observed with *M. haemolytica*. Lipooligosaccharide (LOS) and various outer membrane proteins (OMPs) are the virulence factors of *H. somni*. LOS assists bacterium to escape the host immune response and OMPs are responsible for resistance to complement-mediated serum killing (Confer, 2009). Geertsema *et al*. (2011) showed that DR2 subunit of 270 kDa surface protein IbpA (immunoglobulin binding protein A) is a protective antigen of *H. somni*.

1.1.1.4. *Mycoplasma bovis*

M. bovis causes pneumonia, arthritis, otitis, conjunctivitis and mastitis in cattle. It may also be a predisposing factor leading to the invasion of other bacteria or viruses via weakening the host immune system (Soehnlen *et al*., 2011a). Often the importance of *M. bovis* in BRD has been underestimated but it is widespread within the infected cattle population and cause high economic loss in feedlot animals (Cho *et al*., 2008). It is an extracellular pathogen living on the surface of respiratory epithelial cells and causes cranioventral caseonecrotic bronchopneumonia with abscesses, bronchiectasis and sequestration. Surface proteins and toxin take role in virulence of *M. bovis*. Phenotypic variation among strains is due to variable surface proteins (VSPs) which act as adhesins. Transmission mostly occurs through direct contact with infected animals (Confer, 2009). Since it does not have a cell wall, antimicrobials such as β-lactam antibiotics targeting that region are not effective against *M. bovis*. Resistance for spectinomycin, tilmicosin and tetracycline has been also reported (Soehnlen *et al*., 2011b).

1.1.1.5. *Arcanobacterium pyogenes*

A. pyogenes is the causative agent of mastitis, abortion and a variety of pyogenic infections in domestic ruminants and pigs (Ülbegi-Mohyla *et al*., 2010). It is one of the secondary invaders of a lung which is already infected by other pathogens, and causes chronic abscessing pneumonia characterized by liquefactive necrosis surrounded by a thick fibrous connective tissue. *A. pyogenes* may survive and become the predominant nasopharyngeal isolate after BRD treatment in cattle. Virulence factors are collagen-binding protein (CbpA), pyolysin, and adhesins. (Confer, 2009).

1.1.1.6. *Bibersteinia trehalosi*

B. trehalosi was previously named as *Pasteurella haemolytica* together with *M. haemolytica* but Blackall *et al*. (2007) reclassified it in a new genus, *Bibersteinia* and described the species as "catalase and oxidase-negative, weak haemolysis on bovine blood agar, CAMP-positive, weak growth on MacConkey agar, yellowish pigment, production of acid from cellobiose, raffinose, aesculin, amygdalin, arbutin, gentiobiose and salicin, no acid production from glycerol and *myo*-inositol". It is known to cause severe systemic infections and pneumonia in sheep. Recently, this pathogen has also been reported in cattle pneumonia (Confer, 2009).

1.1.1.7. BRD Associated Viruses

There are also BRD associated viral agents including bovine viral diarrhea virus (BVDV), bovine respiratory syncytial virus (BRSV), bovine herpes virus-1 (BHV-1), parainfluenza 3 virus (PI3V), and infectious bovine rhinotracheitis virus (IBRV) (Wildman *et al*., 2008; Taylor *et al*., 2010a). Viral infections occur via nose to nose contact or aerosols in a short distance. Later, the ciliated epithelium in trachea is lysed and the housekeeping functions of the mucocilliary escalator

are disrupted. Therefore, clearance of bacteria from the upperways fails and the bacteria deposit in alveoli (Ellis, 2009). Figure 1.2 shows the clinical signs of BRD on a calf infected with BRSV.

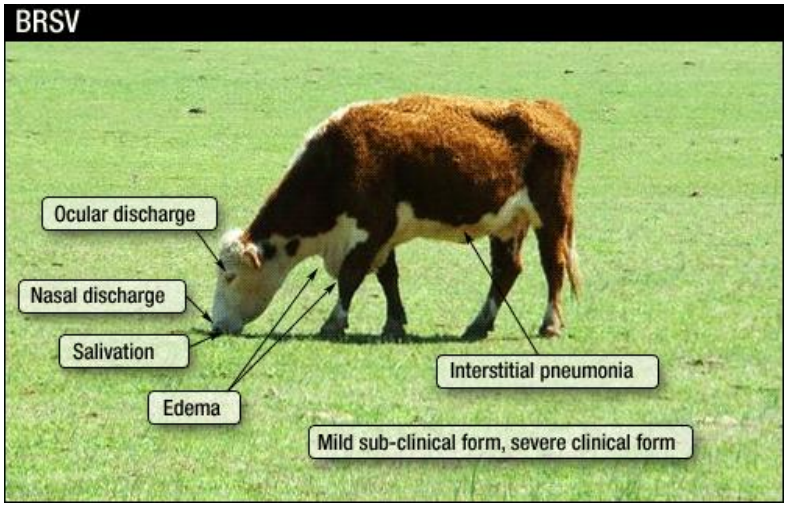

Figure 1.2. Clinical signs of BRD associated BRSV infection (http://homepage.usask.ca/~vim458/virology/studpages2009/VirusWebsite/brsv.html).

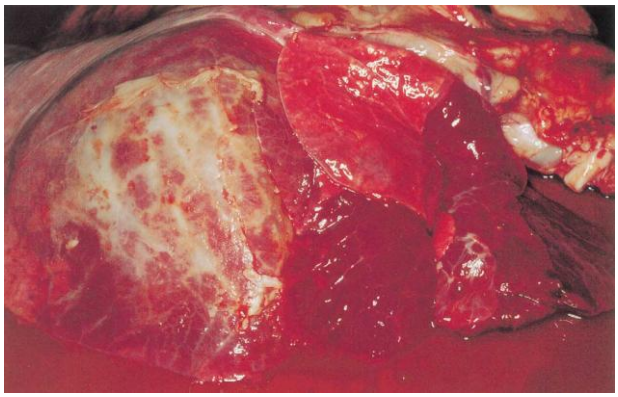

Figure 1.3. Pneumonic lungs from a calf infected with *P. multocida* serotype A:3 (Dowling *et al*., 2002).

1.1.2. Diagnosis

Diagnosis of the pathogens associated with BRD is difficult antemortem especially in the presence of a prior treatment (Taylor *et al*., 2010a) but gross necropsy and histopathological lesions may help to identify the primary bacterial agent. Although necropsy results are not very representative for initiating agent since it takes several days to weeks from disease onset to death, certain microorganisms may be diagnosed antemortem. For instance, chronic respiratory disease studies on feedlot cattle showed that *M. bovis* infections result in lack of weight gain and do not respond treatment. However, distinguishing features of *H. somni* infections are not very clear if other systems are not affected. Clinical signs to characterize a disease as a BRD include increased body temperature, respiratory tract problems (cough, nasal discharge, dyspnea, or tachypnea), depression, and decreased appetite (Taylor *et al*., 2010b).

BRD can be divided into three main categories (Bagley, 1997):
I. Upper respiratory tract infections: the symptoms are inflammation of the nostrils, pharynx and trachea, coughing, nasal discharge, fever and loss of appetite.
II. Diphtheria: clinical signs are load noises during breathing, and swelling which may cause death of the animal by restricting air flow.
III. Lower respiratory tract infection (pneumonia): the infection of the lung may occur due to either migration of the pathogens from the upper respiratory tract or the failure of protection mechanisms which protect lung against infections. Pneumonia is more severe and serious than upper respiratory tract infections. Figure 1.3 shows pneumonic lungs of a calf infected with *P. multocida*.

1.1.3. Control

Young dairy calves considered at high risk for BRD are more likely vaccinated. However, there are some limitations of vaccination against BRD. Antibody titers

may not always be sufficient for protection. Also, timing of the administration is important because stressed calves may not respond to the vaccine appropriately; there is an increased susceptibility to all BRD related pathogens. Vaccination against viral pathogens is more common than against bacterial pathogens because of the higher efficacy (Welsh *et al*., 2004; Taylor *et al*, 2010b). Vista® 5 SQ (Intervet Inc. Millsboro, Delaware) is a Modified Live Vaccine (MLV) containing BVDV (Type 1a and 2), BRSV, PI3V and IBRV. Xue *et al*. (2011) demonstrated that 96% of the heifers vaccinated with Vista® 5 SQ were protected from BVDV Type 1b although the MLV contains Type 1a and 2. Coopers Inc. (New South Wales, Australia) produces vaccines Bovilis® MH against *M. haemolytica* and Bovilis® MH+IBR against both *M. haemolytica* and IBRV.

Preconditioning programs are also practiced in order to prepare the animals for shipment by spreading the stress factors in multiple episodes. These applications include vaccination, weaning, and training of the cattle to drink from a trough and eat from a bunk. Vaccination at preconditioning is important for calves to have time for development of antibodies (Taylor *et al*, 2010b).

Aggressive antimicrobials, tilmicosin, florfenicol, ceftiofur, and enrofloxacin, are used either single or in combination for the control and/or treatment of BRD. Resistance and susceptibility for these antibiotics should be analyzed periodically. The percentage of isolations and the antibiotic susceptibilities of *M. haemolytica*, *P. multocida*, and *H. somni* isolated from the lungs of 6-18-month-old cattle with pneumonia between 1994 and 2002 were shown in Table 1.1. Antimicrobial susceptibilities were significantly decreased for *M. haemolytica* for erythromycin, florfenicol, spectinomycin, and tilmicosin. Susceptibilities for erythromycin, spectinomycin, florfenicol, tetracycline, sulfachloropyridizine, trimethoprim-sulfamethoxazole, and tilmicosin were significantly declined for *P. multocida*. Spectinomycin and sulfachloropyridizine susceptibilities for *H. somni* were variable (Welsh *et al*., 2004).

Table 1.1. Percentages of total isolates of *M. haemolytica*, *P. multocida*, and *H. somni* from the lungs of cattle at Oklahoma Animal Disease Diagnostic Laboratory between 1994 and 2002 (Welsh *et al*., 2004).

	M. haemolytica (%)	*P. multocida* (%)	*H. somni* (%)
1994	62.5	20.0	17.5
1995	58.0	23.9	18.1
1996	64.1	28.2	7.7
1997	50.8	32.3	16.9
1998	30.6	28.6	40.8
1999	40.5	36.5	23.0
2000	35.7	47.4	16.9
2001	44.3	37.1	18.6
2002	50.4	32.4	17.3
Total	46.3	34.7	19.0

1.2. Genus *Pasteurella* and *P. multocida*

The first scientific name for the causative agent of fowl cholera and hemorrhagic septicemia was *Micrococcus gallicidus*, Burrill 1883. Later, the generic name *Pasteurella* was proposed by Trevisan in 1887 to commemorate the work of Luis Pasteur on this microorganism. Many species names were assigned for the genus *Pasteurella* according to their bipolar staining or host organism. In 1939, Rosenbusch and Merchant used *multocida* epithet which was first used by Kitt in 1893 for "*Bacterium bipolare multocidum*" and by Lehmann and Neumann in 1899 for "*Bacterium multocidium*" (Mutters *et al*., 1985). Epithet *gallicida* was also used for this bacterium but Judicial Commission of the International Committee on Systematic Bacteriology requested the use of name *Pasteurella multocida* instead of *P. gallicida* since this species has the same type strain (NCTC 10322) as *P. multocida* in the Approved Lists (Sneath, 1982).

Pasteurella are defined as Gram negative, non-motile and facultative anaerobic bacteria in Bergey's Manual of Determinative Bacteriology. Bipolar staining is observed in the tissue preparations from infected animals. Spherical, ovoid, or rod-shaped cells are 0.3-1.0 μm in diameter and 1.0-2.0 μm in length growing optimum at 37^{o}C (Figure 1.4 A). Their biochemical activities are oxidase and catalase positive, fermenting glucose and other sugars with the production of acid but not gas. Rich media containing ruminant blood (Figure 1.4 B) and brain heart infusion can be used to culture *Pasteurella* but not MacConkey agar or Simmons citrate medium. The members of genus *Pasteurella* are generally found in the oropharyngeal flora of many vertebrates but it is also a parasitic or commensal microorganism found on the mucous membranes of the upper respiratory and digestive tracts of birds and mammals causing many diseases that are of significant economic importance to livestock industries (Bergey and Holt, 2000; Boyce *et al*., 2010) Table 1.2 shows the classification of genus *Pasteurella* and their pathogenicity.

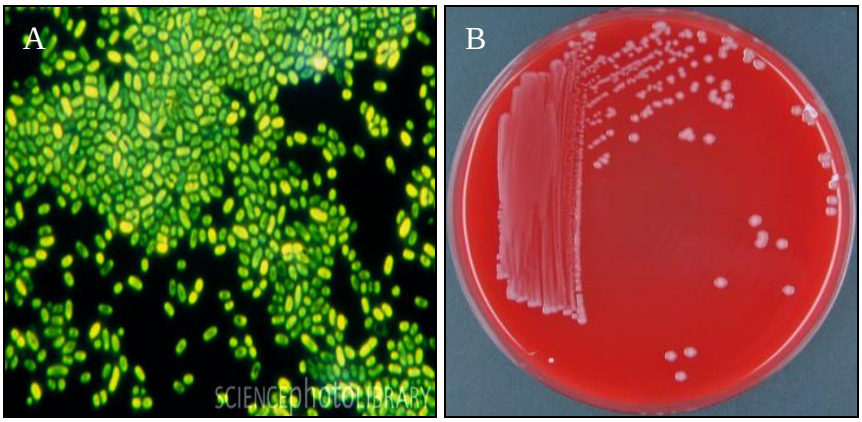

Figure 1.4. Light micrograph of *P. multocida* **(A)**. Darkfield lighting, magnification of 800X at 35 mm size (Abbey, M., http://www.sciencephoto.com/ media/11568/ view). Colony shape of *P. multocida* grown on blood agar **(B)** (http://www.gefor.4t.com/bacteriologia/ pasteurellamultocida.html).

Table 1.2. Currently recognized taxa in the genus *Pasteurella*, host predilection and diseases (Boyce *et al*., 2010).

Species	Hosts	Association/diseases (common serotypes)
P. multocida subsp. *multocida*, *gallicida*, and *septica*	Birds, mammals	FC of birds (A, F, rarely D) Bovine pneumonia (A:3) AR of pigs (toxigenic serotypes A and D) HS of ungulates (B:2; B:2,5; E:2; E:2,5) Bite wound-associated infection in human
P. dagmatis	Dogs	Normal flora in dogs. Cause a range of zoonotic infections in humans
P. canis	Cats and dogs Cattle and sheep?	Normal flora in cats and dogs. Cause a range of mostly bite wound-associated infections in humans. Pneumonia in cattle and sheep?
P. stomatis	Cats and dogs	Normal flora in cats and dogs. Cause a range of mostly bite wound-associated infections in humans.
*[P]. aerogenes**	Pigs	Sepsis, diarrhea and pneumonia
*[P]. bettyae**	Humans	Genitourinary infections
*[P]. caballi**	Horses, pigs	Respiratory infections
*[P].langaaensis**	Birds	Normal flora of respiratory tract
*[P]. pneumotropica**	Cats, dogs, rodents	Pneumonia and various suppurative infections in rodents
*[P]. mairii**	Pigs	Isolated from pig reproductive tract and associated with abortions
*[P]. skyensis**	Fish	Fatal infections in Atlantic salmon
*[P]. testudinis**	Tortoises	Respiratory disease in tortoises

* While these species are currently valid within the *Pasteurella* genus, genomic data suggest that they are not part of the *Pasteurella sensu stricto* group and that they will be moved to new genera in the future.

1.2.1. Classification of *P. multocida*

Currently two methods are being used for classification of *P. multocida*. First one is passive hemagglutination test developed by Carter (1952). In this test erythrocytes are sensitized with capsular antigens that recognize five serogroups (A, B, D, E and F) according to capsular polysaccharides. Genetic and structural studies showed that the bacteria belonging to each serotype express distinct polysaccharides on their capsules (Boyce *et al*., 2010). Serogroup A causes fowl cholera in avian species and enzootic bronchopneumonia or pneumonic pasteurellosis in bovine. In pigs, atrophic rhinitis and pneumonia are associated primarily with toxigenic strains of serogroup D and serogroup A, respectively. Serogroups B and E are associated with hemorrhagic septicemia in ungulates. Serogroup F strains are predominantly isolated from diseased poultry, in particular, turkeys and more recently from a fatal case of fibrinous peritonitis in calves (Catry *et al*., 2005; Al-Hasani *et al*., 2007; Lee *et al*., 2007; Dabo *et al*., 2008b). Human infections with *P. multocida* largely arise from the bite of an infected carnivore, but other types of infections are occasionally reported (Wu *et al*., 2007).

Agglutination (serum/plate agglutination or indirect/passive hemagglutination) tests have some disadvantages for classification of *P. multocida* like:
I) Inability of encapsulated cells to agglutinate without treatment.
II) The lack of antigens for hemagglutination on cells which are not encapsulated.
III) Cross reactions among strains (Heddleston *et al*., 1972).

Therefore, the second test for serotyping, gel diffusion precipitin method was developed by Heddleston *et al*. (1972) in which a precipitate is formed upon antigen-antibody reaction according to lipopolysaccharide (LPS) antigen recognizing 16 somatic serotypes (1–16). Recent studies have revealed that LPS layers in each Heddleston serotypes are structurally different from each other (Boyce *et al*., 2010).

Both of these tests are used for the standard classification of *P. multocida*. First capsule, then the LPS type is stated, e.g. A:3 shows capsular type A and LPS type 3. However, since these methods are laborious and require specific antisera which are difficult to obtain, genetic typing techniques have been developed for more accurate classification of *P. multocida* (Boyce *et al*., 2010).

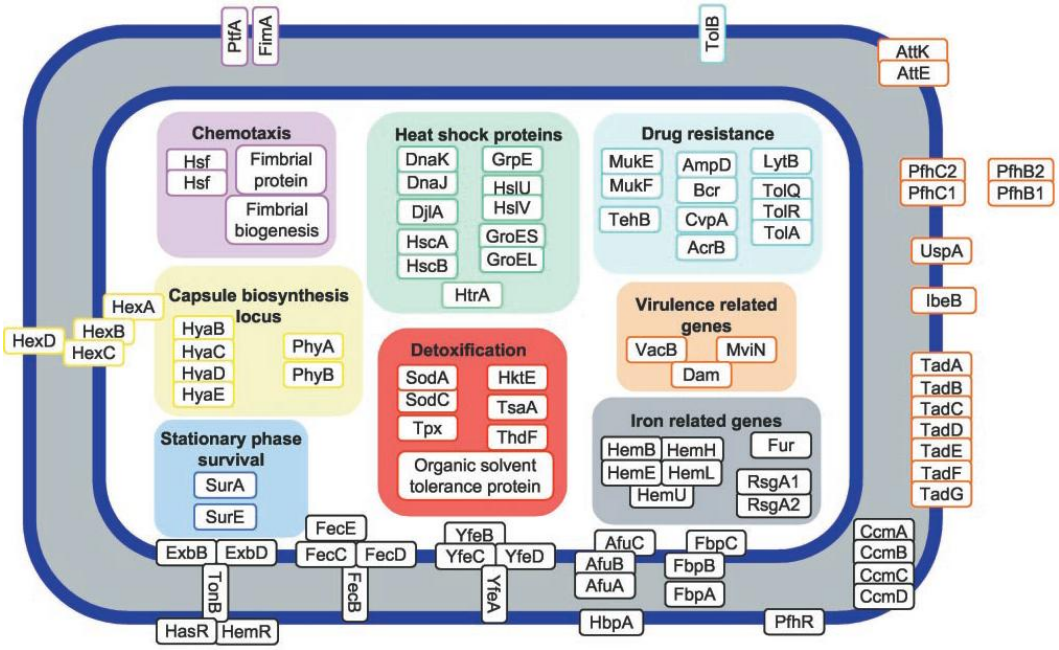

Figure 1.5. Schematic view of the biochemical processes related with *P. multocida* pathogenicity (May *et al*., 2001).

1.2.2. Genome Sequence of *P. multocida*

Genome sequence of an avian isolate of *P. multocida* strain Pm70 serotype A:3 was published by May *et al*. in 2001. The genome consists of 2,257,487 base pairs in length and predicted to contain 2,014 coding sequences (CDSs), 200 (10%) being unique to *P. multocida*, six rRNA operons and 57 tRNAs representing all 20

amino acids. Complete sets of enzymes are encoded for the pathways of Entner–Doudoroff, oxidative pentose phosphate, trichloroacetic acid (TCA) cycle, glycolysis and gluconeogenesis. 104 putative virulence-associated genes (ca. 7% of coding density) were identified. Two genes (*pfhB1* and *pfhB2*) were related with filamentous hemagglutinin. 53 CDSs (more than 2.5% of the genome) were associated with proteins taking role in iron uptake or acquisition (May *et al*., 2001). A schematic view of the biochemical processes taking role in *P. multocida* pathogenicity was shown in Figure 1.5.

1.2.3. Virulence Factors

The major virulence factors of *P. multocida* are capsule, lipopolysaccharide (LPS), toxin (PMT), outer membrane proteins (OMPs), adhesins and type IV fimbriae (pili) (Harper *et al*., 2006).

1.2.3.1. Capsule

Capsule is a highly hydrated polysaccharide protecting the bacterium against phagocytosis and desiccation, and takes role in the bactericidal activity of serum complement. *P. multocida* strains are classified in five (A, B, D, E and F) serogroups with respect to their capsular antigens (Chung *et al*., 2001). Generally, bacterial strains having a capsule are more virulent than their variants lacking the capsule (Harper *et al*., 2006). Acapsular *cexA::tet* strain of *P. multocida* was removed from blood, liver and spleen after i.p. challenge of mice yet wild-type bacteria multiplied rapidly (Boyce and Adler, 2000). Similarly, Chung *et al*. (2001) demonstrated that acapsular *hexA*::*tet* (M) strain of *P. multocida* A:1 (PBA930) was attenuated in both mice and chickens. This acapsular strain was sensitive to bactericidal action of the chicken serum. Mice vaccinated i.m. with PBA930 were cross-protected against A:1 and A:3 strains but s.c. or i.p. vaccinations did not confer any protection (Chung *et al*., 2005). Acapsular strains may be derived from the capsular strains via repeated passage of the bacteria in

the laboratory (Watt *et al*., 2003). Mutation in *fis* gene also resulted in loss of capsule formation and the expression of other virulence factors FHA and PlpE were down-regulated (Steen *et al*., 2010).

1.2.3.2. Lipopolysaccharide (LPS)

In Gram negative bacteria, LPS is an integral component of the outer membrane and in some species, incorporation of lipid A is essential for cell survival. In *P. multocida*, there are two LPS types, glycoform A and B, which have the same outer core but differ in inner core structure (Figure 1.6).

