DEVELOPMENT OF AN EXPERIMENTAL RECOMBINANT VACCINE FORMULATION COMPOSED OF LKTA FROM *MANNHEIMIA HAEMOLYTICA* A1 AND P31 AND LPPB FROM *HISTOPHILUS SOMNI* 8025 AGAINST BOVINE RESPIRATORY DISEASE

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ABSTRACT

DEVELOPMENT OF AN EXPERIMENTAL RECOMBINANT VACCINE FORMULATION COMPOSED OF LKTA FROM *MANNHEIMIA HAEMOLYTICA* A1 AND P31 AND LPPB FROM *HISTOPHILUS SOMNI* 8025 AGAINST BOVINE RESPIRATORY DISEASE

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Two major bacterial pathogens of Bovine Respiratory Disease (BRD), *Mannheimia haemolytica* and *Histophilus somni* are Gram-negative, opportunistic bacteria that live in upper respiratory tracts of ruminants commensally. The one of the most important virulence factor of *M. haemolytica*, leukotoxin, is responsible for lung colonization and establishment of infection. On the other hand, *H. somni* OMPs have a significant contribution to the pathogenicity of the organism. As the disease results in a destroyed animal life, its control is crucial for cattle industries. Inactive bacterin and attenuated bacteria are widely used today against BRD. However, while inactive bacterins are insufficient for protection, attenuated ones can cause outbreak in ruminants. Therefore, there is a need for development of safe and potent recombinant vaccines. The most important epitope region of LktA protein of *M. haemolytica*, and two critical immunogens of *H. somni*, p31 and LppB OMPs, were chosen for the present experimental vaccine. *lktA-p31*, and *lktA-lppB* fusions were constructed, and

expressed in *E. coli* BL21 cells. Three different experimental vaccines; LktA-p31, LktA-LppB, and LktA-p31 + LktA-LppB were formulated with an oil-based adjuvant, and tested on mice to assess the type of immune responses induced. As a result, all three formulations led to a significant increase in the level of total antigen specific IgG antibodies. Also, our combined LktA-p31 + LktA-LppB formulation induced significant levels of IgG2a antibodies indicating the induction of cellular immunity. Additionally, combined vaccine showed 52% bactericidal activity against *H. somni* as compared to the control group, allowing an assessment of the target specificity and functional activity of bactericidal antibodies. The bactericidal killing assay and the antigen-specific IgG2a response proved that our combined vaccine possesses dual protectivity against infection with *M. haemolytica* and *H. somni*.

Keywords: *Histophilus somni* 8025, *Mannheimia haemolytica* A1, LktA, p31, LppB, recombinant fusion vaccines

ÖZ

MANHEIMIA HAEMOLITICA A1'E AİT LKTA İLE *HISTOPHILUS SOMNI* 8025'E AİT P31 VE LPPB'DEN OLUŞAN DENEYSEL REKOMBİNANT AŞI FORMÜLASYONLARININ GELİŞTİRİLMESİ

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BRD'nin iki ana bakteriyel patojeni olan *Mannheimia haemolytica* ve *Histophilus somni*, Gram- negatif, fırsatçı bakteriler olup komensal biçimde sağlıklı koyun ve sığırların üst solunum yollarında yaşarlar. *M. haemolytica*'nın en önemli virülans faktörlerinden biri olan lökotoksin, patojenin akciğerlerde kolonizasyonu ve enfeksiyon geliştirmesinden sorumludur. Öte yandan, *H. somni*'nin dış zar proteinlerinin (OMP'ler) organizmanın patojenitesine önemli bir katkısı vardır. Çoğu zaman ölümlerle sonuçlanan bu hastalığın kontrolü sığır endüstrisi için kritik önem arz etmektedir. Mevcut aşılar inaktif bakterin veya zayıflatılmış bakteri olarak iki çeşittir. Fakat inaktif bakterin içeren aşılar koruyuculuk açısından yetersiz kalırken, atenüe aşılar sığır ve koyunlar arasında salgınlara sebep olabilmektedir. Bu nedenle de, daha güvenilir ve bağışıklama gücü yüksek rekombinant aşılara gereksinim vardır. *M. haemolytica*'nın lökotoksinin (LktA) en önemli epitop bölgesi ile *H. somni*'nin iki kritik immünojeni olan 31 kDa antijen (p31) ve lipoprotein B (LppB) dış zar proteinleri (OMPs), bu çalışmadaki deneysel aşı formülasyonu için

seçilmiş antijenlerdir. *lktA-p31* ve *lktA-lppB* genetik füzyonları oluşturulup, protein ekspresyonları *E. coli* BL21 hücrelerinde gerçekleştirilmiştir. Üç farklı aşı; LktA-p31, LktA-LppB ve LktA-p31 + LktA-LppB, rekombinant proteinlerin yağ bazlı bir adjuvan ile formüle edilmesiyle hazırlanmış ve hangi tip immün yanıtı indüklendiğini belirlemek için farelere uygulanmıştır. Sonuç olarak, üç rekombinant aşı adayı da, antijene özgül IgG antikorlarının seviyesinde anlamlı bir artışa yol açmıştır. Kombine aşı formülasyonumuz (LktA-p31 + LktA-LppB) ise, hücresel immün yanıtının önemli bir göstergesi olan IgG2a seviyelerinde istatistiksel olarak önemli bir artış sağlamıştır. Ayrıca, kombine aşı adayı, kontrol grubuna kıyasla *H. somni*'ye karşı %52 bakterisidal aktivite göstermiş ve bu etki bakterisidal antikorların hedef özgüllüğü ve fonksiyonel aktivitesinin değerlendirilmesine olanak tanımıştır. Bakterisidal aktivite testinin anlamlı sonucu ile antijene özgül IgG2a antikor yanıtının indüklenmesi, kombine aşı adayımızın her iki bakteriyel enfeksiyona karşı koruyuculuğa sahip olduğunu açıkça göstermektedir.

Anahtar Kelimeler: *Histophilus somni* 8025, *Mannheimia haemolytica* A1, LktA, p31, LppB, rekombinant fuzyon aşılar

To my beloved family

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p31	*Histophilus somni* 31 kDa antigen
LppB	*Histophilus somni* lipoprotein B
LktA	*M. haemolytica* leukotoxin
bp(s)	Base pair(s)
BRD	Bovine respiratory disease
LPS	Lipopolysaccharide
IPTG	Isopropyl- β -D-thio-galactoside
ELISA	Enzyme-linked immunosorbent assay
i.p.	Intraperitoneal
IgG	Immunoglobulin G
ATCC	American Type Culture Collection

CHAPTER 1

INTRODUCTION

1.1 Bovine Respiratory Disease

Bovine respiratory disease (BRD) is one of the significant problems in the cattle industry, as it is the most common disease among the feedlot calves. It leads to economic losses due to the high mortality rate. BRD is a complex of conditions, therefore it includes several infections (Snowder *et al*., 2006). Besides several viral and bacterial infections, since they lead to a decrease in cattle immunity, unfavorable environmental factors such as the ones during the transportation of cattle seem to be the precursor of the disease. Table 1.1 summarizes the pathogens related to the disease, and other outside factors.

Two of the major bacterial pathogens involved in BRD, Histophilus somni and Mannheimia haemolytica are actually the members of healthy flora in the upper respiratory tract of bovines. In case of a stress condition, and generally after a viral infection leading to the suppression of the immune system of cattle, these resident bacterial pathogens act as an opportunist in the lower respiratory tracts of animals and lead to the rousing of disease (Yarnall *et al*., 1989; Rice *et al*., 2007).

Table 1.1 Summary of factors lead to BRD (Griffin *et al*., 2010).

Environmental Factors	Nutritional deficiencies, dust, cold and hot weather, dampness, anxiety, fatigue, injury, irritant gases, hunger, shipping distance, dehydration, surgery and ventilation.
Bacterial pathogens	*Histophilus somni*, *Mannheimia haemolytica*, *Mycoplasma bovis*, *Pasteurella multocida, Bibersteinia trehalosi,* and *Arcanobacterium pyogenes.*
Viral pathogens	Parainfluenza type 3 virus (PI3V), infectious bovine rinotracheitis virus (IBRV), bovine viral diarrhea virus (BVDV), Adenovirus, bovine respiratory syncytial virus (BRSV), bovine herpes virus-1 (BHV-1), , Rhinovirus,, Enterovirus, Malignant catarrhal fever (MCF) virus, Reovirus.

1.1.1 Clinical Symptoms and Diagnosis

BRD affects the cattle growth, fertility and grade of carcass; therefore, diagnosis and immediate treatment is critical to decrease these damaging effects. Since the disease process is led by many different factors, diagnosis cannot be achieved by observing a single symptom or a pathogen. An overall evaluation of animal appearance and clinical signs should be performed for characterization of BRD. After clinical

evaluation, lesions from affected organs should be examined under microscope, and the pathogens should be identified prior to any treatment (Fulton and Confer, 2012).

At the beginning of the disease, minor depression and decreased appetite are observed as clinical signs. When the disease progresses, animals start to deny eating as well as nasal and ocular discharges intensify. Other symptoms of BRD are cough, fever, and as the disease becomes more severe, dyspnea, or tachypnea. If the animal does not get the treatment at the early stages of the disease, lungs are permanently destroyed by colonized pathogens, and generally the animal dies (White and Renter, 2009).

1.1.2 Treatment

Although vaccines for bacterial agents of BRD are available, their efficacy is inadequate, and their usage is not very common. Therefore, antimicrobial drugs are highly used for disease control and treatment. For prevention of spreading of BRD, an application called metaphylaxis is usually preferred, and a group of feedlot cattle at risk of getting infected are medicated with antimicrobial agents (Welsh *et al.*, 2004).

On the other hand, since the animals with BRD have apparent clinical signs, symptomatic treatment is also essential. For this purpose, NSAIDs (nonsteroidal anti-inflammatory drugs) are applied together with antimicrobials. When the disease progresses more severely, pneumonia leads to difficulties in breathing. Inflammatory mediators have damaging effects on the lungs, and thus alveolar gas exchange is impaired. Besides the antipyretic activities of NSAIDs, they also inhibit the production or effect of these inflammatory mediators; therefore, they are very important in the treatment to improve the clinical symptoms of the animal (Elitok and Elitok, 2004).

1.2 Physical features of *M. haemolytica* and *H. somni*

M. haemolytica is a gram-negative, aerobic, non-motile, small rod- and coccobacillus-shaped bacterium, and among the BRD-associated bacteria, it is the most important pathogen. It is usually isolated from severe cases of BRD in bovine, and 60% of the isolates belong to the serotype A1 (Confer *et al*., 2006). In Gram's staining, *M. haemolytica* is often stained bipolar. Most strains show faint β-haemolysis on bovine blood agar, and the colonies are 1-2 mm in width after 24 h of incubation at 37 ^{o}C. All strains of *M. haemolytica* ferment D-sorbitol, D-xylose, maltose, and dextrin, but L-arabinose or glucosides are not fermented at all. While strains are negative for ornithine decarboxylase and β-glucosidase, they are positive for α-fucosidase (Angen *et al*., 1999). Table 1.2 shows the biochemical activities of *M. haemolytica.*

H. somni is a gram-negative, facultative anaerobe, non-motile, non-spore-forming, coccobacillus-shaped bacterium, and it is in Pasteurellaceae family, like *M. haemolytica*. It is one of the three important BRD-associated bacteria, and causes thrombotic meningoencephalitis, pneumonia, myocarditis, arthritis, reproductive failure, and septicemia in cattle (Geertsema *et al*., 2011). Haemolysis varies, while some strains are non-haemolytic, some can be α-haemolytic or β-haemolytic on calf blood agar. Colonies are shaped as pinpoint after 24 h incubation at 37 ^{o}C, and they become 1–1.5 mm in diameter after 48 h. Most isolates show indole production and yellowish pigmentation. Thiamin monophosphate is required in the growth medium, and increased growth is observed in the presence of 5-10% CO_2 (capnophilia). *H. somni* does not ferment sucrose, D-galactose, D-fructose, maltose or trehalose. Urease, catalase, Voges–Proskauer, and ornithine decarboxylase tests are negative, and oxidase reaction is positive (Angen *et al*., 2003). In Table 1.3, biochemical properties of *H. somni* are summarized.

Table 1.2 Biochemical properties of *M. haemolytica* (Angen *et al*., 1999).

Haemolysis	(+)
Ornithine decarboxylase	–
L-Arabinose	–
D-Sorbitol	+
D-Xylose	+/(+)
Maltose	+
Dextrin	+
Glucosides	–
Gentiobiose	–
NPG (β-glucosidase)	–
Meso-Inositol	d
ONPF (α-fucosidase)	+
ONPX (β-xylosidase)	d
ONPG (β-galactosidase)	d
Indole	–
D-Melibiose	–

+, Strong or delayed positive

(+), Weak or very weak positive

–, Negative

d, Positive or negative

Table 1.3 Biochemical properties of *H. somni* (Angen *et al*., 2003).

Haemolysis	d
Capnophilia	+
Yellowish pigmentation	+
V-factor dependence	–
X-factor dependence	–
Catalase	–
Oxidase	+
Urease	–
Voges-Proskauer (37 °C)	–
Indole	+*
Ornithine decarboxylase	–
Sucrose	–
D-Galactose	–
D-Fructose	–
Maltose	–
Trehalose	–

*, Deviating strains occur

+, Only positive reactions

–, Only negative reactions

d, Positive or negative reactions

1.3 Classification of *M. haemolytica* and *H. somni*

1.3.1 Classification of *M. haemolytica*

In 1921, identification of three groups of bovine pasteurellae was reported, and atypical strains were classified in group I of *'Bacillus bovisepticus'* (Jones, 1921). Then, by Newsom & Cross (1932), these atypical strains were renamed as *Pasteurella haemolytica.* According to what they ferment, two biotypes were identified; biotype A was L-arabinose fermenter, and biotype T was trehalose fermenter. Throughout 17 serotypes identified, 3, 4, 10, and 15 were associated with

biotype T, and it was classified as *Pasteurella trehalosi* in 1990. Also, biotype A was subclassed into A1, A2, A5, A6, A7, A8, A9, A12, A13, A14, A16, and A17. Except for A17, other serotypes are now identified as *Mannheimia haemolytica*, and serotype 11 is reclassified as *Mannheimia glucosida* (Angen *et al*., 1999).

1.3.2 Classification of *H. somni*

Histophilus has been defined as a novel genus within the family *Pasteurellaceae.* The bacteria previously isolated and described as separate species, *Histophilus ovis*, *Haemophilus somnus,* and *Haemophilus agni*, are all now renamed as *Histophilus somni.* Currently, this novel genus has only one species (Angen *et al*., 2003).

The taxonomic classification of *M. haemolytica* and *H. somni* are given in Table 1.4.

Table 1.4 The taxonomic classification of *M. haemolytica* and *H. somni*.

Kingdom	Bacteria	Bacteria
Phylum	Proteobacteria	Proteobacteria
Class	Gammaproteobacteria	Gammaproteobacteria
Order	Pasteurellales	Pasteurellales
Family	Pasteurellaceae	Pasteurellaceae
Genus	*Mannheimia*	*Histophilus*
Species	*Mannheimia haemolytica*	*Histophilus somni*

1.4 Virulence Factor

1.4.1 Virulence factors of *M. haemolytica*

1.4.1.1 Leukotoxin

M. haemolytica has a range of virulence determinants that enables the bacterium to colonize the respiratory surfaces, and escape from the immune system of the host. One of these determinants, leukotoxin, has a significant role in the pathogenesis and causes lung disruption, which is a characteristic symptom in BRD (Highlander, 2001).

M. haemolytica leukotoxin is a 102 kDa exotoxin, and it is a member of RTX (repeats in toxin) family of bacterial cytolysins. These proteins have several glycine and aspartates amino acids at their C-terminal, which are highly conserved in the RTX family, and they are critical in the stimulation of toxin activity. This toxin is secreted throughout the exponential phase of the growth in all serotypes. Unlike other cytolysins, target cells of leukotoxin are specific. It can only affect ruminant leukocytes and platelets (Lafleur *et al*., 2001). At high concentrations, leukotoxin leads to cytolysin in the bovine neutrophils and macrophages (Hsuan *et al*., 1999). This cytotoxic effect is due to the toxin's ability to form pores on the target cell surface. A calcium influx arises through these pores on the cell membrane, and elevated calcium in the cell results in a series of apoptotic events (Clinkenbeard *et al*., 1989; Maheswaran *et al*., 1992).

