ASSESSMENT OF IMMUNE PROTECTIVE CAPACITIES OF THE RECOMBINANT OUTER MEMBRANE PROTEIN Q, IRON SUPEROXIDE DISMUTASE AND PUTATIVE LIPOPROTEIN FROM *BORDETELLA PERTUSSIS*

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ABSTRACT

ASSESSMENT OF IMMUNE PROTECTIVE CAPACITIES OF THE RECOMBINANT OUTER MEMBRANE PROTEIN Q, IRON SUPEROXIDE DISMUTASE AND PUTATIVE LIPOPROTEIN FROM *BORDETELLA PERTUSSIS*

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Pertussis is a contagious disease which is commonly seen among infants and children and caused by a human pathogen known as *Bordetella pertussis*. There are currently two vaccine types available against the disease; whole-cell (wP) and acellular pertussis (aP) vaccines. Due to the side effects of wP vaccine, aP vaccines are commonly preferred for vaccination. Despite high vaccination coverage, high incidence rates among adolescents and adults have been reported causing the resurgence of pertussis to be the focus of interest in many countries. Main reason is thought to be early waning immunity due to low efficiency of current aP vaccines. Thus, discovery of new antigens and development of new adjuvant systems are promising approaches to improve the efficacy of current aP vaccines.

In the present study, protective capacities of recombinant outer membrane protein Q (OmpQ), putative lipoprotein (Lpp) and iron superoxide dismutase (FeSOD) proteins were evaluated in mice models infected with *B. pertussis*. The genes of interest from *B. pertussis* Tohama I were cloned and introduced into *E. coli* BL21(DE) cells. After purification, recombinant proteins were formulated with alum or monophospholipid A (MPLA) and intraperitoneally introduced to female BALB/c mice twice at intervals of three weeks. While spleens of mice were obtained for evaluation of antigen-specific IFN-γ levels, the lungs of mice were excised after bacterial challenge with *B. pertussis* Saadet strain to evaluate colonization. Sera were also collected to measure IgG1, IgG2a and IL-10 levels. All three recombinant proteins induced high level of antibody responses. While IgG2a-type response was slightly higher in MPLA formulations, Alum-adjuvanted formulations induced higher IgG1 production. Recombinant OmpQ and putative Lpp could not trigger antigen-specific IFN-γ and IL-10 production in both alum- and MPLA-adjuvanted formulations. FeSOD-MPLA vaccination resulted in production of higher level of IFN-γ and IL-10, while there was no significant increase induced by FeSOD-Alum. Moreover, only FeSOD-MPLA vaccination was able to reduce bacterial colonization in mice lungs. In view of these results, recombinant FeSOD adjuvanted with MPLA represents a promising formulation for development of new generation aP vaccines.

Keywords: *Bordetella pertussis*, acellular vaccines, adjuvants, protective immunity, recombinant proteins

ÖZ

BORDETELLA PERTUSSİS'E AİT REKOMBİNANT DIŞ MEMBRAN PROTEİN Q, DEMİR SÜPEROKSİT DİSMUTAZ VE PUTATİF LİPOPROTEİNİN İMMÜN KORUYUCU KAPASİTELERİNİN DEĞERLENDİRİLMESİ

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Boğmaca, yenidoğanlarda ve çocuklarda yaygın şekilde görülen ve insan patojeni olan *Bordetella pertussis*'in neden olduğu bulaşıcı bir hastalıktır. Hâlihazırda tam-hücre (wP) ve aselüler (aP) olmak üzere iki çeşit boğmaca aşısı bulunmaktadır. wP aşısının yan etkilerinden dolayı aşılama için aselüler aşılar yaygın olarak tercih edilmektedir. Yüksek aşılama oranına rağmen, hastalığın gençler ve yetişkinler arasındaki yüksek görülme sıklığına dair raporlar mevcuttur; ayrıca boğmaca hastalığının yeniden ortaya çıkması birçok ülkede endişe konusudur. Ana nedenin mevcut aP aşılarının düşük etkinliği nedeni ile koruyucu bağışıklığın erkenden kaybolması olduğu düşünülmektedir. Bu nedenle, yeni antijenlerin bulunması ve yeni adjuvan sistemlerinin geliştirilmesi mevcut aP aşılarının etkinliğini güçlendirmede umut verici yöntemler olarak görülmektedir.

Mevcut çalışmada, rekombinant dış membrane protein Q (OmpQ), putatif lipoprotein (Lpp) ve demir süperoksit dismutaz (FeSOD) proteinlerinin koruyucu kapasiteleri *B*. *pertussis* ile enfekte edilmiş fare modellerinde değerlendirilmiştir. İlgili genler *B*. *pertussis* Tohama I genomundan klonlanmış ve *E*. *coli* BL21(DE) hücrelerine aktarılmıştır. Saflaştırma sonrasında, rekombinant proteinler alum veya monofosfolipit A (MPLA) ile formüle edilmiş ve dişi BALB/c farelerine intraperitonal yolla üç hafta aralıklarla iki kere uygulanmıştır. Fare dalakları antijene özel IFN-γ seviyelerinin değerlendirilmesi amacıyla alınırken, fare akciğerleri kolonizasyon değerlendirmesi için *B*. *pertussis* Saadet hücreleri ile bağışıklanmadan sonra çıkarılmıştır. IgG1, IgG2a ve IL-10 seviyelerinin ölçülmesi için serum örnekleri toplanmıştır. Rekombinant proteinlerin üçü de yüksek antikor cevabını tetiklemiştir. MPLA formülasyonlarında IgG2a tipi cevap daha yüksek iken, alum formülasyonları daha yüksek IgG1 üretimini tetiklemiştir. Rekombinant OmpQ ve putatif Lpp proteinleri hem alum hem de MPLA ile yapılan formülasyonlarda antijene özel IFN-γ ve IL-10 üretimini arttırmamıştır. FeSOD-MPLA ile yapılan aşılama yüksek seviyede IFN-γ ve IL-10 üretimi sağlarken, FeSOD-Alum formülasyonunda önemli bir artış bulunamamıştır. Ayrıca, sadece FeSOD-MPLA aşılaması fare akciğerlerinde bakteri kolonizasyonunu azaltabilmiştir. Bu sonuçlar göz önüne alındığında, FeSOD ve MPLA'nın yeni jenerasyon aP aşıları geliştirilmesi için sırasıyla umut verici bir antijen ve adjuvan adayı olabileceği görülmektedir.

