

DEVELOPMENT OF RECOMBINANT VACCINE CANDIDATES
COMPOSED OF LTKA FROM MANNHEIMIA HAEMOLYTICA A1 AND
PLPEC-OMPH FROM PASTEURELLA MULTOCIDA A:3 AGAINST BOVINE
RESPIRATORY DISEASE

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COMPOSED OF LTKA FROM MANNHEIMIA HAEMOLYTICA A1 AND
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BOVINE RESPIRATORY DISEASE**

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ABSTRACT

DEVELOPMENT OF RECOMBINANT VACCINE CANDIDATES COMPOSED OF LTKA FROM MANNHEIMIA HAEMOLYTICA A1 AND PLPEC AND OMPH FROM PASTEURELLA MULTOCIDA A:3 AGAINST BOVINE RESPIRATORY DISEASE

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Mannheimia haemolytica A1 and *Pasteurella multocida* serotype A:3 are gram-negative bacterial pathogens which are considered the causative agents of bovine respiratory disease (BRD) or shipping fever in cattle. The foremost of several virulence factors of *M. haemolytica* A1 which allows lung colonization and establishment of infection is leukotoxin (Lkt). The others include adhesin, capsule, outer membrane proteins, and various proteases used for attachment, spreading and evading innate and adaptive host immune responses. Similarly, the major virulence factors of *P. multocida* are capsule, lipopolysaccharide (LPS), toxin (PMT), outer membrane proteins (OMPs), especially PlpE and OmpH, adhesins and type IV fimbriae (pili). BRD has considerable economic importance to the global cattle and feedlot industry. Effective control of BRD is challenging due to inconsistencies in diagnosis and treatment regimes. Extensive antibiotic usage in cattle industry, both prophylactically and therapeutically, leads to development of antibiotic resistance. Therefore, development of vaccine strategies against BRD to demonstrate protection becomes an important issue for cattle industry. *P. multocida* A:3 lipoprotein E (PlpE) and outer membrane protein H

(OmpH) and *M. haemolytica* A1 leukotoxin (LtkA) have been selected as the antigens for vaccine development studies in our laboratory. PlpEC-OmpH and OmpH-LtkA recombinant fusion proteins were previously constructed, expressed and purified. When PlpEC-OmpH was used as an universal vaccine candidate in combination with a mineral oil-based CpG adjuvant, it conferred 100% protection against *P. multocida* A:3 in BALB/c mice. In the present study, OmpH-LtkA recombinant protein was formulated as an oil-based vaccine candidate and BALB/c mice were immunized with this vaccine formulation. In two independent sets of mice challenge-protective potency experiments for each pathogen, %100 and %50 protection was demonstrated against *M. haemolytica* A1 and *P. multocida* A:3, respectively. Additionally, *lktA* fragment was cloned from the genomic DNA of *M. haemolytica* A1 at the upstream of *plpEC-ompH* fusion and the resulting *lktA-plpEC-ompH* triple fusion was constructed. *In vitro* expression of this fusion was shown in *E.coli* BL21 (D3) cells and the purified protein was used for vaccine preparation. The vaccine candidate comprising LtkA-PlpEC-OmpH was formulated with a mineral oil-based adjuvant and the sera collected from the immunized mice were used for the determination of specific antibody and serum IFN- γ titers. Although the antigen-specific IgG1 and IgG2a levels were higher in immunized group, there was no significant difference between control and immunized mice groups in terms of IFN- γ levels. Protective capacity (potency) of this vaccine candidate was also evaluated via mice challenge experiments with lethal doses of *P. multocida* A:3 and *M. haemolytica* A1 separately, which did not give consistent results in two independent sets of experiments for each pathogen.

Keywords: *Pasteurella multocida* A:3, *Mannheimia haemolytica* A1, LtkA, PlpE, OmpH, recombinant fusion vaccines

ÖZ

MANHEIMIA HAEMOLITICA A1'E AİT LKTA İLE PASTEURELLA MULTOCIDA A:3'E AİT PLPEC VE OMPH'DEN OLUŞAN REKOMBİNANT AŞI ADAYLARININ GELİŞTİRİLMESİ

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Mannheimia haemolytica A1 ve *Pasteurella multocida* A:3 sığırlarda solunum yolu enfeksiyonu (BRD) ve nakil humması gibi hastalıkların etmeni olan gram-negatif bakteriyel patojenlerdir. *M. haemolytica* A1'in akciğerlerde kolonizasyonunu ve enfeksiyon geliştirmesini sağlayan virülans faktörlerinin en önemlisi, lökotosin (Lkt) olup diğer virülans faktörleri patojenin konakçıda tutunmasının yanısıra hem doğuştan, hem de kazanılmış bağışıklık sistemlerinden kaçmasını da sağlayan adezin, kapsül, dış zar proteinleri ve çeşitli proteazları içermektedir. *P. multocida* A:3'ün temel virülans faktörleri arasında ise kapsül, lipopolisakkarit (LPS), toksin (PMT), başta PlpE ve OmpH olmak üzere dış zar proteinleri (OMPs), adezinler ve tip IV fimbria (pili) bulunmaktadır. Sığırlarda görülen solunum yolu hastalığı küresel sığır ve besicilik endüstrilerinde dikkate değer bir ekonomik önem arz etmektedir. Tanı ve tedavi sistemleri arasındaki tutarsızlıklar nedeni ile hastalığın etkin kontrolü oldukça zordur. Sığır endüstrisinde antibiyotiklerin profilaktik ya da terapötik açıdan yoğun bir biçimde kullanılması nedeniyle antibiyotiklere karşı direnç gelişmektedir. Bu nedenle söz

konusu solunum yolu enfeksiyonuna karşı aşı geliştirilmesi ve korunma sağlanması yönündeki stratejiler sığır endüstrisi açısından büyük bir öneme sahiptir. *P. multocida* A:3 lipoprotein E (PlpE), dış zar protein H (OmpH) ve *M. haemolytica* A1 lökotoksini (LtkA), bu patojenlere karşı laboratuvarımızda gerçekleştirilen aşı geliştirme çalışmaları için seçilmiş antijenlerdir. PlpEC-OmpH ve OmpH-Lkt füzyon proteinleri daha önceki çalışmalarda oluşturulmuş, ekspres edilmiş ve saflaştırılmıştır. PlpEC-OmpH, mineral yağ bazlı-CPG adjuvanı ile birlikte kullanıldığında bu aşı aday formülasyonunun BALB/c farelerde *P. multocida* A:3'e karşı %100 koruma sağladığı gösterilmiştir. Şimdiki çalışmada, rekombinant OmpH-LktA proteini yağ bazlı adjuvan temelli bir aşı aday olarak formüle edilmiş ve BALB/c fareler bu formülasyonla immunize edilmişlerdir. Her bir patojen için ikişer bağımsız set fare bağımsızlık gücü deneyleri, bu formülasyonun *M. Haemolytica* A1 ve *P. multocida* A:3'e karşı koruyuculuğunun sırasıyla %100 ve %50 olduğunu göstermiştir. İlk kez bu çalışmada, *M. haemolytica* A1 *lktA* fragmenti genomik DNA'dan amplifiye edilmiş ve *plpEC-ompH* füzyonunun üst kısmına klonlanarak *lktA-PlpEC-OmpH* üçlü füzyonu oluşturulmuştur. Bu füzyonun *in vitro* ekspresyonu *E.coli* BL21 (D3) hücrelerinde gerçekleştirilmiş ve saflaştırılan rekombinant protein LktA-PlpEC-OmpH mineral yağ bazlı adjuvan ile formüle edilerek farelere uygulanmıştır. İmmünize edilen farelerden toplanan serumlar, özgül antikor ve serum IFN- γ titrelerini belirlemede kullanılmıştır. Antijene özgül IgG1 ve IgG2a seviyeleri immunize edilmiş fare grubunda daha yüksek bulunurken, IFN- γ seviyeleri kontrol grubu farelere kıyasla istatistiksel olarak anlamlı bir artış göstermemiştir. Bu aşı adayının koruyucu kapasite testleri, *P. multocida* A:3 and *M. haemolytica* A1 için ayrı ikişer bağımsız deney seti halinde yürütülmüş, ancak bu setlerde uyumlu sonuçlar elde edilememiştir.

Anahtar kelimeler: *Pasteurella multocida* A:3, *Mannheimia haemolytica* A1, LktA, PlpE, OmpH, rekombinant füzyon aşılarda

To Me and my "Seagull"

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TABLE OF CONTENTS

ABSTRACT.....	v
ÖZ.....	vii
ACKNOWLEDGEMENTS.....	x
TABLE OF CONTENTS.....	xi
LIST OF TABLES.....	xiii
LIST OF FIGURES.....	xiv
LIST OF ABBREVIATIONS.....	xv
CHAPTERS	
1.INTRODUCTION.....	1
1.1.Bovine Respiratory Disease.....	1
1.2.Pathogenesis and clinical symptoms.....	2
1.3.Diagnosis and Treatment.....	5
1.4.Physical characteristic of <i>P.multocida</i> A:3 and <i>M.haemolytica</i> A1.....	6
1.5.Phylogeny and classification.....	7
1.5.1.Phylogeny and classification of <i>P. multocida</i>	7
1.5.2.Phylogeny and classification of <i>M. haemolytica</i>	9
1.6.Virulence determinants.....	10
1.6.1.Virulence determinants of <i>P. multocida</i>	10
1.6.1.1.Outer membrane proteins.....	10
1.6.1.2. Capsule and adhesins.....	12
1.6.1.3.Lipopolysaccharides (LPS).....	12
1.6.2.Virulence determinants of <i>M. haemolytica</i>	13
1.6.2.1.Toxins and extracellular enzymes.....	13
1.6.2.2.Surface proteins and carbohydrates.....	15
1.7.Vaccine studies.....	16
1.8.Aim of the present study.....	20
2.MATERIALS AND METHODS.....	21
2.1.Bacterial strains and plasmids.....	21
2.2.Culture media.....	22
2.3.Buffers and solutions.....	22
2.4.Chemicals and enzymes.....	22
2.5.Maintenance of bacterial strains.....	22
2.6.Primer design.....	22
2.7.PCR.....	23
2.8.Agarose gel electrophoresis.....	24
2.9.Sequencing reactions.....	24
2.10.Ligation reactions.....	24
2.11.Competent cell preparation and transformation of <i>E.coli</i> cells.....	25

2.12. Plasmid isolation.....	25
2.13. Restriction enzyme digestion.....	26
2.14. Alkaline phosphatase treatment.....	26
2.15. Construction of recombinant plasmids.....	26
2.16. Protein overexpression, purification and dialysis.....	27
2.17. Determination of protein concentration.....	28
2.18. SDS-Page and Comassie Brilliant Blue staining.....	28
2.19. Western blotting.....	29
2.20. Mice experiments.....	30
2.21. IgG ELISA.....	31
2.22. Detection of serum IFN γ levels.....	31
2.23. Statistical analyses.....	32
3. RESULTS AND DISCUSSION.....	33
3.1. Cloning of <i>lktA</i> gene fragment from <i>M. haemolytica</i> into PlpEC-OmpH fusion in <i>E. coli</i>	33
3.1.1. PCR amplification and cloning of <i>lktA</i> fragment into pGEM-T Easy Vector.....	33
3.1.2. Sequence analysis of <i>lktA</i> gene from <i>M. haemolytica</i>	34
3.1.3. Transformation of <i>E. coli</i> BL21 with pET-28a containing <i>lktA</i> gene.....	36
3.1.4. Cloning of <i>lktA</i> into plpEC-ompH fusion in <i>E. coli</i>	37
3.2. Expression of recombinant LktA -PlpEC-OmpH fusion protein in <i>E. coli</i> BL21 (D3).....	38
3.3. Purification of recombinant LktA-OmpH-PlpEC fusion protein by His-Tag affinity chromatography.....	39
3.4. Western blot analysis of recombinant LktA-OmpH-PlpEC fusion protein.....	40
3.5. Mice challenge experiments.....	41
3.5.1. Serum antibody level against recombinant LktA-OmpH-PlpEC fusion protein.....	42
3.5.2. IFN γ in mice vaccinated with recombinant LktA-PlpEC-OmpH fusion protein.....	45
4. CONCLUSION.....	47
5. REFERENCES.....	49
APPENDICES.....	59

LIST OF TABLES

TABLES

Table 1.1 Causative agents of BRD (Snowder et al., 2006).....	2
Table 1.2 Biochemical properties of <i>P. multocida</i> (Namioka, 1978).....	7
Table 1.3 Currently recognized taxa in the genus <i>Pasteurella</i> , host predilection and diseases (Boyce et al., 2010).....	8
Table 1.4 History of the nomenclature of <i>P. multocida</i> (Namioka, 1978).....	9
Table 1.5 Taxonomic classification of both <i>M. haemolytica</i> and <i>P. multocida</i>	10
Table 2.1 Strains used in this study, their characteristics and sources.....	21
Table 2.2 Plasmids used in this study.....	21
Table 2.3 Primers used for amplification of <i>LktA</i> fragment by PCR.....	23
Table 2.4 PCR conditions for amplified genes.....	23
Table 2.5 PCR mixture content.....	23
Table 2.6 Ligation of insert DNA into pGEM-T Easy Vector.....	24
Table 2.7 Ligation of insert DNA into pET-28a Vector.....	25
Table 2.8 Preparation of SDS-polyacrylamide gels.....	29
Table 2.9 Vaccination methodology for <i>P. multocida</i> A:3 and <i>M. haemolytica</i> A1	31

LIST OF FIGURES

FIGURES

Figure 1.1 Scheme representing immune evasions by pathogens of the BRD complex (Srikumaran et al., 2007).....	4
Figure 1.2 Outer membrane proteins of <i>P. multocida</i> (Hatfaludi, 2010).....	12
Figure 1.3 <i>M. haemolytica</i> LktA and its functional domains (Jeyaseelan, 2002). 15	
Figure 2.1 Calibration curve for determination of protein concentrations.....	29
Figure 2.2 Schematic representation of transfer set-up in Western blot.....	30
Figure 3.1 PCR of lktA fragment. Amplification of lktA fragment (lane 1 and 2), Negative control (lane 3), GeneRuler 100bp DNA Ladder (lane 4).....	35
Figure 3.2 Verification of cloning of lktA fragment into pGEM-T. λ DNA/PstI marker (lane 1), pGEM-T vector and lktA fragment (lane 2).....	35
Figure 3.3 (A) Nucleotide, (B) Aminoacid sequence of lktA. Bold characters....	36
Figure 3.4 Computer-based nucleotide BLAST analysis of lktA.....	37
Figure A.1 pGEM®-T Easy Cloning Vector (Promega #A1360).....	60
Figure A.2 pET-28a(+) His-tag Expression Vector (Novagen #69864-3).....	61
Figure A.3 PageRuler™ Plus Prestained Protein Ladder (Fermentas #SM1811) (A) and Unstained Protein Molecular Weight Marker (Fermentas #SM0431)(B).	61
Figure A.4 Lambda DNA/PstI Marker (Fermentas #SM0361).....	62
Figure A.5 100 bp DNA Ladder (GeneRuler # SM0241).....	62

LIST OF ABBREVIATIONS

OmpH	Outer membrane protein H
PlpE	<i>Pasteurella</i> lipoprotein E
PlpEN	Amino terminal of PlpE
PlpEC	Carboxyl terminal of PlpE
bp(s)	Base pair(s)
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

Bovine respiratory disease (BRD) or shipping fever is commonly known as feedlot cattle disease and majorly resulted with destroyed animal life bringing about economic losses for the feedlot dependent industries (Guzmán-Brambila *et al.*, 2012). However, in spite of the numerous researches on the disease since late 1800s, understanding of precisely how and why it happens is uncertain. BRD seems to be cooperation of more than one infectious agents, host immunity and unfavorable ecological factors. Table 1.1 shows the viruses isolated from cattle with BRD, bacterial pathogens connected to the disease and other environmental factors (Bowland, 2000, Taylor, 2010).