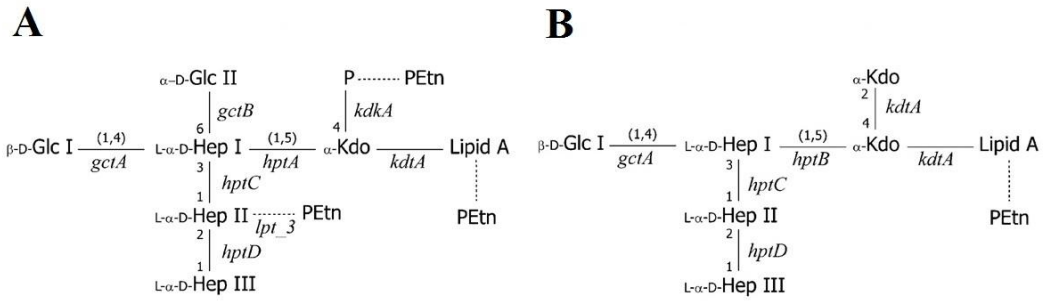

Figure 1.6. Schematic representation of inner core structure of glycoform A **(A)** and glycoform B **(B)**. Glc: glucose, Hep: heptose, P: phosphate, Kdo: 3-deoxy-D-mannooctulosonate, PEtn: phosphoethanolamine (Harper *et al*., 2011).

LPS is an important virulence factor that interacts directly with the innate immune system and induces cellular immune response via toll-like receptor TLR4 (Harper *et al*., 2011). If the host response fails to clear the bacteria and the infection proceeds, accumulation of high amounts of systemic LPS can cause endotoxic shock, and overproduced inflammatory mediators end up with tissue damage, organ failure, septic shock, and death (Harper *et al*., 2004). LPS is the major antigen related with the serotypic classification of *P. multocida*. According to the

antibody responses to LPS, *P. multocida* strains are classified in 16 serotypes (Adler *et al*., 1999). Harper *et al*. (2003) showed that *dcaA* mutant of *P. multocida* was attenuated in mice and chicken due to impaired LPS structure. It is also responsible for the cross protection between serovars 3 and 4 and serovars 2 and 5 (Harper *et al*., 2011).

1.2.3.3. Toxin (PMT)

Pasteurella multocida toxin (PMT) is encoded by *toxA* gene in some strains of serogroup A and D causing progressive atrophic rhinitis (PAR) in pigs (Seo *et al*., 2009) characterized by nasal haemorrhage, bone atrophy and shortening or distortion of snout (Takada-Iwao *et al*., 2007). PMT is a mitogen for fibroblasts and osteoblasts stimulating G protein families G_q and $G_{12/13}$, and activator of phospholipase Cβ (Busch *et al*., 2001; Pullinger and Lax, 2007; Preuß *et al*., 2009). PMT elevates diacylglycerol and inositol 1,4,5-triphosphate levels, protein kinase C activation and Ca^{2+} mobilization in the treated cells (Miyazawa *et al*., 2006). Monomeric 146-kDa protein PMT is composed of 1285 amino acids. N terminus (amino acids 1 to 506) located at the surface and contains cell-binding domain whereas C terminus (amino acids 681 to 1285) contains active site with a mixed α/β domain (Pullinger *et al*., 2001). There are protection studies with mutant forms of PMT against PAR (Petersen *et al*., 1991; Seo *et al*., 2009), however, PMT constitutes only ca. 0.6% of the total bacterial proteins, so toxoid vaccines are not economically efficient (Hsuan *et al*., 2009). Therefore, new vaccine formulations have been studied such as *Bordetella bronchiseptica* and *P. multocida* bacterin-toxoid (Sakano *et al*., 1997) or *P. multocida* bacterin-toxoid (Hsuan *et al*., 2009).

1.2.3.4. Outer Membrane Proteins (OMPs)

The outer membrane proteins (OMPs) of the Gram-negative bacteria are important for the interaction with the extracellular environment. OMPs take role in membrane stability and the transport of various molecules. Non-specific diffusion of solutes and transport of specific ligands are carried out by porins (Boyce *et al*., 2006). The major OMPs of *P. multocida* are shown in Table 1.3.

Table 1.3. The major outer membrane proteins of *P. multocida*.

Designation	Definition	Reference
OmpH	Outer membrane protein H	Luo *et al*., 1997
OmpA	Outer membrane protein A	Dabo *et al*., 2003
Omp16	16 kDa outer membrane protein	Goswami *et al*., 2004
PlpE	*Pasteurella* lipoprotein E	Wu *et al*., 2007
PlpB	*Pasteurella* lipoprotein B	Chomnawang *et al*., 2009
Oma87	87 kDa outer membrane antigen	Ruffalo and Adler, 1996
PCP	Peptidoglycan associated lipoprotein cross-reacting protein	Tabatabai, 2008
GlpQ	Periplasmic glycerophosphodiester phosphodiesterase	Tabatabai, 2008
LctP	Lactate permease P	Tabatabai, 2008
RfaF	Heptosyl transferase F	Tabatabai, 2008
HemR	Heme–hemopexin receptor protein	Tabatabai, 2008

There are protection studies on OMPs of *P. multocida*. OmpH from *P. multocida* B:2 conferred 100% protection in i.p. vaccinated mice but the protection was 80% in s.c. vaccinated animals against challenge with lethal dose of the pathogen (Tan

et al., 2010). Lee *et al*. (2007) reported that recombinant OmpH from a swine isolate of *P. multocida* protected 70% of vaccinated mice. Antisera raised in rabbits against recombinant Oma87 protein protected mice against lethal dose of *P. multocida* A:1 (Ruffalo and Adler, 1996) however, fusion of F1 fragment of Oma87 and GST did not confer any protection in chicken (Mitchison *et al*., 2000). Recombinant OmpA induced Th2-type immune response but did not protect the mice (Dabo *et al*., 2008a). Wu *et al*. (2007) demonstrated that recombinant PlpE from *P. multocida* A:1 cross-protected mice and chickens against challenge with A:1, A:3 and A:4 serotypes, however, protection capacity of PlpB was not that high (Wu *et al*., 2007; Chomnawang *et al*., 2009).

Iron uptake from the environment is also important for the pathogenesis of bacteria because iron is one of the factors regulating the expression of the virulence genes (Puchalski *et al*., 2010). Therefore, iron regulated outer membrane proteins (IROMPs) of *P. multocida* are also mentioned among virulence factors (Garrido *et al*., 2008; Puchalski *et al*., 2010). As discussed earlier, more than 2.5% of *P. multocida* genome codes for proteins related with uptake or acquisition (May *et al*., 2001). They have cross-protection capability. Garrido *et al*. (2008) reported that a *fur* (ferric uptake regulator) mutant of *P. multocida* (serogroup A, ovine isolate) expressing high levels of IROMP conferred cross-protection in mice.

Attachment to host cells or extracellular matrix is important in bacterial infections. Hence, the proteins that mediate this adherence, namely adhesins, are potentially immunogenic (Hatfaludi *et al*., 2010). 39 kDa adhesin, Cp39, in crude capsular extract of *P. multocida* was shown to be cross-protective among serogroup A strains (Sthitmatee *et al*., 2008). Type IV fimbriae (pili) are long, filamentous structures in many Gram-negative bacteria and they also take role in attachment (Hatfaludi *et al*., 2010). Mohd Yasin *et al*. (2011) vaccinated goats with inactivated recombinant *E. coli* expressing fimbrial protein of *P. multocida* B:2 and showed the increase in antibody levels and decrease in the colonization of

pathogen after intratracheal challenge. Fuller *et al*. (2000) identified *pfhaB1* and *pfhaB2* among the virulence genes of *P*. *multocida*. These genes were predicted to encode filamentous hemagglutinin-like proteins taking role in colonization and adherence. Inactivation of *pfhaB2* in an avian isolate of *P*. *multocida* (A:3) caused attenuation of the strain in turkeys after intranasal challenge (Tatum *et al*., 2005).

1.3. Evasion of Pathogens from Host Immune System

The two arms of immune responses are innate and adaptive immunity. Physiological barriers such as skin and mucosal surfaces, tears, normal intestinal flora are the parts of innate immune system found in all multicellular organisms. Macrophages, dendritic cells (DCs), natural killer (NK) cells and neutrophils are the key cellular components. Complement system and the early cytokines are the other key parts of innate immune response. On the other hand, adaptive immune system is constituted by B and T lymphocytes. T cells produce cytokines specific to epitopes of antigens which are presented by B cells, macrophages and DCs. Hence, innate and adaptive immune responses work together.

T cells are divided into T helper (T_h, express CD4, respond MHC class II) and T cytotoxic (T_c, express CD8, respond MHC class I) cells. In response to the antigens, T_h cells secrete cytokines and B cells are differentiated into memory B cells and plasma cells secreting the antibodies, and T_c cells are differentiated into memory T_c cells and effector T_c cells. Secreted antibodies form humoral immunity; on the other hand, cell-mediated immunity is formed by T_c cells against intracellular parasites or by NK cells via antibody-dependent cellular cytotoxicity for extracellular parasites (Srikumaran *et al*., 2007; Elgert, 2009). Despite these protection systems, microbial infections still occur because pathogens develop ways to evade host immunity. A schematic view of immune evasion pathways of BRD associated pathogens was shown in Figure 1.7. The first line of invasion is epithelium and mucosal membranes. BHV-1 infects the epithelial cells of upper respiratory tract resulting in necrosis of epithelium and

adjacent lymphoid tissue. This damage favors bacterial pathogens such as *M. haemolytica* to migrate and colonize in lower respiratory tract.

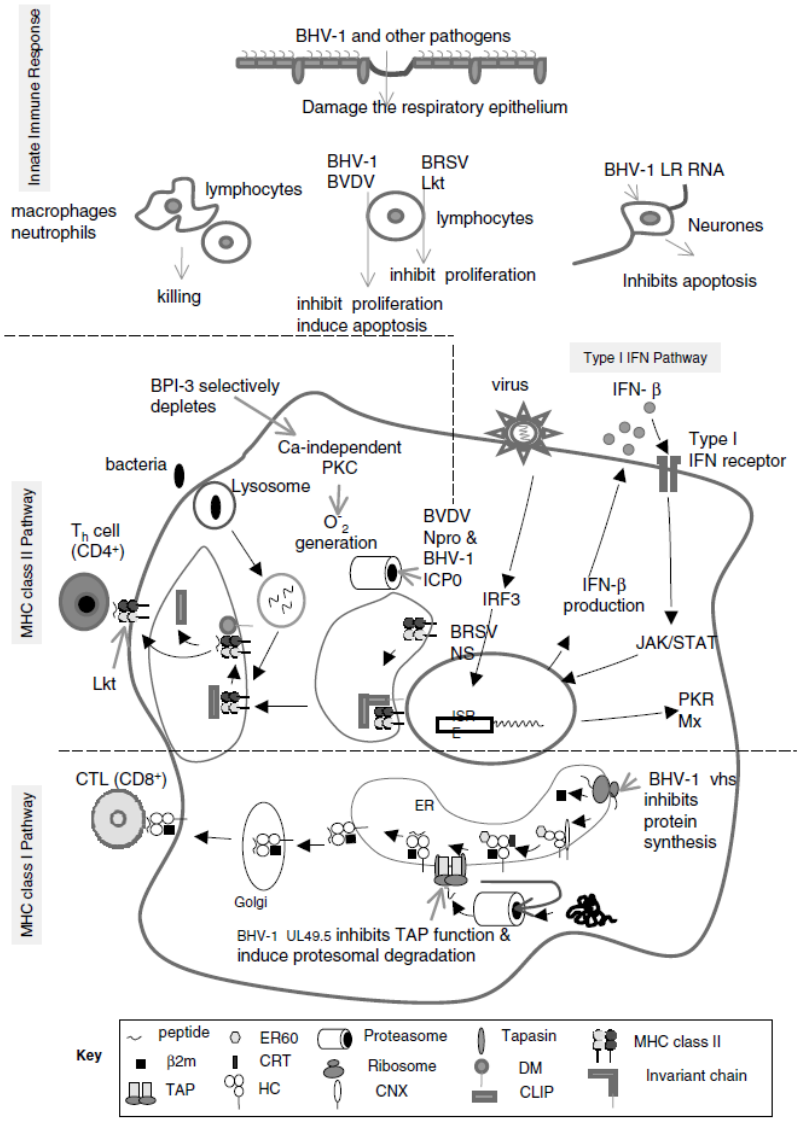

Figure 1.7. Scheme representing immune evasions by pathogens of the BRD complex (Srikumaran *et al*., 2007).

M. haemolytica produces leukotoxin (Lkt) which are cytolytic to leukocytes and evades from phagocytosis, a second barrier. PI3V and *H. somni* are capable of inhibiting the production of superoxide by alveolar macrophages without damaging the cells thus evade from intracellular killing. BHV-1 causes apoptosis in epithelial cells of upper respiratory tract but undergoes a latent infection in sensory neurons by the production of latency-related (LR) RNA. *M. haemolytica*, *M. bovis*, BVDV, BRSV and BHV-1 suppress the proliferation of lymphocytes. BVDV also induces apoptosis of T and B cells or may induce immune tolerance via inhibition of IFN-γ induction. For evasion from humoral response, *M. haemolytica* Lkt down-regulates the expression of MHC class II molecules and *H. somni* blocks binding of immunoglobulins. On the other hand, BHV-1 escapes from T_c cells via down-regulating expression of MHC class I molecules through inhibition of host cell protein synthesis with vhs (virion host shot-off) protein. Many pathogens evade immune responses by rapidly changing the structure of their antigenic surface proteins. For instance, *H. somni* undergoes an antigenic phase variation in its lipooligosaccharide (LOS) components (Srikumaran *et al*., 2007).

1.4. Vaccine Development

The term "vaccine" was derived by Edward Jenner from his less dangerous vaccine strain *Variolae vaccinae* (cowpox virus) adapted from *vaccinus* (*vacca*, cow). Still some of the veterinary vaccines are produced using the same technology introduced by E. Jenner on live vaccines in 1796 and L. Pasteur on killed whole cell vaccines. On the other hand, recent developments in systems biology brought new approaches to vaccine design (Adams *et al*., 2011). Figure 1.8 shows a flow chart on interactions in systems biology for vaccine development. The basic factors that should be considered in design of a successful vaccine are efficacy, safety and the characteristics of the pathogen as summarized in Table 1.4 (Mak and Saunders, 2008). Vaccine types can be categorized as live (attenuated), killed (whole cell), subunit and DNA vaccines.

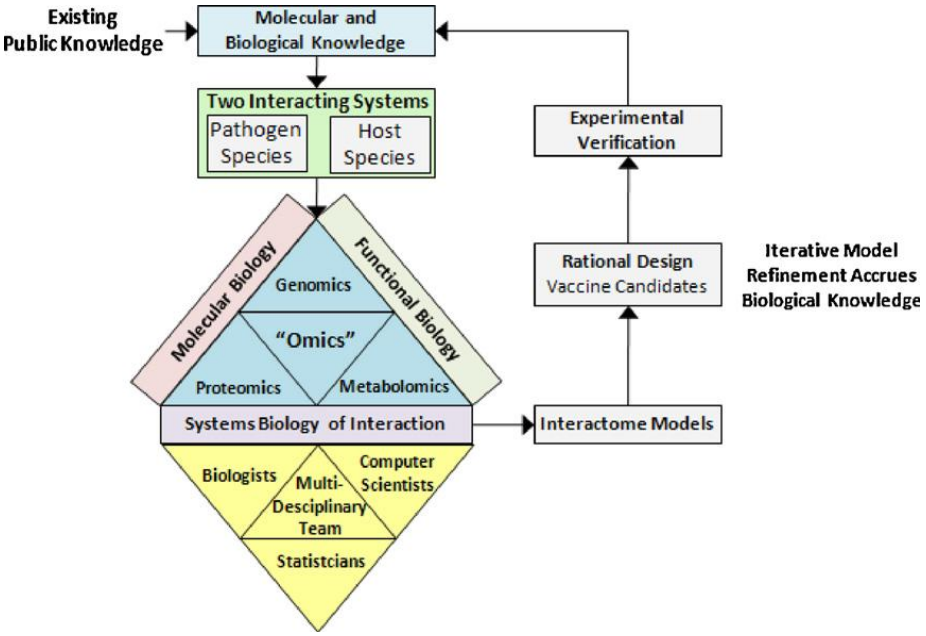

Figure 1.8. The flow chart showing the interactions in systems biology for vaccine design (Adams *et al*., 2011).

Table 1.4. Factors considered in designing of a successful vaccine (Mak and Saunders, 2008).

Characteristic	Description
Efficacy	‣ Induction of appropriate immune responses to eliminate the pathogen ‣ The coverage (percentage of vaccinated individuals protected from the disease) should be 80-95%
Safety	‣ No risk to cause a disease. Detrimental side effects should be very few
Pathogen	‣ Causing an acute rather than chronic infection ‣ Induction of immunity after exposure ‣ Antigenic variation should be little ‣ No attack to the cells of the immune system ‣ Not having an environmental or animal reservoir

1.4.1. Live (Attenuated) Vaccines

As its name implies, a live vaccine is developed using an infectious agent that is still alive but has been somehow weakened (attenuated) not to cause a disease. The most effective viral vaccines are live vaccines attenuated via either heating, continuous passage in tissue culture/animal host, or genetic modifications. A natural pathogen in another host can also be used, e.g. E. Jenner used cowpox virus against smallpox disease in human (Ada, 2003; Lund *et al*., 2005; Arnon, 2011).

The vaccine agent has the same broad spectrum of antigens as the pathogen and since it is able to replicate in the host, exists for longer periods than killed vaccine agent, hence, provides more effective and long lasting immunity generating both humoral and cell-mediated responses. On the other hand, rarely the vaccine agent may revert back and gain pathogenicity or in immune deficient patients even the attenuated form may cause serious infections. Usually, fewer than two per million cases occur annually among vaccinated people (Arnon, 2011).

A successful example of live vaccines is the Bacille Calmette-Guérin (BCG) against tuberculosis. The vaccine was developed via over 200 subculturing of the pathogen by Albert Calmette and Camille Guérin between 1905 and 1921 at Pasteur Institutes in France and started to be used internationally after 1927 (Bonah, 2005; Arnon, 2011).

1.4.2. Killed Whole Cell Vaccines

A killed vaccine contains an organism (a whole bacterium, parasite or virus) that is antigenically intact, but lost the ability of replication or induction of any clinical disease due to the inactivation through heat, gamma irradiation or chemicals such as formaldehyde (formalin), alcohol or alkylating agents (Mak and Saunders, 2008; Day and Schultz, 2011).

Killed vaccines are safer but less effective than live vaccines. If the pathogen is killed properly, it is unable to revert back but the killing process may damage some of the antigens. As the vaccine agent cannot replicate, it is cleared from the body quickly, thus it does not induce the immune responses as well as live organism (Arnon, 2011) and larger amounts of the vaccine should be administered in the primary dose that raises the cost. A weaker response to a given dose is obtained, so frequent boosters are needed (Mak and Saunders, 2008). Most of the killed vaccines require an adjuvant for an adequate immune response. Generally adjuvanted vaccines are considered more likely to have an adverse effect (Day and Schultz, 2011).

Killed vaccines provide better protection than an isolated compartment of the organism. They activate a high inflammatory response inducing an effective antigen presentation by DCs. However, sometimes this may cause side effects like fever, local pain and allergic reactions (Arnon, 2011). Since the vaccine agent is dead it cannot effectively penetrate the host cells. Therefore, they generally induce high levels of neutralizing antibodies but not MHC class I- restricted cytotoxic T-cell (T_c) response. As a result, killed vaccines are not very effective against intracellular pathogens (Ada, 2003; Mak and Saunders, 2008).

In spite of the mentioned drawbacks, killed viral vaccines against influenza, Hepatitis type A, Salk polio and Japanese Encephalitis are wide in use. Preparation of killed whole cell vaccine is easier and less time consuming as well as its safety. These advantages are sometimes very crucial when a vaccine is needed in case of a pandemic disease like H5N1 avian influenza in 2005 and H1N1 influenza in 2009. Bacterial killed vaccines in use for human health are against pertussis (*Bordetella pertussis*) and cholera (*Vibrio cholera*). Studies still proceed to develop an acellular vaccine against pertussis and a toxoid-bacterin vaccine against cholera (Arnon, 2011).

1.4.3. Subunit Vaccines

Subunit vaccines contain specific immunogenic structural proteins or metabolites obtained from the pathogen instead of the entire intact organism. Therefore, they are very safe lacking the risk of reversion or any possible side effects resulting from irrelevant compartments of the pathogen (Mak and Saunders, 2008; Day and Schultz, 2011).

1.4.3.1. Protein Based Subunit Vaccines

Some of the proteins for subunit vaccine production are prepared using conventional purification processes which are very laborious, less effective and increase the cost. Recombinant DNA technology greatly facilitated the vaccine development for pathogens that are very difficult or impossible to grow in vitro and/or whose components are very problematic to purify in sufficient amounts. The gene encoding the antigen is cloned on a vector and introduced to a yeast or bacterial host such as *E. coli*. Recombinant microorganisms are cultured in high volumes in the laboratory and the expression of desired protein is induced using different techniques such as IPTG induction. Protein of interest can easily be isolated from the other proteins of the recombinant organism. However, the host organism may alter the three dimensional conformation of the protein that may decrease or lose its stability and protectivity (Mak and Saunders, 2008). Their advantages are being well characterized and targeting the immune response to specific immunodominant antigens. Nevertheless, they do not activate a broad response as the whole organism does. Like the killed vaccines, it is difficult to get cell-mediated immunity with subunit vaccines. For a better immune response, they require an adjuvant which initiates inflammation and provides prolonged release of the antigen (Arnon, 2011).

1.4.3.2. Polysaccharide Based Subunit Vaccines

Capsular polysaccharides of the pathogenic encapsulated bacteria such as *Salmonella typhi*, *Streptococcus pneumoniae*, *Neisseria meningitidis* or *Haemophilus influenzae* are used for vaccine development. The induction of immunity may occur through two mechanisms: I) polysaccharides serve as haptens and bind to self proteins, then the conjugate is taken up by DCs for activation, II) presentation of non-peptide antigens to T-cells by MHC-like molecule CD1 (Arnon, 2011). Total IgG antibodies against *H. influenzae* type b (Hib) capsular polysaccharides started to increase after 11 months of age and the significant increase in IgG2 levels were observed after 22 months of age in children (Claesson *et al*., 1988). Therefore, immune induction by polysaccharides is not effective in children younger than 2 years of age. In these cases, vaccine efficacy can be increased by joining the capsule polysaccharide to a carrier protein such as diphtheria or tetanus toxoid supplying a T-cell epitope. These types of vaccines are called "conjugate vaccines" (Mak and Saunders, 2008). For an effective vaccine against Hib infection in children, the protective capsular polysaccharide PRP is conjugated to either OMP of Hib or tetanus toxoid. Although conjugate vaccines provide long lasting IgG response, pure polysaccharide vaccines such as Pneumovax-23 containing 23 different polysaccharides from *S. pneumoniae* are still in use for adults (Arnon, 2011).