Anahtar kelimeler: *Bordetella pertussis*, adjuvanlar, aselüler aşılar, koruyucu bağışıklık, rekombinant proteinler

To all the people who always supported and encouraged me…

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LIST OF ABBREVIATIONS

aP	: Acellular pertussis
bp	: Base pair
B. pertussis	: *Bordetella pertussis*
CFU	: Colony forming unit
DTaP	: Diphtheria, tetanus, acellular pertussis
DTwP	: Diphtheria, tetanus, whole-cell pertussis
E. coli	: *Escherichia coli*
FeSOD	: Iron superoxide dismutase
IFN-γ	: Interferon-gamma
Ig	: Immunoglobulin
IL	: Interleukin
IPTG	: Isopropyl-β-D-thiogalactopyranoside
kDa	: Kilodalton
Lpp	: Lipoprotein
MPLA	: Monophospholipid A
OD	: Optical density
OmpQ	: Outer membrane protein Q
PBS	: Phosphate buffered saline
pNPP	: p-nitrophenyl phosphate disodium salt
SDS-PAGE	: Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Th1	: T helper 1
Th2	: T helper 2
TMP-SMZ	: Trimethoprim-sulfamethoxazole
X-gal	: 5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside
wP	: Whole-cell pertussis

CHAPTER 1

INTRODUCTION

1.1. The Disease: Pertussis (Whooping Cough)

Pertussis, or whooping cough, is a highly contagious respiratory tract disease caused by *Bordetella pertussis*, which is a Gram-negative, non-spore forming bacterium isolated by Bordet and Gengou in 1906 for the first time (Bordet and Gengou, 1906). Although pertussis is considered as a disease affecting only infants and children, incidence of the disease in older ages has been recorded. However, young infants still have a higher risk of severe pertussis due to the immaturity of the respiratory and immune systems, and the lack of full vaccination (Mooi *et al*., 2007; Sealey *et al*., 2016). It can be considered as a cough sickness, but severe results including death may be seen, especially among unvaccinated infants (Hewlett *et al*., 2014). Despite the presence of pertussis vaccines and high vaccination coverage (86% in 2015), resurgence of the disease has been recently reported in a number of countries such as England, the U.S., Netherlands and Australia (Burns *et al*., 2014; WHO, 2016). Suboptimal vaccine production, waning immunity, improved diagnosis and antigenic divergence between vaccine and circulating strains are proposed as the leading factors for pertussis resurgence (Berbers *et al*., 2009).

1.1.1. Pathogenesis and Clinical Manifestations

The mechanisms behind pertussis pathogenesis are mainly considered as a team work characterized by scientifically proven or hypothesized contribution of each virulence determinant of the bacterium to the pathogenesis. The steps of pathogenesis are (Hewlett *et al*., 2014):

- Exposure/inoculation: Aerosol transmission of *B*. *pertussis* is possible without any contact between healthy and infected hosts (Warfel *et al*., 2012a). The bacterium locates at the respiratory tract but spreading from the tract rarely occurs.

- Attachment/proliferation: *B*. *pertussis* mainly interacts with ciliated epithelial cells in the lower and upper respiratory tract with contribution of various virulence factors including pertactin, filamentous hemagglutinin and fimbrial proteins (Mattoo and Cherry, 2005).

- Evasion/host defense modulation: Increasing number of virulence factors plays an important role in modulation of host defenses such as serum resistance and ciliary clearance (Mattoo and Cherry, 2005).

- Local/systemic cell and tissue damage: Some of the virulence factors can interrupt endogenous signaling pathways causing increased sensitivity to inflammatory mediators which, in turn, can result in the characteristic cough of the disease (Carbonetti, 2007).

- Chronic infection/death or clearance of symptoms: Contagion of the disease is usually from upper to lower respiratory tract, however

systemic spread rarely occurs. It can last for several months, but mortality rate is quite low (Hewlett *et al*., 2014).

The disease mainly has three stages that can last for weeks or months. At catarrhal stage with duration of 7-10 days, symptoms include runny nose, low-grade fever and mild cough. At this stage, severity of cough can increase during the course of the disease. It is followed by paroxysmal stage which generally lasts from 1-6 weeks to 10 weeks. Coughing fits, paroxymsms, are the characteristic symptoms of the phase which get worse during nighttime. Frequency of paroxysmal attacks increases during the first two weeks before it gradually decreases. In addition to numerous rapid coughs, exhaustion and vomiting can also be observed in the patients. Stage 3, called the convalescent phase, lasts from 10 days to months, the cough alleviates and the recovery gradually begins. However, host can be susceptible to a second respiratory infection (CDC, 2017a).

Apart from the paroxysmal cough, such systemic manifestations as lymphocytosis, post-tussive vomiting, seizures, loss of consciousness may occur after clearance of the infection. The mechanisms of action behind all the signs and symptoms are not clear, however, many are attributed to synergistic effects of virulence determinants of *B*. *pertussis* (Hewlett *et al*., 2014).

1.1.2. Diagnosis and Treatment

The clinical case definition of pertussis is defined as the presence of one or more typical clinical symptoms such as paroxysmal cough lasting for at least 2 weeks. However, its specificity can be negatively affected by patient age, duration between infection and diagnosis, and previous vaccination. In addition to physical examination and history of symptoms, laboratory tests are required for

accurate results. Direct Fluorescent-Antibody Assay (DFA) provides a rapid diagnosis, but its specificity and sensitivity is low. Therefore, it should be accompanied by other methods such as PCR, serology or ELISA (Figure 1.1) (van der Zee *et al*., 2015).