Mannheimia haemolytica and *Pasteurella multocida*, are often secondary bacterial pathogens contributing to arousal of BRD not only in bovine animals but also in several fowl species. Although, both *M. haemolytica* A1 and *P. multocida* A:3 normally reside in upper respiratory tract of domestic animals but not in lower tract they act as opportunist pathogens when the lung is made susceptible against them by viral infections and environmental factors or when microorganisms belong to normal flora of upper respiratory tract find way to avoid phagocytic clearance (Muggli-Cockett, 1992). These are appears to ruin the respiratory mucosa or suppress cattle's immunity, either immediatelly or by means of the effects of endogenous agents such as cortisol, making the calf more vulnerable to opportunistic bacteria (Taylor, 2010, Ahmad *et al.*, 2014).

Table 1.1 Causative agents of BRD (Snowder *et al.*, 2006)

Stress Factors	Viral agents	Bacteria
Heat	PI3	<i>Pasteurella</i>
Cold	IBV	<i>Mannheimia</i>
Dust	BVD	<i>Haemophilus</i>
Dumpiness	BRSV	Other
Injury	Adenovirus	
Fatigue	Rhinovirus	
Dehydration	Herpes virus IV	
Hunger	Enterovirus	
Anxiety	MCF	
Irritant gases	Reovirus	
Nutritional deficiencies		
Surgery		
Climate		
Humidity		
Ventilation		
Shipping distance		
Stocking density		

1.2. Pathogenesis and clinical symptoms

The vertebrate defense system has an advanced system to deal with the multitude of pathogens that are causative agents of infectious diseases. In the meantime, a few pathogens have developed complicated methods as well to avoid the host immunity. The infectious agents of BRD complex are no exemption to this case. In spite of the fact that invulnerable avoidance methods created by viral and bacterial pathogens are diverse, reasonable likenesses do exist. (Srikumaran, 2008).

Fundamentally, immune responses against pathogens are grouped in two; innate and adaptive immunity. In short, macrophages, dendritic cells (DCs), natural killer

(NK) cells and neutrophils are essential cell constituents of innate immune system. Complement system and cytokines are other vital components of innate immunity. On the other hand, B and T lymphocytes are urgent elements of adaptive immune system. Foreign components entered the body can be perceived by antigen presenting cells (B cells, macrophages and DCs) and presented to T cells via major histocompatibility (MHC) complexes. T cells recognize the epitope region of antigens and produce cytokines particular to that epitope. MHC complexes are main components of antigen presentation. T helper (T_h) cells express CD4 glycoprotein on their surfaces and they are activated with MHC class II molecule whereas, Cytotoxic T (T_c) cells are CD8 positive and induced by MHC class I complex. Thus, innate and adaptive systems collaborate while triggering immune responses (Vivier *et al.*, 2011).

Interferon- γ (IFN- γ) is basic for innate and adaptive immunity against viral and intracellular bacterial evasions. The magnitude of IFN- γ is a result of its capacity to improve both MHC class I and class II antigen presentation by expanding expression of subunits of MHC class I and II molecules, either directly or indirectly. IFN- γ is primarily produced by natural killer (NK) cells and natural killer T (NKT) cells, which are effectors of the innate immune response and CD8 and CD4 $Th1$ effector T cells of the adaptive immune system. It is necessary to express that $Th1$ effectors provide cellular immunity against viral and intracellular bacterial infections, conversely, $Th2$ effectors create immune response against extracellular infections (Schoenborn and Wilson, 2007).

Upon activation through antigen-presenting cells the effector T_h cells can be separated into three unique groups as indicated by their cytokine-secretion properties. These groups are alluded as T_h type 1, T_h type 2 and T_h type 17. T_h1 cells secrete cytokines, IFN γ and tumor necrosis factor β (TNF β). These cytokines permit these cells to be effective particularly against intracellular infection of viruses and bacteria. T_h2 cells activates antibody producing B cells by secretion of interleukin (IL)4, 5, 10, 13, T_h17 cells secrete IL-17, 6, 22, 17F, and TNF α and takes role in induction of neutrophils against extracellular

microorganisms furthermore has part in tissue inflammation (Kaiko *et al.*, 2008).

Immunoglobulin isotype profile is greatly influenced by the proportion of T_h1/T_h2 responses. It has been demonstrated that IL-4 dominant T_h2 response comes about a higher level of IgG1/IgG2a though IL-12 and IFN γ mediated T_h1 response has higher IgG2a/IgG1 ratio. As per this information, serum IgG1 levels are being utilized as a marker of T_h2 sort of response and IgG2a levels of the sera is utilized to focus T_h1 kind of immune response (Holdsworth *et al.*, 1999).

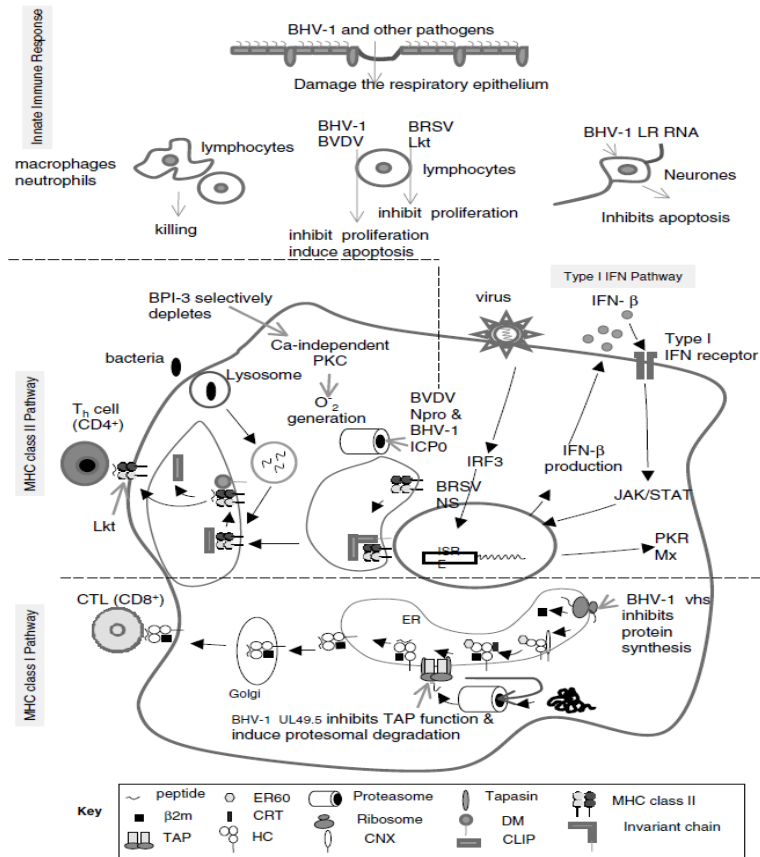


Figure 1.1 Scheme representing immune evasions by pathogens of the BRD complex (Srikumaran *et al.*, 2007).

As Figure 1.1 represents, pathogens of BRD complex have improved a few immunosuppression methods. The system improved by one microorganism not just offer assistance that microorganism, additionally the others, resulting in increase in the severity of the disease (Srikumaran *et al.*, 2007).

It is evident that in spite of all the immune protection mechanisms not all but most of the animals experiencing BRD display various symptoms in the course of the disease. While slight depression, reduced appetite and fever are prior clinical signs as the disease gets severe animal begins to decline eating, nasal and ocular discharges get to be yellowish, thicker and more viscous rather than thin and almost transparent. Unless early indications are noticed and animal does not receive a treatment instantly, disease gets severe, breathing hardens, fever drops under typical body temperature, lungs are permanently demolished owing to the colonization of invasive pathogens and the animal possibly dies (Apley, 2006).

1.3. Diagnosis and Treatment

In order to totally treat the animals suffering from BRD and assess the outcomes of treatment to be able to improve preventative researches accurate diagnosis of disease is essential. There are many factors contributing to the disease process, and, often, the diagnosis depends on combination of clinical signs, rather than analysis of single symptom or pathogenic agent. BRD affects the growth performance of animal, carcass quality and fertility of the influenced cattle; therefore, precise diagnosis and subsequent treatment is vital to decrease negative impacts (White and Renter, 2009).

As mentioned before, a clinical BRD diagnosis relies on observation of clinical illness at the beginning, however, final treatment decision often combines clinical signs with rectal temperature (White and Renter, 2009). During diagnosis, beginning with evaluation of diseased organ following with microscopic analysis of lesions and determination of the etiologic agents upgrade the treatment efficacy (Fulton and Confer, 2012).

Because of the fact that animals having BRD shows distinct clinical signs, medications against the disease are mostly symptomatic. Lung lesions at the further stages of disease hinder the gas exchange and impair breathing. It was declared that NSAIDs ([Non-steroidal anti-inflammatory drug](#)) impede both the

production and effects of inflammatory mediators which are damaging for alveolar gas exchange (Elitok and Elitok, 2004).

1.4. Physical characteristic of *P.multocida* A:3 and *M.haemolytica* A1

P. multocida A:3 is gram negative, non-motile, capsule containing cocobacillary and facultative anaerobic bacterium and notorious causative factor of BRD, fowl cholera, pneumonia and hemorrhagic septicemia in cattle, sheep and goats and swine atrophic rhinitis (Chung *et al.*, 2005; Ahmad *et al.*, 2014). *M. haemolytica* shares the same cultural characteristics with *P. multocida* that is other important agent causing BRD. A few analyses depict that many of the *P. multocida* strains exhibit the same biochemical characteristics (Table 1.5). Biochemical analyses of 27 separate strains of *P. multocida* in terms of catalase activity, indole production, fermentation capabilities against different sugar sources and no hemolytic activity on blood agar unlike weakly hemolytic *M. haemolytica* supported the claims that very nearly all *P. multocida* species are comparable biochemically (Namioka, 1978).

Mannitol, glucose, maltose, sorbitol and sucrose are used for fermentation by all strains of *M. haemolytica* without gas production. Some biochemical reactions such as indole, urease, methyl blue (MB) and Voges-Proskauer (VP) reactions are negative and some are such as catalase (almost always) and oxidase are positive (Smith and Phillips, 1990). Regularly, *M. haemolytica* strains do not use trehalose for fermentation but they use L-arabinose for fermentation. The difference between *Mannheimia* and genus *Pasteurella* is non-producing acid from D-mannose (Angen *et al.*, 1999).

Table 1.2 Biochemical properties of *P. multocida* (Namioka, 1978)

Gelatin hydrolysis	—
Indole production*	+
H ₂ S production†	+
Nitrate reduction	+
VP reaction	—
MR reaction	—
Growth on KCN	+
ONPG‡	d‡
Arginine decarboxylase	—
Lysine decarboxylase	—
Ornithine decarboxylase	+
Citrate utilization	—
Gluconate oxidation	—
Malonate utilization	—
Urease production	—
Catalase	+
Kovac's oxidase	+
Haemolysis	—
Sensitivity to Penicillin	+++

* SIM medium was used.

† Lead acetate paper.

‡ Different reactions by different strains.

§ *O*-nitrophenyl- β -D-galactosidase.

1.5. Phylogeny and classification

1.5.1. Phylogeny and classification of *P. multocida*

In 1952, Carter created the first method to group *P. multocida* species. The method includes a passive agglutination test that can be carried out as either serum-plate agglutination or indirect/passive agglutination or combination of these two agglutination tests. Ordinarily, passive hemagglutination test relies on the utilization of erythrocytes synthesized with capsular antigens that separate *P. multocida* isolates into five groups (A,B,D,E and F) (Adler *et al.*, 1999; Boyce *et al.*, 2010). Nonetheless, this test is not sufficient for exact classification solely by itself just because capsule-containing species fail to offer capacity to agglutinate unless there is a special treatment. As another problem, when a strain does not harbor capsule, but possessing the antigens needed for hemagglutination, the classification is impossible with this method. Moreover, in the course of agglutination test, undesirable cross reactions can take place (Heddleston *et al.*, 1972). Gel diffusion immunoprecipitation test (GDIT) is the other method which was developed by Heddleston *et al.* in 1972. Passive diffusion of antigens toward

each other bringing about precipitation of them in gel matrix is the guideline of GDIT. This serological test takes lipopolysaccharide antigens into consideration and identifies 16 serotypes based on expressed LPS antigens of *P. multocida* species (Boyce *et al.*, 2010; Adler *et al.*, 1999). For instance, *P. multocida* A:3 strain displays type A with respect to its capsule, and type 3 with respect to its LPS antigen. Table 1.3 shows the classification of genus *Pasteurella* and their pathogenicity.

Table 1.3 Currently recognized taxa in the genus *Pasteurella*, host predilection and diseases (Boyce *et al.*, 2010).

Species	Hosts	Association/diseases (common serotypes)
<i>P. multocida</i> subsp. <i>multocida</i> , <i>gallicida</i> , and <i>septic</i>	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
<i>P. dagmatis</i>	Dogs	Normal flora in dogs. Cause a range of zoonotic infections in humans
<i>P. canis</i>	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?
<i>P. stomatis</i>	Cats and dogs	Normal flora in cats and dogs. Cause a range of mostly bite wound-associated infections in humans.
[<i>P.</i> <i>aerogenes</i>]*	Pigs	Sepsis, diarrhea and pneumonia
[<i>P.</i> <i>bettyae</i>]*	Humans	Genitourinary infections
[<i>P.</i> <i>caballi</i>]*	Horses, pigs	Respiratory infections
[<i>P.</i> <i>languaensis</i>]*	Birds	Normal flora of respiratory tract
[<i>P.</i> <i>pneumotropica</i>]*	Cats, dogs, Rodents	Pneumonia and various suppurative infections in rodents
[<i>P.</i> <i>mairii</i>]*	Pigs	Isolated from pig reproductive tract and associated with abortions
[<i>P.</i> <i>skyensis</i>]*	Fish	Fatal infections in Atlantic salmon
[<i>P.</i> <i>testudinis</i>]*	Tortoises	Respiratory disease in tortoises

*These species are currently member of the *Pasteurella* genus yet genomic data states that they are not belong to *Pasteurella sensu stricto* group.