1.4.3.3. Toxoid Vaccines

Several bacteria such as *Corynebacterium diphtheriae* and *Clostridium tetanii* are non-invasive but the toxins they secrete cause pathogenicity. Toxoid vaccines (toxoids) are developed against these types of diseases via inactivating the bacterial toxins by chemicals such as formaldehyde; hence, they lose their toxicity but keep immunogenicity (Mak and Saunders, 2008; Arnon, 2011). For instance, the diphtheria vaccine is produced via growing *C. diphtheriae* in liquid medium, passing the culture from a filter, incubating the filtrate with formaldehyde, and

then adsorption of the toxoid onto aluminum salt (Koslap-Petraco and Hackley, 2011). Antibodies raised upon toxoid immunization neutralize the toxins via binding to them, and then the toxins are cleared from the system. Tetanus and diphtheria toxoids are routinely used against *C. tetanii* and *C. diphtheriae* infections, respectively. Pertussis vaccine also includes *B. pertussis* toxin but special efforts were required in order to eliminate completely its neurotoxicity. The pediatric vaccine DPT (diphtheria-pertussis-tetanus) is a combination of these three toxoids and used worldwide in almost every country (Arnon, 2011).

1.4.3.4. Reverse Vaccinology

In conventional subunit vaccine development, the pathogen is grown in laboratory conditions and separated into individual components. Ability of the each component is tested for induction of the immune responses. This process is time-consuming and only proteins which can be purified in adequate amounts for vaccination tests are identified. Mostly these proteins are not useful for vaccine development because the proteins expressed during infection are not expressed while in vitro culturing. Therefore, it may take several years or decades to identify a protective antigen produced during infection in vivo. Once the suitable antigen is identified, recombinant DNA technology as mentioned previously can be used to obtain the protein in large amounts (Rappuoli, 2001).

A new era started after late 1990s by the publication of genome sequence of a pathogen *H. influenzae* for the first time and by October 2011, 1,670 bacterial genome projects have been finished and 5,478 are ongoing (http://www.genomesonline.org/cgi-bin/GOLD/bin/gold.cgi). By the help of bioinformatics, these genomes are mined for disease related genes; candidate antigens are defined and tested for protectivity. Genomes belonging to multiple strains of a pathogen are mined to obtain a universal vaccine or synthetic peptides are designed mimicking the T-cell and B-cell epitopes. Since this technology proceeds on the opposite way of conventional vaccine development, it is called

"reverse vaccinology". An example of the first applications of reverse vaccinology is the vaccine development studies against *N. meningitidis* which causes sepsis and meningitis in young adults and children. After genome project of *N. meningitidis* started, more than 600 genes coding surface-exposed proteins were identified and half of them were expressed and tested for immunogenicity. 91 antigens were identified and 29 of them were found to be protective (Mora *et al*., 2006). Computer based technologies are very important in reverse vaccinology. There are many software programs for vaccine target prediction like PSORTb (http://www.psort.org/psortb/; Yu *et al*., 2010), LipoP (http://www.cbs.dtu.dk/services/LipoP/; Rahman *et al*., 2008), SVMHC (http://www.sbc.su.se/~pierre/svmhc/new.cgi; Dönnes and Elofsson, 2002), EPIMHC (Reche *et al*., 2005), SMM-align (Nielsen *et al*., 2007), Ensembl (http://www.ensembl.org/index.html). Al-Hasani *et al*. (2007) identified 6 novel immunogenic proteins of *P. multocida* serotype A:1 utilizing PSORTb, LipoP and Protemome Analyst software programs. Recently, He *et al*. (2010) developed a web-based vaccine design system, Vaxign (http://www.violinet.org/vaxign/) that predicts vaccine targets against different pathogens using reverse vaccinology tools.

1.4.4. DNA Vaccines

DNA vaccines are the recombinant bacterial plasmid DNA molecules carrying the genes encoding for foreign (immunogenic) proteins under a eukaryotic promoter for the expression in mammalian cells (Garmory *et al*., 2003). In 1990, Wolff *et al*. showed that the i.m. injection of DNA expression vector carrying the luciferase gene resulted in luciferase production in vivo in the muscle cells of the mouse at least 2 months without a special delivery system. Later, Tang *et al*. (1992) delivered DNA-coated gold microparticles directly into the skin of mice using a biolistic system (gene gun) for eliciting an immune response in mice against a foreign protein. Delivered DNA molecules are taken up by DCs and expressed foreign protein is processed during passage to the draining lymph nodes. Related peptides are attached to the MHC molecule and expressed on the

cells surface. Immunocompetent T-cells in the lymph node recognize the MHC complex and activated. Thus, a type 1 T-cell response is induced in subhuman primates including both humoral and cell-mediated immunity with increased $CD4^+$ and $CD8^+$ effector T-cells (Ada, 2003). In the case of i.m. injection of DNA vaccines, the functional DNA moves to the spleen through blood and antigen-presenting cells (APCs) induce the immune responses (Arnon, 2011).

DNA vaccines are relatively easy in production and cost less with a good safety and preclinical efficacy. Human clinical trials for DNA vaccines against the diseases such as HIV and SARS are ongoing but there are several approved DNA vaccines for use in animals (Larsen *et al*., 2009) such as the one protecting horses against West Nile virus infection. Intradermal injection of a single dose of DNA vaccine containing the plasmid construct carrying the gene for rabies glycoprotein G induced serum neutralizing antibodies and protected the Beagle dogs against challenge with the virulent virus one year after the vaccination (Day and Schultz, 2011). There are DNA vaccine studies against BRD associated pathogens as well. van Drunen Littel-van den Hurk *et al*. (2010) delivered the plasmid construct carrying the gene encoding glycoprotein E2 of BVDV intramuscularly to newborn calves via an electroporation-based system. Induced humoral and cell-mediated immune responses and close to complete protection from clinical signs of the disease were observed in vaccinated calves. In order to increase the efficacy, prime-boost regimens can be applied. Priming with an E2 DNA vaccine and boosting with CpG adjuvanted E2 protein increased both humoral and cell-mediated immune responses and protected the calves from the challenge of BVDV-1 (Liang *et al*., 2006) and BVDV-2 (Liang *et al*., 2008).

1.4.5. Adjuvants

The term “adjuvant” is derived from the Latin word “adjuvare” that means “to help” what exactly the adjuvants do. Most of the vaccines are composed of nonliving material that cannot stimulate a significant immune response.

Therefore, these types of vaccines are formulated with an adjuvant for an increased efficacy. Adjuvants are responsible for a local inflammation as well as tissue destruction, distress and pain which are the key components in induction of humoral and cell-mediated immune responses. Nonliving adjuvants may also act as both delivery systems like liposomes, or as modulators like monophosphoryl lipid A (MPL). Different types of adjuvants have different effects on innate immunity which in turn shapes the adaptive immune response. Therefore, the type of adjuvant has to be selected accordingly. Extracellular pathogens are mostly eliminated by humoral response and Th2 skewing whereas infections caused by intracellular pathogens require cell-mediated immunity and Th1 domination (Lycke, 2007).

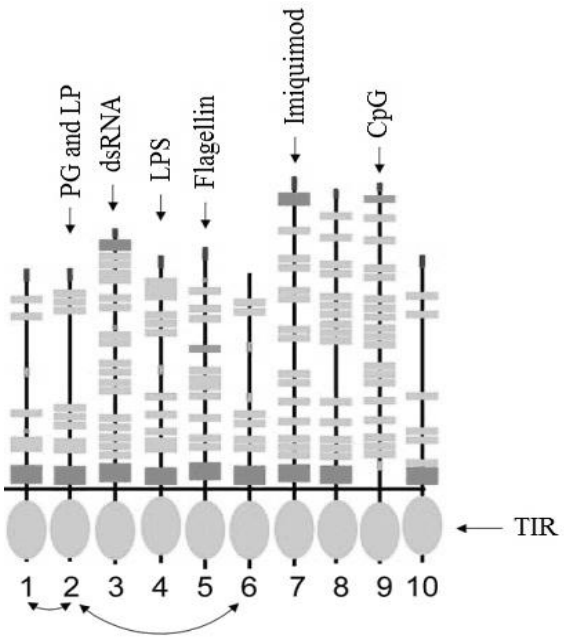

Figure 1.9. Schematic representation of 10 human TLRs and their interaction with molecules of microbial origin. TLRs 1 and 2, and 6 and 2 form heterodimers. PG: peptidoglycan, LP: lipopeptides, Imiquimod: a synthetic antiviral compound, TIR: Toll/Interleukin-1/Resistance domain (Beutler, 2006).

Specific molecules of microbial origin such as LPS, DNA, RNA, bacterial lipopeptides and lipoteichoic acid carry out adjuvant effect interacting with mammalian Toll-like receptors (TLRs) (Figure 1.9). In *Drosophila*, Toll protein is responsible for the development of ventral structures. The molecules taking role in Toll and TLR signaling pathways are the members of the same family (Beutler, 2006).

Many different compounds such as aluminum hydroxide, aluminum phosphate, oil-in-water emulsions, nucleotides (CpG), polyphosphazenes, liposomes, and cytokines have been used for adjuvant purposes (Singh, 2007).

The story of immunological adjuvants started over 85 years ago. In 1924, Lewis showed that i.p. injection of live tuberculosis (TB) pathogen a few days prior to the vaccination with antigens dramatically increased the immune responses against those antigens (Ott and van Nest, 2007). Later, in 1925 G. Ramon reported that it was possible to increase the levels of tetanus or diphtheria antitoxin by adding bread crumbs, starch oil, agar, tapioca, saponin, or lecithin to the vaccines (Edelman, 2000). Two important progresses were carried out by Freund in 1930s by the formulation of "incomplete Freund's adjuvant" (IFA) which is a water-in-oil emulsion containing 10% mannide monooleate (Arlacel A) and 90% mineral oil, and "complete Freund's adjuvant" (CFA) containing water-in-oil emulsion mixed with killed TB. By the mid-1940s, the potency of water-in-oil systems was increased and low-reactogenic alum systems were developed (Ott and van Nest, 2007). Alum compounds such as aluminum hydroxide [$Al(OH)_3$] and aluminum phosphate ($AlPO_4$) are all known as "alum" but their physical characteristics and adjuvant properties are different. Currently, the most prevalent method for preparation of aluminum adjuvanted vaccines is adsorption of antigens onto preformed $Al(OH)_3$ or $AlPO_4$ gels under controlled conditions (Gupta and Rost, 2000). There are some limitations in use of aluminum adjuvants: I) impropriety for boosting immunizations with tetanus and diphtheria antigens, II) formation of granulomas at the injection site, III) occasional erythema, IV) increase in IgE

levels and V) lack of biodegradability. On the other hand, oil adjuvants also have some disadvantages like I) intense inflammation, II) formation of granulomas, III) not retaining at the injection site, IV) carcinogenesis risk by poorly metabolized mineral oils, and V) toxicity of CFA (Ott and van Nest, 2007). Activation of immune cells by synthetic oligodeoxynucleotides (ODNs) was first reported by Yamamoto *et al*. in the beginning of 1990s. Later, Krieg *et al*. (1995) showed that unmethylated CpG dinucleotides induce innate immune system via interaction with TLR9 in endocytic vesicles of the immune cells; then swelling and acidification of vesicles result in production of reactive oxygen species (Klinman *et al*., 2004).

1.4.6. Development of Vaccine Strategies against *P. multocida*

Animal experiments and field studies have shown that utilization of *M. haemolytica-P. multocida* bacterin-toxoids in feedlot animals decreases BRD occurrence and/or overall mortality rates (Wildman *et al*., 2008) and live (attenuated) vaccines provide cross-protection against varying *P. multocida* serotypes. Nevertheless, since the attenuation mechanism is not clear, outbreaks come out in flocks vaccinated with live (attenuated) vaccines. Moreover, serotype-specific immunity is the drawback of killed vaccines (Lee *et al*., 2007). In recent years, chimeric genes created by genetic fusion have been studied for use in recombinant vaccine development because of their versatility (Ayalew *et al*., 2008). DNA vaccine technology is another alternative mean to improve vaccine efficacy. Register *et al*. (2007) conducted a DNA vaccine study using either a 5'-truncated or full length, genetically detoxified *toxA* gene from *P. multocida* serogroup D in two different vectors. The construct carrying a signal sequence with the full length toxin increased both antibody and interferon-γ levels in pigs. Seo *et al*. (2009) reported that a truncated form of PMT was protective in mice and reduced the clinical signs of AR in pigs.

Outer membrane proteins (OMPs) are potential targets for novel antimicrobial drugs as well as vaccines against Gram-negative bacteria (Gatto *et al*., 2002; Carpenter *et al*., 2007). Basagoudanavar *et al*. (2006) showed that mice immunized with OMPs of *P. multocida* 6:B had high antibody titers and Montanide adjuvanted OMPs protected 94% of the animals against bacterial challenge. In *P. multocida*, OmpH is one of the major antigenic, surface-exposed and conserved OMP porin that is detected in 100% of bovine isolates investigated and has potential as a vaccine candidate (Dabo *et al*., 2008b). Protection capacities of recombinant OmpH from *P. multocida* serotype D and B:2 in mice (Lee *et al*., 2007 and Tan *et al*., 2010, respectively) and A:1 in chicken (Luo *et al*., 1997) were reported. Antigenicity and protection capability of OmpA from *P. multocida* were also studied. A strong Th2-type immune response was observed with recombinant OmpA from *P. multocida* serotype A:3 but no protection could be obtained in mice (Dabo *et al*., 2008a). Likewise, OmpA-equivalent Omp28 from *P. multocida* A:3 did not confer any protection in mice (Gatto *et al*., 2002). *Pasteurella* lipoprotein E (PlpE) is another immunogenic OMP of *P. multocida*. Recombinant PlpE from *P. multocida* A:1 was cross-protected the vaccinated mice against challenge with *P. multocida* serotypes A:1, A:3 and A:4, and the vaccinated chicken against A:1 and A:4 (Wu *et al*., 2007). However, protectivity of recombinant PlpB from *P. multocida* A:1 was very low (20-30%) in mice (Chomnawang *et al*., 2009). Sthitmatee *et al*. (2008) reported that recombinant adhesive protein rCp39 from *P. multocida* A:3 was cross protective in chicken against challenge with A:3 and A:1 serotypes. Non-lipidated or lipidated forms of two recombinant lipoproteins GlpQ and PCP from *P. multocida* A:1 induced antibody titers but did not confer any protection in mice (Lo *et al*., 2004).

P. multocida serotypes used in vaccine development have been predominantly avian or swine isolates. There is no vaccine study to date utilizing recombinant OmpH and/or PlpE from a bovine isolate of *P. multocida* A:3. Hence, there is a need of studies on bovine isolates of *P. multocida* for vaccine development against shipping fever.

1.5. The Present Study

The goal of this study is to develop novel vaccine formulations against shipping fever using PlpE and OmpH from a bovine isolate of *P. multocida* (A:3). The chimeras of two *plpE* fragments and *ompH* were used as DNA vaccines. The recombinant proteins and their fusions expressed in *E. coli* were purified and used for immunization of mice. Effects of different adjuvants on protection were also studied. Humoral and cell-mediated immune responses in mice induced by DNA and protein based vaccines and their protective efficacies were evaluated.

CHAPTER 2

MATERIALS AND METHODS

2.1. Bacterial Strains and Plasmids

The sources and characteristics of bacterial strains are listed in Table 2.1. Plasmids used in cloning experiments are given in Table 2.2. The structures of plasmid vectors and the size markers are presented in Appendix A.

Table 2.1. Sources and characteristics of the bacterial strains used in this study.

Strain	Characteristics	Source and Reference
P. multocida P-1062	Serotype A:3, bovine strain	American Type Culture Collection (ATCC 15743)
E. coli DH5α	F' ϕd*lac*ZΔ(*lac*ZY A-argF)U169 *sup*E44λ$^-$ *thi*-1 *gyr*A *rec*A1 *rel*A1 *end*A1 *hsd*R17	American Type Culture Collection
E. coli BL21(DE3)	F$^-$ *ompT gal dcm lon hsd*S_B(r_B^- m_B^-) λ(DE3 [*lac*I *lac*UV5-T7 gene 1 ind1 sam7 nin5])	Novagen, Merck (Germany)

Table 2.2. Plasmids used in cloning and expression.

Plasmid	Size	Markers	Source and Reference
pCMV-LII	3.8 kb	*amp* (Amp^r)	Dr. F. Rodriguez (Rodriguez *et al*., 2001)
pET-28a(+)	5.3 kb	*kan* (Kan^r)	Novagen, Merck (Germany)
pGEM®-T Easy	3.0 kb	*amp* (Amp^r), *lac*Z	Promega Inc. (Madison, WI)

2.2. Culture Media

The composition and preparation of culture media are given in Appendix B.

2.3. Solutions and Buffers

The composition of solutions and buffers are given in Appendix C.

2.4. Chemicals and Enzymes

The chemicals and enzymes used and their suppliers are listed in Appendix D.

2.5. Growth Conditions and Maintenance of Bacterial Strains

P. multocida were grown in Brain Heart Infusion (BHI) Broth (Appendix B) and Blood agar (BA, Appendix B) plates whereas *E. coli* BL21 and *E. coli* DH5α strains were grown in Luria Broth (LB, Appendix B) medium and stored on Luria agar (LA, Appendix B) plates. The cultures were stored at 4^{o}C and subcultured monthly. Cultures grown in LB until mid-log phase were covered with 50% glycerol for long term storage at –80^{o}C.

LB and LA media were supplemented with the appropriate antibiotics, whenever necessary. The concentrations of antibiotics included in media were as follows: Ampicillin, 100 μg/mL; kanamycin, 30 μg/mL.

2.6. Primer Design

Primers for amplification of *ompH* and *plpE* genes of *P. multocida* P-1062 were designed according to the complete genome sequence of *P. multocida* subsp. *multocida* strain Pm70 (NCBI accession number NC_002663) (Table 2.3).

Table 2.3. Primers used in PCR. Restriction enzyme cut sites are underlined.

Gene name	Primer name	Nucleotide sequence	Size of the PCR products
ompH	ompHF	5' agatct**atg**gcaacagtttacaa 3'	984 bp (with ompHR)
ompH	ompHR	5' agatctttagaagtgtacgcgta 3'	984 bp (with ompHF)
ompH	ompHRN	5' agatctgaagtgtacgcgtaaac 3'	981 bp (with ompHF)
plpE	plpEF	5' ggatcc**atg**tgtagcggtggtgg 3'	948 bp (with plpER)
plpE	plpER	5' agatctttgtgcttggtgactt 3'	948 bp (with plpEF)
*plpE*N	plpENR	5' cggagatcttccataacttccataat 3'	489 bp (with plpEF)
*plpE*C	plpECF	5' ggatcc**atg**ccttcagcagattacaa 3'	474 bp (with plpER)

Table 2.4. PCR conditions for amplified genes.

Product	Primers used	PCR conditions (35 cycle)
ompH (without signal sequence)	ompHF and ompHR	Initial denaturat.: 3 min at 94°C Denaturation: 1 min at 94°C Annealing: 1 min at 50°C Extension: 1 min at 72°C Final extention: 10 min at 72°C
ompH (without signal sequence and stop codon)	ompHF and ompHRN	Initial denaturat.: 3 min at 94°C Denaturation: 1 min at 94°C Annealing: 1 min at 50°C Extension: 1 min at 72°C Final extention: 10 min at 72°C
plpE (without signal sequence and stop codon)	plpEF and plpER	Initial denaturat.: 3 min at 94°C Denaturation: 1 min at 94°C Annealing: 1 min at 50°C Extension: 1 min at 72°C Final extention: 10 min at 72°C
*plpE*N (N terminal fragment of *plpE* without signal sequence and stop codon)	plpEF and plpENR	Initial denaturat.: 3 min at 94°C Denaturation: 30 sec at 94°C Annealing: 30 sec at 55°C Extension: 30 sec at 72°C Final extention: 10 min at 72°C
*plpE*C (C terminal fragment of *plpE* without signal sequence and stop codon)	plpECF and plpER	Initial denaturat.: 3 min at 94°C Denaturation: 30 sec at 94°C Annealing: 30 sec at 55°C Extension: 30 sec at 72°C Final extention: 10 min at 72°C

2.7. Polymerase Chain Reactions (PCR)

Final concentrations in PCR mixture were as 1X PCR buffer (Fermentas), 0.2 mM dNTP mix (Fermentas), 0.4 mM of each primer, 2.5 mM $MgCl_2$ (Fermentas), 2

Units of *Taq* polymerase (Fermentas), and 10 ng of template DNA. The volume was completed to 50 µL with dH_2O. The sequences of primers and the size of products are given in Table 2.3. Table 2.4 shows primers and PCR conditions used for the amplification of the genes of interest.

After PCR, amplicons were run on 1% agarose gel. Desired bands from PCR products were cut from the gel and extracted using Qiagen Gel Extraction Kit.

2.8. Agarose Gel Electrophoresis

Electrophoresis was carried out on a horizontal submarine electrophoresis apparatus. 1% agarose gel was prepared in TAE buffer (Appendix C) and run at 90 Volts for 45-60 min. The gel was stained with ethidium bromide solution (0.5 µg/mL of TAE buffer). The DNA bands were visualized on a shortwave UV transilluminator (UVP, Canada) and photographed using Vilber Lourmat Gel Imaging System (Vilber Lourmat, France). *Pst*I digested Lambda DNA marker (Fermentas, Appendix A) was used to determine the molecular weights of DNA bands.

2.9. Sequencing Reactions

DNA sequencing was carried out either by RefGen Biotechnology Inc. (Ankara, Turkey) or MCLAB DNA Sequencing Department (San Francisco, CA) using the chain termination method with BigDye Cycle Sequencing Kit V3.1 (Applied Biosystems) in ABI 3130xl Genetic Analyzer (Applied Biosystems).

Deduced nucleotide and amino acid sequence data were analyzed using National Center for Biotechnology Information (NCBI) database using the BLAST search at the web site (http://www.ncbi.nlm.nih.gov/BLAST). Phylogenetic analyses were conducted using MegAlign (DNASTAR, Madison, WI) and MEGA4 (Tamura *et al*., 2007) computer programs.

2.10. Ligation Reactions

Ligation of PCR products to pGEM-T Easy vector (Promega) was performed as supplier's recommendation. Briefly, 5 μL of 2X ligase buffer, 50 ng of pGEM-T Easy, 100 ng of PCR product and 3 Weiss units of T4 DNA ligase was mixed and the volume was completed to 10 μL with H_2O. Ligation was carried out as overnight incubation at 4^oC. When the vectors pCMV-LII or pET28a(+) were used, vector and insert DNA were mixed in 1:3 molar ratio, 5 Weiss units of T4 DNA Ligase (Fermentas) and 1 μL of 10X ligase buffer were added and the mixture was incubated 16 h at 4^oC.