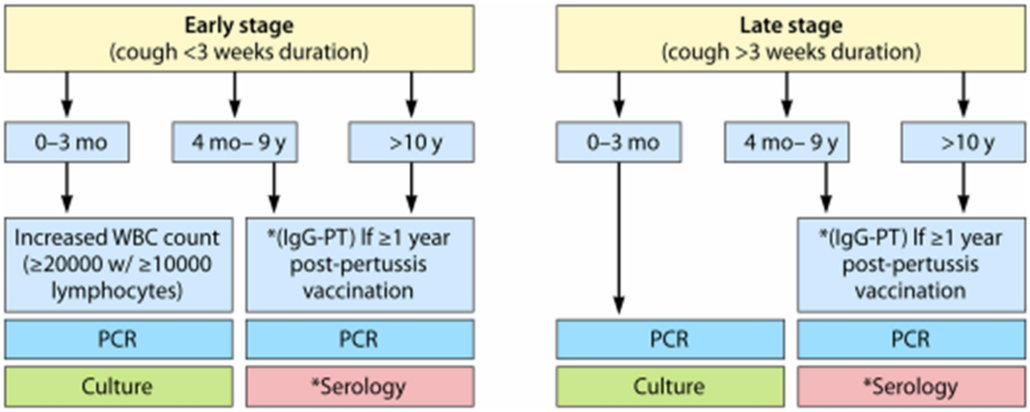


Figure 1.1. Recommended flow diagram for *Bordetella pertussis* diagnosis in the laboratory (van der Zee *et a*l., 2015).

Pertussis can be prevented by vaccination, but the disease can be severe, even lethal, in infants who are too young to be vaccinated. Patients with severe symptoms and complications and infants younger than 3 months should be hospitalized for treatment. It is necessary to provide sufficient hydration, nutrition and oxygen support if necessary (Otar *et al*., 2014). The disease can be treated with antibiotics which can clear the bacteria in nasopharynx and limit the spread of the infection. The efficiency of antibiotics is mainly based on the stage of pertussis, and the optimal time for antibiotic therapy for effective treatment is the first 3 weeks which represents the catarrhal stage. Different antimicrobial agents including erythromycin, azithromycin, clarithromycin and trimethoprim-

sulfamethoxazole (TMP-SMZ) are recommended mainly based on the availability, resistance, patient age and status (Kilgore *et al*., 2016) (Table 1.1). In addition to antibiotics, supplementary treatment options including anti-pertussis toxin immunoglobulin, bronchodilators, steroids and corticosteroids, and antihistamines are investigated. Moreover, search for novel alternative therapeutic targets such as airway anion channels and sphingosine-1-phosphate pathway is in progress (Scanlon *et al*., 2015).

1.1.3. Epidemiology of Pertussis

Pertussis is one of the diseases that should be reported to the health authorities when it is diagnosed. Clinical case definitions of pertussis have been presented by the World Health Organization (WHO) and the Centers for Disease Control and Prevention (CDC). However, many countries apply different clinical case definitions and this causes problems in routine pertussis reports. Therefore, difficulties in evaluation of global incidence of pertussis as well as inconsistencies among countries have emerged (Kurugöl, 2009).

The disease has non-negligible morbidity and mortality rates worlwide, especially among infants. However, it still threatens public health even when high immunization coverage is achieved with effective vaccination programs in developed countries such as Canada, the United States, the Netherlands and Australia (Figure 1.2) (Edwards and Decker, 2013; Kilgore *et al*., 2016; WHO, 2017a). Although pertussis has been primarily affecting children less than 6 years old, a shift towards adolescents and adults has been observed in the past 20 years (Figure 1.3) (Kilgore *et al*., 2016).

Table 1.1. The antimicrobial agents used for treatment of pertussis (Adopted from Kilgore *et al*., 2016).

Agent	Age	Dosing information
Erythromycin	> 1 month	40-60 mg/kg/day in 3 or 4 divided doses for 7-14 days
	Adults	500 mg every 6 or 8 h for 7-14 days
Azithromycin	< 6 months	10 mg/kg daily for 5 days
	≥ 6 months	10 mg/kg on day 1, followed by 5 mg/kg on days 2-5
	Adults	500 mg on day 1, followed by 250 mg on days 2-5
Clarithromycin	> 1 month	15 mg/kg/day in 2 divided doses for 7 days
	Adults	500 mg every 12 h for 7 days
TMP-SMZ	> 2 months	TMP at 8 mg/kg/day plus SMZ at 40 mg/kg/day every 12 h for 14 days
	Adults	TMP at 320 mg/day plus SMZ at 1600 mg/day every 12 h for 14 days

* TMP-SMZ: trimethoprim-sulfamethoxazole

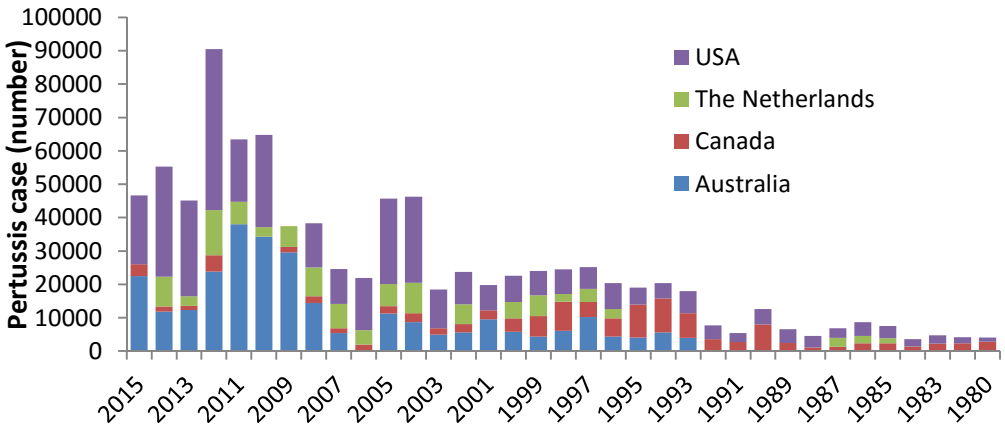


Figure 1.2. Incidence time series of pertussis for USA, the Netherlands, Canada and Australia (WHO, 2017a).