1.5.2. Phylogeny and classification of *M. haemolytica*

Bacterium bipolare multocidum was the first name of *Mannheimia (Pasteurella) haemolytica* (Kitt, 1885) then renamed as *P. haemolytica* in 1932. Initially, there was simply two subclasses A and T for *P. haemolytica*. In 1990, T serotype was categorized as *Pasteurella trehalosi* and also A serotypes were renamed (A1, A2, A5, A6, A7, A8, A9, A12, A13, A14, A16, A17) relying on the DNA-DNA hybridization and 16SRNA sequencing as *M. haemolytica* solely A11 serotype was called as *M. glucosida*. In order to tribute Walter Mannheim, a German biologist whose research intended to clarify and improve the taxonomy of the *Pasteurellaceae* family. The taxonomic classification of both *M. haemolytica* and *P. multocida* are given in Table 1.4 (Namioka, 1978).

Table 1.4 History of the nomenclature of *P. multocida* (Namioka, 1978)

Name of scientist	Year	Name	Literature cited
Bollinger	1879	—	Microparasiten bei eine neue Wild und Rinderseuche, München, 1879.
Pasteur	1880	—	<i>C.R. Acad. Sci.</i> , Paris 90 , 230, 952, 1030, 1880.
Burril	1883	<i>Micrococcus gallicidus</i>	<i>Amer. Naturalist</i> 17 , 320, 1883; <i>J. Roy. Micro. Soc.</i> , London 3 , 339, 1883.
Zopf	1885	<i>Micrococcus cholerae-gallinarum</i>	<i>Die Spaltpilze</i> 3 , Aufl. 57, 1885.
Kitt	1885	<i>Bacterium bipolare multocidium</i>	<i>Sitz. Gesell. Morph. u. physiol.</i> , München 1 , 24, 1885.
Trevisan	1887	<i>Pasteurella cholerae-gallinarum</i>	<i>Rendiconti Reale Istituto Lombardo di Scienze e Lettere</i> 94 , 1887.
Lehmann & Neumann Sternberg	1889	<i>Bacterium multocidum</i>	<i>Bact. Diag.</i> , 2. Aufl. 2 , 196, 1889.
	1893	<i>Bacterium septicaemiae haemorrhagicae</i>	<i>Man. of Bact.</i> 408 , 1893.
Lignières	1900	Zoological classification: <i>Pasteurella avisepctica</i> <i>Pasteurella bovisepctica</i> <i>Pasteurella oviseptica</i> etc.	<i>Ann. Inst. Pasteur</i> 15 , 734–736, 1900.
Topley & Wilson	1931	<i>Pasteurella septica</i>	<i>Princip. Bact. Immunol.</i> , 1st ed. 1 , 488, 1931.
Rosenbusch & Marchant	1939	<i>Pasteurella multocida</i>	<i>J. Bact.</i> 37 , 85, 1939.
Bergey's Manual	1948–	<i>Pasteurella multocida</i>	Since 6th Edn, 1948.

Table 1.5 Taxonomic classification of both *M. haemolytica* and *P. multocida*

Kingdom	Bacteria
Phylum	<i>Proteobacteria</i>
Class	<i>Gammaproteobacteria</i>
Order	<i>Pasteurellales</i>
Family	<i>Pasteurellaceae</i>
Genus	<i>Pasteurella</i>

1.6. Virulence determinants

1.6.1. Virulence determinants of *P. multocida*

1.6.1.1. Outer membrane proteins

It is known that lipopolysaccharides, several minor proteins and some limited number of major proteins are present in the outer membrane of gram-negative bacteria in very high copy numbers. Bacterial porins which are the primary outer membrane proteins serve as channels for transportation of hydrophilic low-molecular-weight substances. Moreover, porins can make epitopes visible on the bacterial surface because they have high immunogenicity as well as antigenicity. Those bacterial porins share strong taxonomic associations that have high homology in both primary amino acid sequence and secondary structure so they are most commonly conserved in bacterial families and in bacterial species (Jeanteur *et al.*, 1991; Bording *et al.*, 1994). For heterologous immunity against Gram-negative bacterial infections, bacterial porins may be preferable vaccine candidates because of mentioned properties (Tabaraie *et al.*, 1994; Rimler, 1996).

In the surrounding of *P. multocida*, Porin H is the primary outer membrane protein and for the several serotypes of *P. multocida*, Porin H is conserved (Chevalier *et*

al., 1993). Molecular weight of denatured monomers of Porin H varies from 34 to 42 kDa which exists as homotrimer in the outer membrane (Chevalier *et al.*, 1993; Lubke *et al.*, 1994). Outer membrane protein H gene (*ompH*) can be diverse for the serotypes of *P. multocida* therefore this gene have variable regions functioning as specific epitopes because of mentioned variations in length and sequence.

Rimler (2001) has reported that 39 kDa *Pasteurella* lipoprotein B (PlpB) displayed antigenic property and showed cross-protection (Tabatabai and Zehr, 2004). Nevertheless, it was noticed via proteome analysis that the cross-protective antigen in fact was PlpE. Blast analysis declared that primary amino acid sequences were 90-100% identical among different strains of *P. multocida*. On the other hand, solely 24% identity was observed once *M. haemolytica* PlpE and *P. multocida* PlpE were compared (Wu *et al.*, 2007). Hatfaludi (2010) stated that PlpE belonging to *P. multocida* was the first recombinant protein which triggers cross-protection against *P. multocida* serotypes.

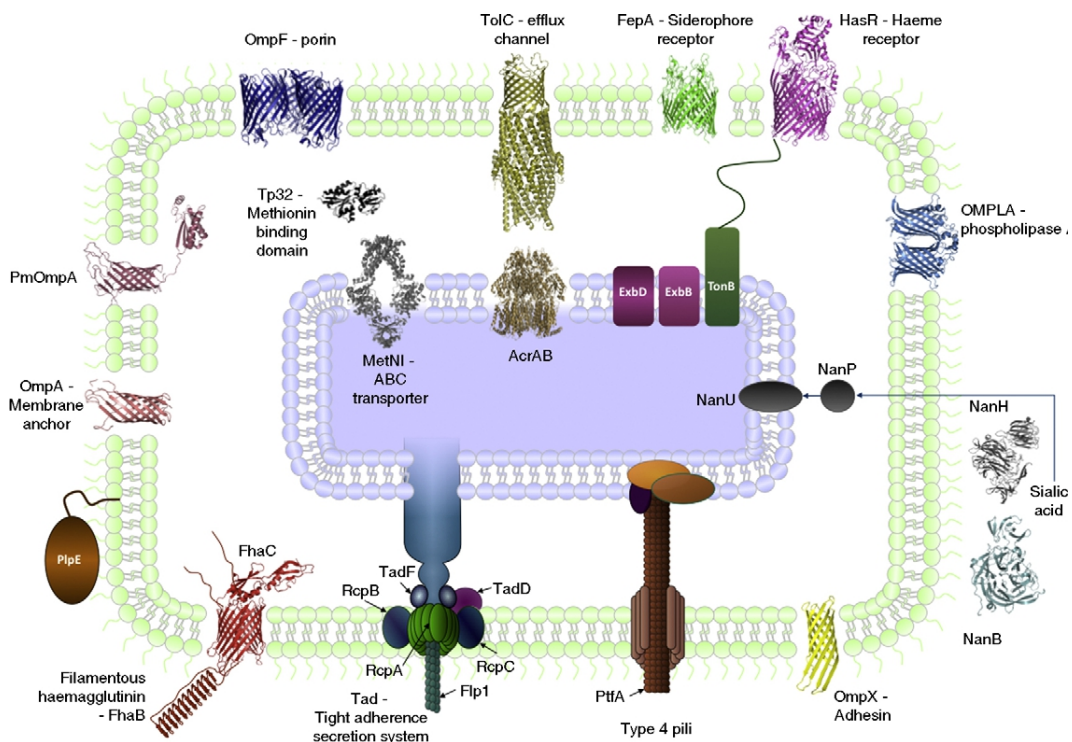


Figure 1.2 Outer membrane proteins of *P. multocida* (Hatfaludi, 2010)

1.6.1.2. Capsule and adhesins

P. multocida can be divided into five groups which are named as A, B, C, D, E and F depending on the capsular properties. Capsular material present in *P. multocida* serotypes A, D and F are very similar according to their structural organization and capsule content besides, strains of *P. multocida* that have a capsule are more pathogenic than their acapsular serotypes (Heddleston *et al.*, 1964; Snipes *et al.*, 1987. *P. multocida* serogroup A and B strains were mutated to obtain non-capsulated strains and attenuated mutants displayed the genetically essential role of capsule in the pathogenesis of *P. multocida* (Harper, 2006; Chung *et al.*, 2001). Non- capsule containing mutant of serogroup A was shown to be non-pathogenic for chickens and was fail to grow in chicken muscle (Chung *et al.*, 2001). It was unexpected that protection can be achieved by immunization of chickens with high doses of this non-capsular mutant in spite of its obvious lack of persistence (Chung *et al.*, 2005). According to in vitro results of Harmon *et al.* (1991), it was stated that capsule provides crucial resistance against phagocytosis. There are more supporting reports that existence and capsule thickness are related with phagocytosis sensitivity (Harper, 2006).

As another outer membrane protein fimbria on the surface of some *P. multocida* serotype A strains were capable of attaching to mucosal epithelium, and the strains that were unable to adhere lacked fimbriae. It was suggested that fimbria serves as an important agent in surface adhesion (Dabo, 2008). Nevertheless, whether fimbria provides virulence in *P. multocida* or not is still unknown (Harper, 2006)

1.6.1.3. Lipopolysaccharides (LPS)

LPS is major constituents of the gram (-) outer membrane and are essential for the organization and functioning of this important structure. As mentioned before, *P. multocida* serogroup classification relies on the LPS antigens. *P. multocida* expresses LPS molecules that do not have the polymeric O-antigen, so-called rough LPS (Rimler, 1990). When host is infected by gram negative

microorganism, the availability of LPS is critical to trigger the innate immunity by which the immune response takes a crucial role to get rid of pathogenic agent and prevent infection as mentioned before. This prior response might be triggered by lipid A which is the core component of LPS layer. Unless immune response remains insufficient to get rid of the pathogen and infection proceeds accumulated LPS can possibly cause sepsis in which large amounts of inflammatory mediators result in tissue damage, organ collapse and finally death (Harper, 2004). Activities of LPS also include connection with other outer membrane components to form a barrier against deleterious substances (Conrad, 1996). Further studies displayed that *P. multocida* LPS has role in attachment to neutrophils, by this way makes transmigration possible throughout the endothelial cells (Galdiero, 2000). In addition to the role of the capsule, LPS also takes an essential role in virulence, such that mutants having truncated LPS are generally attenuated (Harper *et al.*, 2007).

1.6.2. Virulence determinants of *M. haemolytica*

1.6.2.1. Toxins and extracellular enzymes

Lkt is a 102-105 kDa exotoxin of *M. haemolytica* and it is secreted in all serotypes of the bacterium all through the exponential phase of the in vitro growth (Shewen *et al.*, 1985). It is prior pathogenesis factor of *M. haemolytica* and it causes breakdown of all leukocytes (Dassanayake, 2008). Since nowadays after Lkt discovery numerous researches have done, centered around Lkt itself and its function in pathogenesis of BRD. Lkt belongs to RTX (Repeats in Toxin) family in which C-terminal parts of proteins contains several glycine and aspartate aminoacids. These glycine and aspartate rich motifs are highly conserved and they are essential in stimulation of toxicity.

The *lkt* gene is composed of four gene fragments as *lktC* and *lktA* which are at the upstream of *lktB* and *lktD* gene fragments (Highlander *et al.*, 1989). During translation, Lkt-prototoxin (pro-LktA), which is biologically inactive in this form, is

firstly produced via expression of *lktA*, however, after posttranslational modifications including fatty acid acetylation pro-LktA is converted to active LktA protein. Transacylase that is responsible for fatty acid acetylation is encoded by *lktC* gene fragment. Additionally, LktB and LktD proteins are necessary for the transformation of LktA through membrane from cytoplasm.

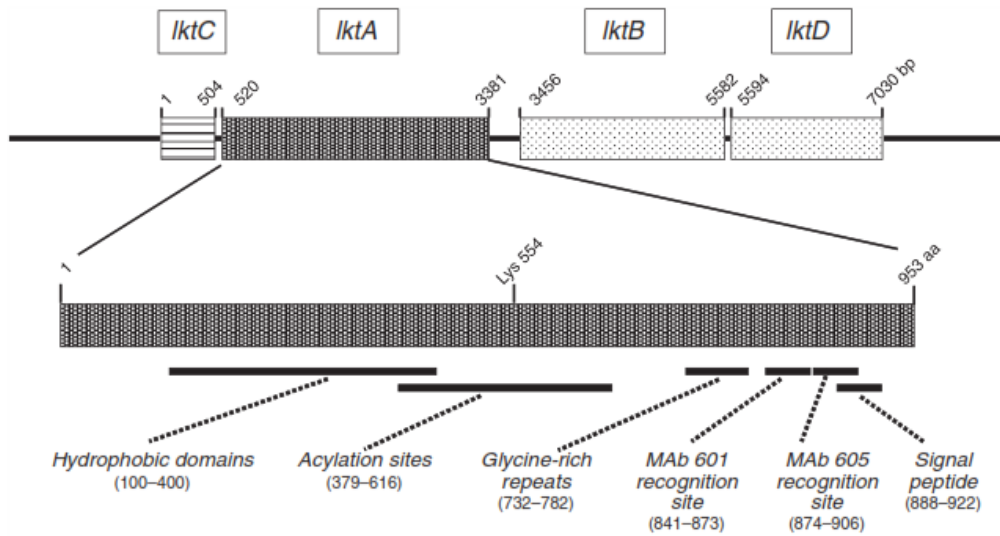


Figure 1.3 *M. haemolytica* LktA and its functional domains (Jeyaseelan, 2002)

LktA contains receptor binding, pore formation and calcium binding domains (Cruz *et al.*, 1990, Sun *et al.*, 1999). Essentially, the N-terminal is involved in receptor binding and apparently a series of hydrophobic residues take role in pore formation (Forestier *et al.*, 1991). 229 amino acid regions in C-terminal of LktA carries most of the epitope sequences (Highlander *et al.*, 1989).