A polycistronic operon containing four genes (*lktC*, *lktA*, *lktB*, and *lktD*) is responsible for the synthesis, activation, and secretion of leukotoxin (Figure 1.1). While *lktC* is at the upstream of the structural gene *lktA*, *lktB* and *lktD* are at its downstream. The *lktA* gene encodes for the 953 amino acid protein LktA, which is first synthesized as an inactive form, proLktA, and after posttranslational

modifications including fatty acid acylation, it becomes a biologically active toxin. The enzyme responsible for this acylation process is transacylase, and it is encoded by the *lktC* gene. The products of *lktB* and *lktD* genes are required for the transportation of acylated LktA to the extracellular environment.

The LktA has some important domains like calcium binding, pore forming, and receptor binding. The N-terminus of LktA is involved in receptor binding, and hydrophobic regions are responsible for the cytolytic activity of the toxin. There is a ~70 amino acid signal peptide at the C-terminus of LktA essential for the secretion of toxin. Also, most of the epitope sequences take place in the C-terminus of LktA, especially in a 229 amino acid region in there (Sun *et al*., 1999; Highlander, 2001; Jeyaseelan *et al*., 2002).

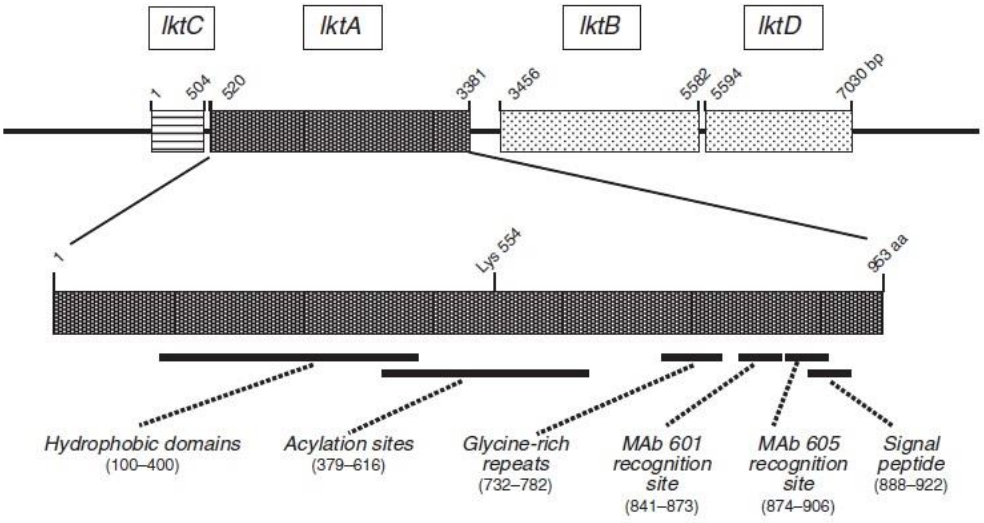

Figure 1.1. Linear model of LktA and several functional domains of it (Jeyaseelan *et al*., 2002).

1.4.1.2 Surface proteins and LPS

Attachment of pathogenic agents to epithelial surfaces and their colonization are required for the development and progress of BRD. The capsule structure of *M. haemolytica* has importance in pathogenesis by different mechanisms. One of them is the ST capsular polysaccharide (CP) providing adherence to the mucosal surfaces of the lung. Also, this ST1 CP gives resistance against complement mediated lysis of bacteria and prevents phagocytosis by neutrophils (Confer *et al*., 1990).

Adhesins are also essential for the attachment of pathogens to surfaces in the upper respiratory tract. Interaction of a 68 kDa adhesin (MhA) of *M. haemolytica* with tracheal epithelial cells was demonstrated. Besides this interaction, MhA specifically binds to bovine neutrophils with glycoprotein receptors. This binding activates neutrophils, which results in oxidative burst (Mora *et al*., 2006).

Outer membrane proteins (OMPs) and lipoproteins are other important virulence determinants of *M. haemolytica.* Some of the OMPs are; a 38-kDa surface-exposed lipoprotein (Lpp38), a 35-kDa PomB, a 32-kDa OmpA-like protein (PomA), and iron-regulated OMPs (IROMPs) including a 77 kDa protein and transferrin-binding proteins, Tbp1 and Tbp2. It is shown that these IROMPs are chemotactic substances, and they impair the phagocytosis capacity of neutrophils and inhibit intracellular killing. Thus, proliferation of pathogenic bacteria in the respiratory tract becomes easier. Also, antibodies raised against some of these OMPs confer resistance in experimental *M. haemolytica* infections, and stimulate phagocytosis and complement-mediated killing.

Lastly, LPS is an essential outer membrane component of the cell walls of gram-negative bacteria, and another virulence determinant of *M. haemolytica*. LPS contributes to the pathogenicity of bacterium by several mechanisms such as

induction of leukocytes to produce several inflammatory cytokines. LPS promotes the expression of several proinflammatory cytokine genes. It is a triggering factor for increment of IL-1β and IL-8 via TNF-alpha, leading to the neutrophil influx, resulting in inflammation. Finally, LPS damages the endothelial cells in the bovine lung (Iovane *et al*., 1999; Highlander, 2001; Jeyaseelan *et al*., 2002).

1.4.2 Virulence factors of *H. somni*

H. somni has various virulence factors which take roles in the attachment to host cells, inhibition of the function of phagocytic cells and the complement system, immunoglobulin binding, and uptake of iron from host. Also, outer membrane proteins (OMPs) have huge contribution to the pathogenicity of the organism.

1.4.2.1 Outer Membrane Proteins

Surface-exposed proteins play an essential role in the *H. somni* pathogenicity and immune response of the host. Antigenic proteins were searched by determining their reactivity against convalescent sera from cattle. Also, challenge studies were conducted to understand which of these immunoreactive antigens are protective. Two important OMPs of *H. somni* identified are a 40 kDa antigen (LppB), and a 31 kDa antigen (p31). It was shown that the hyperimmune serum against the virulent *H. somni* was able to detect the LppB (Theisen *et al*., 1993). In another work, antisera collected from bovines experimentally infected with *H. somni* thromboembolic meningoencephalitis isolates gave reaction to p31 (Won and Griffith, 1993). A 78 kDa OMP antigen was also found to be immunoreactive in Western blots done with convalescent-phase serum, but this antigen did not confer any protective effect. On the other hand, recombinant LppB and p31, together with a commercial vaccine for different bacterial diseases, elicited 100% protection against *H. somni* challenge in mice (Guzmán-Brambila *et al*., 2012).

There are also IROMPs, critical for the survival of pathogen in iron deficient environments. In case of a lack of available iron, *H. somni* produces transferrin-binding proteins (TBP) that are capable of binding to bovine transferrin, but not to any other ruminant transferrins (Corbeil, 2015). This showed that TBPs can be accounted for the host specificity of *H. somni*. It was also demonstrated that five minutes incubation of *H. somni* in fetal bovine serum before its inoculation into mice enhanced the virulence of pathogen (Corbeil, 2007).

1.4.2.2 Immunoglobulin-binding proteins

Other major antigenic proteins recognized by convalescent serum are immunoglobulin-binding proteins (IgBPs) which are high molecular weight proteins. These IgBPs were found to be placed on the surface of all tested pathogenic *H. somni* strains and bind to the Fc portion of bovine IgG2. An antigenic 270 kDa protein called IbpA was also identified which appeared as more than one bands of varying size between 76 to350 kDa on the SDS-PAGE (Sandal and Inzana, 2010). Subsequent studies showed that IbpA forms a fibrillar network on the cell surface and also leaks out to the culture supernatant (Corbeil *et al*., 1997). Therefore, the IbpA is described as a secreted protein as well as a surface protein.

Three important domains of IbpA are A3, A5, and DR2. The IbpA3 subunit has an RGD motif that provides attachment to mammalian cells (Geertsema *et al*., 2008). On the other hand, Geertsema *et al*. (2011) showed that animals immunized with the IbpA DR2 subunit vaccine had negative lung cultures for *H. somni.* Therefore, they concluded that IbpA DR2 subunit conferred protection against *H. somni* induced bovine pneumonia.

1.5 General Host Immune Responses to Pathogenic Bacteria

There are two types of immune responses against pathogens, innate and adaptive immunity. Skin and mucosal surfaces, and intestinal flora are the components of the innate immune system. They protect the organism against pathogens physically. Cellular members of innate immunity are dendritic cells (DCs), macrophages, neutrophils, and natural killer (NK) cells. On the other hand, B and T lymphocytes are the key members of the adaptive immune system. Antigen-presenting cells (APCs), DCs, macrophages, and B cells notice the foreign molecules, and APCs present specific epitopic regions to T cells via major histocompatibility complexes (MHCs). After that, T cells start to produce cytokines specific to these epitopes. Therefore, innate and adaptive immunity work together in defense of host against pathogens (Vivier *et al*., 2011).

According to the class of MHC that is to be recognized, T cells are separated into two main groups. One type of T cells can only be activated by MHC-II molecule and named as T helper (T_h) cells, whereas the ones stimulated by the MHC-I complex are called cytotoxic T (T_c) cells. Th1 type immune response is correlated with an increased level of IFN-gamma and IgG2a. Since increased levels of IFN-gamma induce macrophages, the induction of Th1 type response is vital for the clearance of intracellular microorganisms. Th2 type immunity, on the other hand, is essential for the protection against extracellular pathogens (Elgert, 2009). IFN-gamma also stimulates B cells for class switch to IgG2a which is critical for fixation complement through Fc portions of IgG2a antibodies, thus, for the antibody-mediated clearance of bacteria (Charoenvit *et al*., 2004; Baldwin *et al*., 2009).

1.5.1 Host-BRD Pathogens Interaction

BRD pathogens have evolved to develop different mechanisms to evade the protection systems of the host. Figure 1.2 summarizes these immune evading mechanisms of BRD-related pathogens. As the figure represents, BHV-1 damages the epithelial cells of the respiratory tract by infecting them, hence, physical barriers of immune system are evaded. Opportunistic bacteria like *M. haemolytica* and *H. somni* take advantage of this necrotic, physically damaged epithelium, to migrate toward the lower respiratory tract, and to colonize. A significant virulence factor of *M. haemolytica*, leukotoxin has cytolytic effect on leukocytes, allowing it to escape from phagocytosis. On the other hand, IgBPs of *H. somni* attach to immunoglobulins, thereby preventing their binding to surfaces of the pathogen (Corbeil, 2015).

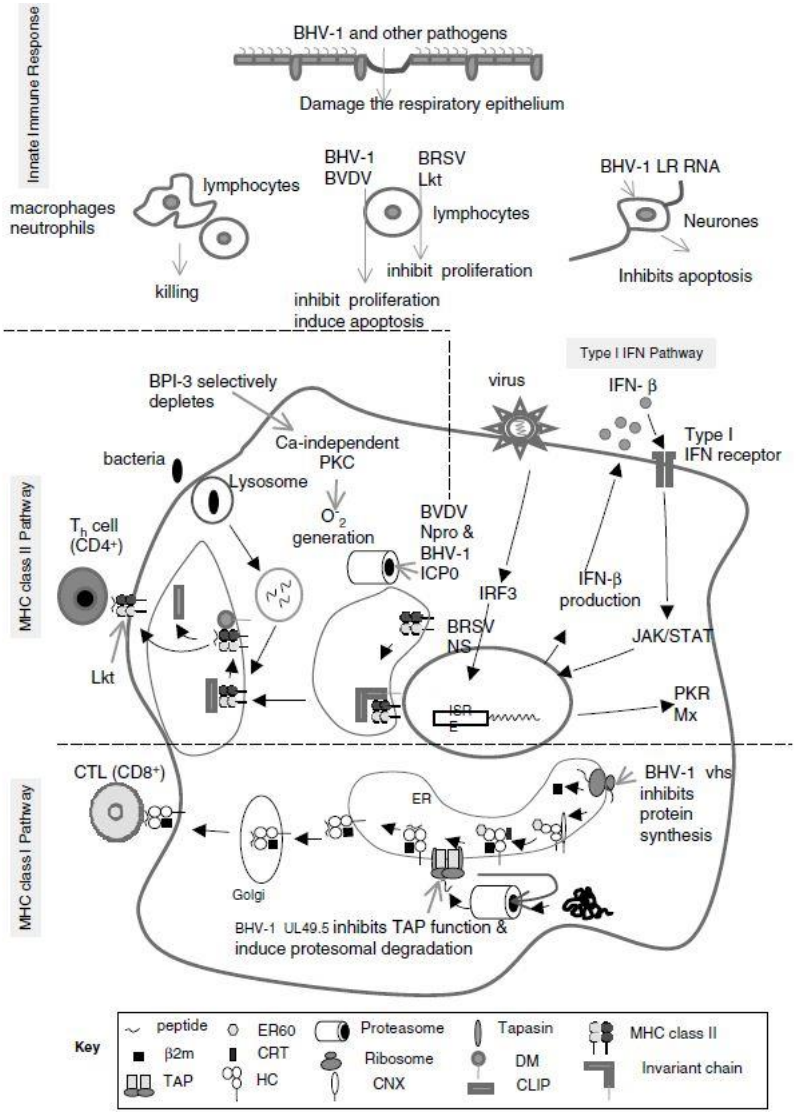

Figure 1.2. Immune evasion by pathogens of the bovine respiratory disease complex (Srikumaran *et al*., 2007).

1.6 Vaccine Studies against *M. haemolytica and H. somni*

Besides the traditional vaccine approaches like attenuated or killed whole-cell vaccines, there are also subunit vaccines that contain specific immunogenic proteins or other metabolites of the pathogen. Since these subunit vaccines do not include any unrelated parts of the pathogen, they induce the host's immune system more precisely

and have much fewer side effects. Novel vaccine approaches against especially gram (-) agents of BRD are usually focused on the OMPs (Carpenter *et al*., 2007).

M. haemolytica has various virulence factors that help lung colonization and have a role in the evasion of host defense mechanisms. Among these factors, LPS, OMPs, and especially leukotoxin induce the production of inflammatory mediators. Therefore, they can be included in prospect vaccines against BRD. Studies to prevent BRD focused on the use of bacterins, live vaccines, and antisera at the beginning of the twentieth century. Later, it was shown that *M. haemolytica* bacterins were inefficient to protect, and they even could lead to augmentation of disease course. Then, antigens without bacteria were studied to develop an improved vaccine (Durham *et al*., 1986). Culture supernatant of *M. haemolytica* A1 was purified from bacteria by centrifugation, and this supernatant, including leukotoxin, together with an adjuvant, was given to calves subcutaneously. This vaccination provided some protection to calves against challenge with live *M. haemolytica* (Shewen and Wilkie, 1988). In another study, SAC86, SAC87, SAC88, and SAC89 chimeric proteins composed of some immunogenic regions from PlpE and Lkt were constructed. Mice were vaccinated with various amounts of these recombinant proteins. Sera from the vaccinated mice had both leukotoxin neutralizing activity and complement-mediated bactericidal effect. rPlpE- and native Lkt-specific antibodies were detected in the sera of these mice. It was also shown that a high level of neutralizing antibodies against Lkt, protected calves from the experimental *M. haemolytica* challenge (Ayalew *et al*., 2008). Also, Confer *et al*. (2009) showed that when cattle were immunized with killed whole-cell bacteria together with a PlpE-Lkt chimeric protein (SAC89) in an oil-based adjuvant, lungs of vaccinated cattle had 75% lower lesion scores than controls. This study demonstrated that chimeric proteins composed of a surface antigen and native Lkt epitopes could prevent shipping fever, and can be good candidates for experimental vaccines against BRD. In a more recent study conducted by Ayalew *et al*., immunization of calves with *M. haemolytica* vesicles

raised antibodies against surface antigens as well as leukotoxin, and significant decrease in clinical signs and lesion scores after challenge was observed (2013).

Furthermore, sera of *M. haemolytica* and *H. somni* bacterins immunized calves were used to identify some immunogenic proteins of these two pathogens. Among them, the ABC system proteins have similarities between both organisms, and have been associated with virulence (Alvarez *et al*., 2015). Therefore, these proteins can also be considered as good candidates for future experimental BRD vaccines. Some of the *M. haemolytica* vaccines tested are listed in Table 1.5.

Table 1.5 Various *Mannheimia haemolytica* vaccines tested under experimental and field trials (Confer & Ayalew, 2018).