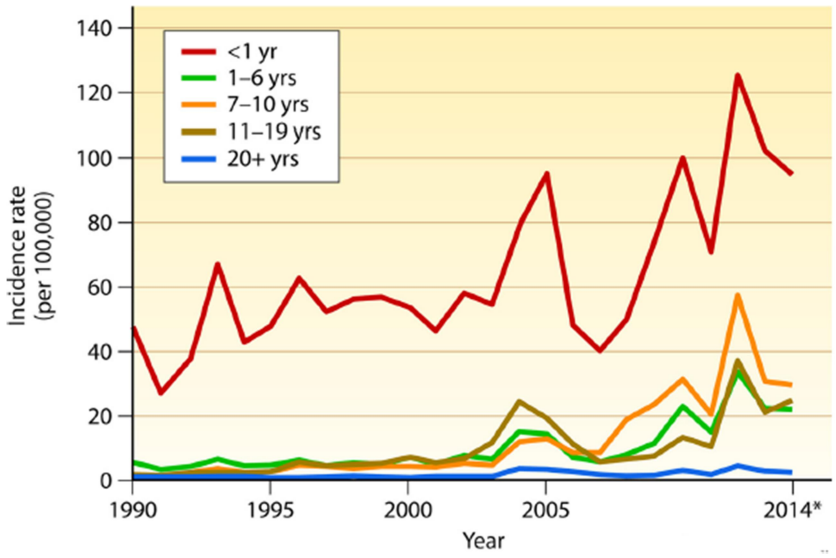


Figure 1.3. Pertussis incidence rates according to age groups (1990-2014) (Kilgore *et al*., 2016).

161,889 global pertussis cases have been reported during 2013. WHO reported 172,940 cases in 2014, corresponding to approximately 7% increase wihtin a year (WHO, 2017a). Despite widespread vaccination efforts, pertussis outbreaks have occurred in many countries. In 2010, the disease killed 10 babies in California, U.S. and a pertussis epidemic was declared by the California Department of Public Health in 2014 with reported 9,935 pertussis cases including 2006 cases in adolescents aged 14-16 years (CDC, 2014). Also, a large pertussis epidemic was experienced in Minnesota, U.S in 2012 with 4,144 cases reported. In the same year, a major outbreak was reported in the United Kingdom with 14 infant deaths among 10,000 laboratory-confirmed cases (Sealey *et al*., 2015). Moreover, 4,387 pertussis cases were reported by the Washington State Secretary of Health in 2012 and the occurence was quite high among children as well as infants less than 1 year old (CDC, 2012). In 2015, Canada had also experienced pertussis outbreaks in different areas including Manitoba, New Brunswick and Mauricie-Central Quebec region (Promed Mail, 2015).

Pertussis cases in Turkey are given in Figure 1.4 according to the data published by WHO (WHO, 2017a). Through vaccination, number of cases has been reduced, but there are still slight increases in 3- to 4-year cycles which require attention due to the severity of the disease especially in infants (Otar *et al*., 2014).

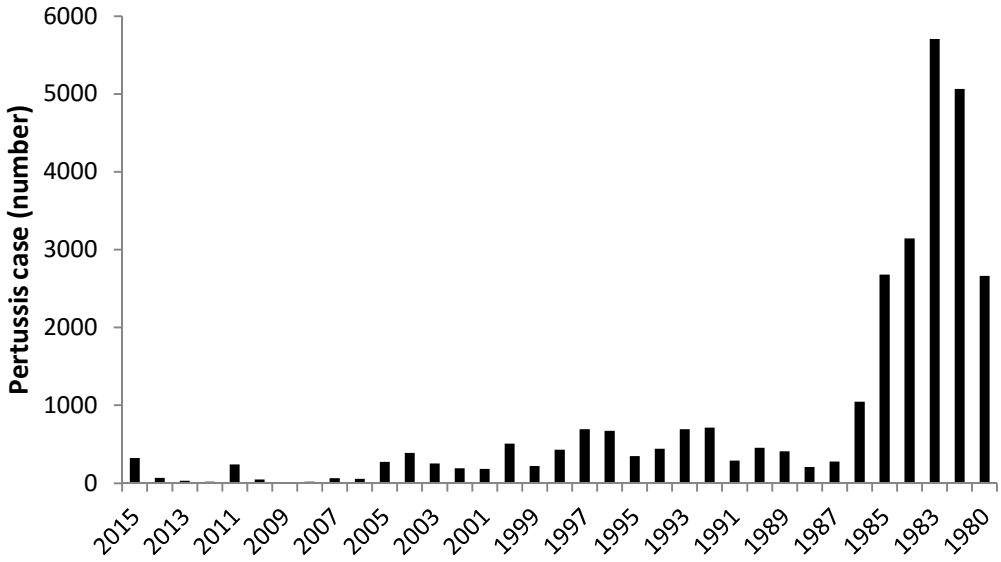


Figure 1.4. Incidence time series of pertussis for Turkey (Adopted from WHO, 2017a).