Concentration of Lkt released into the host system is essential in terms of the immune system activation. Low amount of Lkt induces both neutrophils and macrophages, which results in cytokine (TNF α , IL-1 and IL-8) burst and histamine production by mast cells. (Maheswaran *et al.*, 1992, Hsuan *et al.*, 1999). On the other hand, high concentration of Lkt in host system leads to apoptosis and subsequently necrosis of cells by creating hydrophilic pores on their membranes via insertion of its N-terminal sequence into the membrane (Clinkenbeard *et al.*,

1989, Atapattu *et al.*, 2005).

After the essence of Lkt for virulence of *M. haemolytica* was noticed, *M. haemolytica* A1 supernatant has started to be used for vaccination against pasteurellosis (Rice, 2008).

1.6.2.2. Surface proteins and carbohydrates

For the development of BRD and during the progress of the disease, attachment of pathogenic agents on epithelial surface and proliferation of them are essential prerequisite of the disease. De la Mora *et al.*, (2006) displayed that adhesin protein which is 68 kDa via in vitro cell culturing taken from the nasopharynx of infected host. Besides, neutrophils bear glycoprotein receptor to which *M. haemolytica* adhesin proteins can bind. This binding stimulates neutrophils, which leads to oxidative stress and burst of the cells (De la Mora *et al.*, 2006). Retzer *et al.*, (1998) stated that, *M. haemolytica* outer membrane proteins functioning in iron obtaining (TbpA and TbpB) are probably function also as an adhesion molecule.

M. haemolytica LPS is another pathogenic factor for BRD. Most recent investigations displays that LPS possesses several mechanisms including induction of leukocytes to make them produce cytokines, complement activation and stimulation of cell lysis (Lafleur, 2001, Malazdrewich, 2001). Previous and preliminary studies suggest that injection of LPS intravenously leads to hypotensive shock (Adlam, 1989). Additionally, chimeric mixture of Lkt and LPS cooperates synergetically to increase the pathogenicity of each other. (Li, 1999, Lafleur, 2001). Furthermore, subsequent challenge of alveolar macrophages derived from cattle with both Lkt and LPS brings about high amount of TNF α and IL-8 secretion than cells challenged with Lkt and LPS one by one (Lafleur, 2001).

As mentioned in *P. multocida* virulence factors, capsule is essential for *M. haemolytica* as well. 12 serotypes (S1, S2, S5-S9, S12-14, S16 and S17) were classified depending on the differences of capsular polysaccharide antigen in *M.*

haemolytica. Good encapsulation was observed in exponential phase of bacteria whereas poor encapsulation was observed in stationary phase bacteria. (Corstvet, 1982).

As another virulence factors *M. haemolytica* OMPs are generally grouped in iron-regulated outer membrane proteins (IROMPs). This classification is reasonable since these proteins function in iron acquisition (Ogunnariwo, 1997). Furthermore, Iovane *et al.*, (1998) displayed that OMPs can attract neutrophils via chemotaxis and impairs their phagocytosis capability by this way pathogenic agent can easily colonize in upper respiratory tract.

45 kDa PlpE is one of the OMP of *M. haemolytica* S1 which is immunogenic in bovine species. Apart from *M. haemolytica* S11, all of the *M. haemolytica* species display antibody recognition against PlpE protein. Complement mediated killing assays showed that there is a noteworthy decrease in clearance of *M. haemolytica* unless sera collected from cattle includes PlpE antibody and additionally the study stated the crucial contribution of anti-PlpE antibodies to host immune response (Confer, 2003).

Finally, there are several different proteases which are associated with *M. haemolytica*. A few of them were demonstrated to be included in both innate and adaptive immunities and thus improving lung colonization and occurring of the disease. In defense mechanism against *M. haemolytica*, both IgG1 and IgG2 are believed to be important. IgG1 can be hydrolyzed by a glycoprotease derived from the culture supernatant of *M. haemolytica* hence decrease in opsonization results in induction of phagocytosis and clearance of bacteria. Fyrthermore, protection against the BRD was observed in the case of vaccination of calves with recombinant glycoprotease plus Lkt combination (Lee, 1996).

1.7. Vaccine studies

As this study focused on, outer membrane proteins (OMPs) are generally preferred components for novel vaccine development especially against gram (-)

microorganism (Gatto *et al.*, 2002; Carpenter *et al.*, 2007). Former studies stated that antibody titers after immunization of mice with OMPs of *P. multocida* 6: B was satisfactory as a vaccine candidate (Basagoudanavar *et al.*, 2006). One of the subject component of this study, OmpH, is commonly preferred major antigenic and conserved porin and it is the best all of the bovine species examined express OmpH on their cell surfaces, which places OmpH into major vaccine candidates (Dabo *et al.*, 2008b). Marandi and Mittal (1997) carried out another study in which murine backpack tumor model was developed in order to obtain high levels of IgG MAbs specific for OmpH and OmpA of *P. multocida*. Results indicated that MAbs produced against OmpH inhibited proliferation of *P. multocida* in the lungs of mice and thus provided strong protection.

Another subject of this study PlpE surface-exposed outer membrane protein like OmpH that is important in complement-mediated killing of *M. haemolytica*. In order to describe *M. haemolytica* PlpE Is whether serve as a vaccine antigen or not. As a result, PlpE was seen more likely to create cross-protection. Immunization with a single dose of purified r-PlpE conferred protection on mice against challenge with *P. multocida* strains X-73 (A:1), P-1059 (A:3) and P-1662 (A:4) (Wu *et al.*, 2007).

LPS, OMPs and especially Lkt of *M. haemolytica* are the best known stimulators of inflammation in bovine pneumonic pasteurellosis (Lee *et al.*, 2000). Thus, they are primarily targeted vaccine candidates against BRD. In 1988 Sheewen, stated that subcutaneous vaccination of calves with adjuvanted bacteria-free leukotoxic culture supernatant from log phase cultures of *P. haemolytica* AI was shown to induce some protection against intrabronchial challenge with live *P. haemolytica* (Shewen and Wilkie, 1988). According to one other study vaccination of cattle with partially purified native Lkt stimulated low, yet occasionally significantly elevated, primary antibody responses to formalinized *P. haemolytica* or to OMPs. High neutralizing antibody responses to Lkt correlated with resistance to experimental challenge of cattle vaccinated with live *P. haemolytica* (Confer *et al.*, 1997). In recent studies, SAC86, SAC87, SAC88 and SAC89 chimeric proteins

containing certain regions of PlpE and LKT which are essential immunologically were designed and utilized in vaccine studies. Mice were immunized with different amounts of these proteins. As a result rPlpE and native LKT specific antibody responses were recorded. Also sera from the immunized mice showed complement-mediated bactericidal and LKT-neutralizing activities (Ayalew *et al.*, 2008). Similarly, Confer, *et al.* (2009) improves SAC89 composed of two copies of the immunodominant epitopes of PlpE (R2) and NLKT and this chimeric protein shows significant increment in antibody titers with both SAC89 and SAC89/bacterin composition. Study reveals that addition of chimeric surface antigen/NLKT epitopes to *M. haemolytica* vaccines has the potential to prevent shipping fever.

Based on what is stated by recent studies conducted in our laboratory BALB/c mice were inoculated IP twice with 100 µg/500µl of PlpE, OmpH and PlpEC-OmpH proteins formulated with oil-based and oil-based CpG ODN adjuvants. The sera were collected prior to both booster inoculation and challenge (days 20 and 30, respectively) and used for the measurement of IgG titers and IFN γ levels. Serum IgG levels in mice vaccinated with PlpE and PlpEC-OmpH significantly ($p < 0.05$) increased after first and second immunizations. On the other hand, the increment in IgG level upon injection with OmpH formulated with oil-based or oil-based CpG ODN was only significant after second vaccination and at lower dilutions. Serum IFN- γ levels in mice IP vaccinated with PlpE, OmpH and PlpEC-OmpH formulated with oil-based and oil-based CpG ODN adjuvants were determined by ELISA. Oil-based CpG ODN adjuvanted formulations significantly ($p < 0.05$) increased serum IFN- γ titers after first and second vaccinations while the increment was not statistically significant with oil-based adjuvant alone. The protective efficacy of PlpE, OmpH and PlpEC-OmpH proteins formulated with oil-based or oil-based CpG ODN adjuvants was investigated after IP challenge of the immunized mice with 10 LD of live *P. multocida* A:3. Vaccine formulations composed of PlpE with oil-based or oil-based CpG ODN conferred 80% and 100% protection, respectively. Protectivity of PlpEC-OmpH fusion proteins

formulated with oil-based or oil-based CpG ODN was 60% and 100%, respectively. However, formulations containing rOmpH provided 40% protection, not statistically significant (Okay, *et al.*, 2012). In addition to Okay, *et al.* studies, erstwhile in our laboratory, *ompH* and *lkt* neutralizing epitope genes, *lktA*, were amplified via PCR using chromosomal DNA of *P. multocida* A:3 and *M. haemolytica*. PCR products were ligated to pGEM-T Easy vector and introduced into *E. coli* DH5 α . Recombinant plasmids were verified with restriction enzyme digestion and the genes of interest were cloned in pET28a. After the orientation of insertion and integrity of the constructs was confirmed by restriction enzyme analysis, expressions and purification of His-tagged recombinant proteins (pET28a-OmpH, pET28a-OmpH-Lkt) were carried out for immunization studies. Vaccination of mice to determine the protective potency of vaccine exhibited that OmpH-Lkt vaccine formulated with oil-based adjuvant possesses 100% protection against *P. multocida* and 50% for *M. haemolytica* challenge. (Öğülür, unpublished data).

Preference of injecting recombinant fusion protein instead of mixture of all three proteins is another perspective of this study. There are a few reasonable advantages to use fusion protein for vaccine development. First of all, purification of fusion in a lump rather than one by one is both cost effective and time saving. Additionally, use of longer peptide in fusion with a smaller peptide might make the small peptide much more soluble (Terpe, 2003). Secondly, fusion proteins might possibly be better target for APCs to trigger the specific immune response since it probably involves more than one epitope regions (Cuadros *et al.*, 2004)

In general, predominantly, avian or swine were utilized in vaccine development and hence there is no vaccine study up to now utilizing recombinant OmpH and PlpE from a bovine isolate of *P. multocida* A:3 and Lkt from *M. haemolytica* together. Therefore, there is a need of studies on bovine isolates of *P. multocida* for vaccine development against shipping fever.

1.8. Aim of the present study

The present study aims at developing a novel recombinant vaccine formulation in order to establish 100% protection against both *P. multocida* A:3 and *M. haemolytica* as the causative agents of shipping fever. For this aim, PlpEC and OmpH proteins of *P. multocida* A:3 and Lkt protein of *M. haemolytica* A1 were selected as the potential vaccine components. There were two pioneering studies in our laboratory regarding the development of such a vaccine. In one of these (Okay *et al.*, 2012), *ompH* gene and a *plpE* gene fragment, namely *plpEC* were cloned from *P. multocida* A:3, fused and expressed in *E. coli*. In that work, PlpEC-OmpH fusion was purified and formulated in order to generate a new acellular prototype vaccine. Upon vaccination, antibody responses in mice were significantly increased. Moreover, intraperitoneal challenge of mice with 10 LD50 of *P. multocida* A: 3 conferred 100% protection. *lktA* fragment was subsequently cloned from the genomic DNA of *M. haemolytica* A1 and another fusion protein, OmpH-LktA was subsequently constructed for being used as a universal vaccine against both *P. multocida* A:3 and *M. haemolytica* A1 (Ögüller, unpublished results). In the present study, as the first task, mice challenge experiments against both pathogens were performed using this recombinant fusion. Then, by cloning *lktA* fragment in frame with *plpEC-ompH* fusion, the *lktA-plpEC-ompH* triple recombinant fusion was generated, purified, formulated and the resulting vaccine candidate was tested for its immunogenicity and potency against challenge with the lethal doses of the both pathogens.

CHAPTER 2

MATERIALS AND METHODS

2.1. Bacterial strains and plasmids

Bacterial strains with their properties and their reference sources and plasmids utilized as a part of this study were given in Table 2.1 and Table 2.2, individually.

Table 2.1 Strains used in this study, their characteristics and sources

Strain	Characteristics	Source and Reference
<i>P. multocida</i> P-1062	Serotype A:3, bovine strain	American Type Culture Collection (ATCC 15743)
<i>E. coli</i> DH5 α	F' fdlacZ Δ (lacZY A-argF)U169 supE44 λ^- thi-1 gyrA recA1 relA1 endA1 hsdR17	American Type Culture Collection
<i>E. coli</i> BL21(DE3)	F ⁻ ompT gal dcm lon hsdS _B (r _B ⁻ m _B ⁻) λ (DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 nin5])	Novagen, Merck (Germany)

Table 2.2 Plasmids used in this study

Plasmid	Size	Markers	Source and Reference
pET-28a(+)	5.3 kb	kan (Kan ^r)	Novagen, Merck (Germany)
pGEM [®] -T Easy	3.0 kb	amp (Amp ^r), lacZ	Promega Inc. (Madison, WI)

2.2. Culture media

The substances included in media preparation were stated in Appendix B.

2.3. Buffers and solutions

The substances included in solutions and buffers were listed in Appendix C.

2.4. Chemicals and enzymes

The chemicals and enzymes utilized as a part of this study were given in Appendix D with their supplier companies.

2.5. Maintenance of bacterial strains

In order to prepare -80 stock for long term storage of cells, Brain Heart Infusion (BHI) Broth (Appendix B) was used to grow both for *P. multocida* A:3 and *M. haemolytica* A1. The same growth condition was used for challenge experiments of *M. haemolytica* A1 as well. On the other hand, for challenge experiments with *P. multocida* A:3 Blood Agar (BA) (Appendix B) was preferred to grow cells. *E. coli* BL21 and *E. coli* DH5 α strains were preferred in cloning experiments and for inoculation of them Luria Broth (LB) (Appendix B) was utilized. For long term storage of *E. coli* strains that were used in this study they were grown in LB till the mid-log of their growth and 2mL of culture were centrifuged at 5000rpm for 5 min. After that supernatant was discarded and precipitated cells were resuspended in 0,5mL 50% glycerol. For +4 storage of *E. coli* strains they were inoculated on LA for overnight incubation at 37°C. For plasmid containing strains of *E. coli* culture media were added with the proper antibiotics (100 μ g/mL for ampicillin or 30 μ g/mL for kanamycin) whenever required.