		Efficacy		
Vaccine type	Timeframe	Experimental	Field	Commercially available
Bacterin	Prior to 1990s	Variable	No	No longer
Sodium Salicylate extract	1980s	Variable	Unknown	No
Potassium thiocyanate extract	1980s	Variable	Unknown	No
Saline extract	1980s	Efficacious	Unknown	No
Live – attenuated	1980s	Efficacious	Efficacious or ineffective	No longer
Live – streptomycin-dependent	1985-present	Variable	Efficacious or ineffective	Parenteral or intranasal vaccination
Culture supernatant	1988 to present	Efficacious	Variable	Yes
Bacterin toxoid	1989 to present	Efficacious	Variable	Yes
Capsular polysaccharide	1990s	Variable or poor	Unknown	No
Proprietary extract	1990s	Efficacious	Somewhat efficacious	No longer
Recombinant chimeric protein	2001-present	Partially efficacious	Unknown	No
Ghosts	2003	Efficacious	Unknown	No
Recombinant single protein	2003–3006	Partially efficacious	Unknown	No
LKT-deficient mutant	2012–2013	Efficacious	Unknown	No
Vesicles	2013-present	Efficacious	Unknown	No

Commercially available bacterin vaccines are commonly preferred against *H. somni* infections. Although the protection ability of these bacterins against thrombotic meningoencephalitis was shown, protectivity against other *H. somni*-

related disease is debatable, and there are only very few studies on their protection against respiratory disease (Stephens *et al*., 1982; Humphrey and Stephens, 1983; Geertsema *et al*., 2011). Also, several recombinant subunit vaccine studies for this animal pathogen have been conducted and tested in mice models, but they are found to be moderately effective, as inactive whole cell vaccines. Thus, recombinant vaccines including multi-subunit antigens can be increase the effectiveness of vaccines (Madampage *et al*., 2015). It is today well-established that infection with *H. somni* indeed leads to an increase in IgE antibodies, and immune response based on IgE induction is associated with the severity of disease (Gershwin *et al*. 2005). It was also shown that killed *H. somni* vaccines induce specific IgE response that eventually ends up with serious side effects (Ruby *et al*. 2000). Also, loss of protective surface proteins during the preparation of commercial bacterins is one problem reducing the effectiveness of these products. Moreover, the use of *H. somni* bacterins might induce endotoxic reactions, and even results in death of the animal (Ellis and Yong 1997; O'Toole and Sondgeroth, 2015). This endotoxicity effect of bacterins becomes even more drastic when endotoxin from multiple bacterial sources are combined during successive administration of bacterin vaccines against different gram (-) pathogens (Richeson *et al*., 2019). Due to these adverse effects, and inefficient protection of traditional bacterins, novel vaccine studies started.

Among various virulent determinants of *H. somni*, OMPs and IgBPs draw high attention as candidates for prospect *H. somni* vaccines. In a recent study, three subunits of IbpA; IbpA3, IbpA5, and IbpADR2 were recombinantly produced, and their protectivity were compared with formalin-killed *H. somni*, live *H. somni* cells (convalescent immunity), and culture supernatant containing IbpA shed. Interestingly, the *H. somni* culture supernatant was found to be the most successful in terms of immunization and protection (Geertsema *et al*., 2008). Due to the extracellular exposure of OMPs, OMP-specific antibodies can bind to the cell, and lead to bacterial lysis via complement-mediated killing. Guzman-Brambila *et al*.

(2012) recombinantly produced two *H. somni* OMPs, LppB (40 kDa antigen) and p31 (31 kDa antigen) proteins, and formulated them with a commercial bacterin containing six *Clostridium* species, and aluminum hydroxide adjuvant. Rabbit and sheep subjects immunized with this experimental vaccine produced antibody responses, and immunized mice were protected against *H. somni* septicemia. This study showed the importance and protective capability of OMPs against *H. somni* infections.

Lastly, it should be considered that the two most important pathogens of upper respiratory tract infections in cattle, *M. haemolytica* and *H. somni* are gram (-) bacteria, thus, their whole-cell vaccines have a huge disadvantage in terms of the endotoxin amount contained. This situation is the biggest drawback of the bacterins, especially in the vaccination of pregnant cattle. Therefore, development and use of less reactive subunit vaccines with minimal endotoxin are needed.

1.7 The Present Study

In the present study, it was aimed to develop a highly protective and less-reactionary, novel and safe recombinant subunit vaccine candidate that confers high protection against two major bacterial pathogens of BRD, *M. haemolytica* and *H. somni*. In a previous study conducted in our laboratory, an epitope region from *lkt* gene of *M. haemolytica* A1 was cloned to create a fusion with the *ompH* gene from *P. multocida*. Then, OmpH-LktA fusion protein was purified and formulated with an oil-based adjuvant. Mice immunized with this vaccine were 100% and 50% protected from lethal *M. haemolytica* and *P. multocida* challenges, respectively (Çırçır, 2014). On the other hand, combination of two *H. somni* OMPs, recombinant LppB and p31 exerted a protective effect against *H. somni* (Guzman-Brambila *et al*., 2012). In view of these former works, the most important epitope region of LktA protein of *M. haemolytica*, and two critical immunogens of *H. somni*, p31 and LppB OMPs, were chosen to be included in the present experimental vaccine. Then, two different

genetic constructs, *lktA-p31*, and *lktA-lppB* were developed in pET28a(+) expression vector. Recombinant fusion proteins were expressed in *E. coli*, purified, and formulated with an oil-based adjuvant, and employed on mice. In this way, two experimental vaccine candidates consisting of the fusion protein constructs that can be used against both *M. haemolytica* and *H. somni* were obtained. The vaccines were next tested in mice either together and separately to assess the type of immune responses induced. As a result, it was observed that the vaccine candidate composed of the combination of two fusion proteins successfully elicited humoral and cellular immune responses in mice.

CHAPTER 2

MATERIALS AND METHODS

2.1 Bacterial Strains and Plasmids

Bacterial strains with their characteristics and sources are listed in Table 2.1. Plasmids employed in this study with their key features are given in Table 2.2. The structures of cloning and expression vectors were presented in Appendix A.

Table 2.1 Bacterial strains used in this study and their properties.

Strain	**Characteristics**	**Source and Reference**
H. somni 8025	Bovine strain	American Type Culture Collection (ATCC 43625)
M. haemolytica	Serotype A1, bovine strain	American Type Culture Collection
E. coli DH5α	F^- ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *supE44*λ*- thi-1 gyrA recA1 relA1 endA1 hsdR17*	American Type Culture Collection
E. coli BL21 (DE3)	F^- *ompT gal dcm lon hsd*S_B(r_B^- m_B^-) λ(DE3 [*lacI lacUV5-T7 gene 1 ind1 sam7 nin5*])	Novagen, Merck (Germany)

Table 2.2 Cloning and expression plasmids used in this study.

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

2.2 Culture media

The components of culture media and media preparations were given in Appendix B.

2.3 Buffers and solutions

The components of buffers and solutions were stated in Appendix C.

2.4 Chemicals and enzymes

The enzymes and chemicals used in this study were listed in Appendix D with their suppliers.

2.5 Growth and Maintenance Conditions of bacterial strains

H. somni and *M. haemolytica* were grown on blood agar plates (Orlab, TURKEY). For liquid culture, Brain Heart Infusion Broth (BHI broth, Merck, Germany) supplemented with yeast extract and thiamine HCl (supplemented BHI broth, Appendix B) was preferred to grow *H. somni*, and BHI broth was used for the growth of *M. haemolytica*. *H. somni* cultures either on blood agar plates or in supplemented BHI broths were grown in candle jars at 37 ^oC. *M. haemolytica* cultures in BHI broth

were grown with shaking at 37 ^{o}C. The same liquid media and growth conditions were used for challenge experiments of both organisms. Two different strains of *E. coli*, DH5α and BL21 were used in cloning and protein expression experiments, respectively, and they were grown in Luria agar (LA, Merck, Germany) plates or Luria Broth (LB, Merck, Germany) media. The cultures were stored on blood agar plates or LA plates at 4^{o}C and passaged to new plates monthly. For long term storage of strains, cultures were grown in their liquid media until mid-log phase, mixed with 50% glycerol in 2 ml Eppendorf tubes and kept at -80 ^{o}C. LA and LB media were combined with antibiotics whenever necessary. For preparation of such culture media, a final concentration of 100 μg/ml was used for Ampicillin and 30 μg/ml was used for Kanamycin.

2.6 Primer Design

Primers utilized in the amplification of genes encoding 31 kDa antigen and LppB of *H. somni*, and *lktA* gene fragment of *M. haemolytica* A1 were designed according to complete genome sequences of organisms (GenBank accession numbers NC_010519.1 and NC_021082.1 respectively).

While designing primer pairs, suitable restriction cut sites were added. Restriction enzyme site of *Bam*HI (5'-GGATCC-3') was added into forward primers of the genes encoding 31 kDa antigen and LppB, and into reverse primer of *lktA* gene fragment. Also, *Sac*I cut site, (5'-GAGCTC-3') was added into reverse primers of the genes encoding 31 kDa antigen and LppB, and *Nhe*I cut site (5'-GCTAGC-3') was added into forward primer of *lktA* gene fragment (Table 2.3).

Table 2.3 Primers used in PCR. Restriction enzyme cutsites are bolded.

Gene name	**Primer name**	**Nucleotide sequence**	**Size of PCR product**
31 kDa antigen gene	p31_FP	5’ GGT**GGATCC**ATGAAACTATCACG 3’	847 bp (with primers)
31 kDa antigen gene	p31_RP	5’ CCT**GAGCTC**AGAGAGATTATTTTT C 3’	
lppB gene	lppB_FP	5’ TAAAGTAACGGAGA**GGATCC**ATG AA 3’	872 bp (with primers)
lppB gene	lppB_RP	5’ GTTTAA**GAGCTC**TTAAATTACCAT ATCCACG 3’	
lktA	lktA_FP	5’ ATTC**GCTAGC**TCTGATTCGAACTT A 3’	412 bp (with primers)
lktA	lktA_RP	5’ CAA**GGATCC**CATTGAAGTTGGAG C 3’	

2.7 Polymerase Chain Reactions (PCR)

For the amplification of the genes encoding 31 kDa antigen and LppB, as well as *lktA* gene fragment, Phire Green Hot Start II PCR Master Mix (Thermo Scientific, USA) was utilized. PCR mixture and PCR conditions were designed according to the manufacturer's recommendations (Table 2.4, Table 2.5).

Next, PCR products were run on 1% agarose gel in order to verify the size of fragments. Amplicons that had the expected bands were purified by PCR clean up kit (Macherey-Nagel, Germany).

Table 2.4 PCR mixture composition.

Ingredient	**Amount**
2X Phire Green Hot Start II PCR Master Mix	10 µl
Forward and Reverse Primers	1 µl from 10 µM stock
Genomic DNA	0.5 µg
Nuclease free water	Complete to total volume
Total volume of mixture	20 µl

Table 2.5 PCR conditions for 31 kDa antigen gene, *lppB* gene and *lktA* gene fragment.

Product	**Primers used**	**PCR conditions (40 cycle)**
31 kDa antigen gene	p31_FP and p31_RP	Initial denaturation: 30 s at 98 °C Denaturation: 5 s at 98 °C Annealing: 5 s at 50 °C Extension: 10 s at 72 °C Final extention: 1 min at 72 °C
lppB gene	lppB_FP and lppB_RP	Initial denaturation: 30 s at 98 °C Denaturation: 5 s at 98 °C Annealing: 5 s at 53 °C Extension: 10 s at 72 °C Final extention: 1 min at 72 °C
lktA gene fragment (without stop codon)	lktA_FP and lktA_RP	Initial denaturation: 30 s at 98 °C Denaturation: 5 s at 98 °C Annealing: 5 s at 53 °C Extension: 10 s at 72 °C Final extention: 1 min at 72 °C

2.8 Agarose Gel Electrophoresis

Agarose gels were prepared with 1X TAE Buffer (Appendix C) and this buffer was also used as running buffer in the electrophoresis tank. Agarose gel concentrations were arranged according to the size of DNA that was run on the gel, and 1% gel was generally preferred in this study. The gels were stained with ethidium bromide (EtBr) (0.5 μg/ml a final concentration). Samples were run at 100 Volts for 45-50 minutes. Prior to loading, sample DNAs were mixed with 6X loading dye to track them while

running on the gel (5 µl sample + 1 µl loading dye). GeneRuler 1 kb DNA ladder (Thermo Scientific, USA) was also loaded to the same gel as a reference to determine the size of the DNA of interest. Gel Extraction Kit (Macherey-Nagel, Germany) was utilized to purify the gene fragments from the agarose gel for further use in cloning experiments.

2.9 Ligation Reactions

PCR products were ligated into pGEM-T Easy Vector (Promega) as stated in the user guide of the supplier. Reaction mixture components and amounts for ligation into both pGEM-T Easy Vector and pET-28a (+) Vector were stated in Table 2.6 and Table 2.7. For ligation to occur, reaction mixtures were incubated overnight at 4^{o}C.

Table 2.6 Reaction mixture for ligation into pGEM-T Easy Vector

Ingredient	**Amount**
2X Ligase Buffer	5 µl
pGEM-T Easy Vector	1 µl
Insert DNA	50 ng
T4 DNA Ligase	1 µl
dH_2O	Complete to total volume
Total volume of mixture	10 µl

Table 2.7 Reaction mixture for ligation into pET-28a (+) Vector

Ingredient	**Amount**
10X Ligase Buffer	1 µl
pET-28a (+) Vector	1 µl
Insert DNA	500 ng
T4 DNA Ligase	1 µl
dH_2O	Complete to total volume
Total volume of mixture	10 µl

2.10 Preparation and Transformation of Competent *E. coli* cells

Competent cells of both of the *E. coli* strains, DH5α and BL21 (DE3) were prepared for cloning and expression purposes, respectively. The method described by Hanahan (1985) was used for this purpose. Three ml of overnight *E. coli* culture was transferred into a freshly prepared 250 ml LB medium. The culture was incubated in a shaker (200 rpm) at 37 °C. When OD_{600} reached a value between 0.4-0.6, the culture was put on ice for 15 minutes and centrifuged at 3500 rpm for 5 min at 4 °C. The cell pellet was dissolved in 20 mL of ice-cold Buffer 1 (Appendix C). Next, the cells were harvested by centrifugation at 3,500 rpm for 5 min at 4°C, and resuspended gently in 8 ml of ice-cold Buffer 2 (Appendix C). Finally, 100 µl of aliquots were separated and stored at -80°C.

In order to use for transformation, 100 µl of competent *E. coli* cells were melted on ice for 5-10 minutes until completely thawed. 10 µl of ligation products were mixed with 50 µl of competent cells in a separate microcentrifuge tube and mixed gently by flicking the tube. The mixture was placed on ice for 20 min. The cells were heat-shocked by putting them in a water bath at exactly 42 ºC for 45-50 seconds, and tubes were immediately returned to ice for 2 minutes. 950 µl sterile room-

temperature LB was added to the transformed cells, and incubated at 37ºC for 1.5 hours with shaking (150 rpm). Then, the cells were harvested by centrifugation at 3000 rpm for 10 minutes and upper 900 µl of supernatant was removed. The cell pellet was dissolved in the remaining 100 µl supernatant. Finally, this 100 µl transformed cells were spread on LA containing appropriate antibiotic [100 µg/ml ampicillin for pGEM-T Easy Vector or 30 µg/ml kanamycin for pET-28a (+) Vector]. For selection of recombinant pGEM-T Easy Vector, blue-white selection was made. Thus, besides 100 µg/ml ampicillin addition, LA plates were supplemented with 80 µg/ml X-gal and 0.5 mM IPTG.

2.11 Plasmid Isolation

GeneJET Plasmid Miniprep Kit (Thermo Scientific, USA) was used for the isolation of both pGEM-T Easy and pET-28a (+) vectors. Isolated plasmids were run on the agarose gel for verification and stored at -20°C for further experiments.

2.12 Restriction Enzyme Digestion

The isolated plasmid was mixed with a restriction enzyme together with its appropriate working buffer in a microcentrifuge tube. According to the manufacturer's recommendation, the mixture was incubated at 37°C for 2-3 hours. The plasmids were then run on a gel for analysis, extracted from gel, and kept at -20°C for further use.