2.11. Transformation of *E. coli* Cells

E. coli competent cells were prepared according to the protocol described by Hanahan D. (1985). A single *E. coli* colony from a fresh LA plate was inoculated in 3 mL of LB and incubated overnight with shaking at 37°C to obtain a stationary phase culture. Three ml of this seed culture was inoculated into a fresh flask containing 200 mL LB medium. The culture was incubated at 37 °C at 200 rpm in an orbital shaker until the OD_{600} reaches 0.4-0.6. Then the culture was incubated on ice for 15 min. After centrifuging at 3500 rpm for 5 min at 4 °C, supernatants were decanted and the pellet was resuspended in 20 mL of ice-cold Buffer 1 (Appendix C). The cells were spun down at 3,500 rpm for 5 min at 4°C. Finally, supernatants were decanted and the pellet was resuspended gently in 8 mL of ice-cold Buffer 2 (Appendix C). 100 μL of aliquots were incubated on ice for 15-30 min and the cells were frozen in liquid nitrogen. The competent cells were stored at – 80°C.

For transformation, 100 μl aliquot of competent *E. coli* cells were thawed on ice for 10 min. 10 μL of ligation products or 0.5 ng of appropriate plasmid DNA was added to the cells and mixed gently. The mixture was incubated on ice for 30 min. After a heat shock at 42^oC for 60 sec, it was incubated on ice for 5 min. 900 μL of

LB was added to the mixture and incubated at 37^{o}C for 80 min by gentle agitation (100 rpm). The cells were centrifuged at 3000 rpm for 10 min and resuspended in 100 µL of LB. Transformed cells were plated on selective medium containing appropriate antibiotic (100 µg/mL ampicillin or 30 µg/mL kanamycin). For blue – white colony selection, they were plated on LB agar media containing 80 mg/mL X-gal, 0.5 mM IPTG and 100 µg/mL ampicillin.

2.12. Plasmid Isolation

QIAprep Spin Miniprep Kit (Qiagen) or GeneJET Plasmid Miniprep Kit (Fermentas), were used for the isolation of *E. coli* plasmid DNA as described by the manufacturers.

E. coli plasmids were also isolated by the miniprep method described by previously (Kieser *et al*, 2000). Each strain was grown as a patch on LB agar containing 100 µg/mL ampicillin or 30 µg/mL kanamycin. Ca. 1 cm^2 of bacterial cell mass was scraped with a sterile toothpick and put into Eppendorf tube containing 100 µL cold STE buffer (Appendix C). The cells were resuspended by vortexing and the tubes were incubated on ice for 20 min. 3/5 volume of lysis buffer (Appendix C) was added to each tube and vortexed immediately. The cells were lysed by incubation at room temperature for 10 min and then at 70°C for 10 min to denature DNA. Afterwards, tubes were cooled rapidly in cold water. An equal amount of phenol/chloroform/isoamylalcohol (Amresco, OH) was added and vortexed hard until a homogeneous and milky white mixture was obtained. Finally, the samples were centrifuged for 5 min at 13,000 rpm to separate phases. 20 µL of supernatant was loaded directly on an agarose gel for electrophoresis.

Endotoxin free plasmids for DNA vaccine preparation were isolated using Endofree Plasmid Mega Kit (Qiagen) as described by the manufacturer.

2.13. Restriction Enzyme Digestion

Restriction enzyme was added in a suitable buffer to the DNA to introduce 1 Unit per μg of DNA. The mixture was incubated at a temperature appropriate for that restriction enzyme for 3-5 h. The sample was stored at -20^{o}C when needed.

2.14. Construction of Recombinant Plasmids

plpE and *ompH* genes were amplified via PCR using chromosomal DNA of *P. multocida* P-1062. *plpE* gene was amplified with PlpEF and PlpER primers (Table 2.3); it was also cloned as two fragments, N-terminal (*plpE*N) without a signal sequence and C-terminal (*plpE*C). Primers for N-terminal fragment were PlpEF and PlpNR, and for C-terminal fragment were PlpCF and PlpER (Table 2.3). Primers for *ompH* amplification were OmpHF and OmpHR (Table 2.3). PCR products were ligated to pGEMT Easy vector and introduced into *E. coli* DH5α. *plpE*N-*ompH* and *plpE*C-*ompH* fusions were obtained in pGEMT Easy. Recombinant plasmids were verified with restriction enzyme digestion and nucleotide sequence analysis. The genes of interest were cloned in pCMV to be used as a DNA vaccine (pCMV-ompH, pCMV-plpEN-ompH and pCMV-plpEC-ompH) and in pET28a to express His-tagged proteins (pET28-plpEN-ompH, pET28-plpEC-ompH, pET28-plpEN, pET28-plpEC, pET28-plpE and pET28-ompH). In order to visualize the expression of the genes in a eukaryotic system, gfp gene was also cloned at the 3' of the genes in pCMV (pCMV-ompH-gfp, pCMV-plpEN-gfp, pCMV-plpEC-gfp). A total of 17 (6 in pGEM-T, 5 in pCMV and 6 in pET28a) constructs were obtained.

2.15. Transient Transfection of Mammalian Cells

Human embryonic kidney (HEK) 293 cells were kindly provided by Dr. A. Elif Erson Bensan (METU, Ankara, Turkey) and maintained in complete Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM glutamine, 15%

heat inactivated fetal bovine serum (FBS), 2 mM non-essential amino acids, 100 U/ml penicillin and 0.1 mg/ml streptomycin (all from Biochrom AG, Germany).

HEK 293 cells were transfected with pCMV-ompH-gfp, pCMV-plpEN-gfp and pCMV-plpEC-gfp plasmids using FuGENE 6 Transfection Reagent (Roche Applied Science, Germany) according to manufacturer's recommendations. pCMV-gfp and pCMV were used as positive and negative controls, respectively. $2x10^5$ cells were grown in each well of a six-well plate for 24 h at 37°C in a 5% CO_2 atmosphere in complete DMEM. The cells were washed and 2 ml of DMEM was added to wells. 1 µg or 2 µg of each plasmid was mixed with 3 µl of FuGENE 6 in DMEM and the mixture was added onto the cells. After incubation for 5 h, the medium was changed with DMEM supplemented with FBS and antibiotics. The cells were incubated for 48 h and visualized under Zeiss LSM 510 fluorescence confocal microscope (Carl Zeiss AG, Germany).

2.16. Purification of His-tagged Proteins

Recombinant *E. coli* BL21 cells carrying pET28-plpE, pET28-plpEN, pET28-plpEC, pET28-ompH, pET28-plpEN-ompH or pET28-plpEC-ompH were grown in Luria Broth (LB, Merck) supplemented with kanamycin (30 µg/ml final concentration). Expression was induced at OD_{600} of 0.6 by adding isopropyl-β-D-galactopyranoside (IPTG, Sigma) to 1 mM final concentration and incubated at 37°C for 5 h in a shaker incubator at 200 rpm. Cells were harvested by centrifugation at 6,000 *g* at 4°C for 15 min and resuspended in LEW buffer (Appendix C). Following the sonication using a CP70T Ultrasonic Processor (Cole-Parmer, Vernon Hills, IL) for 6×10 sec at 60% amplitude, cellular debris was removed by centrifugation at 15,000 *g* for 15 min. For solubilization of PlpE, 30 mM of 2-mercaptoethanol (BME) was added to the supernatant. The supernatants containing the recombinant proteins were purified using Protino Ni-TED 2000 packed columns (Macherey-Nagel, Germany) or Ni-NTA Spin Kit (Qiagen) according to suppliers' recommendations. Eluted proteins were dialyzed

using a cellulose dialysis tube (Sigma) in 1 L of DB buffer (Appendix C) at 4^{o}C stirring o/n and sterilized by 0.2 µm membrane filter. The purity of proteins was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were adsorbed onto indicated adjuvant for the preparation of vaccines.

2.18. Determination of Protein Concentration

Protein concentrations were measured by the Bradford quantification method (1976). The assay is based on the observation at 595 nm when the absorbance is maximal for an acidic solution of Coomassie Brilliant Blue G-250 while binding to a protein.

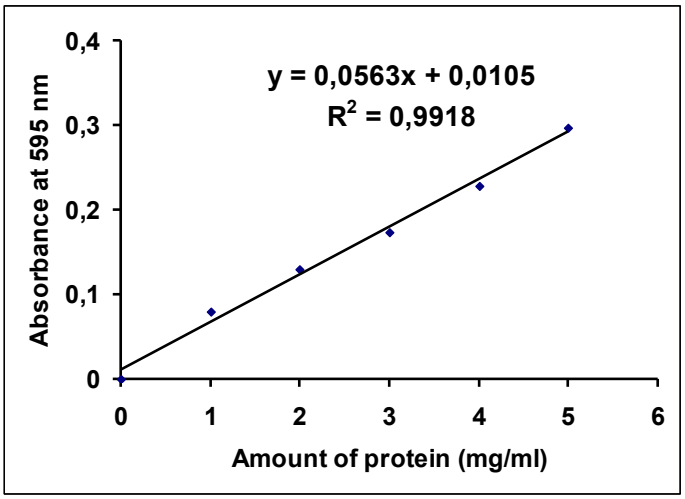

Figure 2.1. Calibration curve for quantification of protein concentrations.

Assay reagent was made by dissolving 100 mg of Coomassie Blue G-250 in 50 mL of 95% ethanol. The solution was then mixed with 100 mL of 85% phosphoric acid and made up to 1 L with distilled water. The reagent was filtered

through Whatman No. 1 filter paper. Bovine serum albumin (BSA) was used as the standard for preparation of protein calibration curve. Volumes of 2, 4, 6, 8 and 10 µL of BSA (1 mg/mL) were added to Eppendorf tubes and volumes were completed to 100 µL with dH_2O. 100 µL of distilled water was added into a tube as reagent blank. 900 µL of assay reagent was added to each tube and vortexed. Optical densities (O.D.) of solutions at 595 nm were measured on a Shimadzu UV-1208 spectrophotometer. A calibration curve was plotted using O.D. values of standards (Figure 2.1) and the amounts of proteins were calculated using equation obtained from the curve.

2.19. Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-polyacrylamide gels were prepared according to Laemmli (1970) (Table 2.5). Ca. 10 µg of protein was mixed with sample loading buffer (Appendix C) and the gel was run at 16 mA in 1X running buffer (Appendix C) using a Mini-Protean electrophoresis apparatus (Bio-Rad) until the loading dye reached to the end of the gel.

2.20. Coomassie Blue R-250 Staining of Polyacrylamide Gels

After electrophoresis, the gel was incubated in fixation solution (Appendix C) for 45 min and then soaked in 100 mL of freshly prepared Coomassie Blue R-250 stain (Appendix C) for 15 min at room temperature. The gel was then destained by keeping it in destaining solution (Appendix C) for at least 1 h.

Table 2.5. Preparation of SDS-polyacrylamide gels.

	Stacking Gel 0.125 M Tris, pH 6.8	Separating Gel 0.375 M Tris, pH 8.8
Monomer concentration	4.5%	12%
Acrylamide/bis	0.65 mL	4 mL
dH_2O	3.05 mL	3.35 µL
1.5 M Tris-HCl, pH 8.8	-	2.5 mL
0.5 M Tris-HCl, pH 6.8	1.25 mL	-
10% (w/v) SDS	50 µL	100 µL
10% Ammonium persulphate	25 µL	50 µL
TEMED	5 µL	5 µL
TOTAL MONOMER	5 mL	10 mL

2.21. Western Blot

3MM Whatman® papers and the 0.2 µm nitrocellulose membrane (Bio-Rad, Hercules, CA) were soaked in 1X transfer buffer (Appendix C) and laid out as shown in Figure 2.2.

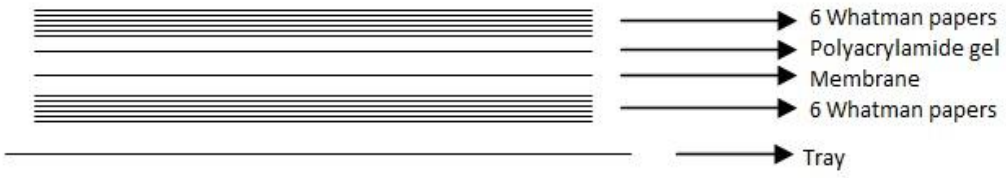

Figure 2.2. Schematic representation of transfer set-up in western blot.

The transfer was performed at 1.5 μA per cm^2 of the membrane for 1 h 15 min using a semi-dry blotter (Cleaver Scientific Ltd, Warwickshire, UK) according to a modified method of Towbin *et al*. (1979). Later, the membrane was incubated in 10% skim milk in 1X TBS (Appendix C) at 4°C o/n or at 37°C for 2 h and then washed with 1X TTBS (0.5% Twin in 1X TBS) for 10 min. Primary antibody was applied at 1/400 dilution in 5% skim milk incubating the membrane 1 h at RT. The membrane was washed with 1X TTBS for 10 min and secondary antibody (antimouse IgG, Sigma) was applied at dilution of 6 μl/100 ml of 5% skim milk for 1 h at RT. Then the membrane was washed with 1X TBS for 10 min and the AP conjugate substrate (Bio-Rad, Hercules, CA) was applied until the bands were visualized.

2.22. Experiments with Mice

Female BALB/c mice weighing between 15 and 18 g were obtained from Faculty of Medicine of Ankara University, Experimental Animals Unit (Ankara, Turkey). The experimental design for the Methodology I vaccination experiments were shown in Table 2.6. The mice were bled from the tail vein 14 days after each immunization and the sera were kept at -20°C.

Immunization of mice for the experiments of adjuvant effect was administered via Methodology II (Table 2.7). CpG adjuvant was kindly provided by Dr. İhsan Gürsel (Bilkent University, Ankara, Turkey) and aluminum hydroxide (alum)-CpG mix, oil based (Montanide ISA 206 VG, Seppic, France) and oil based-CpG adjuvants were kindly provided by Dr. Erkan Özcengiz (VBR Inc., Ankara, Turkey). 100 μg of PlpEC-OmpH was adsorbed onto different adjuvants were administered to each mouse. Control mice received PBS. The second injection was performed three weeks after the first injection and the pathogen challenge was applied 10 days after the second injection. The vaccines were injected through intraperitoneal (i.p.) route. The mice were bled from the tail vein before the second immunization and the challenge; the sera were kept at -20°C. The same

methodology was performed for vaccination of PlpE, PlpEC and OmpH formulated with oil based and oil based-CpG adjuvant. Control mice were received adjuvant only.

Table 2.6. Experimental design for Methodology I vaccination experiments.

Type of experiment	Vaccine	Amount	Route	Number of dose*	Number of mice
DNA vaccines	pCMV-ompH	50 µg	i.m.	3	6
		100 µg	i.m.	3	6
	pCMV-plpEN-ompH	100 µg	i.m.	3	6
	pCMV-plpEC-ompH	100 µg	i.m.	3	6
	pCMV (control)	100 µg	i.m.	3	6
Fusion protein vaccines	PlpEN-OmpH	50 µg	s.c.	2	5
	PlpEC-OmpH	50 µg	s.c.	2	5
	PlpEN-OmpH	100 µg	s.c.	2	5
	PlpEC-OmpH	100 µg	s.c.	2	5
	PBS (control)	500 µl	s.c.	2	5

* Each dose was administered in 15 days intervals.

After vaccination regimen, the mice were challenged with intraperitoneal injection of 10 LD_{50} (55 CFU) of *P. multocida* A:3 in 500 µl of saline solution. *P. multocida* was grown o/n on blood agar plates. The colonies was scratched from the plate and resuspended in sterile saline solution to an OD_{630} of 0.6. A serial dilution was performed and 10^8 dilution was used as the 10 LD_{50} of the pathogen. Survivors were recorded 7 days after challenge. Animal experiments were performed under the approval of Ethical Committee on Animal Experiments of Middle East Technical University, Ankara (Etik-2009/11, 09.07.2009).

Table 2.7. Experimental design for Methodology II vaccination experiments.

Type of experiment	Protein*	Adjuvant	Route	Number of dose**	Number of mice
Adjuvant effect	PlpEC-OmpH	CpG	i.p.	2	5
	PlpEC-OmpH	Alum-CpG	i.p.	2	5
	PlpEC-OmpH	Oil based	i.p.	2	5
	PlpEC-OmpH	Oil based-CpG	i.p.	2	5
	Control	PBS	i.p.	2	5
Single protein vaccines	PlpE	Oil based-CpG	i.p.	2	5
	PlpEC	Oil based-CpG	i.p.	2	5
	OmpH	Oil based-CpG	i.p.	2	5
	Control	Oil based-CpG	i.p.	2	5

* 100 μg of each protein was used.
** The vaccines were administered at day 0 and 21.

2.23. Enzyme-Linked Immunosorbent Assay (ELISA)

Purified recombinant proteins were used as ligands to coat ELISA plates at concentrations of 1 μg/well prepared in carbonate buffer (Appendix C). The plates were covered with parafilm and incubated at 4^{o}C overnight. Afterwards, the plates were washed three times with washing solution (WS, Appendix C). 50 μl of blocking solution (BS, Appendix C) was added to the wells and the plates were incubated at 37^{o}C for 1 h. The sera collected from the mice immunized with DNA and protein based vaccines were diluted as 1:50, 1:100, 1:200, 1:400, 1:800, 1:1600 and 1:3200 in BS to use as primary antibodies and incubated at 37^{o}C for 1 h. The plates were washed four times with WS. Alkaline phosphatase conjugated rabbit anti-mouse IgG (Sigma) was used as secondary antibody at a dilution of 1:1000 in BS and incubated at 37^{o}C for 1 h. The plates were washed four times with WS. 100 μl of AP Conjugate Substrate Kit (Bio-Rad) was used as

colorimetric substrate reagent prepared adding 400 µl of development buffer and 100 µl of solution A and B in 10 ml of dH_2O. The plates were incubated 30 min at RT at a dark place and the reaction was stopped with 50 µl of 1 M NaOH. Optical densities were measured at 405 nm on a RT-2100C Microtiter plate Reader (Rayto, Shenzhen, China). Absorbance values for 1:100 and 1:1600 dilutions were used for the comparison of antibody responses in mice vaccinated with DNA and protein vaccines, respectively.

2.24. Detection of Serum Interferon-gamma (IFN-γ) Levels

Mouse IFN-γ Minikit (Pierce, Thermo Scientific) was used for the detection of serum IFN-γ levels of vaccinated mice. The protocol was applied according to manufacturer's recommendations. Briefly, 96-well plates were coated with 100 µl of diluted Coating Antibody by overnight incubation at room temperature (RT). Next, 300 µl of Blocking Buffer (Appendix C) was added to each well and incubated for 1 h at RT. After aspirating Blocking Buffer, the plate was allowed to dry for 1 h at RT. The plate was sealed and kept at 4^oC. Serum samples were added at 1:4 dilutions in Assay Buffer (Appendix C) and incubated at RT overnight. The plate was washed three times with Wash Buffer (Appendix C) and 100 µl of diluted Detection Antibody was added to each well. After incubation of 1 h at RT, the plate was washed and 100 µl of Streptavidin-HRP (Pierce, Thermo Scientific) was added at a dilution of 1:10000. The plate was incubated at RT for 30 min and washed three times with Wash Buffer. 100 µl of TMB substrate (Thermo Scientific) was added to each well and incubated at RT for 30 min. The reaction was stopped by adding 100 µl of 0.18 M sulfuric acid (H_2SO_4) and the absorbance was read at 450 nm on a RT-2100C Microtiter plate Reader (Rayto, Shenzhen, China).

2.25. Statistical Analyses

An analysis of variance (ANOVA) and the Tukey's test were used for mean comparison of antibody response between groups. Survival data were compared using the chi-square test (two-sided). Statistical analyses for immune responses and survival were performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, USA). The significance level (*p*) for all analyses was set at 0.05. Standard deviations were calculated using Microsoft Office Excel 2010 program.

2.26. Nucleotide Sequence Accession Numbers

GenBank accession numbers of *plpE* and *ompH* genes from *P. multocida* P-1062 are GU247966and GQ914772, respectively.

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Cloning of *ompH* and *plpE* Genes

Genomic DNA of *Pasteurella multocida* P-1062 (A:3, ATCC 15743) was used for PCR amplification of *ompH* and *plpE* genes (Figure 3.1). *plpE* gene was cloned without a signal sequence and a stop codon to obtain a genetic fusion with *ompH*.

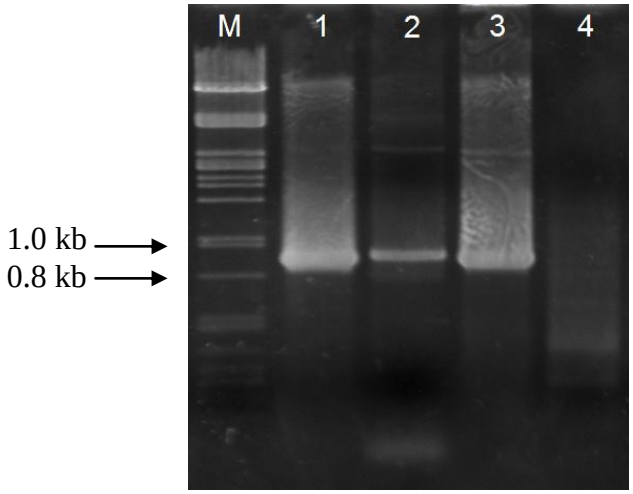

Figure 3.1. Amplification of *plpE* (lane 1), *ompH* (lane 2) and *ompH*NS (lane 3) genes. Lane 4: Negative control, M: Lambda DNA/*Pst*I marker.

Lee *et al*. (2007) reported that cloning of entire *ompH* gene from *P. multocida* was failed probably because of the lethality of protein product; hence, signal sequence of the gene was deleted for the expression of *ompH* in *E. coli*. Therefore, signal sequences were eliminated while designing of primers for amplification of *ompH* and *plpE* genes in this study. One copy of *ompH* was also cloned without a stop codon (*ompH*NS) to obtain a *gfp* fusion which was later used in transfection of mammalian cells.

PCR products were cut and extracted from the agarose gel and ligation to pGEMT Easy vector was performed. Ligation products were introduced into *E. coli* DH5α competent cells. Putative colonies were screened by manual plasmid isolation and verified by restriction enzyme digestion (Figure 3.2).

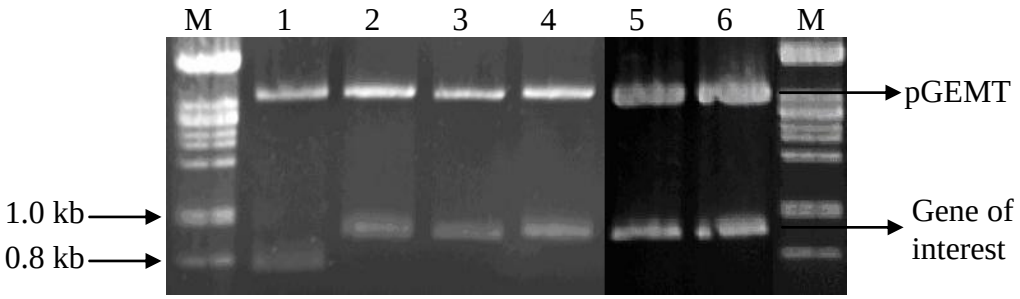

Figure 3.2. Verification of putative colonies for cloning of *plpE* (lanes 1 and 2), *ompH* (lanes 3 and 4) and *ompH*NS (lanes 5 and 6) in pGEMT via restriction enzyme digestion of plasmids. M: Lambda DNA/*Pst*I marker.