1.2. The Genus *Bordetella* and *Bordetella pertussis*

1.2.1. Phylogeny of *Bordetella* and Evolution of *Bordetella pertussis*

The genus *Bordetella* consists of a group of Gram-negative, small, non-spore forming, aerobic coccobacilli, and contains both human and animal pathogens which have an important place in human and veterinary medicine due to their capacity of causing various bronchial and pulmonary infections. Pyhlogenetic analyses of 16S gene sequences revealed that *Bordetella* species collected from soil, water, plants and sediments have evolved from animal-associated species. It was suggested that both soil and water can be possible environmental factors for the evolution of pathogenic *Bordetella* species. Based on these findings, it was suggested that wide distribution of pathogenic *Bordetella* species and

frequent outbreaks of the disease may be due to bacteria spreading out from an environmental source such as water and soil (Soumana *et al*., 2017).

The genus currently contains ten species; *B*. *pertussis*, *B*. *avium*, *B*. *parapertussis* (human-adapted), *B*. *parapertussis* (ovine-adapted), *B*. *holmesii*, *B*. *bronchiseptica*, *B*. *hinzii*, *B*. *ansorpi*, *B*. *trematum* and *B*. *petrii* (Mattoo and Cherry, 2005). Among them, *B*. *pertussis*, *B*. *parapertussis* (human-adapted), *B*. *holmesii*, *B*. *petri* and *B*. *bronchiseptica* can cause respiratory tract infections in humans, however *B*. *pertussis* can provoke more severe infection in the human host (Zlamy, 2016). The comparative genomic analysis uncovered independent evolution of *B*. *parapertussis* and *B*. *pertussis* from distinct *B*. *bronchiseptica*-like ancestors (Parkhill *et al*., 2003) and multilocus sequence typing revealed a recent evolution of *B*. *pertussis* from *B*. *bronchiseptica* (Diavatopoulos *et al*., 2005). In addition, it seems that *B*. *pertussis* has a smaller genome than *B*. *parapertussis* and *B*. *bronchiseptica,* mainly due to high levels of gene loss. It is suspected that these genes are mostly related with bacterial colonization in non-human hosts whereas *B*. *pertussis* pathogenesis is now restricted to humans (Sealey *et al*., 2016).

1.2.2. Virulence Determinants of *Bordetella pertussis*

Virulence determinants of *B*. *pertussis* are grouped in two main categories; adhesins and toxins. Adhesins consist of fimbriae, pertactin, tracheal colonization factor, serum-resistance protein and filamentous hemagglutinin. Toxins, on the other hand, contain pertussis toxin, tracheal cytotoxin, adenylate cyclase toxin, lipooligosaccharide and dermonecrotic toxin (Figure 1.5) (Melvin *et al*., 2014; Zlamy, 2016).

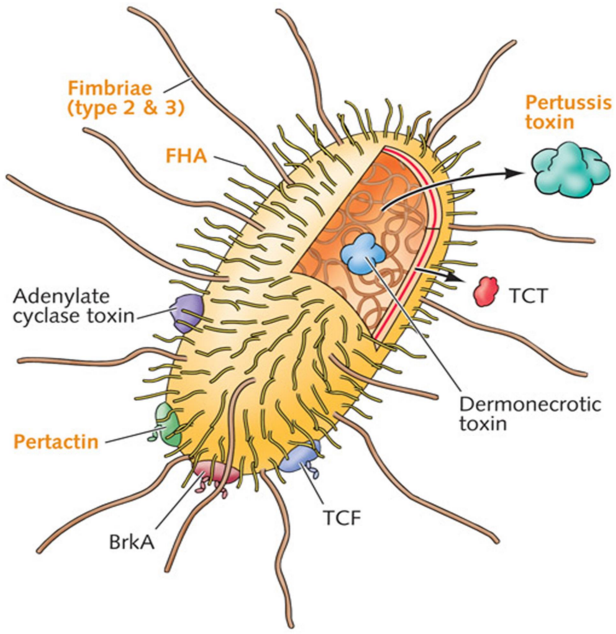


Figure 1.5. Virulence determinants of *Bordetella pertussis* (Adopted from Weiss, 1997).

Genome-wide analyses have uncovered that the *bvgAS* locus encoding a two-component system, BvgAS, is responsible for controlling the expression of hundreds of genes which are involved in numerous functions such as pathogenesis, survival outside the host, and physiological and regulatory systems (Cummings *et al*., 2006; Melvin *et al*., 2014).

1.2.2.1. The BvgAS Phosphorelay

BvgA and BvgS proteins encoded by *bvgAS* locus function as a two-component signal transduction system to sense many different signals coming from the environment (Smith *et al*., 2001). While BvgA protein is a DNA-binding response regulator, BvgS is a transmembrane polydomain sensor kinase which is found in cytoplasmic membrane. Autophosphorylation of BvgS leads to the

activation of BvgA through phosphorylation. Activated BvgA binds to alpha subunit of RNA polymerase which then either represses or activates the transcription of various virulence genes except the one encoding tracheal cytotoxin (Melvin *et al*., 2014).

BvgAS system regulates at least three phenotypic phases named as Bvg- (inactive), Bvg+ (active) and Bvgi (intermediate), leading to control of differential expression of virulence genes (Deora *et al*., 2001). When BvgAS system is functional, phosphorylated BvgA leads to transcriptional activation of *virulence* (*vir*) activated gene (*vag*) loci which are responsible for the expression of crucial proteins including virulence factors of the pathogen. While *vag* is transcriptionally activated, transcription of *vir* repressed gene (*vrg*) loci encoding outer membrane proteins is repressed by active BvgA. Under Bvg- phase, transcriptional repression of *vrg* loci is removed and transcription of *vag* loci is not activated. The third phase, Bvgi, is distinguished from other two phases by the absence of toxin production and the presence of adhesins. In addition, it seems that some genes such as *bipA* are maximally expressed in this phase (Cotter and Jones, 2003). Studies propose that BvgAS functions as a sensor to detect whether the pathogen is inside of a host where it can activate Bvg+ phase or outside where Bvg- phase is under control (Cotter and Miller, 1994; Merkel *et al*., 1998).