2.13 Construction of Recombinant Plasmids

While the genes encoding 31 kDa antigen and LppB were amplified via PCR from chromosomal DNA of *H. somni* 8025, *lktA* gene fragment was amplified from the genomic DNA of *M. haemolytica* A1. p31_FP and p31_RP primers (Table 2.3) were used to amplify the full-length gene for 31 kDa antigen. Similarly, *lppB* was

amplified with lppB_FP and lppB_RP primers (Table 2.3). Moreover, *lktA* gene fragment containing 32 amino acids epitope region near its C-terminus was amplified with lktA_FP and lktA_RP primers (Table 2.3) without a stop codon. PCR products were ligated into pGEM-T Easy Vector and then transformed into competent *E. coli* DH5α cells. Transformants were spread onto LA plates including IPTG, X-gal, and ampicillin to determine the recombinant plasmids by blue-white selection. From white colonies, plasmids were extracted using GeneJet Plasmid Miniprep Kit (Fermentas) and digested with restriction enzymes for verification of constructs. The verified genes were cloned into pET-28a (+) for expression, and putative recombinant pET-28a (+) vectors were screened and verified via restriction enzyme digestion. Then, in order to construct *lktA-p31* double fusion, pET-28a (+)-*p31* was digested with *Bam*HI-*Sac*I enzymes to extract *p31* gene with sticky cut sites at their ends. pET-28a (+)-*lktA* was also digested with *Bam*HI-*Sac*I enzymes, and then, *p31* gene was ligated at the downstream of the *lktA* in pET-28a (+). The same restriction enzyme digestion and ligation protocol were followed for the construction of *lktA-lppB* fusion in pET-28a (+). Lastly, to further confirm two genetic fusions, pET-28a (+)-*lktA-p31* and pET-28a (+)-*lktA-lppB* recombinant vectors were digested with *Nhe*I-*Sac*I enzymes.

2.14 Protein Overexpression and Purification of His-tagged Proteins

Recombinant *E. coli* BL21 cells carrying pET28-*lktA-p31* and pET28-*lktA-lppB* were inoculated in LB (Merck, Germany) supplemented with 30 μg/ml kanamycin as final concentration, and grown in 200 rpm shaker at 37°C until OD_{600} reaches 0.6. At this point, overexpression was induced by the addition of IPTG to 1 mM final concentration, and the culture was continued to incubate in 200 rpm shaker at 37°C for another 5 hours. After this incubation, the culture was centrifuged at 6000 g at 4°C for 15 minutes, and the cell pellet was dissolved in LEW buffer (Appendix C). Resuspended cells were freezed-thawed thrice, and sonicated to lyse cells mechanically by using a CP70T Ultrasonic Processor (Cole-Parmer, Vernon Hills,

IL) for 6×10 seconds at 60% amplitude. Cellular debris and the non-protein components were removed by centrifugation at 15000 g at 4°C for 15 minutes. The supernatants containing expressed fusion proteins were collected and run on the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to check the overexpression. Next, supernatants were purified via Protino Ni-TED 2000 packed columns (Macherey-Nagel, Germany) according to the protocol supplied with the kit. Purified recombinant fusion proteins were sterilized by passing through a 0.2 μm membrane filter. Finally, the purity of proteins was analyzed by SDS-PAGE, and the sterile proteins were kept in -20 ^{o}C for further use in vaccine preparations.

2.15 Determination of Protein Concentration

Concentrations of expressed and purified proteins were determined by both Bradford method (Bradford, 1976) and measuring OD_{280} value. A calibration curve was constructed with OD_{595} measurements of standards prepared (Figure 2.1) for the calculation of the protein concentrations. OD_{280} values were interpreted as the concentration units, in mg/ml, and values similar to the ones found by Bradford method were obtained.

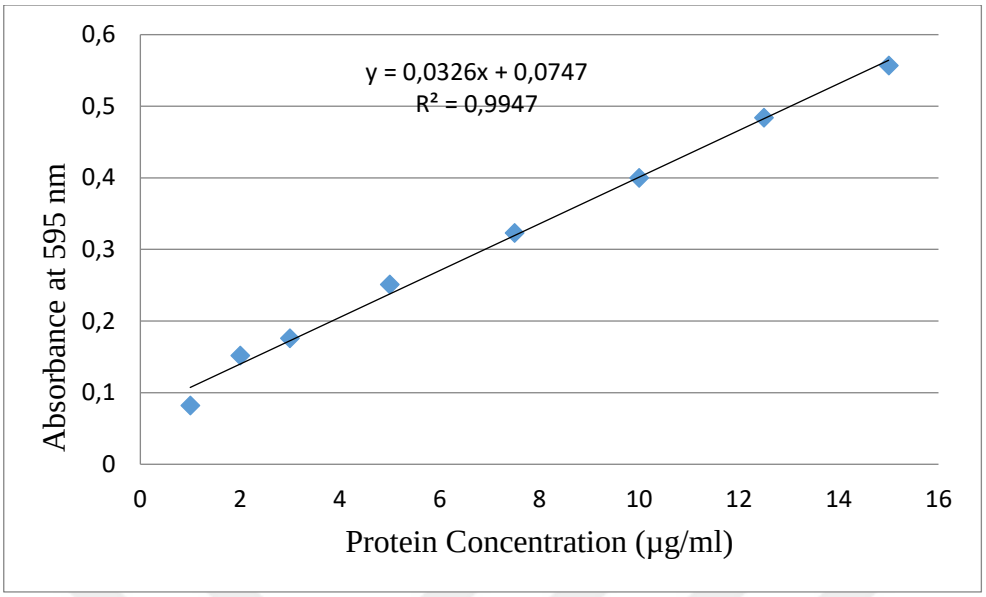

Figure 2.1. Calibration curve for determination of protein concentrations.

2.16 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-polyacrylamide gels were prepared as stated in Laemmli (1970). The components and their amounts were given in Table 2.8. Samples were added with sample loading buffer (Appendix C) to track them on the gel. After loading the samples into the wells, the gel was run at 100 V in 1X running buffer (Appendix C) for 1.5-2 hours by using a Mini-Protean electrophoresis apparatus (Bio-Rad) until the loading dye was seen at the bottom of the gel.

2.17 Coomassie Brillant Blue Staining of Polyacrylamide Gels

Following SDS-PAGE, the gel was stained 15 min using Coomassie Blue R-250 (Appendix C) supplemented with methanol freshly. Then, the stained gel was incubated in destaining solution (Appendix C) until the background staining was cleared.

Table 2.8 Preparation of SDS-polyacrylamide gels.

	Stacking Gel 0.125 M Tris, pH 6.8	**Separating Gel** 0.375 M Tris, pH 8.8
Monomer concentration	4.5%	12%
Acrylamide/bis	0.65 ml	4 ml
dH2O	3.05 ml	3.35 ml
1.5 M Tris-HCl, pH 8.8	-	2.5 ml
0.5 M Tris-HCl, pH 6.8	1.25 ml	-
10% (w/v) SDS	50 µL	100 µL
10% Ammonium persulphate	25 µL	50 µL
TEMED	5 µL	5 µL
Total Volume	**5 ml**	**10 ml**

2.18 Western Blotting

3MM Whatman papers and 0.2 µm nitrocellulose membrane (Bio-Rad, Hercules, CA) were cut according to the size of the gel. Then, together with unstained SDS-polyacrylamide gel, they were soaked into 1X transfer buffer (Appendix C) and placed on the tray, as shown in Figure 2.2. 1.5 mA current per cm^2 of the membrane was applied for 1 hour using a semi-dry blotter (Cleaver Scientific Ltd, Warwickshire, UK). After transfer, blocking of the membrane was carried out in 10% skim milk in 1X TBS (Appendix C) at room temperature for 2 hours. Then, membrane washed in 1X TBS-T (0.5% Tween in 1X TBS) for 10 minutes. Sera from Lkt-p31 and Lkt-LppB vaccinated mice were used as primary antibody and prepared as 1/300 dilution in 5% skim milk. Membrane was incubated in this primary antibody for 1 hour at room temperature. After another 10 minutes wash in 1X TBS-T, 4 µl

secondary antibody (anti-mouse IgG, Sigma) was added into 50 ml of 5% skim milk, and the membrane was incubated in it for 1 hour at room temperature. Lastly, the membrane was washed with only 1X TBS for 10 minutes and put into freshly prepared AP conjugate substrate (Bio-Rad, Hercules, CA) until the protein bands were visualized.

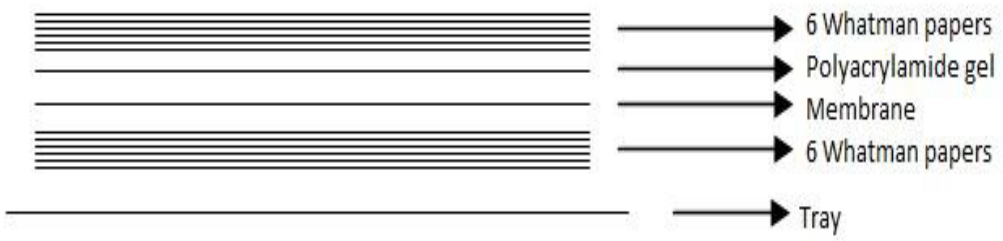

Figure 2.2. Schematic representation of transfer set-up in Western blot.

2.19 Mice experiments

For mice experiments, 18-20 g male BALB/c mice were obtained from KOBAY Experimental Animals Laboratory (Ankara, Turkey). Mice were injected with recombinant fusion proteins adjuvanted (oil-based adjuvant; Seppic ISA 201) with experimental design tabulated in Table 2.9. Each dose of injections was applied at 15 days interval. 14 days after second injections, tails of mice were bled. After that, blood samples were incubated for 1 hour at room temperature and centrifuged at 5000 rpm for 20 minutes. Sera were taken and stored in -20 ^{o}C for further use.

Table 2.9 Experimental design for vaccination experiments.

Vaccine	Amount	Route	Number of doses	Number of mice
Lkt-p31	100 µg	i.p.	2	10
Lkt-lppB	100 µg	i.p.	2	10
Lkt-p31 + Lkt-lppB	100 µg each	i.p.	2	10
PBS + adjuvant (Control)	500 µl	i.p.	2	10

Animal experiments were performed under the approval of Ethical Committee on Animal Experiments of KOBAY Experimental Animals Laboratory, Ankara (02.08.2019).

2.20 Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA plates were coated with each of the recombinant proteins, p31, LppB, Lkt-p31, and Lkt-LppB at concentrations of 4 µg/well in carbonate buffer (Appendix C), and incubated at 4 ^{o}C overnight. Coated plates were washed three times with washing solution (Appendix C), and air dried. 100 µl of blocking solution (Appendix C) was added to the wells, and the plates were kept at 37 ^{o}C for 1 hour. After blocking, sera were diluted as 1:100, 1:200, 1:400, and 1:800 in blocking solution, added to the wells, and plates were incubated at 37 ^{o}C for 1 hour. The plates were washed three times with washing solution. After wells were dried, alkaline phosphatase conjugated rabbit anti-mouse IgG (Sigma), or rat anti-mouse IgG2a (SouthernBiotech, USA) were added into wells as secondary antibody at a dilution of 1/1000 in blocking solution. Plates were kept at 37 ^{o}C for 1 hour. The plates were

washed four times with washing solution. As alkaline phosphatase substrate, p-nitrophenyl phosphate (PNPP) tablets (Thermo Scientific, USA) were prepared according to the manufacturer's recommendation. After the addition of substrate, plates were incubated 15 minutes at room temperature in the dark. The plates were read at 405 nm on a RT-2100C Microtiter plate Reader (Rayto, Shenzhen, China). Absorbance values at 1:800 and 1:400 dilutions were used to compare total IgG and IgG2a antibody responses in mice, respectively. However, for IgG2a response in the LppB coated plate, 1/100 dilutions were compared.

2.21 Complement-Mediated Bacterial Killing Assay (Bactericidal Assay)

Complement-mediated killing assays were performed according to method of Ayalew *et al*. with some modifications (2008). As complement source, sera from unvaccinated mice were collected, aliquoted, and immediately put into -80 ^{o}C for fresh use in each assay. Vaccinated and control serum samples were heated at 56 ^{o}C for 30 minutes to inactivate resident complement. Also, 24 hours *H. somni* culture was scraped from a blood agar plate and diluted in PBS solution until OD_{600} was 0.5. 1/10000 dilution from this OD_{600}: 0.5 culture in PBS was prepared and used directly in the assay. Then, 25 µl from this 1/10000 diluted bacteria, 25 µl vaccinated or control serum, and 25 µl complement source were mixed and incubated at 37 ^{o}C for 45 minutes. After that, 25 µl from this 75 µl mixture was spread onto plates in three replicates. Number of colonies grown in control and test groups was counted after 18-20 hours of incubation at 37 ^{o}C in a candle jar. Percent survival was calculated as: (Test growth/Control growth) ×100% for each replicate.

2.22 Statistical Analyses

Statistical analyses were carried out with t-test and performed using GraphPad Prism version 8.00 for Windows (GraphPad Software, USA). The significance level (p-value) for all analyses was set at 0.05.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Cloning of 31 kDa Antigen (p31) Gene and lipoprotein B (LppB) gene from *H. somni* and Leukotoxin A (*lktA*) Gene Fragment from *M. haemolytica*

Genomic DNA of *H. somni* 8025 (ATCC 43625) was utilized for PCR amplification of genes encoding 31 kDa antigen and LppB (40 kDa antigen), while the genomic DNA of *M. haemolytica* A1 was used for PCR amplification of the 393 bp *lktA* fragment of *lkt* gene including epitope region. *lktA* fragment was cloned without a stop codon to be able to generate two different genetic fusion with the genes for 31 kDa antigen and LppB, respectively.

3.1.1 PCR Amplification and Cloning of *p31* Gene into pGEM-T Easy Vector

Since the *p31* gene encoding 31 kDa antigen in *H. somni* was cloned into the downstream of *lktA* fragment, it was cloned including its stop codon for the termination of translation. Primers designed by Guzmán-Brambila *et al*. (2012) were used in our study with appropriate restriction enzyme cut sites for successful amplification of 822 bp *p31* gene to cover the whole 273 amino acid protein (Figure 3.1).

After PCR amplification, the *p31* gene was ligated into pGEM-T Easy vector, and introduced *E. coli* DH5α cells. Putative recombinant plasmids were extracted from *E. coli* and cut with *Bam*HI-*Sac*I enzymes for verification of cloning (Figure 3.2).

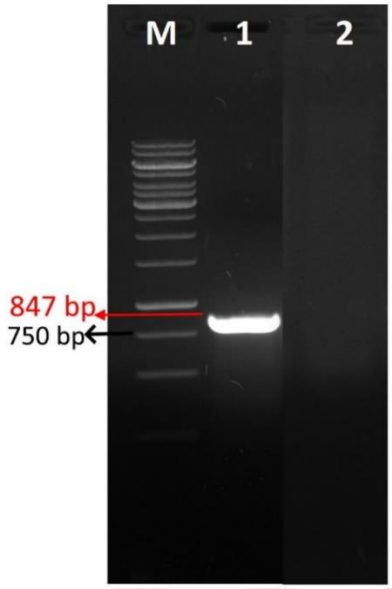

Figure 3.1. PCR amplification of *p31* gene. Lane 1: Amplification of *p31* gene, Lane 2: No template control. M: GeneRuler 1 kb DNA ladder.

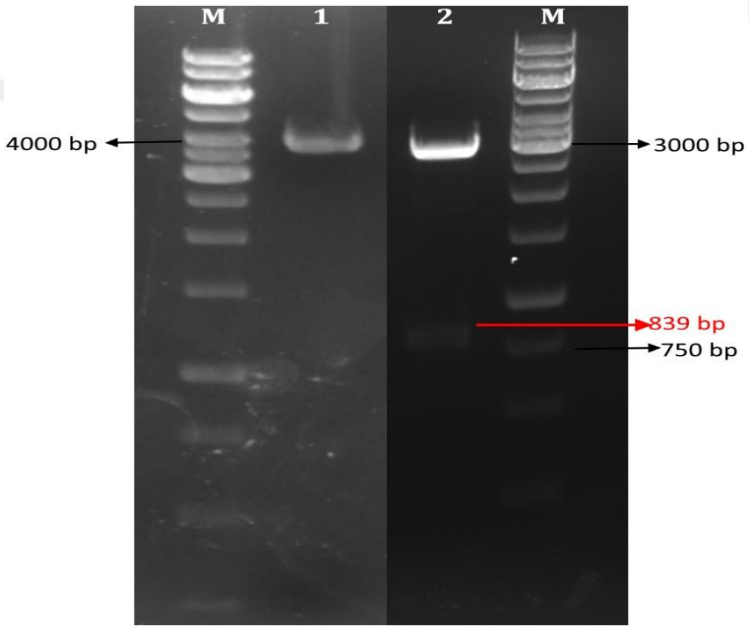

Figure 3.2. Verification of cloning of p31 gene into pGEM-T. Lane 1: *Bam*HI digested pGEM-T-*p31*, Lane 2: *Bam*HI-*Sac*I digested pGEM-T-*p31*. M: GeneRuler 1 kb DNA ladder.