3.2. Sequence Analysis of *ompH* and *plpE* Genes from *P. multocida* P-1062

Positive clones for pGEMT-ompH and pGEMT-plpE were used for sequencing of the genes; the sequences were compared with previously sequenced genes in BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) database and analyzed using

DNASTAR (DNASTAR Inc., Madison, WI) and Mega4 (Tamura *et al*., 2007) programs.

A

```
GCAACAGTTTACAATCAAGACGGTACAAAAGTTGATGTAAACGGTTCTGTAC
GTTTAATCCTTAAAAAAGAAAAAAATAAGCACGGTGATTTAGTGGATAACGG
TTCACGCGTTTCATTCAAAGCGTCTCATGATTTAGGCGAAGGCTTAAGCGCA
TTAGCTTATACAGAACTTCGTTTCAGTAAAAATGTAACAAAGCAAAAAAAGA
CCAAAGCAGGAAAAGACAAGAATTATGTTGTTGAACGACTTGGTAACAATGT
CCACGTAAAACGTCTTTATGCCGGTTTCGCGTATGAAGGTTTAGGAACATTA
ACTTTCGGTAACCAATTAACTATCGGTGATAATGTTGGTGTGTCTGATTACA
CTTACTTCTTAGGTGGTATCAACAACCTTCTTTCTAGCGGTGAAAAAGCAAT
TAACTTTAAATCTGCAGAATTCAACGGTTTCACATTTGGTGGTGCGTATGTC
TTCTCAGCGGATGCTGACAAACAAGCATCACGTGATGGTCGCGGTTTCGTTG
TAGCGGGTTTATACAACAGAAAAATGGGCGATGTTGGTTTCGCACTTGAAGC
AGGTTATAGCCAAAAACAAAAATATGTAACAGCAGCTAAACAAGAAAAAGCC
TTTATGGTCGGTACTGAATTATCATATGCTGGTTTAGCACTTGGTGTTGACT
ATGCACATACAGTGACTAACAAAGAAAAAGTAGAAGGTAAAAAACGCGCACT
TGAAGTAGGTTTAAACTATGACATTAATGACAAAGCAAAAGTTTACACTGAC
TTGATTTGGGCAAAAGAAAGTTCAAAAGGTGTTACTACAAGAGATTCTAGCA
TCTTATTAGGTGCGGGCTACAAGCTTCACAAAAAAGTTGAAACCTTTGTTGA
AGGTGGCTGGAGCAGAAAGAAAGCTGCTGTTGGCGTAACAACAAAAGATAAC
AAAGTTGGTGTTGGTTTACGCGTACACTTCTAA
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B

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ATVYNQDGTKVDVNGSVRLILKKEKNKHGDLVDNGSRVSFKASHDLGEGLSA
LAYTELRFSKNVTKQKKTKAGKDKNYVVERLGNNVHVKRLYAGFAYEGLGTL
TFGNQLTIGDNVGVSDYTYFLGGINNLLSSGEKAINFKSAEFNGFTFGGAYV
FSADADKQASRDGRGFVVAGLYNRKMGDVGFALEAGYSQKQKYVTAAKQEKA
FMVGTELSYAGLALGVDYAHTVTNKEKVEGKKRALEVGLNYDINDKAKVYTD
LIWAKESSKGVTTRDSSILLGAGYKLHKKVETFVEGGWSRKKAAVGVTTKDN
KVGVGLRVHF-
```

Figure 3.3. Nucleotide **(A)** and amino acid **(B)** sequences of *ompH* gene.

Sequence similarity of the proteins among different strains is important in vaccine development for cross protection. For instance, the cross-reactive proteins of

Clostridium tetani shared 65–78% sequence similarity with their closest homologues in *C. perfringens* (Alam *et al*., 2008). Accordingly, the sequence of *ompH* (GenBank accession number: GQ914772, Figure 3.3) was analyzed in the present study. Computer-aided analysis (EditSeq, DNASTAR) of 969 base pairs (bp) *ompH* gene without the signal sequence was shown in Table 3.1. Luo *et al*. (1997) reported that *ompH* from *P. multocida* X-73 (serotype A:1) is 1,059 bp long coding for 353 amino acids, including a 20 amino acid signal peptide. The mature protein has a calculated molecular mass of 36.6 kDa.

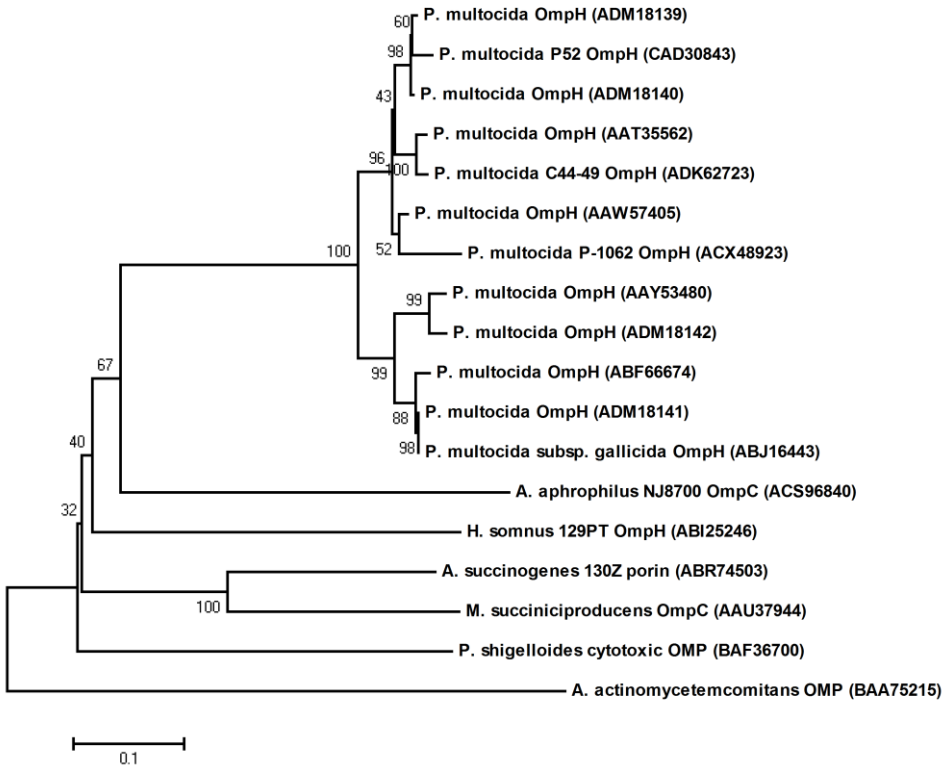

Figure 3.4. Phylogenetic relationships of OmpH sequences. The phylogenetic tree was constructed by using MEGA4 with Neighbor-Joining method (Saitou and Nei, 1987). The numbers show the percentage that the related taxon grouped together in 1000 repeats. GenBank accession numbers are given in parentheses.

Table 3.1. Computer-aided analyses of *ompH*, *plpE*N and *plpE*C genes.

Gene name	Number of nucleotides (bp)	G+C content (%)	Number of amino acids	Molecular mass (kDa)
ompH	969	38.29	322	35.0
*plpE*N	474	35.86	158	17.5
*plpE*C	459	34.20	153	17.4

Mature OmpH from *P. multocida* P-1062 was 10 amino acids shorter than that of X-73, and it had 81.7–90.7% sequence similarity to OmpH sequences belonging to other *P. multocida* serotypes of avian, bovine or swine origin and 23.9 – 29.8% identity to outer membrane protein sequences of *Actinobacillus actinomycetemcomitans*, *Haemophilus somnus*, *Plesiomonas shigelloides*, *Mannheimia succiniciproducens*, *Actinobacillus succinogenes*, *Aggregatibacter aphrophilus*. In order to show the identity of P-1062 OmpH to other *P. multocida* OmpH sequences, a phylogenetic tree was constructed in MEGA4 program (Figure 3.4).

plpE gene was cloned via PCR as full-length and an N-terminal (*plpE*N, bases from 1 to 483) and a C-terminal (*plpE*C, bases from 487 to 945) fragment (Figure 3.5A). Nucleotide sequences of *plpE*N and *plpE*C were identical to *plpE* sequence (GenBank accession number GU247966). Computer-aided analyses (Edit-Seq, DNASTAR) of *plpE*N and *plpE*C were shown in Table 3.1. The alignment of deduced amino acid sequence of the PlpE from *P. multocida* P-1062 (Figure 3.5B) with other bacterial outer membrane proteins (MegAlign, DNASTAR) showed that the sequence had 89–100% identity to PlpE sequences from other *P. multocida* strains and 24–29% identity to PlpE sequences of *Mannheimia haemolytica*.

A

TGTAGCGGCGGTGGCGGTAGCGCTGGAAATCGTGCTGACCGTGTAGA
GGAAAAAGCACAACCGGTTCAATCAAATAGTGAGCCTTCTTCCGCTC
CAATCAAAAATCCTACTAATACCGCTACGAATGATTCTCTTCATGAC
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ATCCTCCTTTAAAGCCCCTCTAGAACAAGAAAAAAACCAACCTGCAC
AAGAAAATCTCACTTGGACAGGTTATCATGTTTCAGAAGTGGGAAAT
GCGAGTAATAATGTAGATAAAGATAACGTTACGGTATTCACTTTCGT
AAAATATAATTCTCAATACAATGATGATCCAGTTTTTGATAAAACAA
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TGGAAGTTATGGA(CAA)CCTTCAGCAGATTACAAAAAAGTAGAAAA
AAATTATATTTATGCAATTAAACCAGATGCAATAAATAATGAGAACC
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GTATGGCAATGTGACTCTTACTTTCCGAAATGGCAAGATTTATGGTG
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GGAGAAGGACAAAACTTAACTATAACACCACACAAGGACAATCCCCA
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TTATCAACGCAGAAAAAACTGATAAAAAATACGTTGTTGGTGTAGGA
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AGCACAA

B

CSGGGGSAGNRADRVEEKAQPVQSNSEPSSAPIKNPTNTATNDSLHDK
LSMSSHDTSKENSQQSSFKAPLEQEKNQPAQENLTWTGYHVSEVGNAS
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YNRGRDDLFQLSGEGQNLTITPHKDNPHKLSPTGPDNMAMELNFINAE
KTDKKYVVGVGKAEKYYGLLFAEKSHQAQ

Figure 3.5. Nucleotide **(A)** and amino acid **(B)** sequences of *plpE* gene. N terminal fragment was underlined and C terminal fragment was shaded.

In order to show the identity of P-1062 PlpE to other *P. multocida* PlpE sequences, a phylogenetic tree was constructed in MEGA4 program (Figure 3.6). As discussed by Wu *et al*. (2007) and Singh *et al*. (2010), with more than 90% sequence similarity among *P. multocida* strains, PlpE is a cross protective antigen.

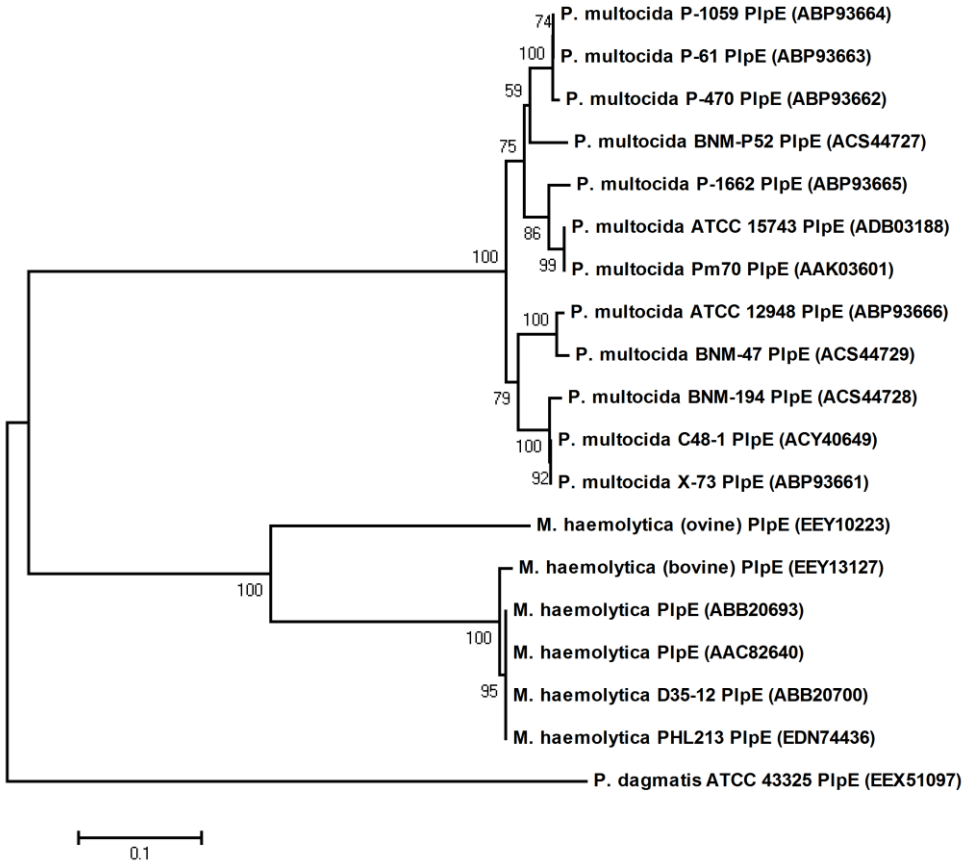

Figure 3.6. Phylogenetic relationships of PlpE sequences. The phylogenetic tree was constructed by using MEGA4 with Neighbor-Joining method (Saitou and Nei, 1987). The numbers show the percentage that the related taxon grouped together in 1000 repeats. GenBank accession numbers are given in parentheses.

3.3. Construction of DNA Vaccines and Expression of *ompH*, *plpE*N and *plpE*C Genes in Mammalian Cells

In DNA vaccine studies, transfection efficiency of the plasmid constructs and the gene expression in a eukaryotic system are checked out prior to vaccination (Doroud *et al*., 2010; Zhu *et al*., 2011). A cDNA encoding green fluorescent protein (GFP) from the jellyfish *Aequorea victoditis elegans* can be used as a reporter gene to visualize the expression of genes and the localization of proteins in eukaryotic cells (Chalfie *et al*., 1994; Watanabe *et al*., 1999). In this study, first, *ompH*NS, *ompH*, *plpE*N and *plpE*C were cloned in eukaryotic expression vector pCMV under the human cytomegalovirus (CMV) promoter (Figure 3.7).

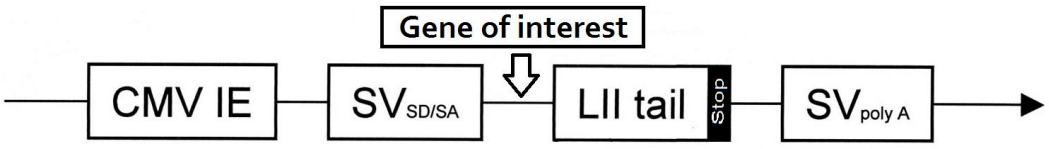

Figure 3.7. Scheme of the DNA vaccine plasmid pCMV. *ompH*, *plpE*N, *plpE*C, *gfp* and their fusions were cloned in *Bgl*II site between $SV_{SD/SA}$ and LII tail. CMV IE: human cytomegalovirus immediate-early promoter; $SV_{SD/SA}$ and SV_{polyA}: SV40 splice donor-acceptor and transcription terminator-polyadenylation signal, respectively; LII tail: targeting signal of lysosomal integral membrane protein-II (modified from Rodriguez *et al*., 2001).

Then, fusions of *ompH*NS, *plpE*N and *plpE*C with *gfp* at 3' in pCMV were obtained using *E. coli* DH5α. To observe the expression of these bacterial genes in a mammalian host, human embryonic kidney (HEK) 293 cells were transiently transfected with pCMV-ompHNS-gfp, pCMV-plpEN-gfp, pCMV-plpEC-gfp. pCMV-gfp and pCMV alone were also used as positive and negative controls,

respectively. After 48 h incubation, *gfp* expression in transfected cells was monitored under fluorescence confocal microscope (Zeiss LSM 510) (Figure 3.8).

GFP expression was successfully observed in HEK 293 cells transfected with pCMV-ompHNS-gfp, pCMV-plpEN-gfp, pCMV-plpEC-gfp and pCMV-gfp while there was no GFP expression in negative control. Later, in addition to *ompH*, *plpE*N-*ompH* and *plpE*C-*ompH* fusions were also obtained in pCMV. Finally, endotoxin free pCMV-ompH, pCMV-plpEN-ompH and pCMV-plpEC-ompH were purified from *E. coli* cells to use as DNA vaccines.

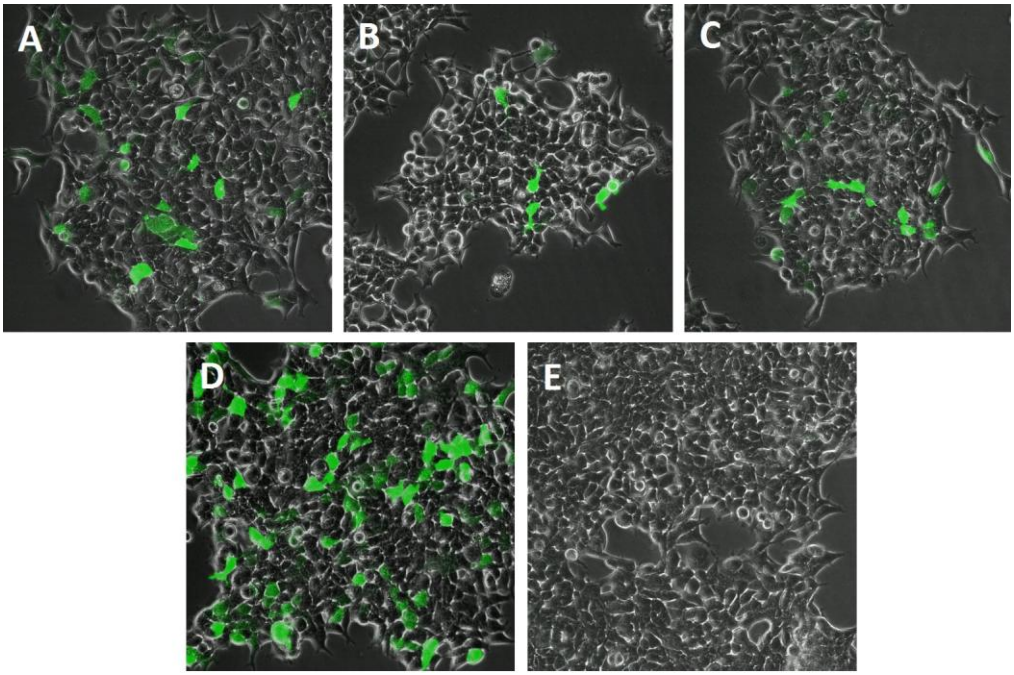

Figure 3.8. In vitro expression of *ompH*, *plpE*N and *plpE*C in mammalian cells. HEK 293 cells were transiently transfected with pCMV-ompH-gfp **(A)**, pCMV-plpEN-gfp **(B)**, pCMV-plpEC-gfp **(C)**, and pCMV-gfp **(D)** and pCMV **(E)** as a positive and negative control, respectively. Green color shows the expression of the gene of interest together with *gfp* gene at 3'. The cells were visualized under 20x objective of Zeiss LSM 510 fluorescence confocal microscope.

3.4. Immune Responses against DNA Vaccines pCMV-ompH, pCMV-plpEN-ompH and pCMV-plpEC-ompH

Six BALB/c mice per group were i.m. immunized three times in two weeks intervals with 50 µg or 100 µg of pCMV-ompH DNA vaccine. Serum IgG levels were measured via ELISA technique (Figure 3.9). As compared to control group mice vaccinated with pCMV, there was not a significant increase in antibody levels in mice vaccinated with 50 µg of pCMV-ompH whereas significant increment (p<0.05) was obtained with 100 µg of pCMV-ompH after third vaccination.

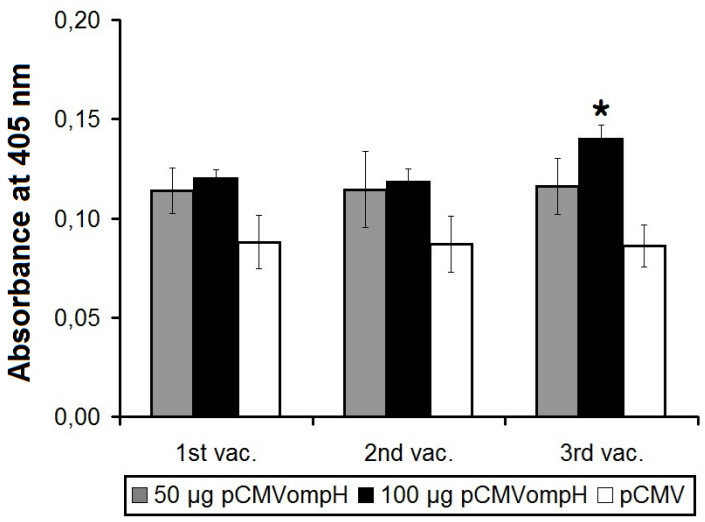

Figure 3.9. Total IgG levels in 1:100 diluted sera from the mice vaccinated with pCMV-ompH DNA vaccine. Statistically significant (p<0.05) increase as compared to control was shown with an asterisk.

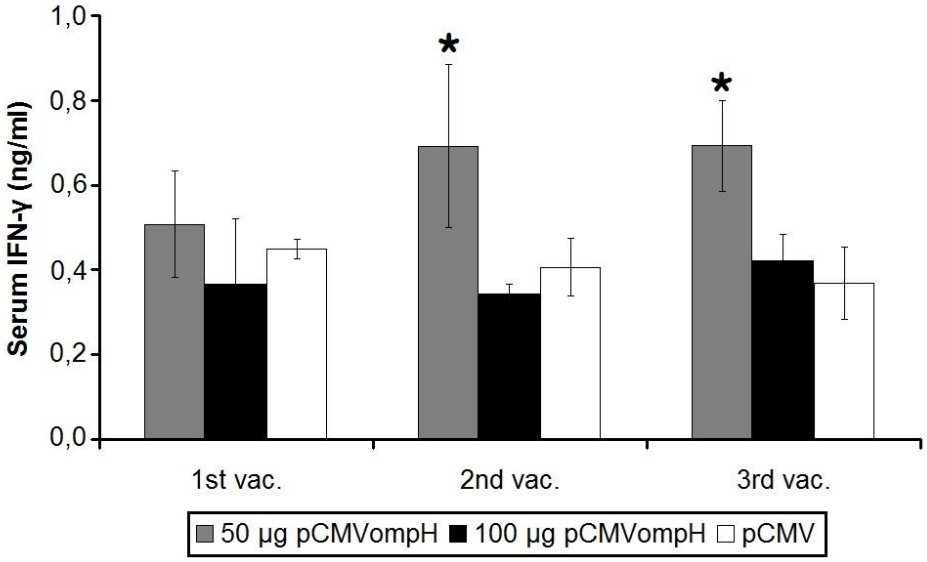

Figure 3.10. IFN-γ levels in 1:4 diluted sera from the mice vaccinated with pCMV-ompH DNA vaccine. Statistically significant (p<0.05) increase as compared to control was shown with an asterisk.