Natural signals which can cause autophosphorylation of BvgS are not known, but avirulent state of *B. pertussis* can be obtained by the presence of nicotinic acid or sulfate ions in the culture media or low incubation temperature which inactivate BvgS (Melvin *et al*., 2014).

1.2.2.2. Toxins

Among five known toxins of the pathogen, pertussis toxin (PT) is one of the most important virulence factors solely expressed from *ptx* genes of *B*. *pertussis*. The toxin has an A monomer with a catalytic activity and a B oligomer with five membrane-binding-transport subunits (Higgs *et al*., 2012). The B oligomer can interact with surface glycoproteins on host cells allowing PT to enter via receptor-associated endocytosis (Stein *et al*., 1994). The A monomer has no lysine residues which is the target for uniquitination and this allows PT to avoid degradation in the cytosol. The A subunit has a role in deactivation of cytoplasmic G proteins which leads to corruption of immune response regulation (Higgs *et al*., 2012). Studies have revealed that PT causes lymphocytosis and can inhibit phagocytosis by antigen-presenting cells (APC) and chemotaxis of immune cells (Spangrude *et al*., 1985; Shumilla *et al*., 2004). Purified PT is detoxified by treating with chemicals such as formaldehyde to constitute an important component of commercially available acellular pertussis (aP) vaccines (Seubert *et al*., 2014).

Adenylate cyclase toxin (ACT) is another toxin that has two modules: N terminal domain, that functions in conversion of ATP to cyclic AMP (cAMP) and C terminal domain, which has a role in binding to target cells to form pores (Melvin *et al*., 2014). ACT has a high affinity for binding to complement receptor-3 (CR3) complex of immune cells such as macrophages and it can induce apoptosis of innate immune cells through pore formation, ATP depletion and unusual calcium influx (Guermonprez *et al*., 2001; Higgs *et al*., 2012). It was shown that ACT immunization can provide a level of protection in mice models, suggesting that it may be considered as a potential component of a vaccine (Betsou *et al*., 1995). However, it was later demonstrated that detoxified ACT prevents complement-mediated phagocytosis by neutrophil-like cells

through blocking CR3 complex which in turn might be a drawback if used as a vaccine antigen (Prior *et al*., 2006).

The common endotoxin called lipopolysaccharide (LPS) has no O antigen in *B*. *pertussis*, so it is called lipooligosaccharide (LOS) more conveniently. It was shown that this toxin has roles in colonization and survival of the pathogen (Harvill *et al*., 2000; Higgs *et al*., 2012). In addition, it was reported that stimulation of dendritic cells by LOS leads to production of anti-inflammatory regulatory T cells (Higgins *et al*., 2003). Another study demonstrated that LOS prevents apoptosis of monocyte-derived dendritic cells and extends their longevity (Fedele *et al*., 2007). Therefore, its inclusion into aP vaccines can be considered and tested in future.

Tracheal cytotoxin (TCT) is a low-molecular weight fragment produced from peptidoglycans during cell wall remodeling and secreted into extracellular environment in large amounts due to inefficient recycling by *B*. *pertussis* (Melvin *et al*., 2014). *In vitro* studies reveal that TCT can cause inhibition of DNA synthesis in tracheal epithelial cells. Also, its synergistic action with LOS induces pro-inflammatory cytokine synthesis, leading to destruction of ciliated epithelial cells. Therefore, it is suggested that TCT may have a role in survival of the pathogen in the airways (Cookson *et al*., 1989; Flak *et al*., 1999).

Dermonecrotic toxin (DNT) is the toxin that causes necrotic lesions in the mice subcutaneously infected with *B*. *pertussis* (Cowell *et al*., 1979). DNT functions by activating intracellular Rho GTPases by deamination or polyamination. Its entry into the cytoplasm is facilitated by dynamin-mediated endocytosis, and it also has a role in stimulating vasoconstriction (Walker and Weiss, 1994; Fukui-Miyazaki *et al*., 2010).

1.2.2.3. Adhesins

The major adhesin factor of *B*. *pertussis* is filamentous hemagglutinin (FHA) which is a large and highly immunogenic protein. FHA is found on bacterial cell surface and responsible for adherence of the pathogen to host ciliated epithelial cells (Higgs *et al*., 2012). The protein itself cannot elucidate protective immunity which suggests that FHA is not a key protective antigen but is still one of the components of current aP vaccines due to its protective action with other antigens (Knight *et al*., 2006). Although it is mostly expressed on cell surface, a substantial amount is secreted into the extracellular environment where it can show immunomodulatory actions including induction of interleukin (IL) 10 and suppression of interferon-gamma (IFN-γ) production. Moreover, generation of IL-10 producing T regulatory cells leads to inhibition of T helper type 1 (Th1) cells (Inatsuka *et al*., 2005).

Pertactin (PRN) is another adhesin factor having role in adherence of the pathogen to epithelial cells and monocytes (Higgs *et al*., 2012). PRN belongs to basic outer membrane autotransporter family and is involved in aP vaccines due to its protective efficacy demonstrated in mice and humans (Novotny *et al*., 1991; Taranger *et al*., 2000). It was shown that defects in both FHA and PRN cause more rapid clearance of the bacteria from the respiratory tract (Locht, 1999). In recent years, PRN-negative *B*. *pertussis* strains have been isolated from patients, suggesting a selective pressure of aP vaccines (Pawloski *et al*., 2013).

The *Bordetella* resistance to killing (BrkA) protein is an autotransporter protein sharing homology with PNR. BrkA has a role in adherence to host cells and provides resistance to killing by inhibiting antibody-mediated classical pathway of the complement system (Barnes and Weiss, 2001). BrkA-deficient mutants of

B. *pertussis* have shown defects in their colonization ability in mice (Elder and Harvill, 2004). When added to two-component (PT and FHA) acellular vaccine, it improved the protectivity of the vaccine as much as the classical third component pertactin (PRN), suggesting that BrkA can be used for a new aP vaccine formulation with other antigens (Marr *et al*., 2008).