3.1.2 PCR Amplification and Cloning of Lipoprotein B (*lppB*) Gene into pGEM-T Easy Vector

Since the *lppB* gene was to be cloned into the downstream of the *lktA* fragment, it was cloned together with its stop codon to stop protein synthesis during the expression of the *lktA*-*lppB* fusion. Primers used for the amplification of *lppB* gene in this study were again the same as the ones in Guzmán-Brambila *et al*. (2012) with restriction enzyme cut sites *Bam*HI and *Sac*I. The entire 840 bp *lppB* gene expressing 279 amino acids was successfully amplified by PCR (Figure 3.3).

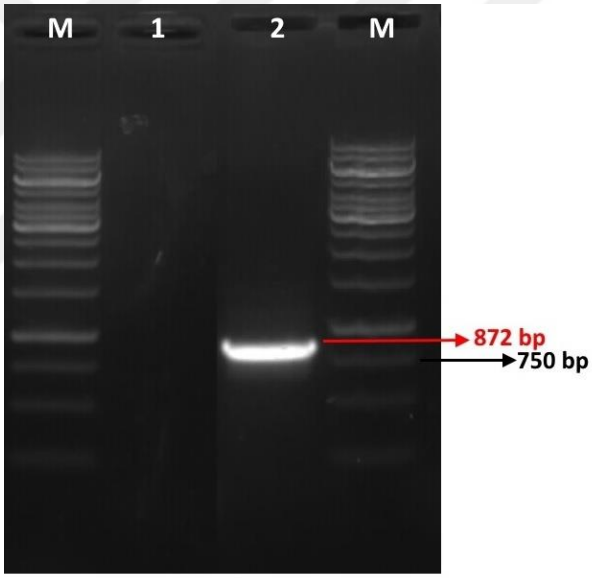

Figure 3.3. PCR amplification of *lppB* gene. Lane 1: No template control, Lane 2: Amplification of *lppB* gene. M: GeneRuler 1 kb DNA ladder.

The *lppB* gene was ligated into pGEM-T Easy vector and competent *E. coli* DH5α cells were transformed with the recombinant plasmid. To verify cloning, pGEM-T-*lppB* constructs were double digested with BamHI-SacI and run on 1% agarose gel (Figure 3.4).

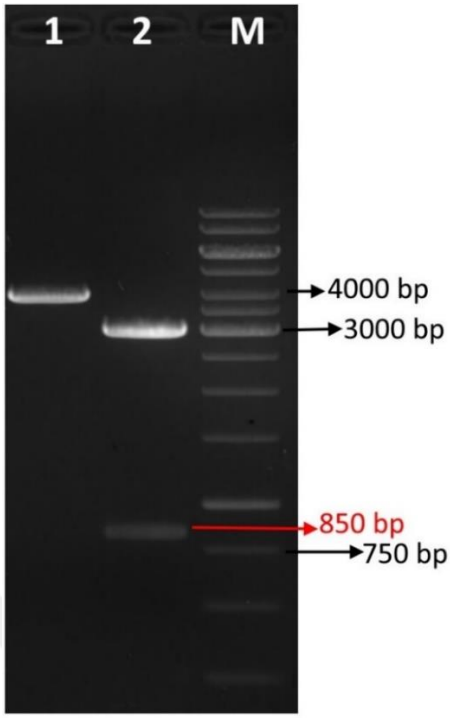

Figure 3.4. Verification of cloning of *lppB* gene into pGEM-T. Lane 1: *Bam*HI digested pGEM-T-*lppB*, Lane 2: *Bam*HI-*Sac*I digested pGEM-T-*lppB*. M: GeneRuler 1 kb DNA ladder.

3.1.3 PCR Amplification and Cloning of *lktA* Gene Fragment into pGEM-T Easy Vector

lktA epitope fragment of the *lkt* gene was cloned at the upstream of the *p31* and *lppB* genes. This *lktA* fragment contains 32 amino acids epitope region near its C-terminus. This epitope region was reported to be recognized by strongly neutralizing monoclonal antibodies (Lainson *et al*., 1996). During the cloning of *lktA* fragment, its stop codon was excluded when generating genetic fusions. Primers were designed to be compatible with the primers used for the *p31* and *lppB* genes. While *Nhe*I cut site was added into the forward primer, *Bam*HI cut site was added to the reverse primer of *lktA* fragment. The 393 bp *lktA* fragment was amplified successfully (Figure 3.5).

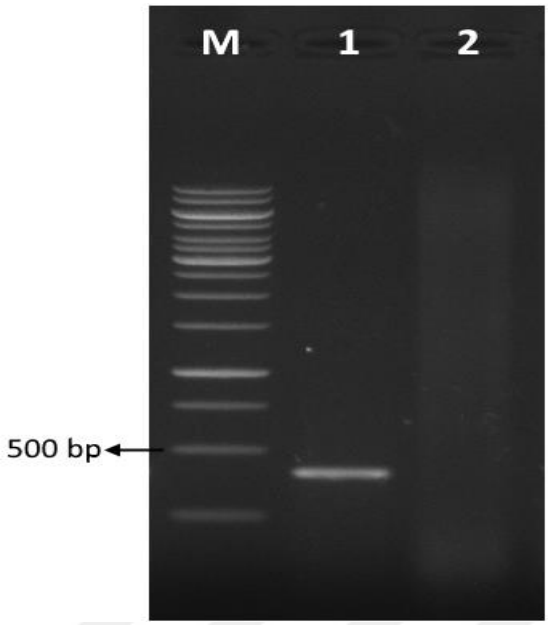

Figure 3.5. PCR amplification of *lktA* fragment. Lane 1: Amplification of *lktA* fragment, Lane 2: No template control. M: GeneRuler 1 kb DNA ladder.

After PCR amplification, the *lktA* fragment was ligated into pGEM-T Easy vector. Then, the ligation product was introduced into *E. coli* DH5α competent cells. The isolated plasmids were double cut with *Nhe*I-*Bam*HI enzymes for verification of cloning (Figure 3.6).

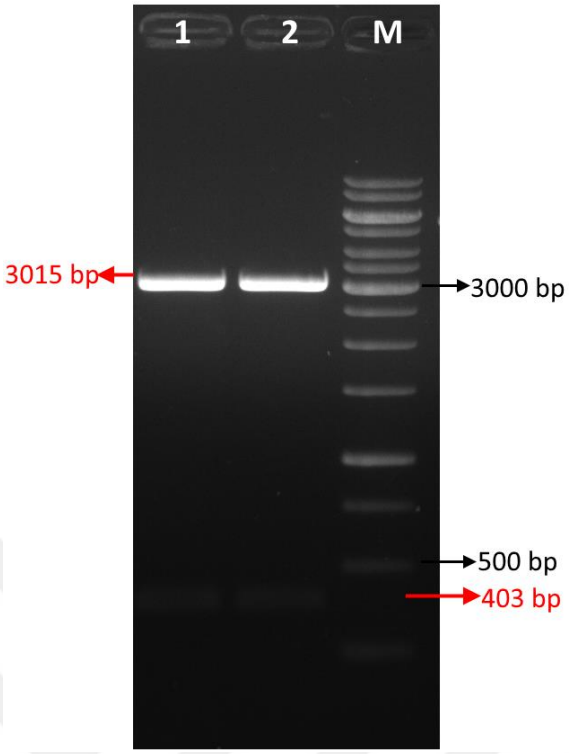

Figure 3.6. Verification of cloning of *lktA* fragment into pGEM-T. Lane 1 and 2: *Nhe*I-*Bam*HI digested *lktA* fragment cloned pGEM-T. M: GeneRuler 1 kb DNA ladder.

3.1.4 Cloning of 31 kDa Antigen (*p31*) Gene, Lipoprotein B (*lppB*) Gene and leukotoxin A (*lktA*) Gene fragment into pET-28 a (+) Vector

pET-28a (+) expression vector has six histidine sequences at C and N termini of its multiple cloning site, and the genes cloned into this site are under the control of the T7 promoter which requires T7 RNA polymerase for transcription of the genes. Also, lacUV5 promoter direct the transcription of T7 RNA polymerase gene in the expression host, *E. coli* BL21. Since lacUV5 promoter is inducible with IPTG, expression of cloned genes also depends on IPTG induction. pGEM-T-*p31* and pGEM-T-*lppB* as well as pET-28a (+) vectors were digested with *Bam*HI and *Sac*I enzymes, and gene of interests were ligated into linearized pET-28a (+). The same

procedure with *Nhe*I-*Bam*HI enzymes was performed for the ligation of *lktA* fragment into pET-28 a (+). Thus, pET-28a (+)-*p31*, pET-28a (+)-*lppB* and pET-28a (+)-*lktA* recombinant vectors were successfully obtained in *E. coli* DH5α, and verified by restriction enzyme digestion (Figure 3.7 and 3.8). After verification, plasmids containing the expected fragments were transformed into *E. coli* BL21 cells.

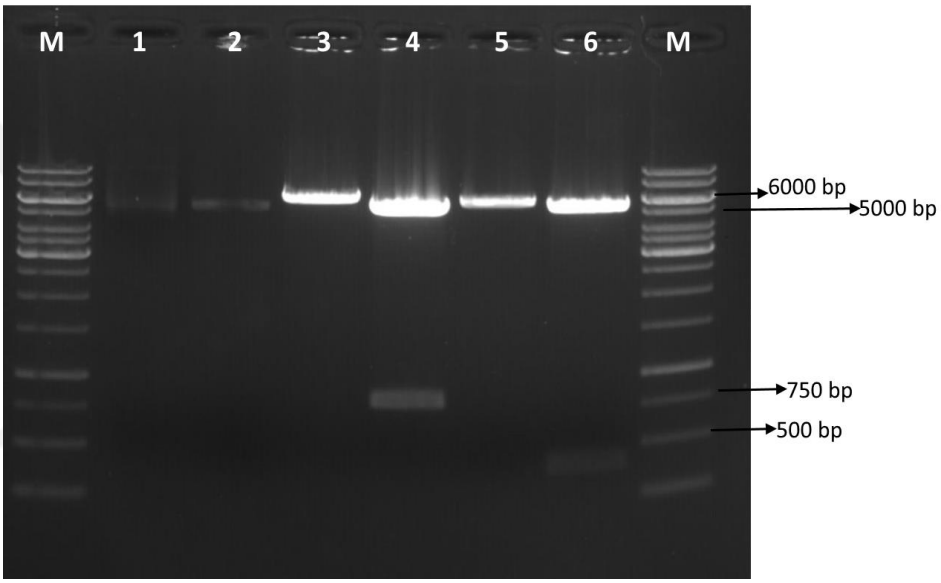

Figure 3.7. Verification of cloning of *p31* gene and *lktA* gene fragment into pET-28a (+). Lane 1: Undigested pET-28a (+), Lane 2: *Bam*HI digested pET-28a (+), Lane 3: *Bam*HI digested pET-28a (+)-*p31*, Lane 4: *Bam*HI-*Sac*I digested pET-28a (+)-*p31*, Lane 5: *Bam*HI digested *lktA* gene fragment cloned pET-28a (+), Lane 6: *Bam*HI-*Nhe*I digested *lktA* gene fragment cloned pET-28a (+). M: GeneRuler 1 kb DNA ladder.

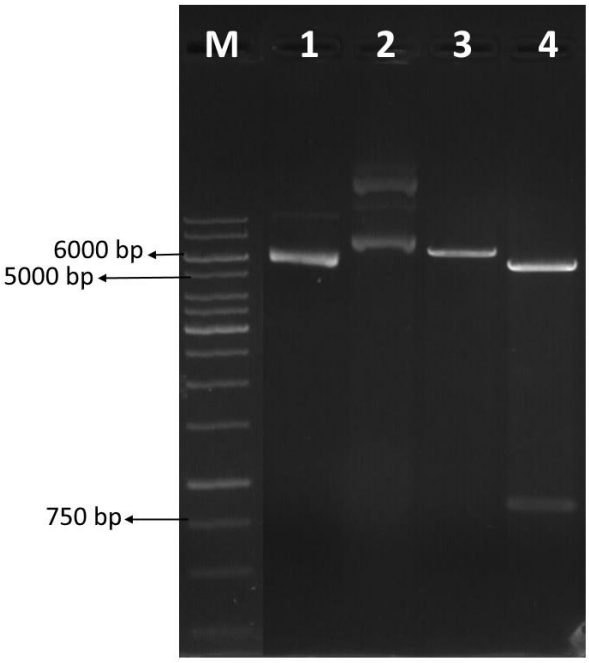

Figure 3.8. Verification of cloning of *lppB* gene into pET-28a (+). Lane 1: Undigested pET-28a (+), Lane 2: *Bam*HI digested pET-28a (+), Lane 3: *Bam*HI digested pET-28a (+)-*lppB*, Lane 4: *Bam*HI-*Sac*I digested pET-28a (+)-*lppB*. M: GeneRuler 1 kb DNA ladder.

3.1.5 Derivation of pET28a (+)-*lktA-p31*

pET-28 a (+)-*p31* and pET-28 a (+)-*lktA* recombinant vectors were double digested with *Bam*HI-*Sac*I enzymes to construct fusion. *p31* gene was ligated into pET-28a (+)-*lktA* at the downstream of *lktA* fragment. Following transformation, putative recombinant plasmids were isolated from *E. coli*, and *lktA-p31* fusion was verified via double digestion with *Nhe*I-*Sac*I enzymes (Figure 3.9). The *lktA-p31* gene fusion was observed as 1238 bp fragment, as expected. After verification of the construct on the gel, plasmids containing *lktA-p31* fusion were transformed into *E. coli* BL21 cells.

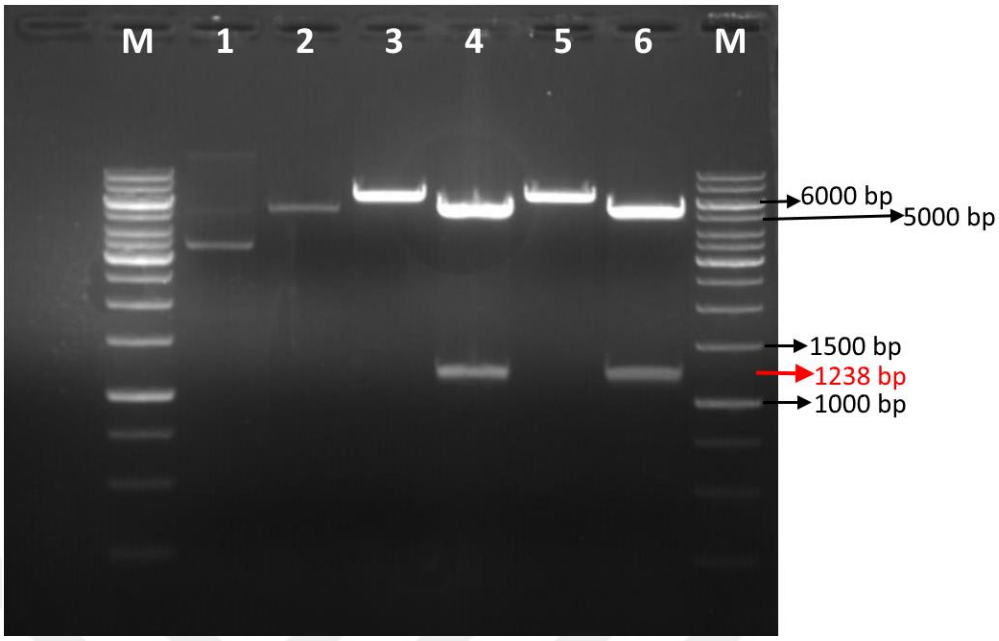

Figure 3.9. Verification of pET-28a (+)-*lktA-p31* construct. Lane 1: Undigested pET-28a (+), Lane 2: *Bam*HI digested pET-28a (+), Lanes 3&5: *Bam*HI digested *lktA-p31* cloned in pET-28a (+), Lanes 4&6: *Nhe*I-*Sac*I digested *lktA-p31* cloned in pET-28a (+). M: GeneRuler 1 kb DNA ladder.

3.1.6 Derivation of pET28a (+)-*lktA-lppB*

The same procedure explained in the Section 3.1.5 was used in the cloning of *lppB* gene into pET-28 a (+)-*lktA. Nhe*I-*Sac*I digested plasmids were run on 1% agarose gel and the expected 1249 bp *lktA-lppB* fragment was observed (Figure 3.10). After verification, the pET-28 a (+)-*lktA-lppB* vector was transformed into *E. coli* BL21 cells.