2.6. Primer design

Whole genome sequence *M. haemolytica* A1 was used to design primers to amplify *lktA* gene fragment. The forward primer was added 5'-ggatcc-3'

restriction site of *Bam*HI, On the other hand, the reverse primer was added 5'- agatct-3' restriction site of *Bgl*II (Table 2.3).

Table 2.3 Primers used for amplification of *lktA*

Gene name	Primer name	Nucleotide sequence	Size of the PCR products
<i>LktA</i>	Forward	5'- attc ggatc tctctgattcgaactta- 3'	400 bp
<i>LktA</i>	Reverse	5'- caa agatc tctcattgaagtggagc- 3'	

2.7. PCR

Table 2.4 PCR conditions for *lktA* gene fragment

Product	PCR conditions (35 cycle)
<i>lktA</i>	Initial denaturant.: 3 min at 94°C Denaturation: 1 min at 94°C Annealing: 1 min at 50°C Extension: 1 min at 72°C Final extension: 10 min at 72°C

Table 2.5 PCR mixture content

Component	Amount
PCR buffer (Fermentas)	5 µl from 10X buffer
dNTP mix (Fermentas)	1 µl from 10mM mix
Primers	2 µl from 10 µM primers
MgCl ₂ (Fermentas)	5 µl from 25 mM MgCl ₂
<i>Taq</i> polymerase (Fermentas)	1 µl
Genomic DNA	0,5 µg
dH ₂ O	Complete to total volume
Total mixture volume	50 µl

2.8. Agarose gel electrophoresis

1X TAE (Tris-Acetate-EDTA) Buffer (Appendix B) was used to prepare agarose gel. Depending on the gene that were run on electrophoresis, agarose gel concentration varied but generally 1% gel was preferred. Optimal conditions for running samples on gel were 100 Volts for 40-45 minutes. Sample DNAs were added 6X Loading Dye which becomes 1X in final concentration before running them on gel. Together with DN samples Gene Ruler™ 100bp DNA Ladder was loaded in one separate well to determine the size of *lktA* gene. Qiagen Gel Extraction Kit was used to extract the gene fragment of interest from the gel.

2.9. Sequencing reactions

Sample DNAs were sent to RefGen Biotechnology Inc. (Ankara, Turkey). To be sequenced and sequenced genes and their primary protein sequences were compared with database via National Center for Biotechnology Information (NCBI) by BLAST program at the web site (<http://www.ncbi.nlm.nih.gov/BLAST>).

2.10. Ligation reactions

4°C for 16 hours is optimal condition for ligation to takes place. Table 2.6 and 2.7 respectively shows mixture contents of ligation reactions for both pGEM-T Easy Vector and pET-28a Vector.

Table 2.6 Ligation of insert DNA into pGEM-T Easy Vector

Mixture Content	Amounts
1X Ligase Buffer	1 µl
pGEM-T Vector	1 µl
DNA insert	500 ng
T4 DNA Ligase	1 µl
dH ₂ O	Complete to final volume
Total Mixture Volume	10 µl

Table 2.7 Ligation of insert DNA into pET-28a Vector

Mixture Content	Amounts
10X Ligase Buffer	1 μ l
pET-28a Vector	2 μ l
DNA (insert)	500 ng
T4 DNA Ligase	1 μ l
dH ₂ O	Complete to final volume
Total Mixture Volume	10 μl

2.11. Competent cell preparation and transformation of *E.coli* cells

Competent cells were prepared from *E. coli* depending on the method stated in Hanahan D. (1985). After preparation they were kept at at -80°C to be used in transformation procedure.

First step of the transformation was melting of 100 μ l *E. coli* competent cells on ice until they were completely thawed. Then 10 μ L of ligation product containing either recombinant pGEM-T or pET-28a vectors were mixed with competent cells with gentle pipeting and left on ice for 30 minutes. In following heat shock step, competent cells incubated at at 42°C for 60 seconds and cells were immediately put on ice for 5 mins. 900 μ L fresh and sterile LB was added in the mixture after heat shock and left in 37°C, 180 rpm shaker for 1,5 hours. On next step, transformed cells were precipitated via centrifugation at 3500 rpm for 10 min and remaining 900 μ L supernatant at top of the pellet was taken to be discarded. Later pellet was resuspended in 100 μ L of the supernatant remained in eppendorf tube . Finally spread plate technique was applied to inoculate the transformed cells on LA added with appropriate antibiotic (100 μ g/mL ampicillin for pGEM-T Vector or 30 μ g/mL kanamycin for pET-28a Vector). To screen colonies with the blue-white screening method, right before inoculation of pGEM-T vector containing transformed cells, LA plates were spreaded with 4 μ l of 20 mg/ml X-gal stock and 40 μ l of 100 mg/ml IPTG stock.

2.12. Plasmid isolation

Both pGEM-T and pET28a vectors were isolated from *E. coli* by using GeneJET

Plasmid Miniprep Kit (Fermentas) protocol. After isolation, plasmids were kept in -20°C for storage or run on agarose gel for analysis of them.

2.13. Restriction enzyme digestion

17 µl of plasmid DNA mixed with 1 µl of restriction enzyme (BamHI, BglII, NotI or HindIII) and mixture was added with suitable restriction enzyme buffer supplemented with the related enzyme in eppendorf tube. The mixture was incubated at 37°C for 1,5-2 hours and the sample was kept at -20°C for following studies.

2.14. Alkaline phosphatase treatment

Alkaline phosphatase treatment was carried out according to the protocol of rAPid Alkaline Phosphatase Kit (Roche). The sample kept at -20 °C for following experiments.

2.15. Construction of recombinant plasmids

plpE and *ompH* genes were amplified via PCR using chromosomal DNA of *P. multocida* P-1062. *plpE* was also cloned as C-terminal (*plpEC*) fragment. PCR products were ligated to pGEM-T Easy vector by using *BamHI* and *BglII* enzymes and introduced into *E. coli* DH5α and *plpEC-ompH* fusions have been obtained earlier in pGEM-T Easy by Dr. Sezer Okay, a previous member of our Laboratory. This recombinant pGEM-T vector was digested with *NotI* to verify the construct of interest. After extraction of *plpEC-ompH* fusion by restriction digestion with *BamHI* and *BglII*, for expressing His-tagged proteins it was ligated into pET-28a vector, which was already digested with *BamHI* in order to express His-tagged proteins. To design the *LktA-plpEC-ompH* triple fusion *LktA* fragment was amplified via PCR using chromosomal DNA of *M. haemolytica* by using primers depicted at Table 2.3. After amplification, *LktA* gene was directly cloned at the upstream of the *plpEC-ompH* fusion in pET-28a via *BamHI* restriction digestion.

To further confirm the construction of fusion within the recombinant vector BamHI and *NotI* digestion was applied. As the next step, expression and purification of His-Tag proteins were carried out.

2.16. Protein overexpression, purification and dialysis

Recombinant *E. coli* BL21 cells carrying pET28- *lktA-plpEC-ompH* were inoculated in LB added with kanamycin (30 µg/ml final concentration) and grown in 37°C 180 rpm shaker. IPTG was added when optical density of cells reached 0.6 at 600nm as a 1 mM final concentration and left for incubation at 37°C for 5 h at 180 rpm. After 5 hours growth cell were centrifuged at 6000g for 15 minutes at 4°C and dissolved in LEW buffer (Appendix C). Sonication was applied to resuspended cells via CP70T Ultrasonic Processor (Cole-Parmer, Vernon Hills, IL) for 6 times 10 sec duration at 60% amplitude and cell debris was precipitated by spinning cells 15,000 g for 15 min. Remaining supernatant containing expressed protein at top of the precipitated cell debris was taken to be purified via Protino Ni-TED 2000 packed columns (Macherey-Nagel, Germany) following the supplied protocol. Purified recombinant protein was dialyzed with overnight stirring by using cellulose dialysis membrane (Sigma), whose molecular weight cut-off is 14kDa, in 1 L of dialysis buffer (DB) (Appendix C) at 4°C. On next step, dialyzed protein was passed through the 0.2 µm membrane filter to be sterilized. Finally sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was applied to analyze protein.

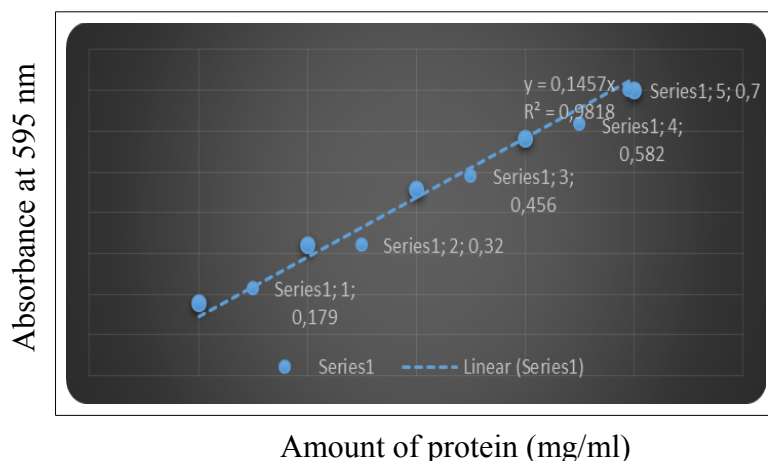


Figure 2.1 Calibration curve for determination of protein concentrations.

2.17. Determination of protein concentration

Concentration of protein was determined comparatively by both Bradford method (Bradford, 1976) and by measuring OD₂₈₀ value. According to Bradford assay, calibration curve was obtained from the OD595 values of standards (Figure 2.1) and protein concentrations were computed by using the calibration curve equation.

2.18. SDS-Page and Comassie Brilliant Blue staining

Laemmli (1970) was used for SDS-polyacrylamide gels content of which was given in Table 2.5. Before loading samples on gel they were mixed with loading buffer given in Appendix C at a 1X final concentration and the gel was run at 18 mA in 1X running buffer (Appendix C) using a Mini-Protean electrophoresis apparatus (Bio-Rad) till the loading dye came close to the bottom of the gel.

Table 2.8 Preparation of SDS-polyacrylamide gels.

	Stacking Gel	Separating Gel
	0.125 M Tris, pH 6.8	0.375 M Tris, pH 8.8
Monomer concentration	4.5%	12%
Acrylamide/bis	0.65 mL	4 mL
dH ₂ O	3.05 mL	3.35 μ L
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 μ L	100 μ L
10% Ammonium persulphate	25 μ L	50 μ L
TEMED	5 μ L	5 μ L
TOTAL MONOMER	5 mL	10 mL

2.19. Western blotting

3MM Whatman[®] papers and the 0.2 μ m nitrocellulose membrane (Bio-Rad, Hercules, CA) were wet with 1X transfer buffer (Appendix C) and superposed as displayed in Figure 2.2.

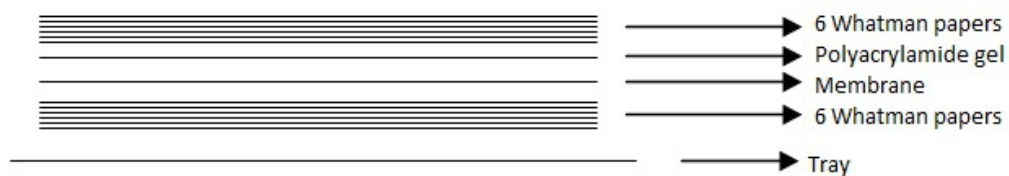


Figure 2.2 Schematic representation of transfer set-up in Western blot.

Ssemi-dry blotter (Cleaver Scientific Ltd, Warwickshire, UK) apparatus was used for blotting. After set up preparation following procedure was applied as stated in Okay (2011). During procedure, as primary antibody sera obtained from mice injected with Lkt-PlpEC-OmpH protein was used.

2.20. Mice experiments

16g weight BALB/c mice were purchased from Faculty of Medicine of Ankara University and injected with 100 µg per dose of Lkt-PlpEC-OmpH fusion protein (Table 2.9) mixed with oil-based adjuvant. Control mice were injected only with PBS-oil-based adjuvant mixture. All vaccinations were applied intraperitoneally. After 10 days from both first and second injections, blood was taken from the mice to obtain sera. For sera collection, blood was incubated in room temperature for 1 hour and spinned down at 4000 rpm for 10 min. Sera remained at upper part of tube was collected to be stored at -20°C.

Table 2.9 Vaccination methodology for *P. multocida* A:3 and *M. haemolytica* A1

	Pathogen Challenge	
	Vaccinated	Control
Total mice #	13	4
Vaccination route	i.p.	i.p.
Amount	100µg/500 µl	100µg/500 µl

After vaccination process, mice were challenged with *P. multocida* A:3 in saline solution. Previously agar plate inoculated cells were collected from plate and dissolved into saline solution up to OD₆₃₀ of 0.6. Prepared stock was diluted to 10⁷ and each mice challenged with 500µl of this diluted solution. For *M. haemolytica* challenge the organism was grown in Brain Heart Infusion Broth until OD₆₃₀ of 0.9 and 500 µl of culture was directly injected to mice. Survivors were recorded 4 days after challenge. Animal experiments were performed under

the approval of Ethical Committee on Animal Experiments of Middle East Technical University, Ankara (Etik-27.08.2012).

2.21. IgG ELISA

For both IgG1 and IgG2 ELISA analysis protocol stated in Okay (2011) was followed with a set of changes. Firstly, at initial step plates were coated with Lkt-PlpEC-OmpH protein. Secondly, sera utilized as primary antibody in this analysis were obtained from mice vaccinated with Lkt-PlpEC-OmpH protein and dilutions at related steps were applied as 1:100, 1:200, 1:400, 1:800, 1:1600, 1:3200, 1:6400, 1:12800, 1:25600, 1:51200, 1:102400. Plates were analyzed via RT-2100C Microtiter plate Reader (Rayto, Shenzhen, China) at 405nm. For IgG1 antibody response 1:100 (vaccinated) and 1:51200 (control) dilutions were taken into consideration whereas, 1:100 (vaccinated) and 1:3200 (control) dilutions were used for the titer of IgG2a antibody responses. Additionally, antibody titers of both IgG1 and IgG2a antibodies of only vaccinated mice were compared on the relevant graphs.

2.22. Detection of serum IFN γ levels

ELISA for Mouse IFN- γ Kit (Mabtech) 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 μ l of diluted coating antibody for 4 h at room temperature (RT). Next, 200 μ l of Blocking Buffer (5% BSA and 0,025 Tween 20 in 1X PBS) was added to each well and incubated for 2 h at RT. After washing with Wash Buffer (0,05 Tween 20 in PBS) serum samples were added without dilution and incubated at RT for 2 h. The plate was washed again and 100 μ l of diluted biotin labeled detection antibody was added to each well. After incubation of 2 h at RT, the plate was washed and 100 μ l of Streptavidin-AKP was added at a dilution of 1:5000. The plate was incubated at RT for 1 h and washed again. 100 μ l of PNPP (p-nitrophenyl phosphate) substrate (Thermo Scientific) was added to each well and incubated at

RT till the color formation and the absorbance was read at 405 nm on a RT-2100C Microtiter plate Reader (Rayto, Shenzhen, China).