Serum interferon gamma (IFN-γ) levels were also measured to reveal any cell-mediated immune response conferred by pCMV-ompH DNA vaccines (Figure 3.10). In contrast to antibody response, there was not a significant increase in serum IFN-γ levels in mice vaccinated with 100 µg of pCMV-ompH whereas the increment by 50 µg of pCMV-ompH was significant (p<0.05) after second and third vaccinations.

Vaccinated mice were challenged with 10 LD_{50} (55 CFU) of live *P. multocida*. However, neither 50 µg nor 100 µg of pCMV-ompH DNA vaccine conferred protection.

Later, six mice per group were vaccinated with 100 µg of pCMV-plpEN-ompH or pCMV-plpEC-ompH fusion DNA vaccines. Significant increase (p<0.05) in antibody levels was obtained in mice vaccinated with pCMV-plpEC-ompH after first vaccination and remained after second and third injections. On the other

hand, in spite of higher mean values than that of control group, a significant increase (p<0.05) in antibody level was obtained after third vaccination with pCMV-plpEN-ompH (Figure 3.11).

Serum IFN-γ levels in mice vaccinated with pCMV-plpEN-ompH or pCMV-plpEC-ompH DNA vaccines were measured (Figure 3.12). Serum IFN-γ titers were significantly high (p<0.05) in mice immunized with pCMV-plpEN-ompH vaccine after first and second immunizations. However, a significant increase in serum IFN-γ levels could not be obtained by pCMV-plpEC-ompH construct. As a result, pCMV-plpEN-ompH DNA vaccine induced both humoral and cell-mediated immune responses against *P. multocida* A:3 in mice.

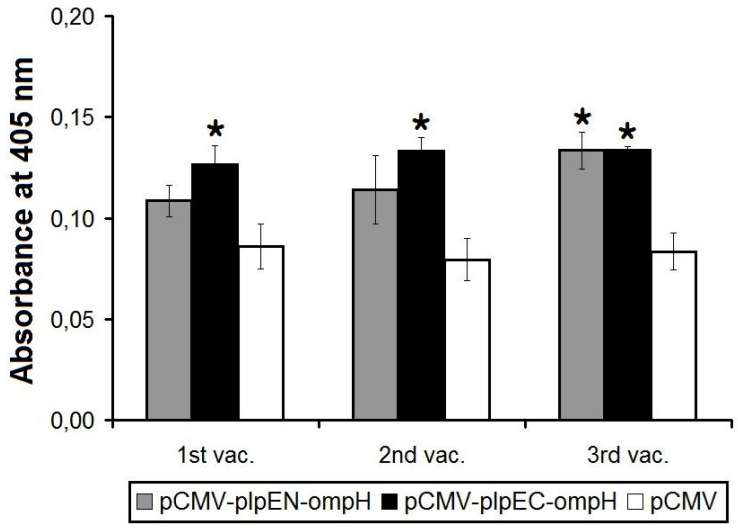

Figure 3.11. Total IgG levels in 1:100 diluted sera from the mice vaccinated with pCMV-plpEN-ompH or pCMV-plpEC-ompH DNA vaccines. Statistically significant (p<0.05) increase as compared to control was shown with an asterisk.

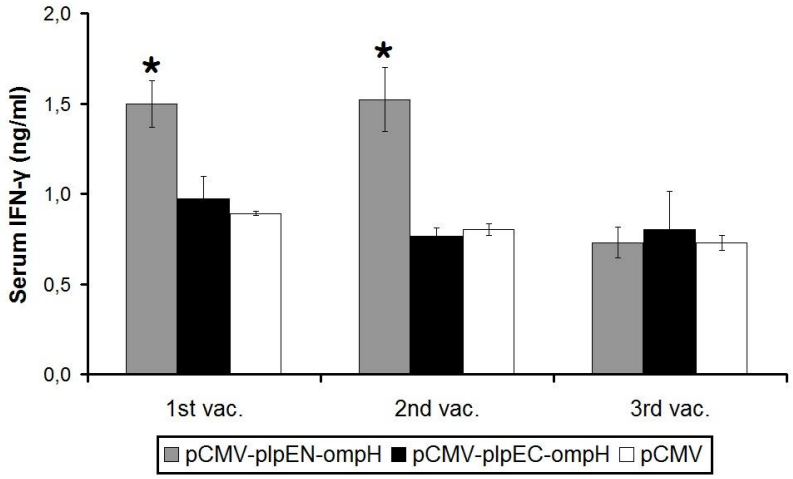

Figure 3.12. IFN-γ levels in 1:4 diluted sera from the mice vaccinated with pCMV-plpEN-ompH or pCMV-plpEC-ompH DNA vaccines. Statistically significant (p<0.05) increase as compared to control was shown with an asterisk.

The mice immunized with pCMV-plpEN-ompH or pCMV-plpEC-ompH were challenged with 10 LD_{50} of live *P. multocida* but no protection was obtained despite of induced antibody and/or IFN-γ levels. Therefore, they are not individually suitable for use as a vaccine but can be used for prime-boost studies, i.e. priming with a DNA vaccine and boosting with a protein formulation. Liang *et al*. (2008) immunized calves priming with a plasmid encoding E2 protein of BVDV-2 and boosting with E2 protein formulations. Both humoral and cell-mediated immune responses were induced in vaccinated animals and protection was recorded upon viral challenge.

There is only one DNA vaccine study on *P. multocida* conducted by Register *et al*. (2007). They constructed four DNA vaccines composed of 5'-truncated or full-length, genetically detoxified *toxA* gene (encoding PMT) from a swine isolate of *P. multocida* in two different vectors which have or lack a signal sequence for secretion. The construct encoding full-length gene with a signal sequence induced

both humoral and cell-mediated immune responses in mice and pigs. However, protection studies in mice with lethal dose of bacteria were not performed.

3.5. Expression of *plpE*N-*ompH* and *plpE*C-*ompH* Gene Fusions in *E. coli* and Optimization of Recombinant Protein Purification via Affinity Column Chromatography

For gene expression in *E. coli* and protein purification by His-tag affinity column chromatography, fusion gene constructs, i.e. *plpE*N-*ompH* and *plpE*C-*ompH*, were obtained in pET28a(+) vector using *E. coli* DH5α. Later, recombinant plasmids were introduced to *E. coli* BL21 (DE3) cells providing phage T7 RNA polymerase (Kothari *et al*., 2006) and recombination was verified by restriction enzyme digestion. For the production of His-tagged fusion proteins, recombinant *E. coli* BL21 (DE3) cells were grown in Luria Broth containing IPTG to a final concentration of 1 mM for the induction of gene expression under the control of phage T7 promoter (Kothari *et al*., 2006). IPTG-induced cultures were centrifuged and the pellets were resuspended in Binding Buffer (Ayalew *et al*., 2008) containing 6 M urea. The cells were sonicated and the lysates were centrifuged, supernatants containing the over-expressed proteins. Ni-NTA (Qiagen) columns were used for the purification of fusion proteins on the bases of nickel affinity of histidine-tagged proteins. Purity of eluates was monitored on SDS-polyacrylamide gels (Figure 3.13). Over-expression and partial purification of 55-60 kDa His-tagged fusion proteins in denaturing conditions using Ni-NTA columns were successfully obtained.

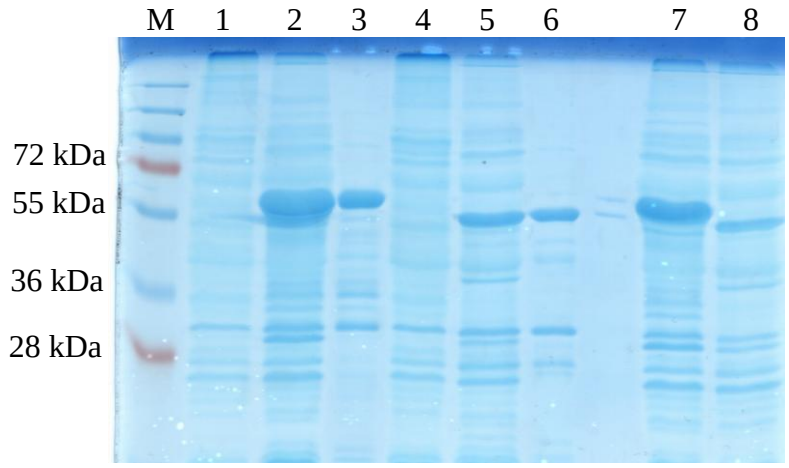

Figure 3.13. Purification of PlpEN-OmpH and PlpEC-OmpH fusion proteins using Ni-NTA columns. M: Prestained protein ladder, Lanes 1, 4: Uninduced culture lysates, Lanes 2, 5: IPTG-induced culture lysates, Lanes 3, 6: Eluates, Lanes 7, 8: Flow-through samples for PlpEN-OmpH and PlpEC-OmpH, respectively.

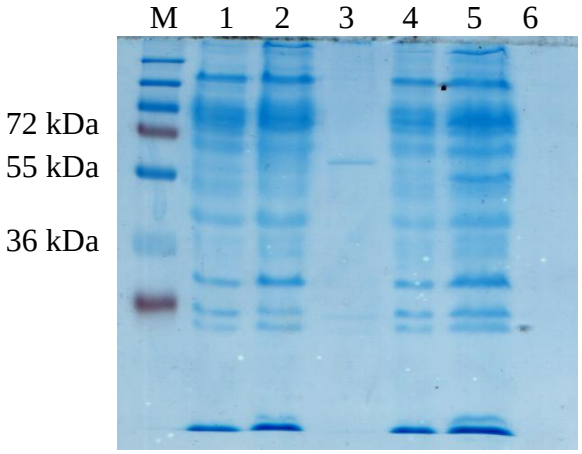

Figure 3.14. Purification of PlpEN-OmpH and PlpEC-OmpH fusion proteins using Ni-TED columns under native conditions. M: Prestained protein ladder, Lanes 1, 3: Uninduced culture lysates, Lanes 2, 4: IPTG-induced culture lysates, Lanes 3, 6: Eluates for PlpEN-OmpH and PlpEC-OmpH, respectively.

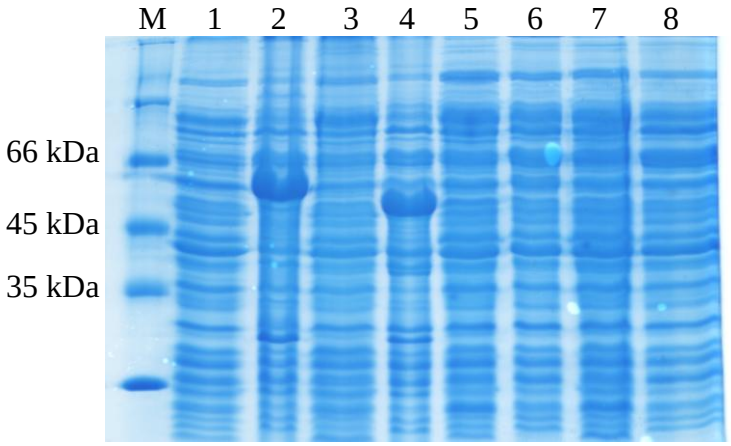

Figure 3.15. Effect of urea on solubilization of inclusion bodies in the cells expressing PlpEN-OmpH or PlpEC-OmpH. M: Unstained protein ladder, Lanes1, 5: Uninduced culture lysates and Lanes 2, 6: IPTG-induced culture lysates for PlpEN-OmpH. Lanes 3, 7: Uninduced culture lysates and Lanes 4, 8: IPTG-induced culture lysates for PlpEC-OmpH. Cells were treated with (lanes 1-4) or without (lanes 5-8) urea.

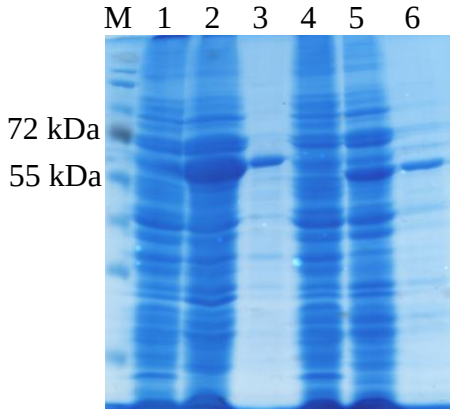

Figure 3.16. Purification of PlpEN-OmpH and PlpEC-OmpH fusion proteins using Ni-TED columns under denaturing conditions. M: Prestained protein ladder. Lanes 1, 4: Uninduced culture lysates, Lanes 2, 5: IPTG-induced culture lysates, Lanes 3, 6: Eluates for PlpEN-OmpH and PlpEC-OmpH, respectively.

Ni-TED (Macherey-Nagel) columns were used to obtain larger amounts of proteins to be used in vaccine preparations. First, native conditions were applied for the purification of proteins. Uninduced and IPTG-induced cells were resuspended in Lysis-Elution-Wash (LEW) Buffer of the commercial kit lacking urea and sonicated. Lysates were centrifuged and the supernatants from induced culture were used as crude extracts for His-tagged fusion proteins. After nickel affinity column chromatography using Ni-TED columns, eluates were screened on SDS-polyacrylamide gel (Figure 3.14). Lack of a difference between uninduced and induced samples and very low level or no protein in eluates probably resulted from unsuccessful solubilization of inclusion bodies in the absence of urea.

Later, effect of urea on solubilization of inclusion bodies was investigated for protein purification using Ni-TED columns. Uninduced and IPTG-induced cultures were centrifuged and the pellets were resuspended in either Binding Buffer (Ayalew *et al*., 2008) containing 6 M urea or LEW Buffer lacking urea. The cells were sonicated and centrifuged. Solubilized proteins were run on SDS-polyacrylamide gel (Figure 3.15). Over-production of fusion proteins was observed in IPTG-induced cells treated with Binding Buffer but LEW Buffer lacking urea was ineffective on solubilization of inclusion bodies.

Finally, His-tagged fusion proteins were decided to be purified under denaturing conditions using Ni-TED columns for use in vaccine formulations. Protein profiles of the samples were visualized via SDS-PAGE (Figure 3.16). Solubilization of inclusion bodies and purification of His-tagged proteins were successfully accomplished. Molecular masses of PlpEN-OmpH and PlpEC-OmpH fusions were ca. 55 and 53 kDa, respectively.

3.6. Immune Responses against Fusion Proteins PlpEN-OmpH and PlpEC-OmpH

Five BALB/c mice per group were immunized twice with either 50 µg or 100 µg of fusion proteins adsorbed to aluminum hydroxide (alum) at day 0 and 14. Before second immunization and challenge, mice were bled from tail veins and serum samples were obtained.

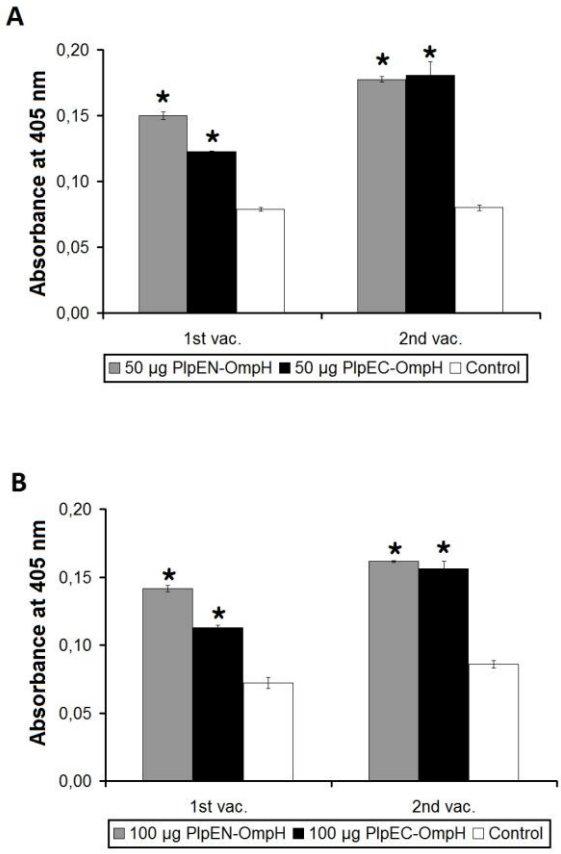

Figure 3.17. Total IgG levels in 1:1600 diluted sera from the mice vaccinated with either 50 µg **(A)** or 100 µg **(B)** of PlpEN-OmpH or PlpEC-OmpH fusion proteins adsorbed on alum adjuvant. Statistically significant ($p<0.05$) increase as compared to control was shown with an asterisk.

Antibody levels in mice immunized with PlpEN-OmpH or PlpEC-OmpH fusion proteins were measured via ELISA. Both of the fusion proteins induced humoral immune response (Figure 3.17). Serum IFN-γ levels in vaccinated mice were also investigated (Figure 3.18). Neither 50 μg PlpEN-OmpH nor 50 μg or 100 μg of PlpEC-OmpH increased cell-mediated immune responses in vaccinated mice. Statistically significant (p<0.05) increase in serum IFN-γ level was only observed in mice vaccinated with 100 μg of PlpEN-OmpH.

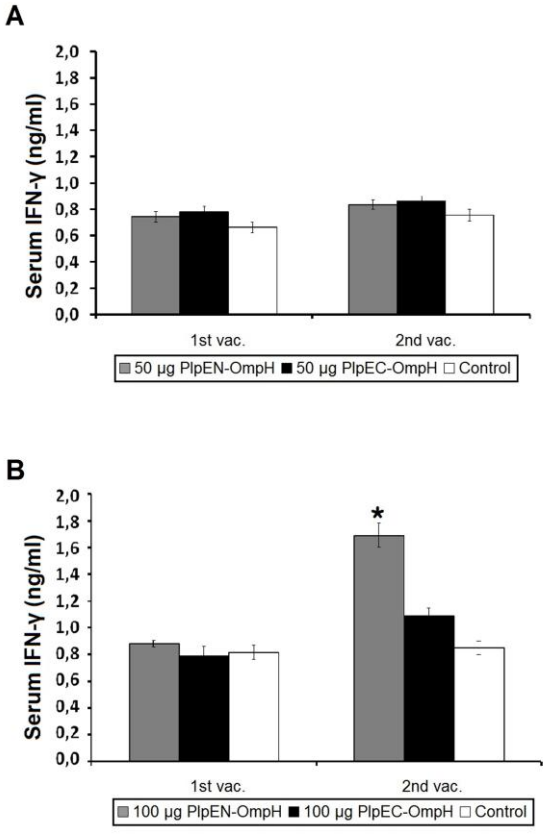

Figure 3.18. IFN-γ levels in 1:4 diluted sera from the mice vaccinated with either 50 μg **(A)** or 100 μg **(B)** of PlpEN-OmpH or PlpEC-OmpH fusion proteins adsorbed on alum adjuvant. Statistically significant (p<0.05) increase as compared to control was shown with an asterisk.

Mice immunized with either 50 µg or 100 µg of fusion proteins were challenged with 10 LD_{50} of live *P. multocida* A:3. Two out of five mice (40%) vaccinated with 100 µg of PlpEC-OmpH survived after challenge which was not statistically significant (p=0.11). Other vaccine formulations did not confer any protection (Table 3.2).

Table 3.2. Protection conferred in mice immunized with PlpEN-OmpH or PlpEC-OmpH protein adsorbed on alum against challenge with *P. multocida* A:3.

Vaccine formulation (protein/adjuvant)	Dose (µg)	No. of mice survived/challenged	Protection %
PlpEN-OmpH/alum	50	0/5	0
PlpEC-OmpH/alum	50	0/5	0
PlpEN-OmpH/alum	100	0/5	0
PlpEC-OmpH/alum	100	2/5	40
Control	-	0/5	0

3.7. Effect of Different Vaccine Adjuvants on Protectivity of PlpEC-OmpH

Selection of appropriate antigen doses and use of potent adjuvants that can boost antigen immunogenicity is a crucial step in vaccine development and is essentially empiric. Hence, the useful approach for determination of safe and efficient vaccine candidates is trial-and-error methodologies (Rojo-Montejo *et al*., 2011).

In vaccine formulations, adjuvants are used to form a depot effect and induce the cytokine production by targeting immune cells. Antibody subclasses and hence, Th1-Th2 balance can change depending on the type of the adjuvant. In mice, IgG1 is related with Th2-type immunity whereas IgG2a, IgG2b, and IgG3 are associated with Th1-type response. Different mechanisms take role in the

clearance of encapsulated bacteria by each IgG subclasses. For instance, binding to Fc receptors is mediated by IgG2a and IgG2b and they can fix complement together with IgG3 better than IgG1 can; protection may be provided by IgG1 or IgG3 through cooperatively binding to bacteria. Thus, an immune response against encapsulated bacteria may be beneficial with a broad subclass distribution (Lefeber *et al*., 2003).

CpG oligodeoxynucleotides (ODNs) has been utilized as vaccine adjuvants in a number of studies on animals including mice and cattle (Klinman *et al*., 2004). Bacterial DNA contains unmethylated CpG dinucleotides at an expected frequency but they are underrepresented and selectively methylated in vertebrates resulting in recognition of bacterial DNA as a pathogen-associated material. Through binding to TLR9, CpG ODNs induce Th1-type responses with secretion of IL-12 and IFN-γ in mice (Liang *et al*., 2006) whereas aluminum hydroxide induces Th2-type cellular immunity (Makidon *et al*., 2008). Chen *et al*. (2011) reported that hepatitis B vaccine formulation with CpG adjuvant induced Th1-type immune response increasing IFN-γ and IL-2 levels while the alum adjuvanted formulation triggered Th2-type response. Oil based adjuvants (emulsions) have been used in vaccine formulations since 1945 (Aucouturier *et al*., 2006). Emulsion type adjuvants are more efficient than alum. They target muscle cells and induce an early and strong immuno-competent environment at the injection site in addition to providing a prolonged duration for the action of the antigen (Huang *et al*., 2010).

In this study, different adjuvants were used in formulations of PlpEC-OmpH to increase its 40% protective efficacy. For this purpose, previously used aluminum hydroxide (alum) was replaced with four different adjuvants: I) CpG, II) alum-CpG, III) oil based and IV) oil based-CpG. The vaccination protocol was changed from two s.c. injections in two weeks intervals to two i.p. injections at day 0 and 21 and bacterial challenge at day 31. Peripheral blood samples were collected

from tail veins of the mice before the second immunization and challenge and the sera were used in ELISA for the measurement of immune responses.