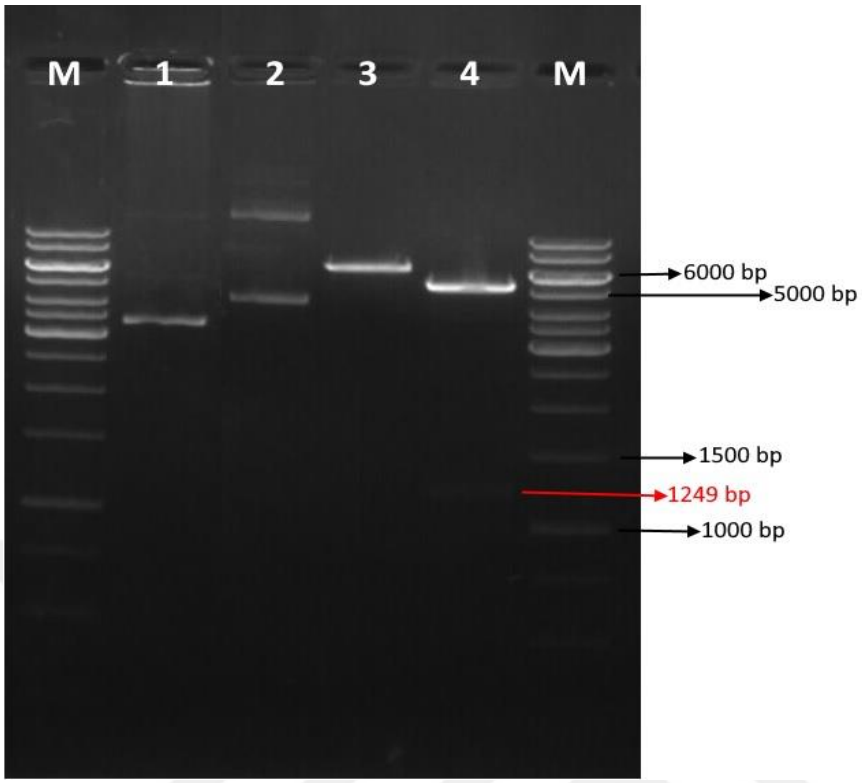

Figure 3.10. Verification of pET-28a (+)-*lktA-lppB* construct. Lane 1: Undigested pET-28a (+), Lane 2: *Bam*HI digested pET-28a (+), Lane 3: *Bam*HI digested *lktA-lppB* cloned pET-28a (+), Lanes 4: *Nhe*I-*Sac*I digested *lktA-lppB* cloned pET-28a (+). M: GeneRuler 1 kb DNA ladder.

3.2 Expression of Recombinant LktA-p31 and LktA-LppB Fusion Proteins in *E. coli* BL21 (D3)

For the expression of each fusion protein, LB supplemented with kanamycin was used for the growth of *E. coli* BL21 (D3) cells containing *lktA-p31* or *lktA-lppB* fusion inserted pET-28a (+). Expression of genetic fusions were induced using IPTG. Protein extraction protocol was performed, and the cell lysate was run on 12% SDS-PAGE (Figure 3.10 and Figure 3.10). The predicted protein size for LktA-p31 fusion was approximately 48 kDa (LktA ≈ 14.6 kDa, p31 ≈ 31 kDa, 23 aa upstream of

fusion protein including 6 His-tag ≈2.5 kDa), and for LktA-LppB was about 58 kDa (LktA ≈ 14.6 kDa, LppB ≈ 40 kDa, 23 aa upstream of fusion protein including 6 His-tag ≈2.5 kDa). The fusion proteins were obtained at the expected molecular weights.

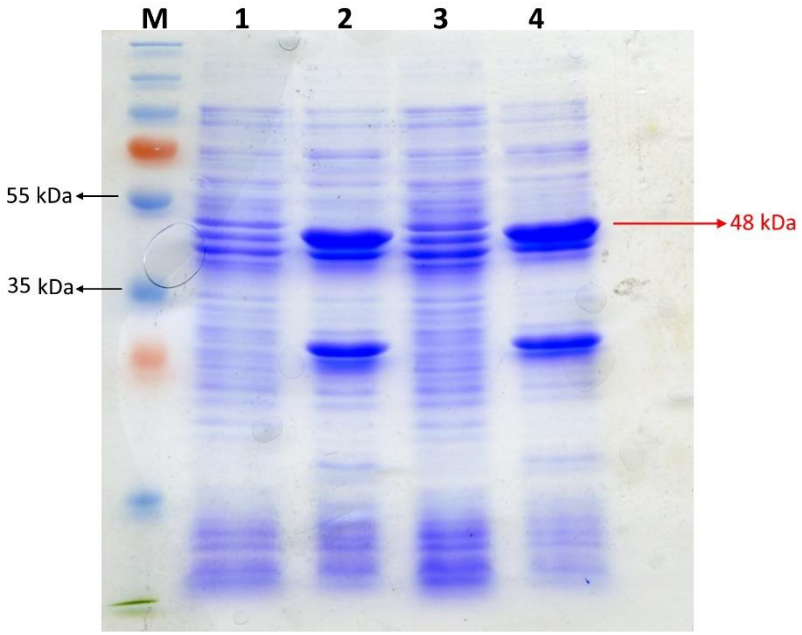

Figure 3.11. SDS-PAGE analysis of LktA-p31 fusion protein expression. Lanes 1 and 3: Uninduced control *E. coli* lysate, Lanes 2 and4: IPTG induced *E. coli* lysate for LktA-p31 fusion protein. M: PageRuler Plus Prestained Protein Ladder.

As also seen in Western blot figure below (Figure 3.15), some of the fusion proteins were observed to undergo cleavage.

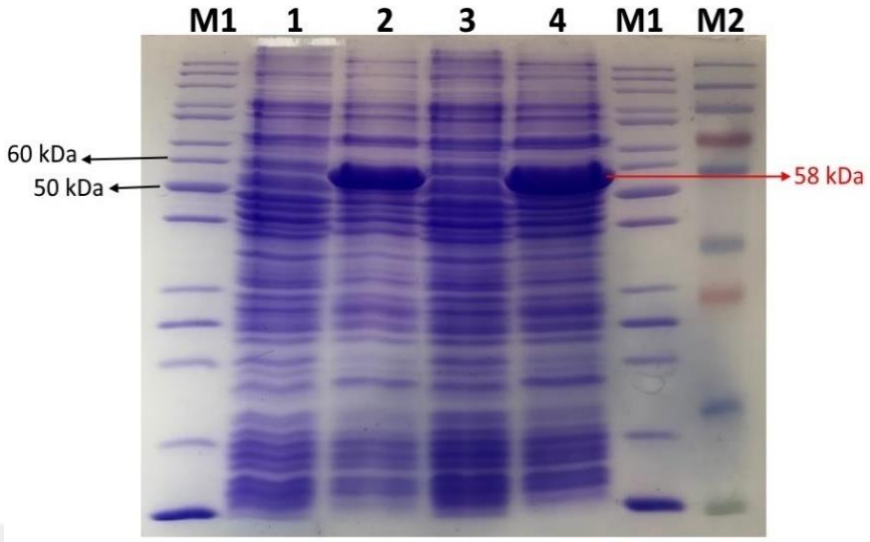

Figure 3.12. SDS-PAGE analysis of LktA-LppB fusion protein expression. Lanes 1 and 3: Uninduced control *E. coli* lysate, Lanes 2 and 4: IPTG induced *E. coli* lysate for LktA-LppB fusion protein. M1: PageRuler Unstained Protein Ladder. M2: PageRuler Plus Prestained Protein Ladder.

3.3 Purification of Recombinant LktA-p31 and LktA-LppB Fusion Proteins via Affinity Chromatography

Protino® Ni-TED 2000 protein purification system was used to purify both of the recombinant his-tagged fusion proteins (Figure 3.13 and Figure 3.14). Urea concentration of cell lysates containing fusion proteins was 8 M, and it was decreased to 2 M before purification using a buffer lacking urea. Other buffers used in the purification protocol contained 2 M urea. After purification, the fusion proteins were sterilized with a 0.22 μm syringe filter and stored in -20 ^{o}C till used for vaccine formulations.

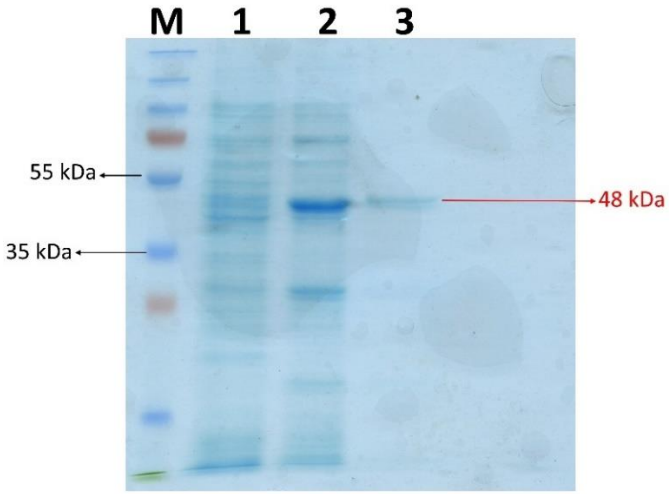

Figure 3.13. SDS-PAGE analysis for the purification of LktA-p31 fusion protein. Lane 1: Uninduced control *E. coli* lysate, Lane 2: IPTG induced *E. coli* lysate for LktA-p31 fusion protein, Lane 3: Purified LktA-p31 fusion protein. M: PageRuler Plus Prestained Protein Ladder.

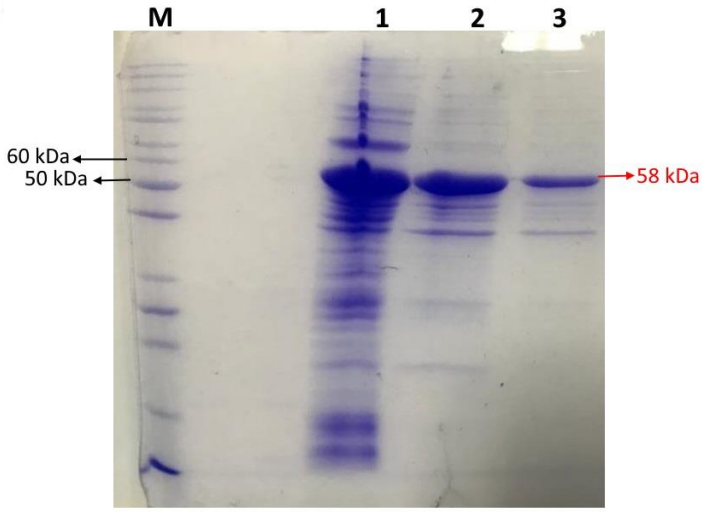

Figure 3.14. SDS-PAGE analysis after the purification of LktA-LppB fusion protein. Lane 1: IPTG induced *E. coli* lysate for LktA-LppB fusion protein, Lane 2: LktA-LppB fusion protein first eluate. Lane 3: LktA-LppB fusion protein second eluate. M: PageRuler Unstained Protein Ladder.

3.4 Western Blot Analysis of Recombinant LktA-p31 and LktA-LppB Fusion Proteins

Sera collected from the mice immunized with LktA-p31 and LktA-LppB were evaluated via Western blot analysis in terms of their interactions with recombinant proteins, p31, LppB, LktA-p31 and LktA-LppB as well as *H.somni* lysate (Figure 3.15 and Figure 3.16).

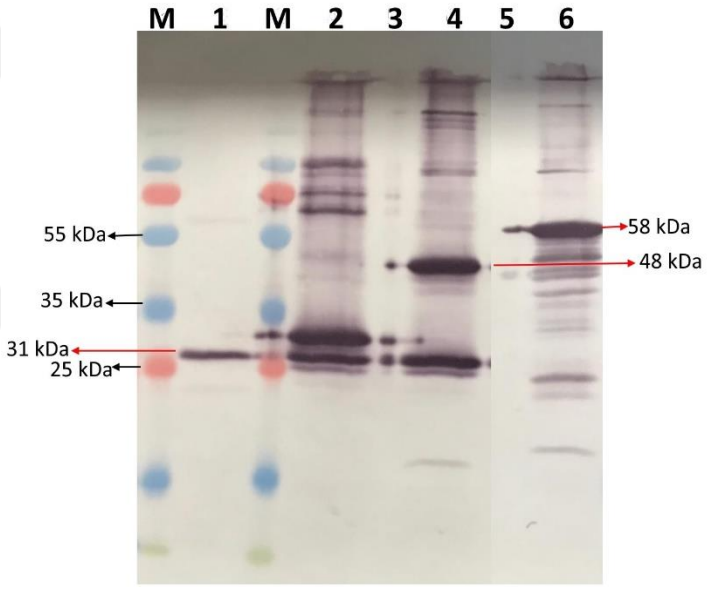

Figure 3.15. Western blot analysis using the serum against LktA-p31 fusion protein. Lane 1: *H. somni* lysate, Lane 2: p31 eluate, Lane 4: LktA-p31 fusion protein eluate, Lane 6: LktA-LppB fusion protein eluate. M: PageRuler Plus Prestained Protein Ladder.

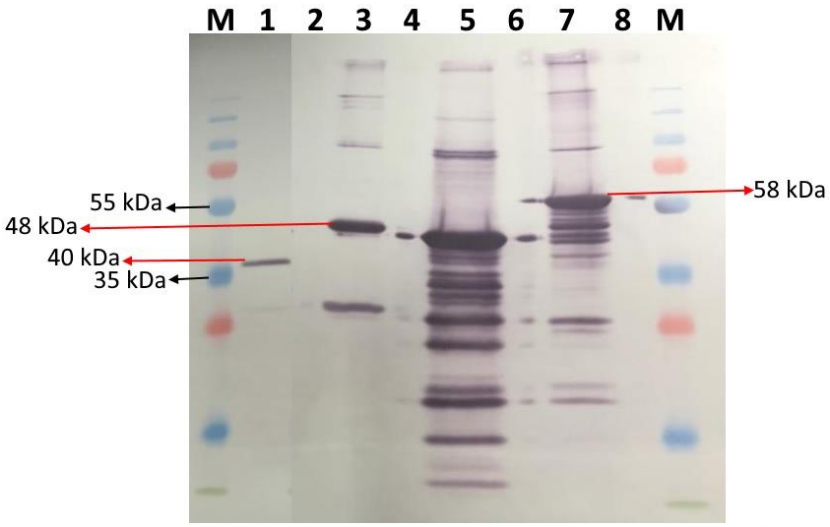

Figure 3.16. Western blot analysis using the serum against LktA-LppB fusion protein. Lane 1: *H.somni* lysate, Lane 3: LktA-p31 fusion protein eluate, Lane 5: LppB eluate, Lane 7: LktA-LppB fusion protein eluate. M: PageRuler Plus Prestained Protein Ladder.

As seen in the figures above, antibodies produced in the vaccinated mice gave positive reactions with recombinant p31 (31 kDa), LppB (40 kDa), LktA-p31 (48 kDa), LktA-LppB (58 kDa), as well as native p31 and LppB found in *H.somni* lysate, as expected.

Anti-LktA serum collected from mice injected with *M. haemolytica* LktA - *P. multocida* OmpH-PlpEC fusion protein (LktA-OmpH-PlpEC) (Çırçır, 2014) was also used as primary antibody in Western blot experiment to identify the recombinant LktA-p31 and LktA-LppB fusion proteins (Figure 3.17 and Figure 3.18). Anti-LktA sera gave positive reaction only with LktA-p31 and LktA-LppB fusions but not with recombinant p31 or LppB proteins, as expected.

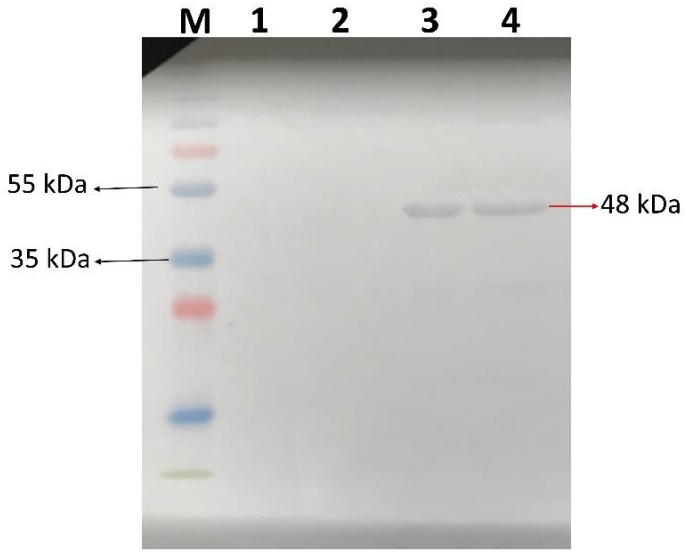

Figure 3.17. Western blot analysis with sera against LktA-OmpH-PlpEC fusion protein. Lane 1: IPTG induced *E. coli* lysate for p31 protein, Lane 2: p31 eluate, Lane 3: IPTG induced *E. coli* lysate for LktA-p31 fusion protein, Lane 4: LktA-p31 fusion protein eluate. M: PageRuler Plus Prestained Protein Ladder.