2.23. Statistical analyses

Statistical analyses were carried out with t-test. p-value was accepted as 0,05.

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Cloning of *lktA* gene fragment from *M. haemolytica* into PlpEC-OmpH fusion in *E.coli*

3.1.1. PCR amplification and cloning of *lktA* fragment into pGEM-T Easy Vector

Genomic DNA of *M. haemolytica* A1 was used for PCR amplification of *lktA* epitope fragment of *lkt* gene (Figure 3.1). This fragment was reported to have the epitope involved in neutralization of LKT and localized to a 32 amino acids region near the C-terminus (Lainson and Murray, 1996). The *lktA* fragment was cloned without a stop codon to obtain a genetic fusion with *PlpEC-ompH* double fusion on which the fragment resides at the upstream of the *plpEC-ompH* fusion.

After PCR amplification (Figure 3.1), *lktA* fragment was extracted from the gel and ligated into the pGEM-T Easy Vector, which was digested with *Bam*HI restriction enzyme. Recombinant plasmid was then transformed into *E.coli* DH5 α . The cells were inoculated into LA plates containing X-gal, IPTG and ampicillin to select the recombinants. Plasmids from white colonies were isolated from transformed cells and double digested with *Bam*HI-*Bgl*III enzymes for verification of cloning and run on gel. As shown in Fig. 3.2, both empty pGEM-T vector and *lktA* fragment are in expected sizes.

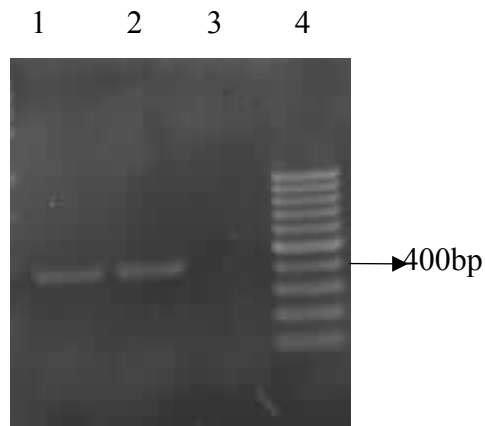


Figure 3.1 PCR of *lktA* fragment. Amplification of *lktA* fragment (lane 1 and 2), Negative control (lane 3), GeneRuler 100bp DNA Ladder (lane 4).

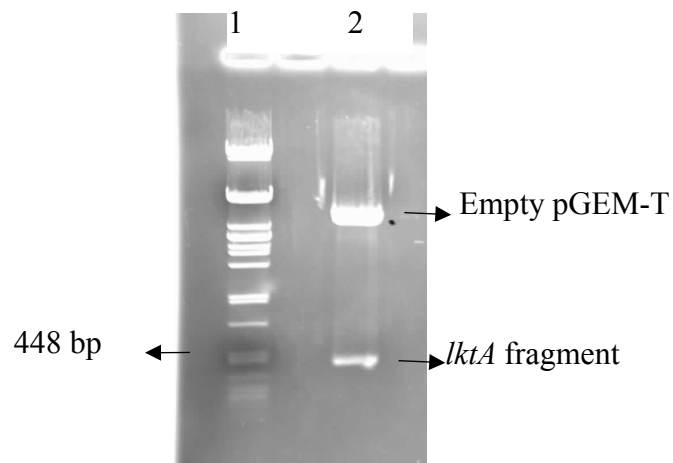


Figure 3.2 Verification of cloning of *lktA* fragment into pGEM-T. λ DNA/PstI marker (lane 1), pGEM-T vector and *lktA* fragment (lane 2)

3.1.2. Sequence analysis of *lktA* gene from *M. haemolytica*

Positive clones for pGEM-T-*lktA* was used for sequencing of the gene and the sequence was compared with previously sequenced genes in nucleotide BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome) database.

(A)
TCTGATTCGAACTTAAAAGATTTAACATTTGAAAAAGTTAAACATAAT
CTTGTCATCACGAATAGCAAAAAAGAGAAAGTGACCATTCAAACACTG
GTTCCGAGAGGCTGATTTTGCTAAAGAAGTGCCTAATTATAAAGCAAC
TAAAGATGAGAAAATCGAAGAAATCATCGGTCAAATGGCGAGCGGA
TCACCTCAAAGCAAGTTGATGATCTTATCGCAAAGGTAACGGCAA
ATTACCCAAGATGAGCTATCAAAGTTGTTGATAACTATGAATTGCTCA
AACATAGCAAAAATGTGACAAACAGCTTAGATAAGTTAATCTCATCTG
TAAGTGCATTTACCTCGTCTAATGATTGAGAAATGTATTAGTGGCTCC
AACTTCAATG

(B)
KDLTFEKVKHNLVITNSKKEKVTIQNWF**READFAKEVPNYKATKDEKI**
EEIIGQNGERITSKQVDDLIAKGNGKITQDELSKVVDNYELLKHSKNVT
NSLDKLISSVSAFTSSNDSRNVLVAPTS

Figure 3.3 (A) Nucleotide, (B) Aminoacid sequence of *lktA*. Bold characters show (MM601) neutralizing anti-LktA MAb binding site of LktA. (Gentry and Srikumaran, 1991)

Pasteurella haemolytica A1 leukotoxin gene, encoding LktA, LktB, LktC and LktD proteins, complete cds
 Sequence ID: [gbIM20730.1IPASA1LKT](#) Length: 7801 Number of Matches: 1

Range 1: 3413 to 3805 [GenBank](#) [Graphics](#) ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
726 bits(393)	0.0	393/393(100%)	0/393(0%)	Plus/Plus
Query 1	TCTGATTCGAACTTAAAAGATTTAACATTTGAAAAAGTTAAACATAATCTTGTCATCACG			60
Sbjct 3413	TCTGATTCGAACTTAAAAGATTTAACATTTGAAAAAGTTAAACATAATCTTGTCATCACG			3472
Query 61	AATAGCAAAAAAGAGAAAAGTGACCATTCAAACCTGGTTCCGAGAGGCTGATTTTGCTAAA			120
Sbjct 3473	AATAGCAAAAAAGAGAAAAGTGACCATTCAAACCTGGTTCCGAGAGGCTGATTTTGCTAAA			3532
Query 121	GAAGTGCCTAATTATAAAGCAACTAAAGATGAGAAAAATCGAAGAAATCATCGGTCAAAAAT			180
Sbjct 3533	GAAGTGCCTAATTATAAAGCAACTAAAGATGAGAAAAATCGAAGAAATCATCGGTCAAAAAT			3592
Query 181	GGCGAGCGGATCACCTCAAAGCAAGTTGATGATCTTATCGCAAAAGGTAACGGCAAAAAT			240
Sbjct 3593	GGCGAGCGGATCACCTCAAAGCAAGTTGATGATCTTATCGCAAAAGGTAACGGCAAAAAT			3652
Query 241	ACCCAAGATGAGCTATCAAAGTTGTTGATAACTATGAATTGCTCAAACATAGCAAAAAT			300
Sbjct 3653	ACCCAAGATGAGCTATCAAAGTTGTTGATAACTATGAATTGCTCAAACATAGCAAAAAT			3712
Query 301	GTGACAAACAGCTTAGATAAGTTAATCTCATCTGTAAGTGCATTTACCTCGTCTAATGAT			360
Sbjct 3713	GTGACAAACAGCTTAGATAAGTTAATCTCATCTGTAAGTGCATTTACCTCGTCTAATGAT			3772
Query 361	TCGAGAAATGTATTAGTGGCTCCAACCTTCAATG		393	
Sbjct 3773	TCGAGAAATGTATTAGTGGCTCCAACCTTCAATG		3805	

Figure 3.4 Computer-based nucleotide BLAST analysis of *lktA*

3.1.3. Transformation of *E.coli* BL21 with pET-28a containing *lktA* gene

pET-28a (+) expression vector carries His-tag sequences at C and N termini and the expression of genes are under the control of T7 promoter. After the digestion of pGEM-T+*lktA* vector with *Bam*HI and *Bgl*II enzymes, *lktA* fragment was extracted from the agarose gel. pET-28a expression vector was restricted with *Bam*HI enzyme and treated with alkaline phosphatase. *lktA* fragment was then ligated into pET-28a (+). Recombinant vector (pET-28a + *lktA*) was transformed into *E.coli* BL21 (D3) cells. The cells were inoculated into LB agar plates containing kanamycin. Plasmids from recombinant colonies were isolated and digested with restriction enzymes to verify cloning.

3.1.4. Cloning of *lktA* into *plpEC-ompH* fusion in *E.coli*

As previously mentioned, pET-28a + *plpEC-ompH* recombinant vector has already been constructed (Figure 3.5) (Okay *et al.*, 2012).

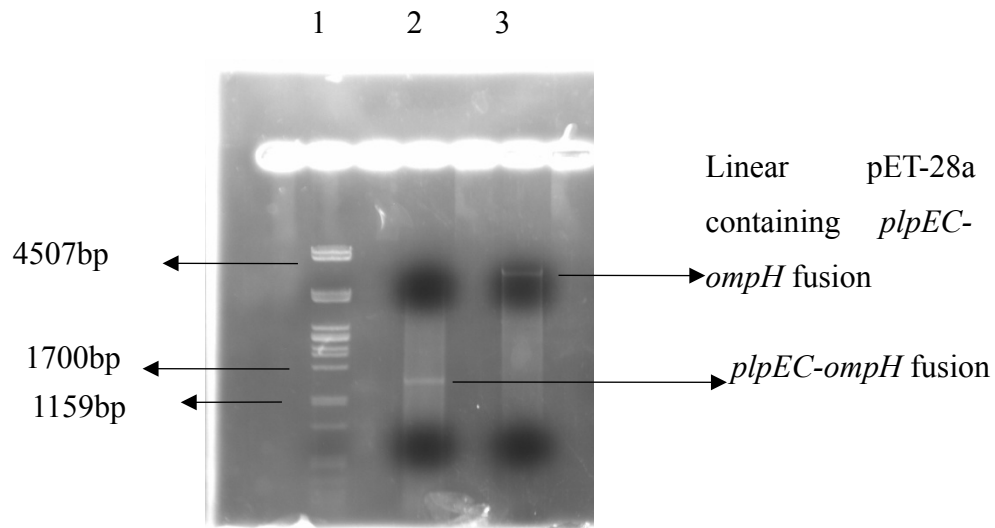


Figure 3.5 λ DNA/PstI marker (lane 1), *plpEC-ompH* fusion (lane 2), Linear pET-28a containing *plpEC-ompH* fusion (lane 3).

lktA fragment digested from pET-28a expression vector was ligated into pET-28a +*plpEC-ompH* vector at the upstream of *plpEC-ompH* fusion after double digestion with *Bam*HI-*Bgl*III enzymes. To verify cloning, transformed cells were grown on kanamycin containing Nutrient agar, and the colonies were selected to isolate recombinant plasmids. Isolated plasmids containing triple fusion were digested with *Bam*HI and *Not*I enzymes. During *lktA* cloning, a cloning site of approximately 200 bp was lost from pET-28a vector due to *Bam*HI digestion; therefore, *lkt-plpEC-ompH* fusion was shown as an 1858 bp fragment, as expected. If there was a reverse insertion then after digestion, only 32bp in between the *Bam*HI/*Not*I restriction sites would be cut and hence whole length of fusion would remain stick to the linear pET-28a. As seen in Fig.3.6, one of the selected colonies (lane 1) gave the expected result. After verification, the plasmid containing *lkt-plpEC-ompH* fusion was transformed into *E. coli* BL21 (DE3) cells for the expression of the fusion protein.

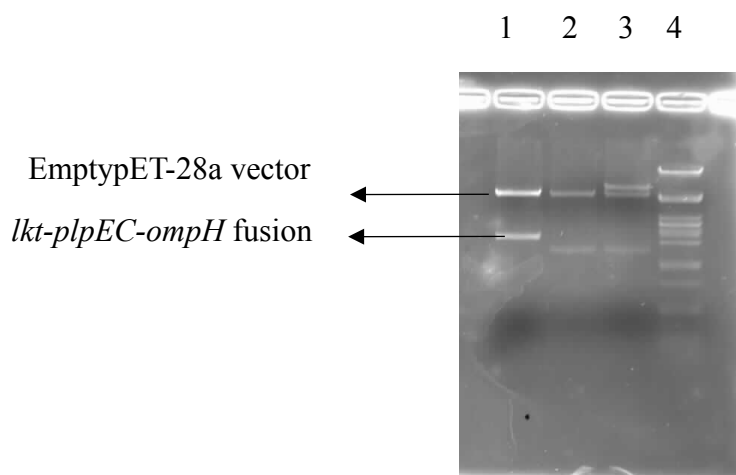


Figure 3.6 Verification of putative colonies for cloning of *lkt-plpEC-ompH* via BamHI and NotI double digestion (lane 1, 2 and 3), λ DNA/PstI marker (lane 4).

3.2. Expression of recombinant LktA -PlpEC-OmpH fusion protein in *E.coli* BL21 (D3)

For the expression of LktA-PlpEC-OmpH protein, 1200 mL of LB (divided into 4 flasks) was cultured with *E.coli* BL21 (D3) cells transformed with the gene of interest. 600 mL of the culture was spared as the control group and not induced. IPTG was added to other two flasks to induce the expression of the fusion protein. Total protein extraction followed by SDS-PAGE. If there were no modification of the protein, its full size was expected as approximately 67 kDa (LktA \approx 14.6 kDa, PlpEC \approx 17.4 kDa, OmpH \approx 35 kDa). Fig. 3.7 shows verification of the expected fusion protein size of ca. 67 kDa.

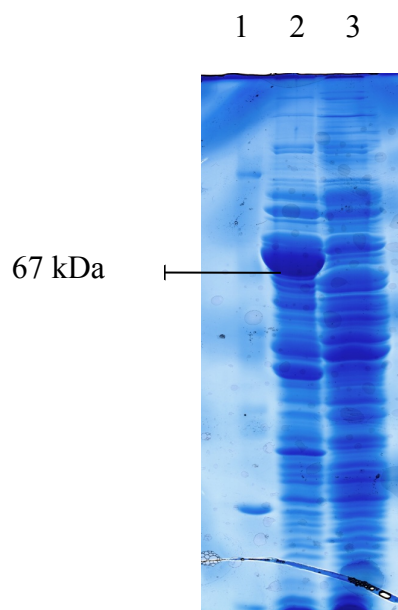


Figure 3.7 SDS-PAGE analysis of LktA-OmpH-PlpEC fusion protein expression. Fermentas Unstained Protein Marker (lane 1), IPTG induced overexpressed LktA-OmpH-PlpEC fusion protein (lane 2), Uninduced control (lane 3).