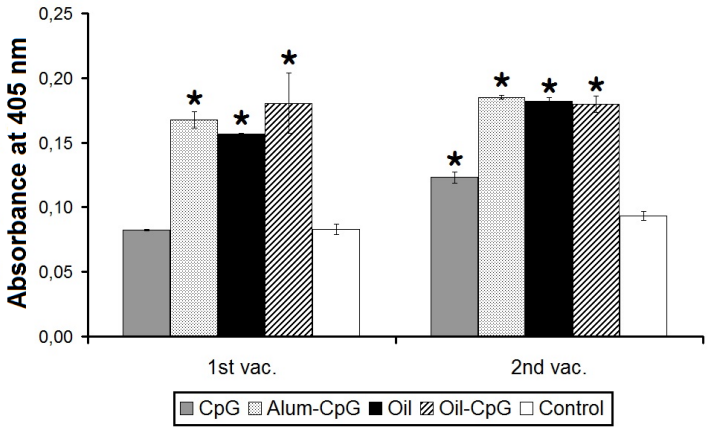

Figure 3.19. Total IgG levels in 1:1600 diluted sera from the mice vaccinated with 100 μg of PlpEC-OmpH fusion protein formulated with CpG, alum-CpG, oil based or oil based-CpG adjuvants. Statistically significant (p<0.05) increase as compared to control was shown with an asterisk.

A statistically significant (p<0.05) increment in antibody response was obtained in mice vaccinated with alum-CpG, oil based and oil based-CpG formulations both after first and second immunizations. There was not a significant difference among these adjuvants in terms of induction of antibody levels. CpG formulation increased antibody titers only after second injection still being lower as compared to those by the other three adjuvants (Figure 3.19). Formulations containing CpG significantly (p<0.05) increased serum IFN-γ levels after first and second vaccination (Figure 3.20). A statistically significant increment in IFN-γ levels in mice vaccinated with oil based formulation with respect to control group was not recorded.

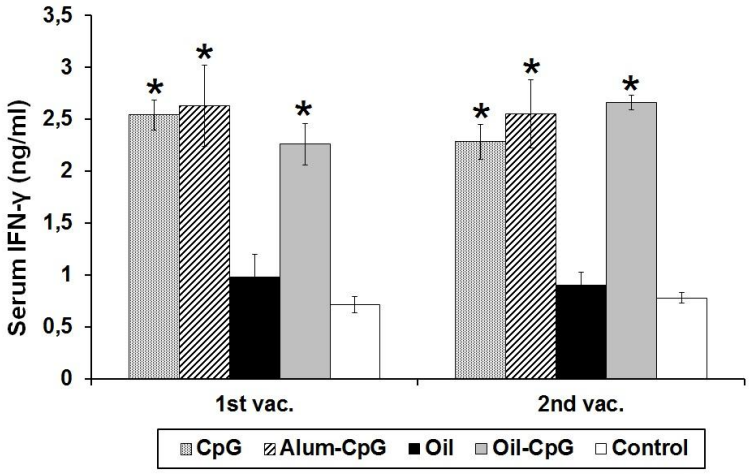

Figure 3.20. IFN-γ levels in 1:4 diluted sera from the mice vaccinated with 100 µg of PlpEC-OmpH fusion protein formulated with CpG, alum-CpG, oil based or oil based-CpG adjuvants. Statistically significant (p<0.05) increase as compared to control was shown with an asterisk.

Although alum induces Th2-type immunity, increased serum IFN-γ levels in mice vaccinated with alum-CpG formulation showed a Th1-type polarization. Likewise, Gentil *et al*. (2010) reported that the vaccine formulation with alum adjuvant induced IgG1 levels responsible for Th2-type response whereas the one with alum-CpG adjuvant had increased titers of IgG2a and IgG2b associated with Th1-type immunity. Lefeber *et al*. (2003) demonstrated that the highest levels of IgG subclasses associated with Th1-type response were obtained with the vaccine formulation with CpG and dimethyl dioctadecyl ammonium bromide combination.

Table 3.3. Protection conferred in mice immunized with PlpEC-OmpH protein formulated with CpG, alum-CpG, oil based or oil based-CpG against challenge with *P. multocida* A:3.

Vaccine formulation (protein/adjuvant)	No. of mice survived/challenged	Protection %
PlpEC-OmpH/CpG	0/5	0
PlpEC-OmpH/alum-CpG	0/5	0
PlpEC-OmpH/oil based	3/5	60*
PlpEC-OmpH/oil based-CpG	5/5	100*
Control	0/5	0

*Statistically significant (p<0.05) as compared to control.

Protective capacities of the vaccine formulations composed of PlpEC-OmpH fusion protein formulated with CpG, alum-CpG, oil based or oil based-CpG adjuvants were also exhibited via challenge of the immunized mice with 10 LD_{50} of live *P. multocida*. Control group mice and those vaccinated with CpG or alum-CpG formulations were died at the second day after challenge (Table 3.3). Formulation with oil based adjuvant conferred 60% (3 out of 5 mice) protection while 100% (5 out of 5) of the mice vaccinated with PlpEC-OmpH formulated with oil based-CpG adjuvant were protected from the bacterial challenge. Thus, the formulation composed of PlpEC-OmpH fusion protein and oil based-CpG adjuvant can be used as a potential vaccine candidate against *P. multocida* A:3.

3.8. Expression of *ompH*, *plpE*N, *plpE*C and *plpE* Genes in *E. coli* and Purification of Recombinant Proteins

For the gene expression and protein purification, *ompH*, *plpE*N, *plpE*C and *plpE* were cloned in pET28a vector and introduced into *E. coli* BL21(DE3) cells. Purification of the recombinant PlpEN, PlpEC and OmpH proteins from *E. coli*

cultures was performed via affinity column chromatography as described in section 3.5. PlpEC and OmpH proteins were successfully purified but recombinant PlpEN could not be recovered from *E. coli* (Figure 3.21).

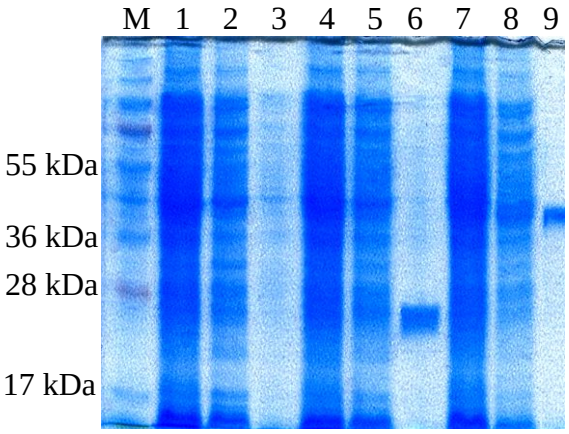

Figure 3.21. Purification of recombinant PlpEN, PlpEC and OmpH using Ni-TED columns under denaturing conditions. M: Prestained protein ladder. Lanes 1, 4, 7: Uninduced control, Lanes 2, 5, 8: IPTG-induced culture lysates and Lanes 3, 6, 9: Eluates for PlpEN, PlpEC and OmpH, respectively.

Standard protocol was applied for the purification of PlpE but no protein was bound on the column (Figure 3.22). Therefore, the effects of increased NaCl concentration as well as addition of β-mercaptoethanol (B-ME) or Triton X-100 to the LEW Buffer with 8 M urea were investigated. Ni-NTA and Ni-TED columns were used in each case (data not shown). Finally, the purification protocol was optimized as addition of 30 mM B-ME to the LEW buffer containing 8 M urea and 1 M NaCl with the utilization of Ni-NTA columns (Figure 3.23).

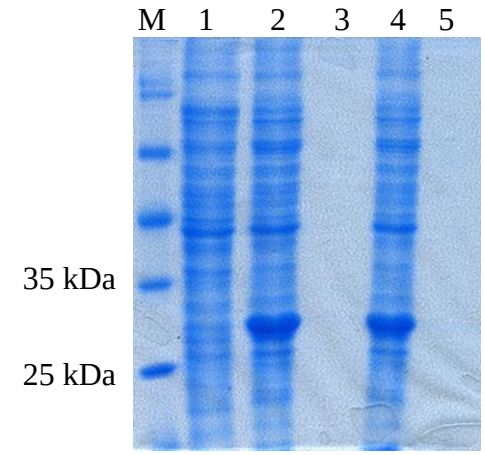

Figure 3.22. Purification of recombinant PlpE using Ni-TED columns under standard conditions. M: Protein ladder, Lane 1: Uninduced control, Lane 2: IPTG induced culture lysate, Lane 3: Eluate, Lane 4: Flow-through, Lane 5: Wash fractions.

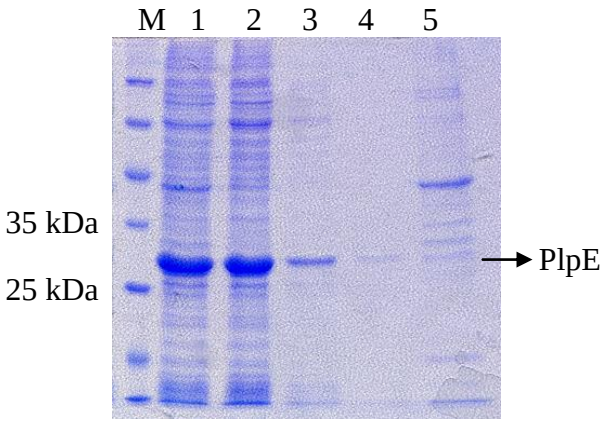

Figure 3.23. Purification of recombinant PlpE using Ni-NTA columns under optimized conditions. M: Protein ladder, Lane 1: IPTG induced culture lysate, Lane 2: Flow through, Lane 3: Wash I, Lane 4: Wash II, Lane 5: Eluate fractions.

3.9. Immune Responses against Recombinant OmpH, PlpEC and PlpE

Recombinant OmpH, PlpEC and PlpE were formulated with oil based-CpG adjuvant that provided the full protection with PlpEC-OmpH. Five BALB/c mice per group were i.p. immunized with 0.5 ml of vaccine formulations containing 100 μg of recombinant protein at day 0 and 21. Antibody levels in vaccinated mice were measured (Figure 3.24). A statistically significant (p<0.05) increase in antibody titers were obtained in mice immunized with PlpEC and PlpE after first and second injections and with OmpH after second vaccination.

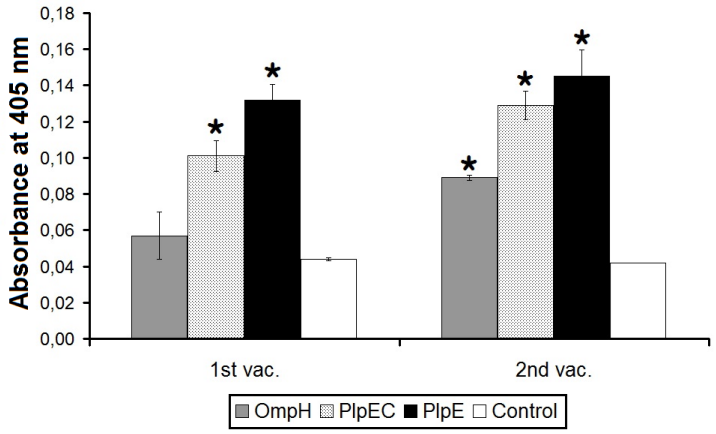

Figure 3.24. Total IgG levels in 1:1600 diluted sera from the mice vaccinated with 100 μg of recombinant OmpH, PlpEC and PlpE formulated with oil based-CpG adjuvant. Statistically significant (p<0.05) increase as compared to control was shown with an asterisk.

Serum IFN-γ levels in mice vaccinated with recombinant OmpH, PlpEC and PlpE formulated with oil based-CpG were significantly (p<0.05) induced after first and second injection (Figure 3.25).

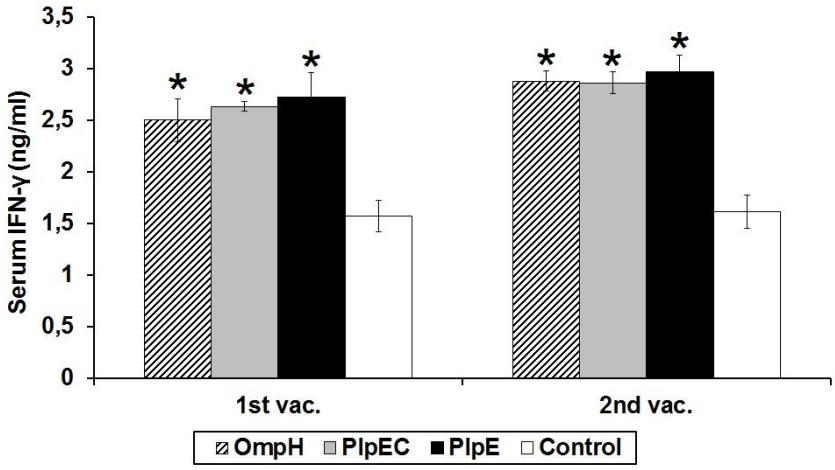

Figure 3.25. IFN-γ levels in 1:4 diluted sera from the mice vaccinated with 100 µg of recombinant OmpH, PlpEC and PlpE formulated with oil based-CpG adjuvant. Statistically significant (p<0.05) increase as compared to control was shown with an asterisk.

Table 3.4. Protection conferred in mice immunized with recombinant OmpH, PlpEC or PlpE protein formulated with oil based-CpG adjuvant against challenge with *P. multocida* A:3.

Vaccine formulation (protein/adjuvant)	No. of mice survived/challenged	Protection (%)
OmpH/oil based-CpG	3/6	50
PlpEC/oil based-CpG	3/5	60*
PlpE/oil based-CpG	5/5	100*
Control (oil based-CpG)	0/5	0

*Statistically significant (p<0.05) as compared to control.

For the detection of protective efficacies of recombinant OmpH, PlpEC and PlpE proteins, immunized mice were challenged with 10 LD_{50} of live *P. multocida* A:3. Control mice were died at day 2 after challenge. OmpH formulation conferred 50% protection while 60% (3 out of 5 mice) protection was provided by PlpEC formulation. On the other hand, a full (100%) protection was obtained with the vaccine formulation composed of 100 μg of recombinant PlpE adsorbed on oil based-CpG adjuvant (Table 3.4).

Outer membrane protein H (OmpH) and *Pasteurella* lipoprotein E (PlpE) are of interest in recombinant vaccine studies against different serotypes of *P. multocida*. Wu *et al*. (2007) reported that recombinant PlpE from *P. multocida* serotype A:1 causing fowl cholera conferred 63-100% protection in mice and chicken against heterologous challenge with the serotypes A:1, A:3 and A:4. Luo *et al*. (1997) vaccinated chickens with native and recombinant OmpH from *P. multocida* serotype A:1 and reported 100% and 18% protection, respectively. However, Sthitmatee *et al*. (2008) showed that both native and recombinant OmpH from serotype A:1 and its identical protein, Cp39 from serotype A:3 conferred 60-100% protection in chickens against challenge with serotypes A:1 and A:3. Tan *et al*. (2010) immunized mice i.p. or s.c. with recombinant OmpH from *P. multocida* serotype B:2 causing hemorrhagic septicemia and obtained 80% and 100% protection, respectively. Moreover, recombinant OmpH from a swine isolate of *P. multocida* causing atrophic rhinitis provided 70% protection in mice (Lee *et al*., 2007). Dabo *et al*. (2008) vaccinated mice with recombinant OmpA from a bovine isolate of *P. multocida* serotype A:3 but no protection was obtained. However, there is no study on protectivity of recombinant PlpE or OmpH proteins isolated from *P. multocida* A:3 targeting shipping fever. In the present study, immunogenicity and protective capacities of OmpH and PlpE from *P. multocida* P-1062 (serotype A:3, a bovine isolate) were investigated. PlpEN-OmpH and PlpEC-OmpH fusions or recombinant OmpH, PlpEC and PlpE proteins were capable of inducing antibody titers in vaccinated mice irrespective of adjuvant used in the formulation. Induction of serum IFN-γ levels in vaccinated mice was

achieved by alum adjuvanted PlpEN-OmpH and CpG containing formulations. However, statistically significant ($p<0.05$) protection was conferred by PlpEC-OmpH formulated with oil based and oil based-CpG and PlpE and PlpEC formulated with oil based-CpG. Dabo *et al*. (2008a) reported that recombinant OmpA from a bovine isolate of *P*. *multocida* A:3 elicited a strong Th2-type immunity but did not provide a protection showing the insufficiency of a sole Th2-type immune response in protection and the need for some specific Th1 response. Therefore, a balance between Th1- and Th2-type immunity and selection of appropriate antigen would be effective in protection against *P*. *multocida*. Both humoral and cell-mediated immune responses are involved in *P*. *multocida* infection (Korde *et al*, 2005; Praveena *et al*., 2010); the former takes role in the suppression of the infection in early stage whereas the latter is probably necessary for the complete elimination of the pathogen in the later stage of the infection (Verma and Jaiswal, 1997). In this study, the vaccines composed of PlpEC-OmpH and PlpE formulated with oil based-CpG increased serum total IgG and IFN-γ levels and conferred 100% protection in mice.

CHAPTER 4

CONCLUSIONS

- There was not a significant increase in antibody titers in mice vaccinated with 50 μg of pCMV-ompH whereas significant increment (p<0.05) was obtained with 100 μg of pCMV-ompH after third vaccination.

- Serum IFN-γ levels in mice vaccinated with 100 μg of pCMV-ompH did not induced while the increment by 50 μg of pCMV-ompH was significant (p<0.05) after second and third vaccinations.

- Neither 50 μg nor 100 μg of pCMV-ompH DNA vaccine confer protection against 10 LD_{50} (55 CFU) of live *P. multocida*.

- pCMV-plpEC-ompH significantly (p<0.05) increased antibody levels after first, second and third injections. On the other hand, a significant increase (p<0.05) in antibody level was obtained after third vaccination with pCMV-plpEN-ompH.

- pCMV-plpEN-ompH significantly (p<0.05) induced IFN-γ titers after first and second immunizations. However, a significant increase in serum IFN-γ levels could not be obtained by pCMV-plpEC-ompH construct. As a conclusion, pCMV-plpEN-ompH DNA vaccine induced both humoral and cell-mediated immune responses against *P. multocida* A:3 in mice.

- No protection could be obtained with pCMV-plpEN-ompH or pCMV-plpEC-ompH DNA vaccines against 10 LD_{50} of live *P. multocida*.

- Antibody levels in mice immunized with PlpEN-OmpH or PlpEC-OmpH fusion proteins were siginicantly (p<0.05) induced.

- Neither 50 μg PlpEN-OmpH nor 50 μg or 100 μg of PlpEC-OmpH increased cell-mediated immune responses in vaccinated mice. Statistically significant (p<0.05) increase in serum IFN-γ level was only observed in mice vaccinated with 100 μg of PlpEN-OmpH.

- Two out of five mice (40%) vaccinated with 100 μg of PlpEC-OmpH were survived after challenge which was not statistically significant (p=0.11).

- A statistically significant (p<0.05) increment in antibody response was obtained in mice vaccinated with PlpEC-OmpH formulated with alum-CpG, oil based and oil based-CpG after first and second immunizations. CpG formulation increased antibody titers only after second injection still being lower as compared to those by the other three adjuvants.

- Formulations containing PlpEC-OmpH and CpG significantly (p<0.05) increased serum IFN-γ levels after first and second vaccination immunization. A statistically significant increment in IFN-γ levels in mice vaccinated with PlpEC-OmpH and oil based adjuvant formulation was not recorded.

- Formulation with PlpEC-OmpH and oil based adjuvant conferred 60% (3 out of 5 mice) protection while 100% (5 out of 5) of the mice vaccinated with PlpEC-OmpH and oil based-CpG adjuvant were protected from the bacterial challenge.

- A statistically significant ($p<0.05$) increase in antibody titers were obtained in mice immunized with PlpEC and PlpE after first and second injections and with OmpH after second vaccination.

- Serum IFN-γ titers were significantly ($p<0.05$) induced by vaccination with OmpH, PlpEC and PlpE formulated with oil based-CpG adjuvant.

- OmpH and oil based-CpG formulation conferred 50% protection while 60% protection was provided by PlpEC formulation. On the other hand, a full (100%) protection was obtained with the vaccine formulation composed of 100 μg of recombinant PlpE formulated with oil based-CpG adjuvant.

- PlpEC-OmpH fusion and recombinant PlpE formulated with oil based-CpG adjuvant provided full protection in mice. These two formulations are potential candidates for acellular vaccine development against shipping fever.