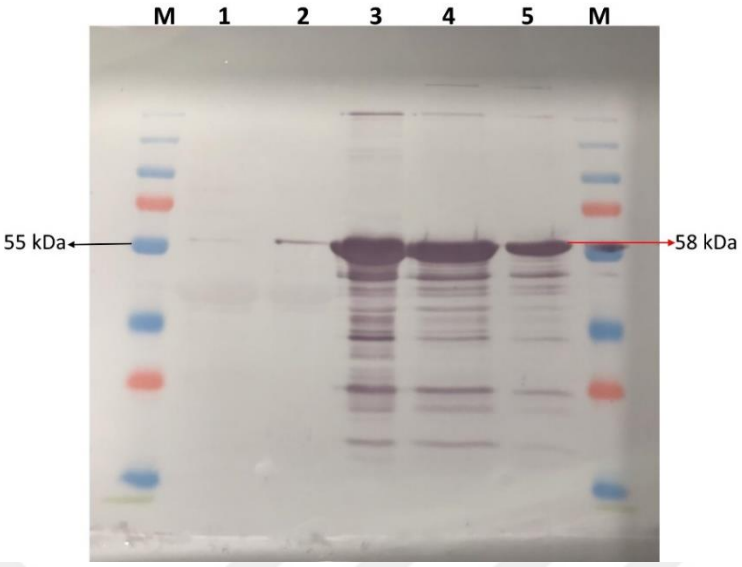

Figure 3.18. Western blot analysis with sera against LktA-OmpH-PlpEC fusion protein. Lane 1: IPTG induced *E. coli* lysate for LppB protein, Lane 2: LppB eluate, Lane 3: IPTG induced *E. coli* lysate for LktA-LppB fusion protein, Lane 4: LktA-LppB fusion protein first eluate, Lane 5: LktA-LppB fusion protein second eluate. M: PageRuler Plus Prestained Protein Ladder

Sera collected from the mice immunized with LktA-p31 and LktA-LppB were mixed and evaluated via Western blot analysis in terms of their interactions with native leukotoxin secreted into culture supernatant of *M. haemolytica* (Figure 3.19). Antibodies raised against fusion proteins positively reacted with native LktA protein of 102 kDa.

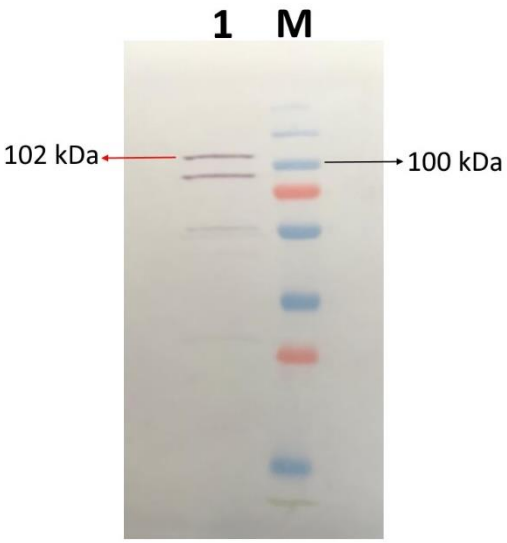

Figure 3.19. Western blot analysis using the sera against LktA-p31 and LktA-LppB fusion proteins. Lane 1: Six hours *M. haemolytica* culture supernatant containing native LktA protein, M: PageRuler Plus Prestained Protein Ladder.

3.5 Experiments on mice

Three different experimental vaccine formulations were prepared with the same oil-based adjuvant for the present study. The first one was formulated with the 100 µg of LktA-p31 fusion protein per injection (0.5 ml), while the second contained 100 µg LktA-LppB. As a third formulation, two fusion protein based combined vaccine consisting of 100 µg of each LktA-p31 and LktA-LppB fusions were prepared. Each ten BALB/c mice group were immunized intraperitoneally (i.p.) twice with each formulation with 14 days interval, and another ten mice were injected with PBS + adjuvant as the control on the same days. Sera were collected from all mice 14 days after the second immunization.

3.5.1 Antibody Responses in mice immunized with the LktA-p31 Fusion Protein and Two Fusion Protein Based Combined Vaccine (LktA-p31 + LktA-LppB)

ELISA was performed to evaluate the antibody levels raised against fusion proteins. Since IgG2a is related with the Th1 response, both total IgG and IgG2a levels were analyzed to interpret the type of immune response induced.

Serum antibody levels were evaluated against recombinant p31 protein and LktA-p31 fusion protein. As shown in Figure 3.20, total IgG raised against both recombinant proteins in mice immunized either with LktA-p31 fusion protein or combined vaccine consisting of two fusion proteins, LktA-p31 + LktA-LppB, were found significantly higher than the control group. IgG2a levels in same sera were also examined, and both of the candidate vaccine formulations significantly induced IgG2a antibodies against recombinant p31 protein and LktA-p31 fusion protein (Figure 3.21).

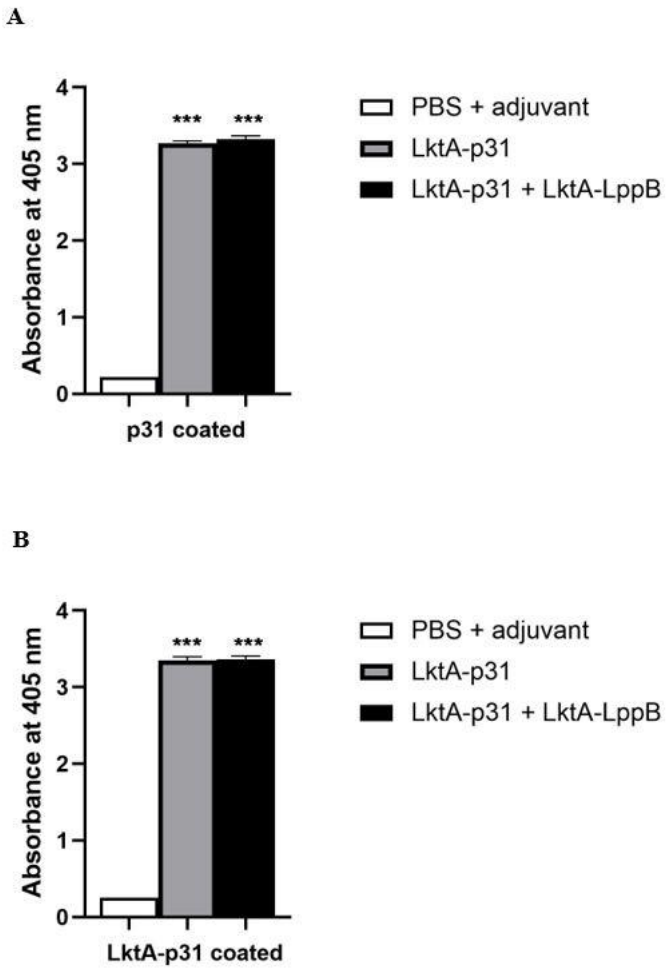

Figure 3.20. Total IgG levels in 1:800 diluted sera from the mice immunized with LktA-p31 or LktA-p31+LktA-LppB against recombinant p31 (A) and LktA-p31 (B). Statistically significant (p<0.001) increase as compared to control was shown with three asterisks.

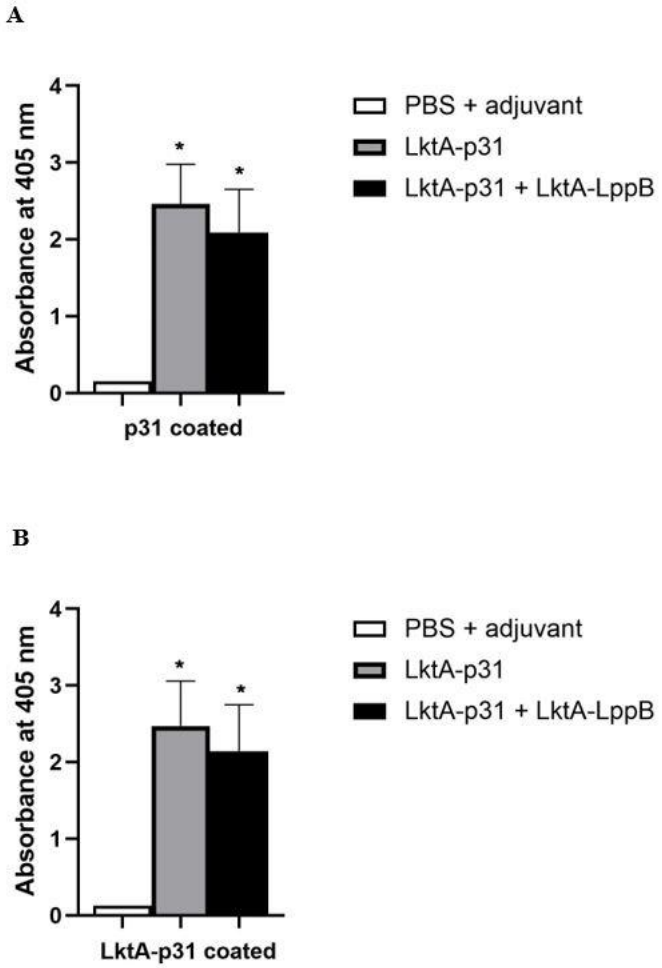

Figure 3.21. IgG2a levels in 1:400 diluted sera from the mice immunized with LktA-p31 or LktA-p31+LktA-LppB against recombinant p31 (A) and LktA-p31 (B). Statistically significant (p<0.05) increase as compared to control was shown with an asterisk.

3.5.2 Antibody Responses in mice immunized with the LktA-LppB Fusion Protein and Two Fusion Protein Based Combined Vaccine (LktA-p31 + LktA-LppB)

ELISA was utilized to evaluate antibody levels in sera of mice immunized with LktA-LppB fusion protein or combined vaccine consisting of two fusion proteins, LktA-p31 + LktA-LppB. In both of the vaccinated groups, a significant increase in total IgG response against recombinant proteins was observed (Figure 3.22). On the other hand, IgG2a antibodies against recombinant LppB protein or LktA-LppB fusion protein were significantly induced only with two fusion based combined vaccine (LktA-p31+LktA-LppB, 100 µg each per injection) formulation (Figure 3.23).

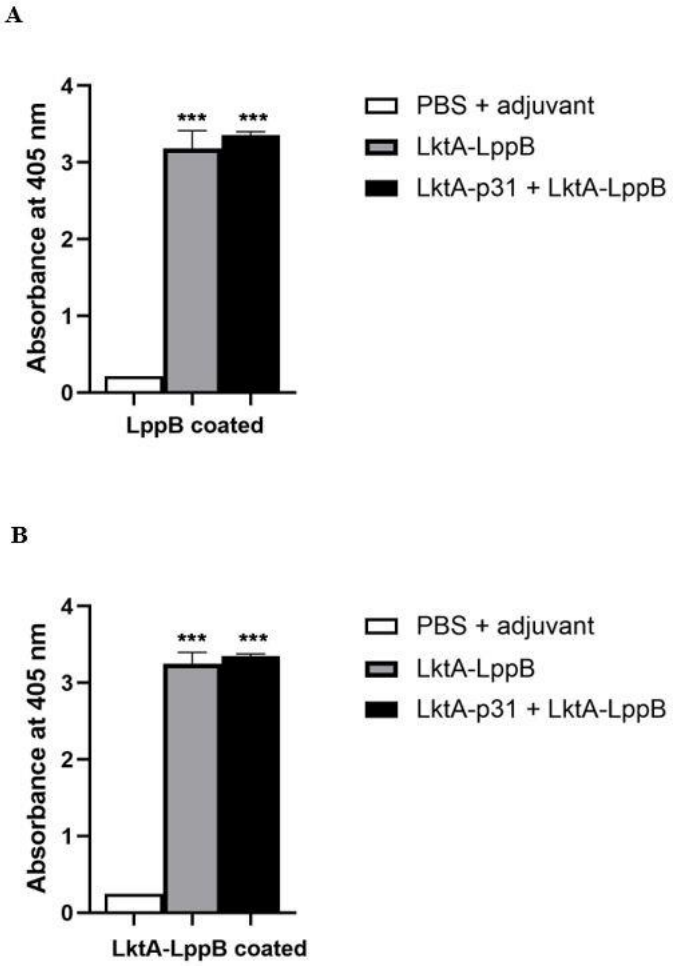

Figure 3.22. Total IgG levels in 1:800 diluted sera from the mice immunized with LktA-LppB or LktA-p31+LktA-LppB against recombinant LppB (A) and LktA-LppB (B). Statistically significant (p<0.001) increase as compared to control was shown with three asterisks.

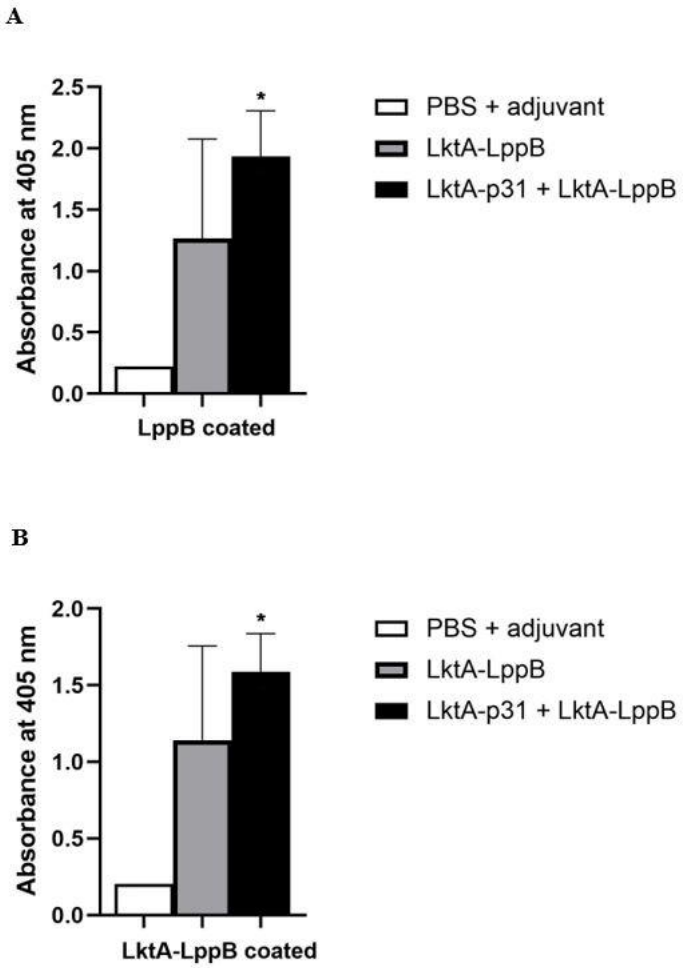

Figure 3.23. IgG2a levels in the sera from the mice immunized with LktA-LppB or LktA-p31+LktA-LppB against recombinant LppB (1:100 dilution) (A) and LktA-LppB (1:400 dilution) (B). Statistically significant (p<0.05) increase as compared to control was shown with an asterisk.

Bovine respiratory disease is caused by many microorganisms, and the two most important bacterial pathogens of this condition are *H. somni* and *M. haemolytica*. Therefore, efforts for development of a potent vaccine against BRD have always been continued.

Development of a potent vaccine against *M. haemolytica* and *H. somni* the two most important bacterial pathogens of BRD, was the purpose of this study. The effectiveness of vaccines containing bacterins of *M. haemolytica* is weak and not clear, and live *M. haemolytica* vaccines have controversy in protection studies (Martin, 1983; Mosier *et al*., 1998). Besides, common use of antibiotics among calves in feedlots inhibits the replication of live vaccine organism, and make the use of live bacterial vaccines unfunctional (Hjerpe, 1990).

Furthermore, Gershwin *et al*. (2005) stated that *H. somni* infection induces IgE antibody response, correlating with the severity of disease state, and it was also shown that commercial killed *H. somni* vaccines produced IgE mediated responses, and enhance disease (Ruby *et al*., 2000; Berghaus *et al*., 2006). However, the evocation of IgG2 antibody response is essential for protection against *H. somni* pneumonia (Corbeil *et al*., 1997). Th1 type immune response includes activation of macrophages, hence, leads to phagocytosis of bacteria. In the Th1 immune response, B cells class switch to IgG2a that is responsible for fixation complement through Fc portions of them, thus, for the antibody-mediated clearance of bacteria (Charoenvit *et al*., 2004; Baldwin *et al*., 2009). Thus, a successful vaccine candidate against BRD should induce Th1 type immune response involving increased level of IgG2a in mice.