3.3. Purification of recombinant LktA-OmpH-PlpEC fusion protein by His-Tag affinity chromatography

Recombinant His-Tagged fusion protein was purified with Protino® Ni-TED 2000 protein purification system (Figure 3.8). After purification, the urea concentration of the protein decreased from 8M to 4M via dialysis with Sigma-Aldrich Dialysis tubing cellulose membrane average flat with 33mm, and sterilized with PALL 0.2 μm Syringe filter (Figure 3.9). After purification, His-Tag parts remained attached to the proteins but they do not interfere with the result because of their small size, weak immunogenicity and not having the ability to affect protein folding.

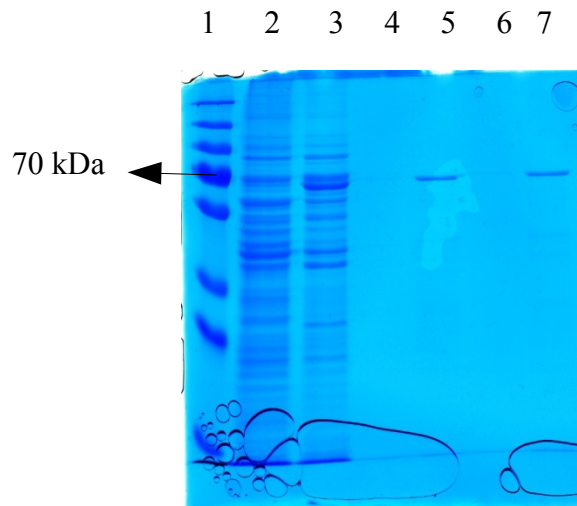


Figure 3.8 SDS-PAGE analysis of the purified LktA-OmpH-PlpEC fusion protein. PageRuler Prestained Protein Marker (lane 1), Control flow through (lane 2), Fusion protein flow through (lane 3), Control 1st elute (lane 4), Fusion protein 1st elute (lane 5), Control 2nd elute (lane 6), Fusion protein 2nd elute (lane 7).

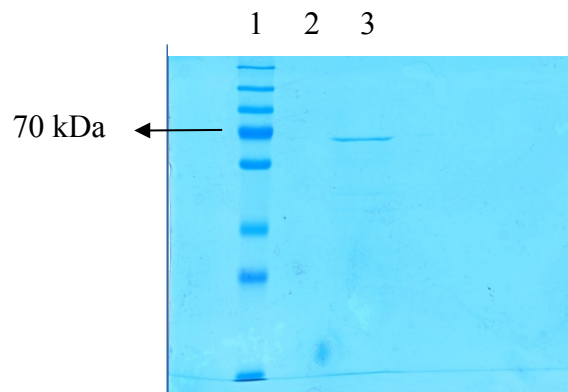


Figure 3.9 SDS-PAGE analysis after the dialysis of LktA-OmpH-PlpEC fusion protein. PageRuler Prestained Protein Marker (lane 1), Control elute (lane 2), Fusion protein elute (lane 3)

3.4. Western blot analysis of recombinant LktA-OmpH-PlpEC fusion protein

Sera were obtained from the mice immunized intraperitoneally with LktA- PlpEC- OmpH fusion protein. In addition, each component of the fusion protein, (except for native LktA in culture supernatant as the positive control, instead of LktA fragment) was analyzed with fusion antisera.

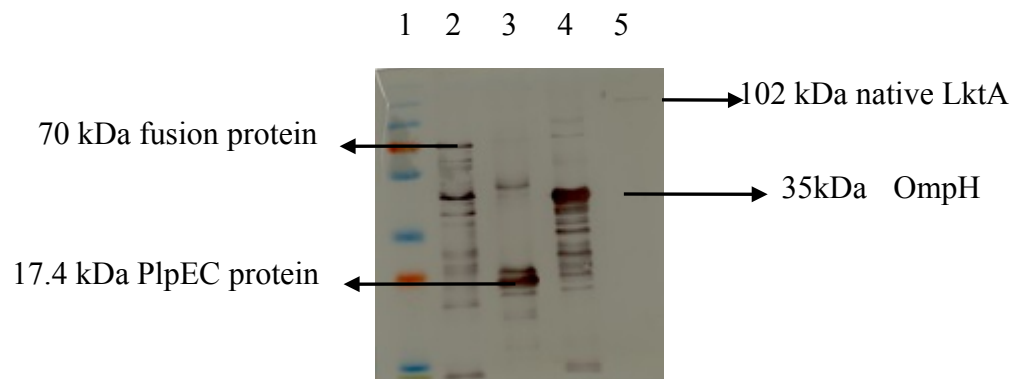


Figure 3.10 Western blot analysis with sera against the fusion protein. PageRuler Prestained Protein Marker (lane 1), Fusion protein (lane 2), PlpEC protein (lane 3), OmpH Protein (lane 4), Native LktA protein (lane 5).

As seen in Fig 3.10 the antibodies raised in the animals positively reacted with PlpEC, OmpH and the fusion, as expected.

3.5. Mice challenge experiments

As a preliminary experiment, fifteen BALB/c mice for each set of experiment were injected two times with 21 days interval. 10 of them vaccinated with Lkt-OmpH+mineral oil-based vaccine (100µg/500µl) and 5 of them immunized only with PBS as a control group. As a result, Lkt-OmpH+mineral oil-based vaccine showed 50% protection against *P. multocida* A:3 challenge and 100% against *M. haemolytica* A1 challenge.

Table 3.1 Dead/survival scores of Lkt-OmpH+mineral oil-based vaccine immunized and control mice

Challenge organism	Control Score	Vaccinated Score
<i>P. multocida</i> A:3	5/5	5/10
<i>M. haemolytica</i> A1	5/5	10/10

For present study mice experiments, thirteen BALB/c mice per group were immunized two times with 100µg/500µl vaccine prepared with fusion protein at

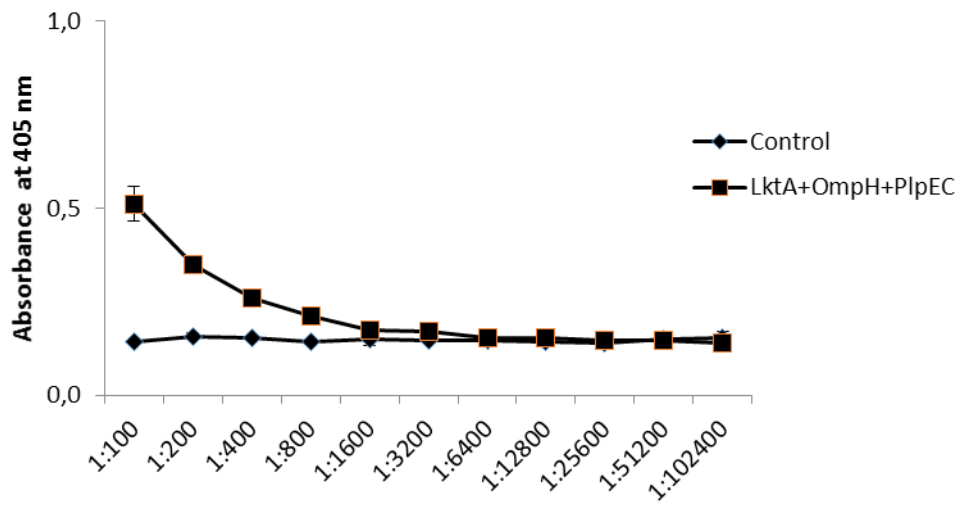
day 0 and 21. To constitute the controls, BALB/c mice per group were injected with PBS+adjuvant at the same time intervals. 10 days after each injection sera were collected from both control and vaccinated mice. Two days after second sera collection, one group of mice was challenged with *M. haemolytica* and the other group was challenged with *P. multocida* A:3. As a result, almost all mice died in both control and immunized groups challenged with both *M. haemolytica* and *P. multocida* A:3 separately.

3.5.1. Serum antibody level against recombinant LktA-OmpH-PlpEC fusion protein

To determine serum-specific antibody levels to the recombinant fusion protein, sera of immunized and control mice were collected before second immunization and challenge and they were pooled. ELISA was carried out to measure antibody levels. The analyses were performed for both IgG1 and IgG2a to reveal Th2 and Th1 responses, respectively.

In Fig. 3.11 and 3.12, serum IgG1 and IgG2a levels are presented as a function of dilution factors. LktA-PlpEC-OmpH immunized mice showed significant ($p < 0.05$) increase in serum IgG1 after first and second vaccination. The increase in the second vaccination was more noticeable. While there was not much significant difference between control and immunized groups of mice in terms of IgG2a level at first vaccination, the level significantly ($p < 0.05$) increased in the second vaccination.

A)



B)

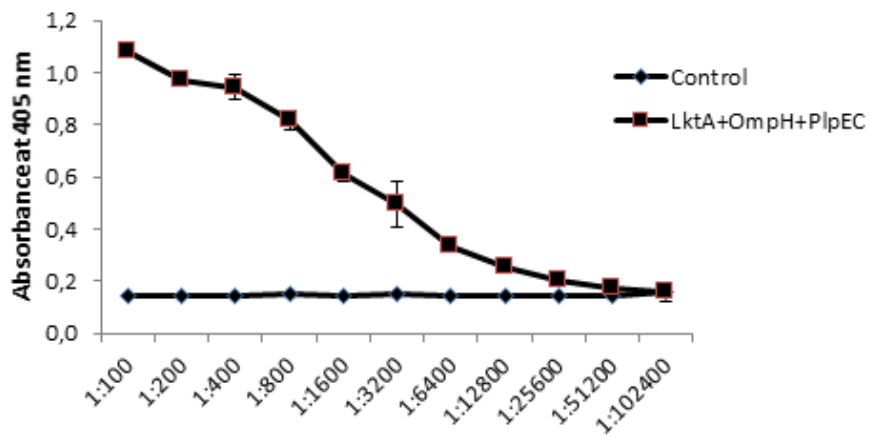
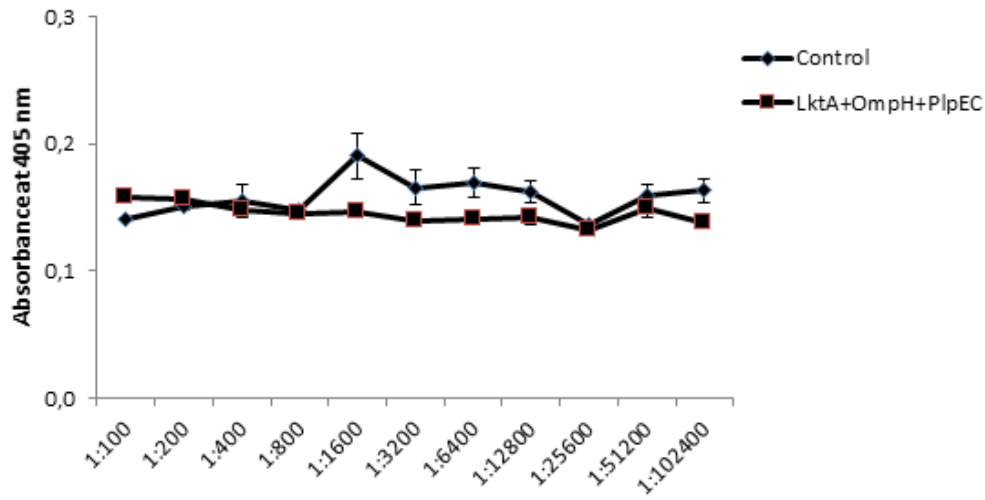


Figure 3.11 Serum IgG1 titers in mice immunized with LktA-OmpH-PlpEC fusion protein. (A) Serum titers after first vaccination and (B) serum titers after second vaccination.

A)



B)

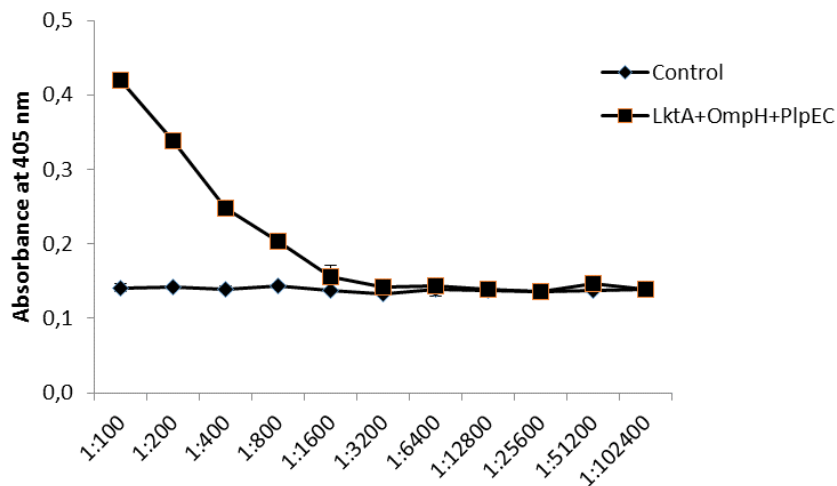


Figure 3.12 Serum IgG2a titers in mice immunized with LktA-OmpH-PlpEC fusion protein. (A) Serum titers after first vaccination and (B) serum titers after second vaccination.

In Fig. 3.11, the mean antibody titers \pm SD are given to evaluate the distribution of IgG1 and IgG2a with respect to first and second vaccination with LktA-OmpH-PlpEC fusion protein. As shown in Fig. 3.12, a nearly balanced IgG1 and IgG2a response was elicited in mice group immunized with LktA-PlpEC -OmpH- fusion

protein in the first vaccination. In the second vaccination, an increase in both IgG1 and IgG2a responses was detected; however, the increment in IgG1 level was higher than that of IgG2a.