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APPENDIX A

STRUCTURES OF PLASMID VECTORS AND SIZE MARKERS

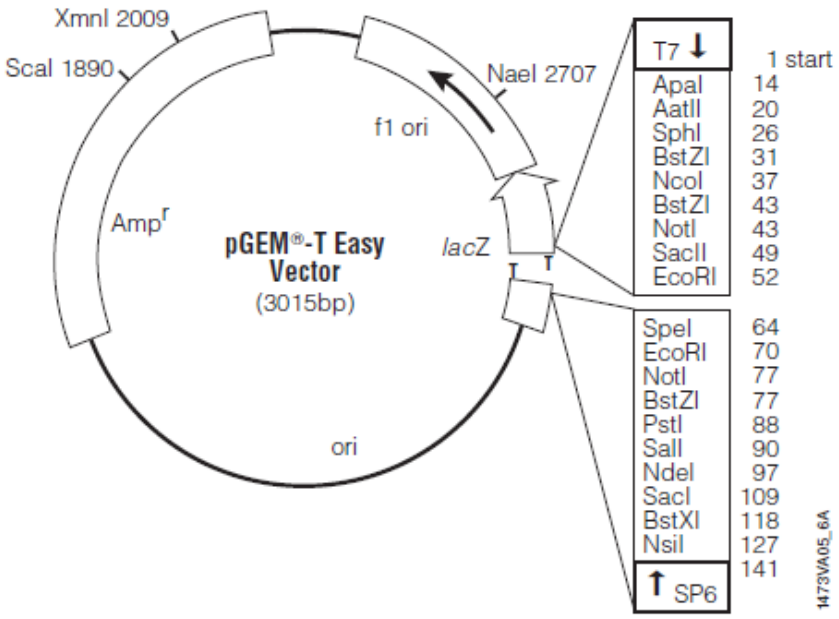

Figure A1. pGEM®-T Easy Cloning Vector (Promega #A1360)

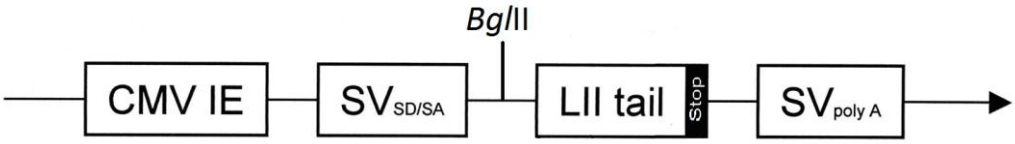

Figure A2. pCMV-LII DNA Vaccine Vector

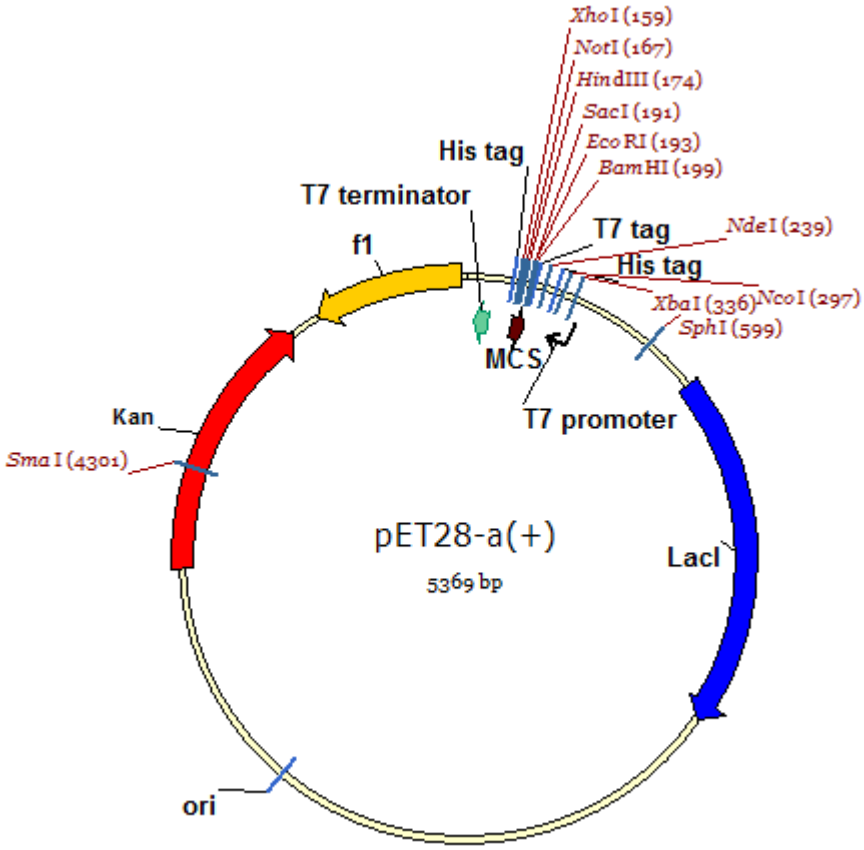

Figure A3. pET-28a(+) His-tag Expression Vector (Novagen #69864-3)

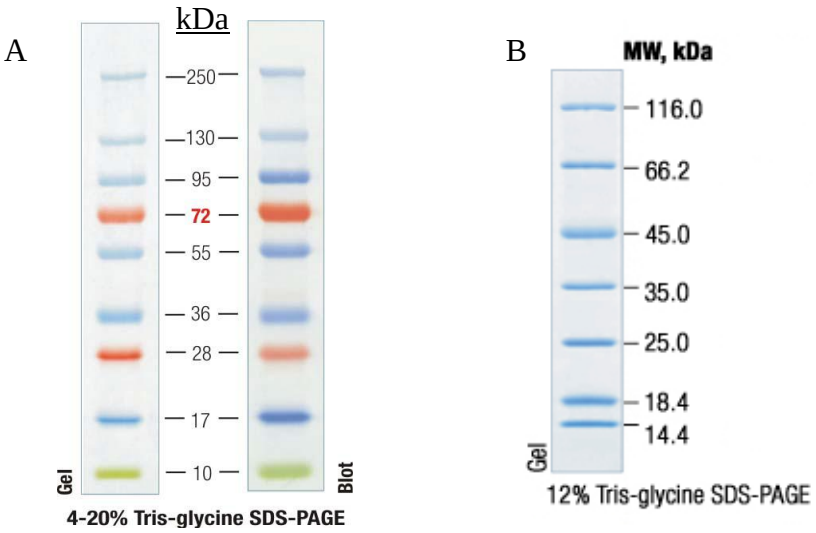

Figure A4. PageRuler™ Plus Prestained Protein Ladder (Fermentas #SM1811) **(A)** and Unstained Protein Molecular Weight Marker (Fermentas #SM0431) **(B)**.

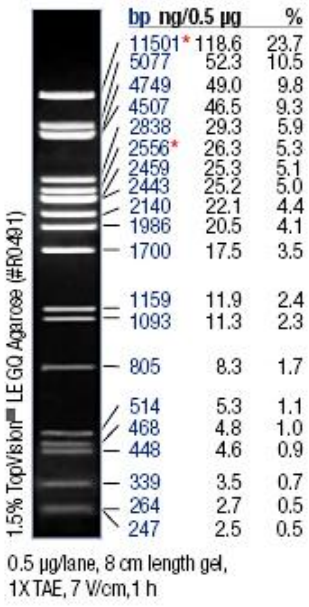

Figure A5. Lambda DNA/*Pst*I Marker (Fermentas #SM0361)

APPENDIX B

COMPOSITION AND PREPARATION OF CULTURE MEDIA

B1. Luria Broth:

- Tryptone 10 g
- Yeast Extract 5 g
- NaCl 10 g
- Distilled water up to 1000 ml

Final pH is 7.0; sterilized at 121^{o}C for 15 min.

B2. Luria Agar:

- Tryptone 10 g
- Yeast Extract 5 g
- NaCl 10 g
- Agar 15 g
- Distilled water up to 1000 ml

Final pH is 7.0; sterilized at 121^{o}C for 15 min.

B3. Brain-Heart Infusion Broth:

- Nutrient substrate 27.5 g
- D(+)Glucose 2 g
- NaCl 5 g
- Na_2HPO_4 2.5 g

- Distilled water up to 1000 ml

Final pH is 7.5; sterilized at 121^{o}C for 15 min.

B4. Blood Agar:

- Pancreatic casein 15 g
- Papaic digest of soy flour 5 g
- NaCl 5 g
- Agar 15 g
- Sheep blood (v/v) 5%
- Distilled water up to 1000 ml

Final pH is 7.3; sterilized at 121^{o}C for 15 min.

APPENDIX C

SOLUTIONS AND BUFFERS

C1. Agarose Gel Electrophoresis

C1.1. TAE Buffer (50X)

• Tris-base	242 g
• Glacial acetic acid	57.1 mL
• EDTA (0.5 M, pH 8.0)	100 mL
• Distilled water	up to 1000 mL

C1.2. Loading Buffer (10X)

• Bromophenol blue (w/v)	0.25%
• Xylene cyanol FF (w/v)	0.25%
• Sucrose (w/v)	40%

C2. SDS-Polyacrylamide Gel Electrophoresis (PAGE)

C2.1. Acrylamide/Bis

• Acrylamide	146 g
• N.N'-Methylene-bis acrylamide	4 g
• Distilled water	up to 500 mL

Filtered and stored at 4°C. Protected form light.

C2.2. Tris HCl (1.5 M)

- Tris-base 54.45 g
- Distilled water 150 ml

pH is adjusted to 8.8 with HCl, distilled water to 300 ml and stored at 4°C.

C2.3. Tris HCl (0.5 M)

- Tris-base 6 g
- Distilled water 60 ml

pH is adjusted to 6.8 with HCl, distilled water to 100 ml and stored at 4°C.

C2.4. Running Buffer (10X)

- Tris-base 30 g
- Glycine 144 g
- SDS 10 g
- Distilled water up to 1000 ml

C2.5. Sample Loading Buffer (4X)

- Tris-HCl (1 M, pH 6.8) 2 ml
- EDTA (0.5 M) 1 ml
- Glycerol 4 ml
- SDS 0.8 g
- β-mercaptoethanol 0.4 ml
- Bromophenol blue 0.008 g
- Distilled water up to 10 ml

C2.6. Fixation Solution

- Ethanol 40%
- Glacial acetic acid 10%
- Distilled water 50%

C2.7. Coomassie Blue R-250 Stain

- Coomassie Blue R-250 — 0.25 g
- Methanol — 125 ml
- Glacial acetic acid — 25 ml
- Distilled water — 100 ml

C2.8. Destaining Solution

- Methanol — 100 ml
- Glacial acetic acid — 100 ml
- Distilled water — 800 ml

C3. Western Blot

C3.1. Transfer Buffer (1X)

- Methanol — 200 ml
- Tris-base — 3.63 g
- Glycine — 14.4 g
- SDS — 0.37 g
- Distilled water — up to 1000 ml

C3.2. Tris-buffered Saline, TBS (1X)

- Tris-base — 2.42 g
- NaCl — 29.2 g
- Distilled water — up to 1000 ml

C4. Protein Purification

C4.1. LEW (Lysis-Elution-Wash) Buffer (pH 8.0)

- Urea 8 M
- NaCl 300 mM
- NaH_2PO_4 50 mM

C4.2. DB (Dialysis Buffer, pH 8.0)

- NaH_2PO_4 50 mM
- NaCl 500 mM
- Urea 4 M

C5. *E. coli* Competent Cell Preparation

C5.1. Buffer 1

- RuCl 100 mM
- KAc 30 mM
- $CaCl_2$ 10 mM
- Glycerol 15%

pH is adjusted to 5.8 with dilute acetic acid and filter sterilized.

C5.2. Buffer 2

- $CaCl_2$ 75 mM
- RuCl 10 mM
- MOPS 10 mM
- Glycerol 15%

pH is adjusted to 6.5 with 0.2 M KOH and filter sterilized.

C6. IPTG (Isopropyl-β-D-thiogalactoside) for Colony Selection

- IPTG 100 mg
- Distilled water 1 ml

The solution was filter sterilized and stored at –20°C.

C7. X-Gal (5-bromo-4-chloro-3-indolyl-B-D-galactoside)

- X-Gal 20 mg
- Dimethylformamide 1 ml

The solution was stored at –20°C protected from light.

C8. Plasmid Isolation

C8.1. STE Buffer

- Sucrose (w/v) 10.3%
- Tris-HCl (pH 8.0) 25 mM
- EDTA (pH 8.0) 25 mM

C8.2. Lysis Buffer

- NaOH 0.3 M
- SDS (w/v) 2%

C9. ELISA for Detection of Antibody Titers

C9.1. Carbonate/Bicarbonate Buffer (0.05 M)

- Na_2CO_3 1.59 g
- $NaHCO_3$ 3.88 g
- Distilled water up to 1000 ml

pH is adjusted to 9.6 and stored at 4^oC.

C9.2. Washing Solution (1X PBS - 0.1% Tween-20)

- NaCl 8 g
- KCl 0.2 g
- Na_2HPO_4 1.44 g
- KH_2PO_4 0.24 g
- Tween-20 1 ml
- Distilled water up to 1000 ml

pH is adjusted to 7.2 and stored at 4^{o}C.

C9.3. Blocking Solution

- 2% (w/v) BSA in 1X PBS - 0.1% Tween-20.

C10. ELISA for Detection of Serum IFN-γ Titers

C10.1. Coating Buffer (1X PBS)

- NaCl 8 g
- KCl 0.2 g
- Na_2HPO_4 1.44 g
- KH_2PO_4 0.24 g
- Distilled water up to 1000 ml

pH is adjusted to 7.4 and stored at 4^{o}C.

C10.2. Blocking Buffer

- 4% (w/v) BSA and 5% (w/v) sucrose in 1X PBS.

C10.3. Assay Buffer

- 2% (w/v) BSA in 1X PBS.

C10.4. Wash Buffer (1X PBS-0.2% Tween-20)

• NaCl	8 g
• KCl	0.2 g
• Na_2HPO_4	1.44 g
• KH_2PO_4	0.24 g
• Tween-20	2 ml
• Distilled water	up to 1000 ml

pH is adjusted to 7.4 and stored at 4^oC.

C10.5. Stop Solution

- 0.18 M sulfuric acid.

APPENDIX D

SUPPLIERS OF CHEMICALS, ENZYMES AND KITS

D1. Chemicals	**Suppliers**
Acrylamide	Sigma
Agar-agar	Merck
Agarose	Biomax (Prona)
Ammonium persulfate	AppliChem
Ampicillin	Sigma
Anti-mouse IgG	Sigma
Bovine serum albumin	Sigma
Brain Heart Broth	Merck
Bromophenol blue	Merck
$CaCl_2.2H_2O$	Merck
Coomassie Blue G-250	Fluka
Coomassie Blue R-250	Fluka
Dimethylformamide	Merck
dNTPs	Fermentas
DTT	Fluka
Dulbecco's modified Eagle's medium	Biochrom
EDTA	Sigma
Ethanol	Sigma
Ethidium bromide	Sigma
Fetal bovine serum	Biochrom

Formaldehyde	Merck
Glacial acetic acid	Merck
Glycerol	Merck
Glycine	Merck
H_2SO_4	Merck
HCl	Merck
IPTG	Sigma
Isopropanol	Merck
Kanamycin	Sigma
KCl	Merck
KH_2PO_4	Merck
Luria Broth	Merck
Methanol	Merck
$MnCl_2$	Merck
MOPS	AppliChem
N.N'-Methylene-bis acrylamide	Sigma
Na_2CO_3	Merck
Na_2HPO_4	Merck
NaCl	Sigma
$NaHCO_3$	Merck
NaOH	Merck
Non-essential amino acids	Biochrom
Penicillin/streptomycin	Biochrom
Phenol/chloroform/isoamylalcohol	Amresco
Phosphoric acid	Merck
Potassium acetate	Merck
RuCl	Merck
SDS	Merck
Skim milk	Fluka
Streptavin-HRP	Pierce
Sucrose	Merck

TEMED	Merck
TMB	Thermo Sci
Tris-base	Sigma
Tris-HCl	Fluka
Tween-20	Merck
Urea	Sigma
X-gal	Sigma
Xylene cyanol FF	Merck
2-mercaptoethanol	Merck

D2. Enzymes

Alkaline phosphatase	Roche
*Bam*HI	Fermentas
*Bgl*II	Fermentas
*Eco*RI	Fermentas
*Hin*dIII	Fermentas
*Not*I	Fermentas
T4 DNA ligase	Fermentas
Taq DNA polymerase	Fermentas

D3. Kits

AP Conjugate Substrate Kit	Bio-Rad
Endofree Plasmid Mega Kit	Qiagen
FuGENE 6 Transfection Reagent	Roche
Gel Extraction Kit	Qiagen
Mouse IFN-γ Minikit	Pierce
Ni-NTA Spin Columns	Qiagen
pGEMT Easy Vector	Promega
Plasmid Midi Kit	Qiagen
Plasmid Mini Kit	Qiagen
Protino Ni-TED 2000 Packed Columns	Macherey-Nagel

CURRICULUM VITAE

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EDUCATION

Degree	Institution	Year of Graduation
M. Sc.	METU Biology	2005
B. Sc.	Anadolu University Biology	2002

WORK EXPERIENCE

Year	Place	Enrollment
2002-2011	METU Biology	Research Assistant

FOREIGN LANGUAGES

Advanced English, Fluent Spanish

PUBLICATIONS

Theses:

M. Sc. Thesis: Cloning of chitinase A (*chiA*) gene of *Serratia marcescens* Bn10 and its expression in Coleoptera-specific *Bacillus thuringiensis*.

B. Sc. Thesis: Kekik (*Origanum onites*) yağının mutajenik ve antimutajenik etkisisnin Ames Testi ile değerlendirilmesi.

Research Articles:

1. Okay, S., Özcengiz, E., Gürsel, İ., Özcengiz, G. Immunogenicity and protective efficacy of the recombinant PlpE and OmpH from *Pasteurella multocida* A:3 in mice. *Clinical and Vaccine Immunology* (submitted).

2. Okay, S., Özcengiz, G. (2011). Molecular cloning, characterization and homologous expression of an endochitinase gene from *Bacillus thuringiensis* serovar *morrisoni*. *Turkish Journal of Biology* 35: 1-7.

3. Özcengiz, G., Okay, S., Ünsaldı, E., Taşkın, B., Liras, P., Piret, J. (2010). Homologous expression of aspartokinase (*ask*) gene in *Streptomyces clavuligerus* and its *hom*-deleted mutant: Effects on cephamycin C production. *Bioengineered Bugs* 1 (3): 191-197.

4. Okay, S., Tefon, B.E., Ozkan M. and Ozcengiz, G. (2008). Expression of chitinase A (*chiA*) gene from a local isolate of *Serratia marcescens* in Coleoptera-specific *Bacillus thuringiensis*. *Journal of Applied Microbiology* 104: 161-170.

5. Ipek, E., Zeytinoglu, H., Okay, S., Tuylu, B.A., Kurkcuoglu M. and Baser, K.H.C. (2005). Genotoxicity and antigenotoxicity of Origanum oil and carvacrol evaluated by Ames Salmonella/microsomal test. *Food Chemistry* 93(3): 551-556.

International Congress Presentations:

Oral presentation:

1. Okay, S., Özcengiz, E., Özcengiz, G. (2010). Development of new vaccine strategies against *Pasteurella multocida*. Prato Conference on the Pathogenesis of Bacterial Diseases of Animals (6-9 October 2010, Prato, Italy). Abstract Book p. 47.

Poster presentations:

1. Okay, S., Ipek, E., Zeytinoğlu, H., Kurkcuoğlu, M. (2003). Antimutagenicity testing of Origanum oil and carvacrol in the Ames assay. 13th Balkan Biochemical Biophysical Days & Meeting on Metabolic Disorders (October 12-15, 2003 Aydın, Turkey) Programme & Abstracts. Turkish Journal of Biochemistry 28(3): 140.

2. Okay, S., Ozkan, M. and Ozcengiz, G. (2006). Expression of an endochitinase (*chiA*) gene from *Serratia marcescens* Bn10 in a Cry3A producer *Bacillus thuringiensis*. 2nd FEMS Congress of European Microbiologists (July 4-8, 2006 Madrid, Spain) Abstract Book p.124.

3. Okay, S. and Ozcengiz, G. (2008). Characterization and homologous expression of endochitinase gene (*chi3023*) from *Bacillus thuringiensis* serovar *morrisoni*. XII. International Congress of Bacteriology and Applied Microbiology (5-9 August 2008, İstanbul, Turkey) Abstract Book p. 75.

4. Ünsaldı, E., Okay, S. and Özcengiz, G. (2008). Cephamycin C overproduction upon releasing relaxing precursor flux by genetic engineering and medium formulation. XII. International Congress of Bacteriology and Applied Microbiology (5-9 August 2008, İstanbul, Turkey) Abstract Book p. 98.

5. Okay, S., Aragon, V., Rosell, R., Pujols, J., Ozcengiz, G., Rodriguez, F. (2009). Use of FHA from *Bordetella bronchiseptica* as an adjuvant to improve DNA vaccination in small and large animals. 3rd annual meeting EPIZONE "Crossing borders" (12-15 May 2009, Antalya, Turkey) Abstract Book p.137.

6. Okay, S., Ünsaldı, E., Taşkın, B., Kurt, A., Piret, J., Liras, P., Özcengiz, G. (2009). Metabolic engineering of aspartate pathway for increased production of cephamycin C in *Streptomyces clavuligerus*. International Symposium on Biotechnology: Developments and Trends (27-30 September 2009, METU, Ankara, Turkey) Abstract Book p. 99.

National Congress Presentations:

Oral presentations:

1. Okay, S., Özcengiz, G. (2007). *Bacillus thuringiensis* alttür *morrisoni* kitinaz geninin karakterizasyonu. 15. Ulusal Biyoteknoloji Kongresi (28-31 Ekim, Antalya). Bildiri Kitabı Sayfa: 303-304.

2. Ünsaldı, E., Okay, S., Özcengiz, G. (2009). Yabanıl tip *Streptomyces clavuligerus* ve *hom* geni delesyona uğramış mutantında aspartokinaz (*ask*) geni çoklu kopyasının homolog ekspresyonu: Hücre içi serbest aminoasit seviyesindeki değişimler. 16. Ulusal Biyoteknoloji Kongresi (13-16 Aralık, Antalya). Bildiri Kitabı Sayfa: 314-317.

Poster presentations:

1. Okay, S., Özkan, M., Özcengiz, G. (2005). Kitinaz A geninin *Serratia marcescens*'ten klonlanması ve Coleoptera-spesifik *Bacillus thuringiensis*'te ifade edilmesi. 14. Ulusal Biyoteknoloji Kongresi (31 Ağustos-2 Eylül, Eskişehir) Bildiri ve Poster Kitabı Sayfa: 586.

2. Okay, S., Tefon, B.E., Özkan, M., Özcengiz, G. (2007). *Serratia marcescens* Kitinaz A Geninin Rekombinant *Bacillus thuringiensis*'te Ekspresyonunun Analizi. 15. Ulusal Biyoteknoloji Kongresi (28-31 Ekim, Antalya). Bildiri Kitabı Sayfa: 44.

3. Okay, S., Özcengiz, E., Özcengiz, G. (2009). *Pasteurella multocida* P-1062 suşuna ait *ompH* geninin klonlanması ve biyoinformatik analizi. 16. Ulusal Biyoteknoloji Kongresi (13-16 Aralık, Antalya). Bildiri Kitabı Sayfa: 15.

4. Okay, S., Baloğlu, M.C., Eroğlu, A., Battal, A., Özcengiz, G., Öktem, H.A., Yücel, M. (2009). *Serratia marcescens*'e ait kitinaz A geninin tütün bitkisine aktarılması. 16. Ulusal Biyoteknoloji Kongresi (13-16 Aralık, Antalya). Bildiri Kitabı Sayfa: 14-15.

5. Yılmaz, Ç., Okay, S., Tefon, B. E., Özcengiz, E., Özcengiz, G. (2009). *Bordetella pertussis* Tohama I ve Saadet suşuna ait PPIase, POMP ve FIMX genlerinin klonlanması. 16. Ulusal Biyoteknoloji Kongresi (13-16 Aralık 2009, Antalya) Bildiri Kitabı, sayfa: 71-72.

PROJECTS:

1. Development of new vaccine strategies of utility in animal health (PCI2005-A7-0092; 2006-2008). Complementary action between Spain and Turkey financed by Spanish Ministry of Education.

2. Genetik manipulasyonlarla *Bacillus thuringiensis* alttür *tenebrionis*'in larvisidal aktivitesinin ve spektrumunun artırılması. TÜBİTAK TBAG Proje 2413 104T023, 2006: 101

3. Cloning of chitinase A (*chiA*) gene from *Serratia marcescens* Bn10 and its expression in Coleoptera-specific *Bacillus thuringiensis* (BAP-08-11-DPT-2002K120510-BTEK12). ÖYP-DPT Project.

RESEARCH ACTIVITY:

Studies on DNA vaccine strategies at CReSA (Animal Health Research Center), UAB (Autonomous University of Barcelona), Barcelona, Spain; for one year starting from January 2008 (supervised by Dr. Fernando Rodriguez).

AWARDS/GRANTS:

1. First Honor in B. Sc., Anadolu University, June 2002
2. Graduate Courses Performance Award, METU, 2005-2006 Academic Year
3. TÜBİTAK International Scientific Publication Awards, 2005, 2008, 2011
4. METU Scientific Publication Award, 2008
5. ÖYP Grant for one year research in CreSA, Barcelona, Spain, 2008
6. Travel Grant, VetPath Conference, Prato Italy, 6-9 October 2010