Consequently, it seems that there is a need for vaccine candidates stimulating Th1 immune response. In our work, serum IgG2a levels in immunized mice were

significantly increased compared to control group, verifying the immunogenicity of the two fusions protein-based combined vaccine (LktA-p31 + LktA-LppB, 100 µg of each per injection).

3.6 Complement-Mediated Bacterial Killing Assay for *H. somni*

Demonstration of bactericidal activities of antibodies against immunogen proteins in the presence of complement is one of the most important indicators for these proteins to be used as vaccines.

Therefore, three sera from mice immunized with combined vaccine consisting of two fusion proteins (LktA-p31+LktA-LppB, 100 µg each per injection) were pooled and used as test serum in the complement-mediated bacterial killing assay. Also, sera from mice injected with PBS + adjuvant were used as control serum. As a complement source, sera from unvaccinated mice were used. As a result, sera from vaccinated mice showed 52% bactericidal activity on average (mean percent of three replicates) in the presence of complement (Figure 3.24), pointing to the possibility of induction of cellular response as well as the humoral one.

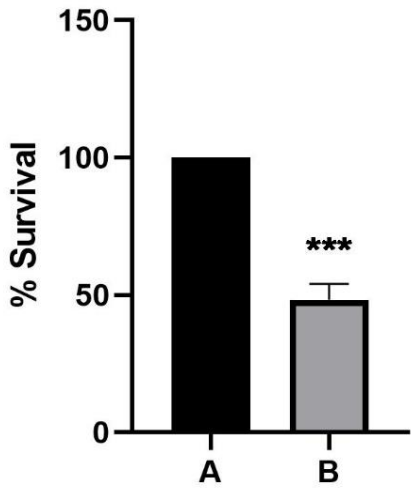

Figure 3.24. Complement-Mediated Bacterial Killing Assay for *H. somni*. A) Complement and sera from 'PBS + adjuvant' injected mice were used as a negative control. B) Complement-mediated bacterial killing activity of sera from mice immunized with the combined vaccine (LktA-p31 + LktA-LppB, 100 µg of each per injection). Statistically significant (p<0.001) difference as compared to control was shown with three asterisks.

Several studies showed the contribution of the antibodies produced against some OMPs of *M. haemolytica* to the complement-mediated killing of bacteria in vitro. A combination of a complement source, and hyperimmune serum from recombinant PlpE immunized calf, induced lysis of bacteria, and decreased the amount of bacteria (Ayalew *et al*., 2008). In another work, antibodies against the polysaccharide of *Haemophilus injluenzae* type b provided protection against the pathogen by complement-mediated lysis of bacteria (Amir *et al*., 1990). Moreover, it was suggested that the existence of bactericidal antibodies correlates with protection against meningococcal infections (Frasch, 1989). Therefore, in a study to define the OMP inducing the production of protective antibodies, monoclonal antibodies

against meningococcal class-1 OMP showed bactericidal effect, and also protected rats from infection (Goldschneider *et al*., 1969; Saukkonen *et al*., 1987). In another study conducted, while serum of calf vaccinated with *M. haemolytica* OMPs led to 100% bacterial killing; serum depleted of anti-PlpE antibodies also resulted in significant amounts of bactericidal effect (75%) (Pandher *et al*., 1998). Also, in three different studies, the serum dilution which led to a ≥50% killing compared to the number of colonies present before incubation with serum and complement was stated as the bactericidal titer, therefore we can agree that the antibodies confered ≥50% killing have significant bactericidal effect (Branefors and Dahlberg, 1980; Maslanka *et al*., 1997; Weerts *et al*., 2019).

In the present work, complement-mediated bacterial killing assay was performed to emphasize the potency of immunization achieved with our two fusion protein-based combined vaccine formulation. As a result, a significant bactericidal effect on *H. somni* cells were obtained, which shows that our fusion proteins are protective, and antibodies raised against candidate combined vaccine are highly potent.

On the other hand, the epitope fragment of LktA was included in our vaccine candidate. Since leukotoxin is an exotoxin, the antibodies raised against LktA fragment cannot bind to the surface of *M. haemolytica*, and stimulate cell lysis. Thus, we performed complement-mediated killing assay for *H. somni*, but not for *M. haemolytica*.

Consequently, our finding that the antibody response evoked in immunized mice elicited a high rate of bactericidal effect *in vitro* in the presence of the complement system emphasizes the protective capacity of our vaccine candidate.

CHAPTER 4

CONCLUSION

- Two different genetic constructs, *lktA-p31* and *lktA-lppB* were cloned in pET28 a (+). Recombinant fusion proteins expressed from these constructs were purified and formulated with an oil-based adjuvant.
- According to ELISA results, LktA-p31 and LktA-p31 + LktA-LppB formulations strongly induced total IgG and IgG2a responses against recombinant p31 and LktA-p31 proteins, significantly. Likewise, both LktA-LppB and LktA-p31 + LktA-LppB formulations led to a significant increase in the level of total IgG antibodies against recombinant LppB and LktA-LppB proteins, indicating the existence of a strong humoral response.
- On the other hand, our combined LktA-p31 + LktA-LppB formulation, but not LktA-LppB induced statistically significant levels of IgG2a antibodies. This provided very strong clue for the induction of not only humoral, but also cellular immunity by the combined experimental vaccine developed in this work.
- In the context of the present study, antigen specific-IFN-γ responses could not be determined to further verify the cellular immunity within the time limits of an M Sc thesis, as this required the establishment of splenocyte cultures.
- Complement-mediated bacterial killing assay was performed for *H. somni*. Sera obtained from mice immunized with fusion proteins-based combined vaccine (LktA-p31+LktA-LppB) showed 52% bactericidal activity when compared to the unvaccinated control group, allowing a positive assessment of the target specificity and functional activity of bactericidal antibodies.

- The results of the bactericidal killing assay and the antigen specific IgG2a response revealed that this prospect vaccine possesses potential against bacterial infections caused by the afore-mentioned pathogens.
- The present study provided an experimental recombinant combined vaccine "LktA-p31 + LktA-LppB" to be used against both *M. haemolytica* and *H. somni* and the former findings on its immunogenicity. Further studies on this potential vaccine will require the challenge experiments with both pathogens for more direct evaluation of its protectivity.

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APPENDICES

A. Structures of Plasmid Vectors and Size Markers

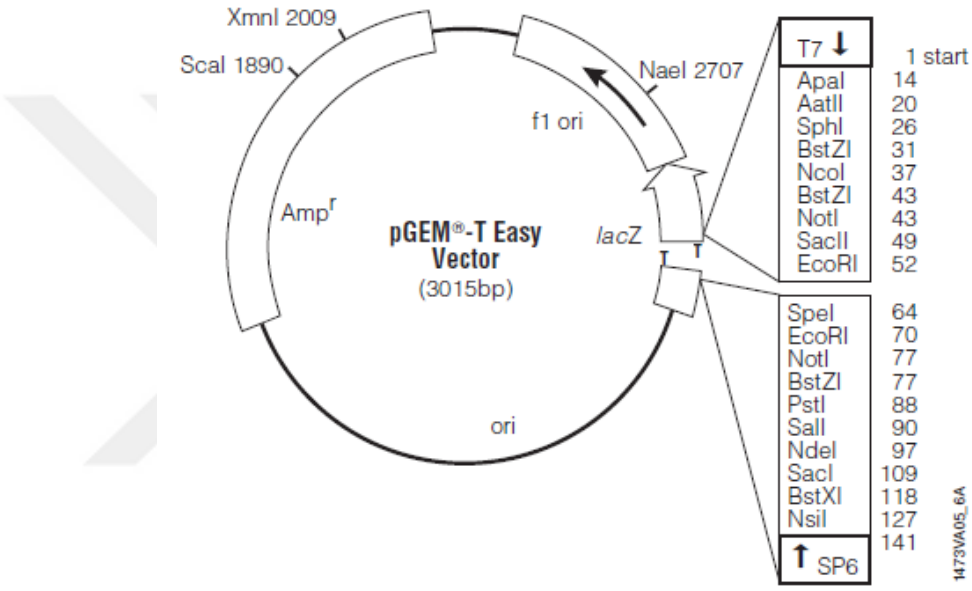


Figure A.1 pGEM®-T Easy Cloning Vector (Promega #A1360)

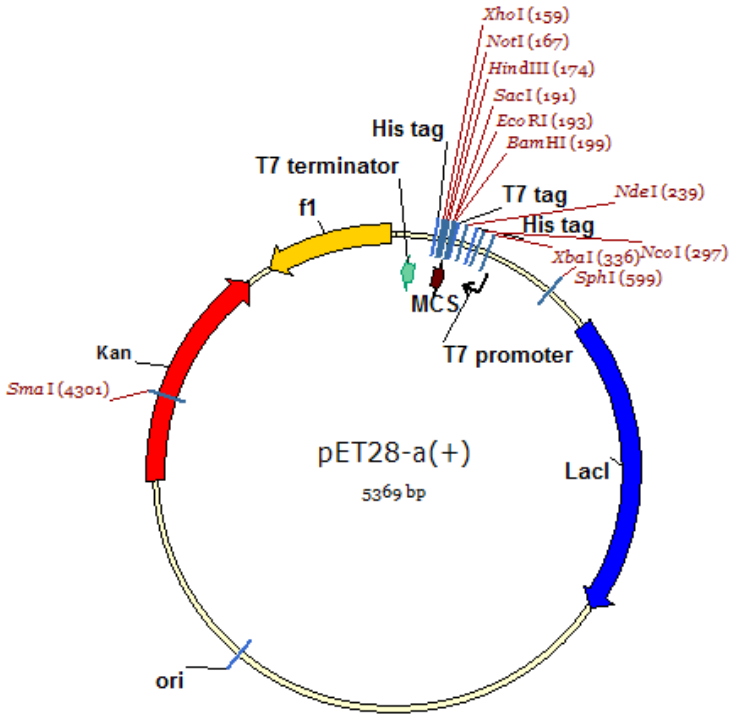
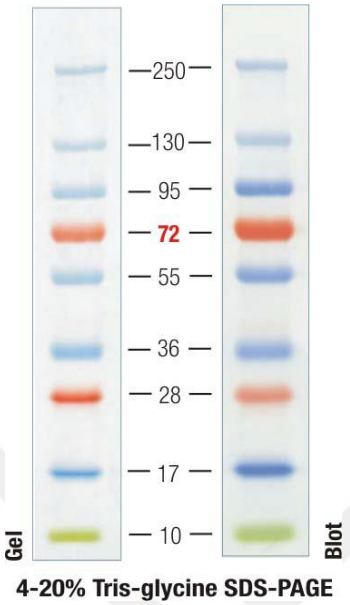


Figure A.2 pET-28a(+) His-tag Expression Vector (Novagen #69864-3)

A



B

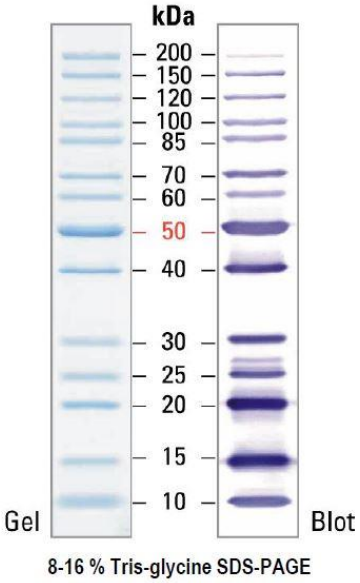


Figure A.3 PageRuler™ Plus Prestained Protein Ladder (Thermo Scientific #26619) **(A)** and PageRuler™ Unstained Protein Ladder (Thermo Scientific #26614) **(B)**.

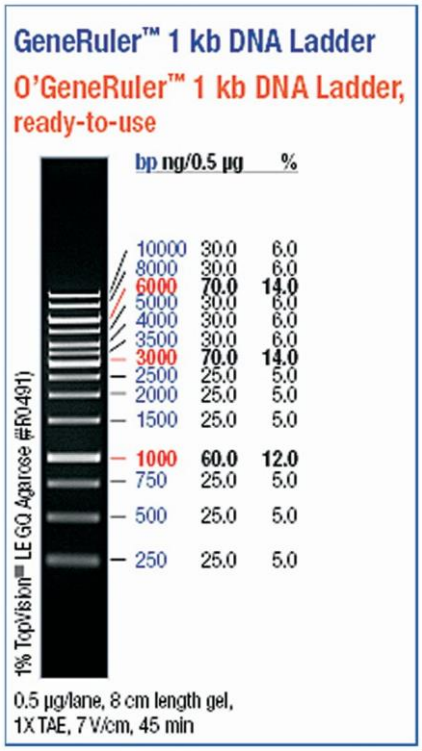


Figure A.4 1 kb DNA Ladder (GeneRuler # SM0311)

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 121oC for 15 minutes.

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 121oC for 15 minutes.

B3. Brain-Heart Infusion Broth:

- Nutrient substrate 27.5 g
- D(+)Glucose 2 g
- NaCl 5 g
- Na_2HPO_4 2.5 g
- Distilled water up to 1000 ml

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

B4. Supplemented Brain-Heart Infusion Broth:

- Nutrient substrate 27.5 g
- D(+)Glucose 2 g
- NaCl 5 g
- Na_2HPO_4 2.5 g
- Yeast Extract 5 g
- Thiamine HCl 0.1 g
- Distilled water up to 1000 ml

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

B5. 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 121oC for 15 min.

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

- **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 (6X)**

- Tris-HCl (1 M, pH 6.8) 3.5 ml
- Glycerol 3.6 ml
- SDS 1.03 g
- β-mercaptoethanol 0.5 ml
- Bromophenol blue 0.0013 g
- Distilled water up to 10 ml

- **C2.6. Fixation Solution**

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

- **C2.7. Coomassie Blue R-250 Stain**

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

- **C2.8. Destaining Solution**

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

C3. Western Blot

C3.1. Transfer Buffer (1X)

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

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

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

C4. Protein Purification

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

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

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

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

C5. *E. coli* Competent Cell Preparation

C5.1.Buffer I

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

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

C5.2. Buffer II

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

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

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

- IPTG 100 mg
- Distilled water 1 ml

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

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

- X-Gal 20 mg
- Dimethylformamide 1 ml

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

C8. Plasmid Isolation

C8.1. STE Buffer

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

C8.2. Lysis Buffer

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

C9. ELISA for Detection of Antibody Titers

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

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

pH is adjusted to 9.6 and stored at 4^{o}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^{o}C.

C9.3. Blocking Solution

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

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
$CaCl_2$.2H2O	Merck
Coomassie Blue G-250	Fluka
Coomassie Blue R-250	Fluka
Dimethylformamide	Merck
dNTPs	Fermentas
DTT	Fluka
Dulbecco's modified Eagle's medium	Biochrom
EDTA	Sigma
Ethanol	Sigma
Ethidium bromide	Sigma

Fetal bovine serum	Biochrom
Formaldehyde	Merck
Glacial acetic acid	Merck
Glycerol	Merck
Glycine	Merck
H_2SO_4	Merck
HCl	Merck
IPTG	Sigma
Isopropanol	Merck
Kanamycin	Sigma
KCl	Merck
KH_2PO_4	Merck
Luria Broth	Merck
Methanol	Merck
$MnCl_2$	Merck
MOPS	AppliChem
N.N’-Methylene-bis acrylamide	Sigma
Na_2CO_3	Merck
Na_2HPO_4	Merck
NaCl	Sigma
$NaHCO_3$	Merck
NaOH	Merck

Non-essential amino acids	Biochrom
Penicillin/streptomycin	Biochrom
Phenol/chloroform/isoamylalcohol	Amresco
Phosphoric acid	Merck
Potassium acetate	Merck
RuCl	Merck
SDS	Merck
Skim milk	Fluka
Streptavin-HRP	Pierce
Sucrose	Merck
TEMED	Merck
TMB	Thermo Scientific
Tris-base	Sigma
Tris-HCl	Fluka
Tween-20	Merck
Urea	Sigma
X-gal	Sigma
Xylene cyanol FF	Merck
2-mercaptoethanol	Merck

D2. Enzymes

*Bam*HI	NEB
SacI	NEB
NheI	NEB
T4 DNA ligase	Fermentas
Taq DNA polymerase	Fermentas

D3. Kits

AP Conjugate Substrate Kit	Bio-Rad
Gel Extraction Kit	Macherey-Nagel
pGEMT Easy Vector	Promega
Plasmid Mini Kit	Thermo Scientific
Protino Ni-TED 2000 Packed Columns	Macherey-Nagel