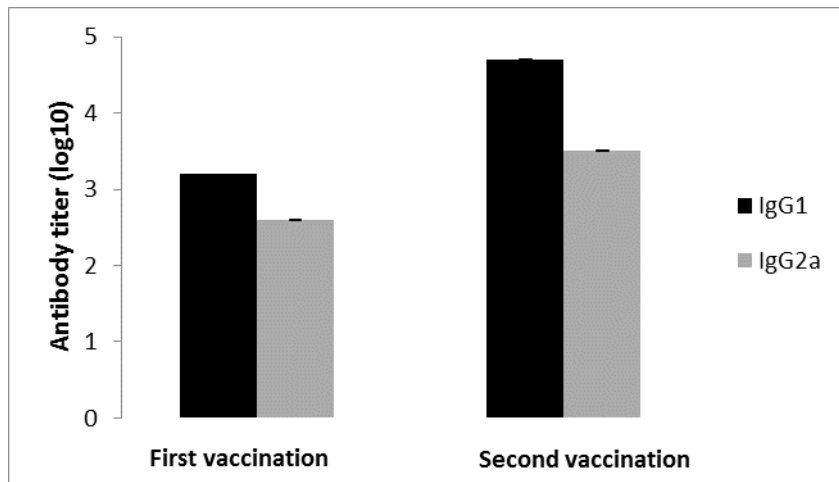


Figure 3.13 Antibody response to recombinant LktA-OmpH-PlpEC fusion protein in terms of IgG1/IgG2a distribution.

Measurement of total IgG levels gives little information about what is actually happening in immune system, and there are studies that show protective activities with low or undetectable antibody responses (Roberts *et al.*, 1990; Shahin *et al.*, 1990; Strugnell *et al.*, 1992). In this study, the antibody levels in immunized mice were much higher than that of the control groups, supporting immunoprotectivity of the constructed vaccine candidate.

3.5.2. IFN γ in mice vaccinated with recombinant LktA-PlpEC-OmpH fusion protein

Cellular immune responses are more consistent than antibody response and they can be characterized as Th1- or Th2-type responses (Kaiko *et al.*, 2008). Th2-type responses are composed of high level of IL-4, IL-10 and IgG1, Th1-type responses involve increased IFN- γ , IgG2a levels and activation of infected macrophages to induce antibacterial mechanisms. IFN- γ can activate macrophages whose phagocytosis ability plays role in bacterial clearance (Fisher *et al.*, 1988).

When disease proceeds Th1 cells release IFN- γ providing recruitment and activation of neutrophils and macrophages for intracellular bacterial killing. In addition, IFN- γ stimulates B cells to produce opsonizing and complement-fixing antibody (IgG2a in the mouse) (Mills, 2001).

In our work, in order to determine IFN- γ levels, the sera were analyzed with ELISA for Mouse IFN- γ kit. The result of cytokine ELISA is presented in Fig. 3.14.

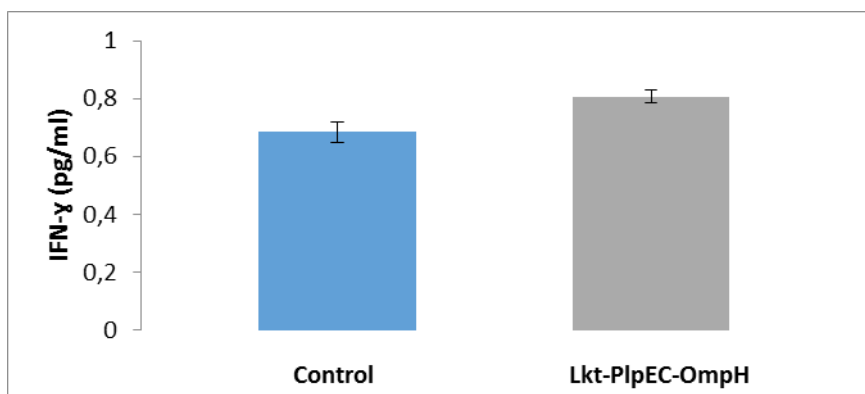


Figure 3.14 IFN- γ levels in the control and fusion protein-vaccinated mice just before challenge.

As seen in Fig. 3.14, there was no significant difference between control and vaccinated groups upon immunization with the fusion protein in terms of IFN- γ levels.

CHAPTER 4

CONCLUSION

- PlpEC-OmpH and OmpH-LktA fusion proteins were obtained throughout our former works in our laboratory. OmpH-LktA oil-based vaccine has been evaluated with respect to its potency in the present study. When formulated with oil-based adjuvant, 100% and 50% protections were recorded against *M. haemolytica* and *P. multocida* A:3, respectively.
- As a novel work in this thesis, *lktA* fragment was cloned into pET-28a (+) vector containing *plpEC* and *ompH* genes, at the upstream, and the resulting LktA-PlpEC-OmpH fusion protein heterologously expressed in *E. coli* was purified.
- ELISA tests revealing IgG1 and IgG2a levels showed that IgG1 response was higher in both first and second immunizations with our vaccine candidate. When the ratio of IgG1/IgG2a is evaluated, it seemed that increase in IgG1 level was higher than that of IgG2a in first and second immunizations.
- Although IgG1 and IgG2a levels were significantly high especially after second immunization, mice survival experiments gave conflicting results. In spite of an increased antibody response recorded in this work, cellular immune responses, specifically Th1-type response which enhances IgG2a and IFN- γ levels might not played a critical role.
- Mice immunized with LktA-PlpEC-OmpH in an oil based adjuvant were challenged with lethal doses of both of the pathogens in separate groups, however, any protection could not be mentioned since the results of

independent sets of animal experiments were rather conflicting. The potency of the current vaccine candidate remains to be clarified through further animal experiments.

- There was no significant difference between control and vaccinated groups in terms of IFN- γ levels. Th2-type response involving high level of IL-4, IL-10 and IgG1 was probably induced instead of Th1-type response during vaccinations. Therefore, in spite of high level of IgG responses against the fusion protein, lack of high survival rate in mice can be explained with low levels of IFN- γ .
- Further studies will involve improvement of purification of the current LktA-PlpEC-OmpH to achieve 100% protection against the targeted pathogens.

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APPENDIX A

STRUCTURES OF PLASMID VECTORS AND SIZE MARKERS

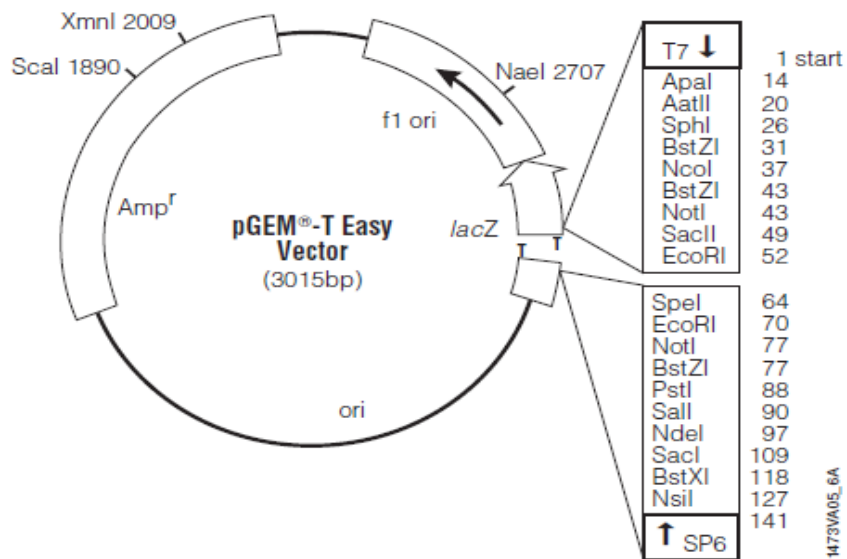


Figure A.1 pGEM[®]-T Easy Cloning Vector (Promega #A1360)

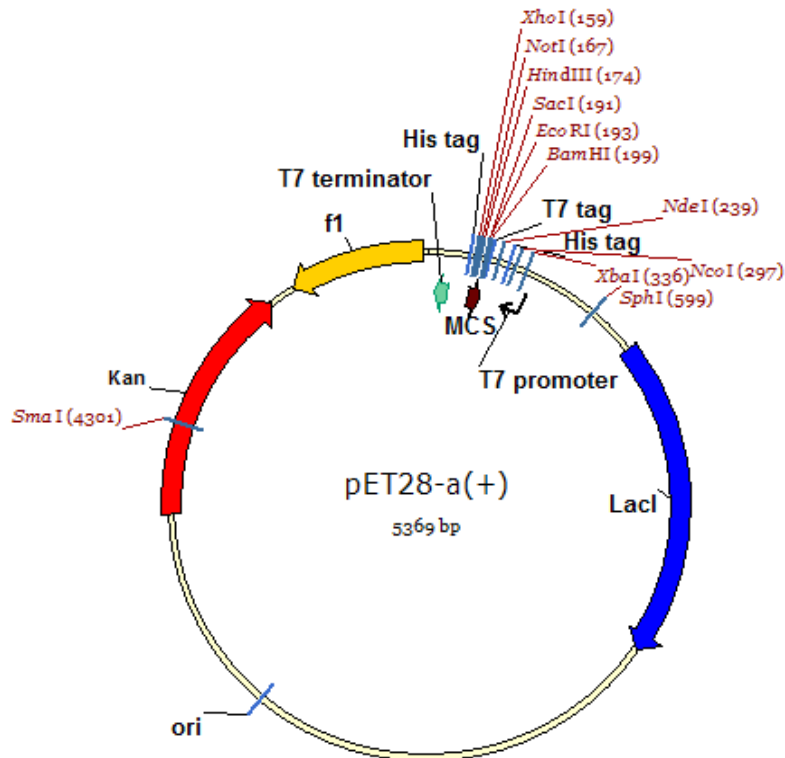


Figure A.2 pET-28a(+) His-tag Expression Vector (Novagen #69864-3)

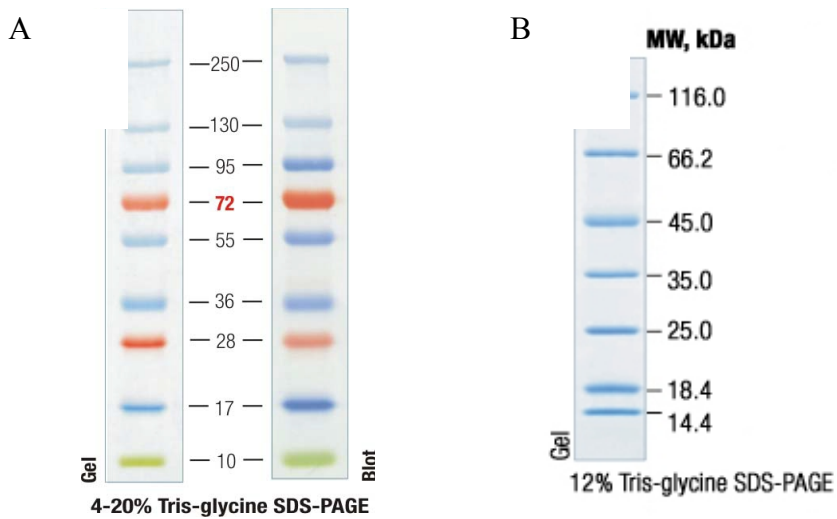


Figure A.3 PageRuler™ Plus Prestained Protein Ladder (Fermentas #SM1811) (A) and Unstained Protein Molecular Weight Marker (Fermentas #SM0431) (B).

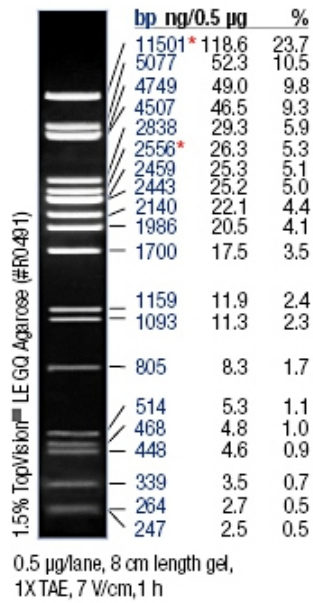


Figure A.4 Lambda DNA/*Pst*I Marker (Fermentas #SM0361)

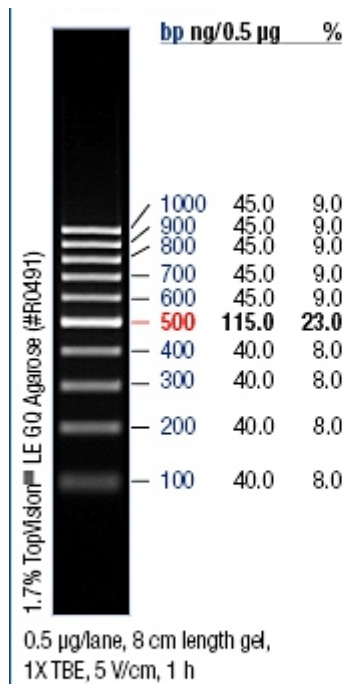


Figure A.5 100 bp DNA Ladder (GeneRuler # SM0241)

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°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°C for 15 min.

B3. Brain-Heart Infusion Broth:

- Nutrient substrate 27.5 g
- D(+)Glucose 2 g
- NaCl 5 g
- Na₂HPO₄ 2.5 g
- Distilled water up to 1000 ml

Final pH is 7.5; sterilized at 121°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°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 from 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
- NaH₂PO₄ 50 mM

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

- NaH₂PO₄ 50 mM
- NaCl 500 mM
- Urea 4 M

• C5. *E. coli* Competent Cell Preparation

C5.1. Buffer 1

- RuCl 100 mM
- KAc 30 mM
- CaCl₂ 10 mM
- Glycerol 15%

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

C5.2. Buffer 2

- CaCl₂ 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)

- Na₂CO₃ 1.59 g
- NaHCO₃ 3.88 g
- Distilled water up to 1000 ml

pH is adjusted to 9.6 and stored at 4°C.

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

- NaCl 8 g
- KCl 0.2 g
- Na₂HPO₄ 1.44 g
- KH₂PO₄ 0.24 g
- Tween-20 1 ml
- Distilled water up to 1000 ml

pH is adjusted to 7.2 and stored at 4°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
- Na₂HPO₄ 1.44 g
- KH₂PO₄ 0.24 g
- Distilled water up to 1000 ml

pH is adjusted to 7.4 and stored at 4°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
- Na₂HPO₄ 1.44 g
- KH₂PO₄ 0.24 g
- Tween-20 2 ml
- Distilled water up to 1000 ml

pH is adjusted to 7.4 and stored at 4°C.

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 ₂ O	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 ₂ SO ₄	Merck
HCl	Merck
IPTG	Sigma
Isopropanol	Merck
Kanamycin	Sigma
KCl	Merck
KH ₂ PO ₄	Merck
Luria Broth	Merck
Methanol	Merck
MnCl ₂	Merck
MOPS	AppliChem
N.N'-Methylene-bis acrylamide	Sigma
Na ₂ CO ₃	Merck
Na ₂ HPO 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
<i>Bam</i> HI	Fermentas
<i>Bgl</i> II	Fermentas
<i>Eco</i> RI	Fermentas
<i>Hind</i> III	Fermentas
<i>Not</i> I	Fermentas
T4 DNA ligase	Fermentas
<i>Taq</i> 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