B. *pertussis* expresses two serologically distinct fimbrial proteins; namely Fim2 and Fim3, both of which are crucial for adhesion to host epithelial cells. Mutations in *fim2* and *fim3* promoter regions lead to serotype switching. Fimbriae-deficient *B*. *pertussis* mutants demonstrated reduction in adherence in a baboon model (Funnell and Robinson, 1993). While some *B*. *pertussis* strains produce both Fim2 and Fim3, some others express either Fim2 or Fim3. The protective activities of Fim2 and Fim3 were demonstrated in mouse infection models, as a result, aP vaccine manufacturers include at least one of them into their vaccines (Robinson *et al*., 1989; Gorringe and Vaughan, 2014).

Tracheal colonization factor (TCF) has been identified as a virulence-associated factor which has structural similarity with PRN and enhances the growth of *B*. *pertussis* in the trachea. TCF has two forms, one of which is small and secreted from the cell whereas the other is large and cell-associated (Finn and Stevens, 1995; Locht, 1999).

1.3. Host Immune Responses to *Bordetella pertussis*

B. *pertussis* was considered as an extracellular pathogen for a long time; however it has been demonstrated that the pathogen can survive in neutrophils and macrophages, indicating that both humoral (antibody) and cellular immune responses are required to clear the bacteria from the host respiratory tract (Figure 1.6) (Lamberti *et al*., 2013). In addition to humoral response, stimulation

of Th1 cells and IFN-γ production seem to be necessary for recovery from pertussis (Mascart *et al*., 2003).

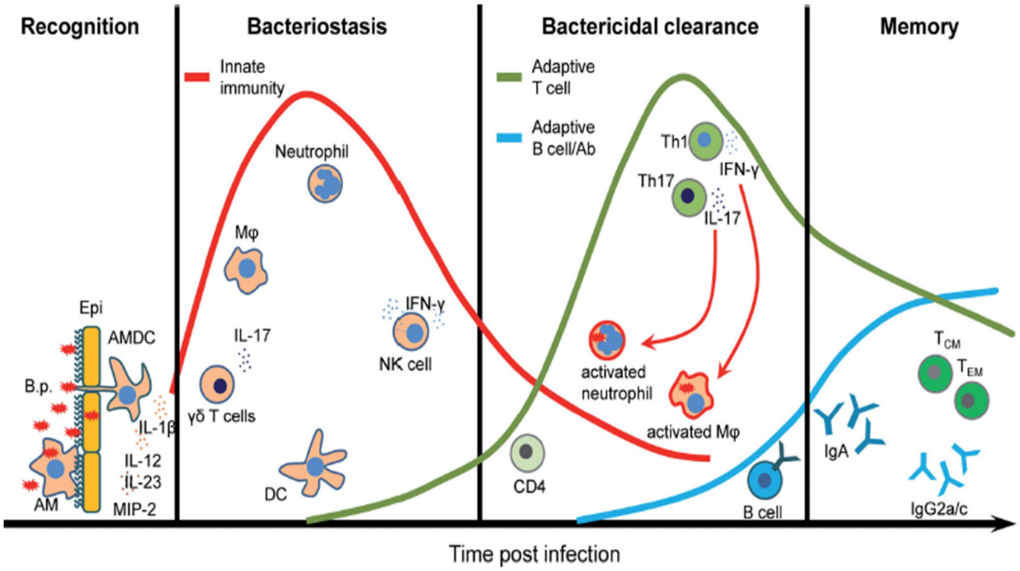


Figure 1.6. Immune response to *Bordetella pertussis* (Adopted from Brummelman *et al*., 2015).

1.3.1. Innate Immunity to *Bordetella pertussis*

When *B*. *pertussis* infects a host, it binds to ciliated epithelial cells of trachea, and bronchioles via synergistic effects of adhesin factors including FHA and PRN. First line of defense against the pathogen is the barrier of epithelial cells which can prevent bacterial penetration (Brummelman *et al*., 2015). Two resident immune cells, immature dendritic cells (DCs) and alveolar macrophages, respond to the infection (McGuirk *et al*., 1998). After phagocytosis of *B*. *pertussis*, macrophages exhibit both nitric oxide (NO)-dependent and NO-independent pathways to eliminate the pathogen. Although a

large amount of the bacteria is destroyed, a small portion can survive in non-acidic compartments of macrophages (Lamberti *et al*., 2013). Phagocytosis of the pathogen by macrophages is boosted by IFN-γ and IL-17 (Higgs *et al*., 2012). Studies have shown that IFN-γ and IL-17 also contribute to eliminate intracellular *B*. *pertussis* (Mahon and Mills, 1999; Higgins *et al*., 2006). When dendritic cells encounter with virulence factors and pathogen-associated molecular patterns (PAMPs) of *B*. *pertussis*, they utilize them to prime T cells at lymph nodes. Toll-like receptor 4 (TLR4) on DCs interacts with LPS, leading to maturation of the cells and IFN-γ secretion which then activate Th1 cells (Jahnsen *et al*., 2006; Higgs *et al*., 2012).

Response of macrophages and DCs to the pathogen is followed by an influx of natural killer (NK) cells and neutrophils to the infection site. Although it is well known that mainly NK cells are responsible for the defense against viral infections, they can also act as an initial source for IFN-γ production which is an important cytokine that mediates Th1 responses for bacterial clearance from the lungs in the case of pertussis infection. Macrophages activated by *B*. *pertussis* secrete IL-12 which induces the production of IFN-γ from NK cells (Byrne *et al*., 2004; Brummelman *et al*., 2015). PT prevents neutrophil recruitment at early period of infection, however synergistic actions of PT, ACT and LOS lead to IL-17 response which drives recruitment of neutrophils at day 10-14 after infection. It is suggested that neutrophil recruitment and activation mediated by IL-17 response has a role in bacterial clearance through antibody-associated phagocytosis and neutrophil extracellular trap (NET) formation (Ross *et al*., 2013; Eby *et al*., 2015).

1.3.2. Adaptive Immunity to *Bordetella pertussis*

1.3.2.1. Cell-Mediated Response

Virulence factors of *B. pertussis* recognized by specific receptors on DCs. Fragmented parts of the pathogen after ingestion by DCs are presented to naïve CD4+ T cells in draining lymph nodes through major histocompatibility complexes (MHC). Depending on the signal quality, peptide specificity, and cytokine types produced by innate immune cells, activated CD4+ T cells will proliferate and differentiate into various subtypes of T cells including Th17, Treg (regulatory), Th1, Th2 or T_{FH} (follicular) while some CD4+ T cells act as memory T cells (Christie and Zhu, 2014). In natural infection, the most dominant subtype is Th1 cells that secrete IFN-γ and IL-2 (Mills *et al*., 1993). IFN-γ and IL-2 mainly activate macrophages and other immune cells to induce phagocyte-dependent protective actions which are necessary to clear the pathogen from the lungs. Also, IFN-γ stimulates production of immunoglobulin G (IgG) 2a antibody which has an opsonic activity. While Th1 cells predominantly function in cell-mediated response, Th2 cells mainly have roles in antibody-mediated immunity (Fedel *et al*., 2015). Th17 cells produce IL-17 which, in turn, activates recruitment of macrophages and neutrophils to the infection site where they effectively phagocytize the pathogen (Higgs *et al*., 2012). T_{FH} cells have a role in formation of germinal centers (GC) where B cells and CD4+ T cells can interact for antibody production. Moreover, it is suggested that T_{FH} cells are important for the production of antigen-specific memory B cells (Crotty, 2011).

During pertussis infection, Treg cells secreting IL-10 are mainly induced by two *B. pertussis* virulence factors; ACT and FHA (McGuirk *et al*., 2002). These cells act as immunomodulators which limit immune responses by inhibiting

functions of Th1 cells. Although suppression of the immune system seems threatening during infection, it might be beneficial for the host to prevent excessive and persistent immune reactions.

Unlike CD4+ T cells, little information is available for CD8+ cytotoxic T cell activity in pertussis infection because of the fact that *B*. *pertussis* has been considered as an extracellular pathogen for a long time. However, it is now well-documented that the pathogen can also act intracellularly. As a result, studies are now being focused on CD8+ T cell response which has a role in antigen presentation after phagocytosis of pathogens. Although it is proposed that CD8+ T cells do not function in protective immunity against *B*. *pertussis*, a recent study demonstrated the production of IFN-γ by CD8+ T cells that help CD4+ T cells to induce a better immune response against *B*. *pertussis* (Dirix *et al*., 2012).

1.3.2.2. Antibody-Mediated (Humoral) Response

When naïve B cells interact with the antigens, they become short-lived plasma cells secreting antibodies. On the other hand, as they encounter with T_{FH} cells, GCs are formed where B cells turn into ether long-lived antibody-secreting plasma cells or antigen-specific memory B cells (van Twillert *et al*., 2015). Naïve B cells have IgM on their cell membranes and IgM is more poly-reactive than other antibodies to give rapid response to various antigens. It can function in fixing complement proteins. In early stages of pertussis infection, IgA secreted from mucosal surfaces is the first antibody detected in high level in the serum. It protects mucosal surfaces from bacterial invasion and from toxins either by blocking their bindings or by neutralization (Schroeder and Cavacini, 2013). Following IgA, titers of *B*. *pertussis*-specific IgG antibodies start to increase in the serum. Among subclasses of IgG antibodies, IgG1 and IgG3 are mainly produced after interaction of B cells with protein antigens, while IgG2a

and IgG4 antibodies are induced as a result of polysaccharide antigens. In addition, it is thought that IgG1 antibodies mainly reflect Th2 type immune response while IgG2a is associated with Th1 response (Hayes *et al*., 2011). A balance between IgG1 and IgG2a seems to be significant in the fight against *B. pertussis* in terms of bacterial clearance (Hjelholt *et al*., 2013; van Twillert *et al*., 2015).

As previously stated, Th2 cells have predominant roles in antibody-mediated immunity and the cytokines IL-4 and IL-5 secreted by Th2 cells activate B cell maturation, leading to the production of antibodies in high amounts (Horikawa and Takatsu, 2006).

1.4. Vaccines

1.4.1. History and Types of Vaccines

It has been over 200 years since vaccinology was introduced in 1798 by Edward Jenner who discovered a protection against smallpox disease when the materials from a lesion caused by *Variola* virus were obtained and inoculated into the arm of an eight-year-old boy (Figure 1.7). While the protection launched by Edward Jenner has taken a part in the literature as 'variolation', Louis Pasteur has been known as the pioneer of 'vaccine' concept (Plotkin and Plotkin, 2011). The discovery of smallpox vaccine was followed by Pasteur who explored how vaccines could be made from attenuated or weakened pathogens and invented 'first generation' vaccines against fowl cholera, anthrax, and rabies. These discoveries paved the way for 'second generation' vaccine production against different diseases including measles, polio, rubella and mumps throughout the history (Plotkin, 2014). Although conventional methods such as attenuation or killing of pathogens have been popular for vaccine production since the

beginning of the vaccine history, there are some limitations that should be overcome. Firstly, production of vaccines via empirical approaches is a very slow process and does not meet the need for a rapid vaccine development as is the case with influenza pandemics. Moreover, it is proven to be difficult to apply these approaches to uncultivable pathogens including papilloma virus type 16 and 18, and *Mycobacterium leper*. Finally, vaccines produced via traditional methods may not confer broad protection against pathogens having high antigenic variability such as HIV and serogroup B meningococcus or against intracellular pathogens such as *M. tuberculosis* (Finco and Rappuoli, 2014).

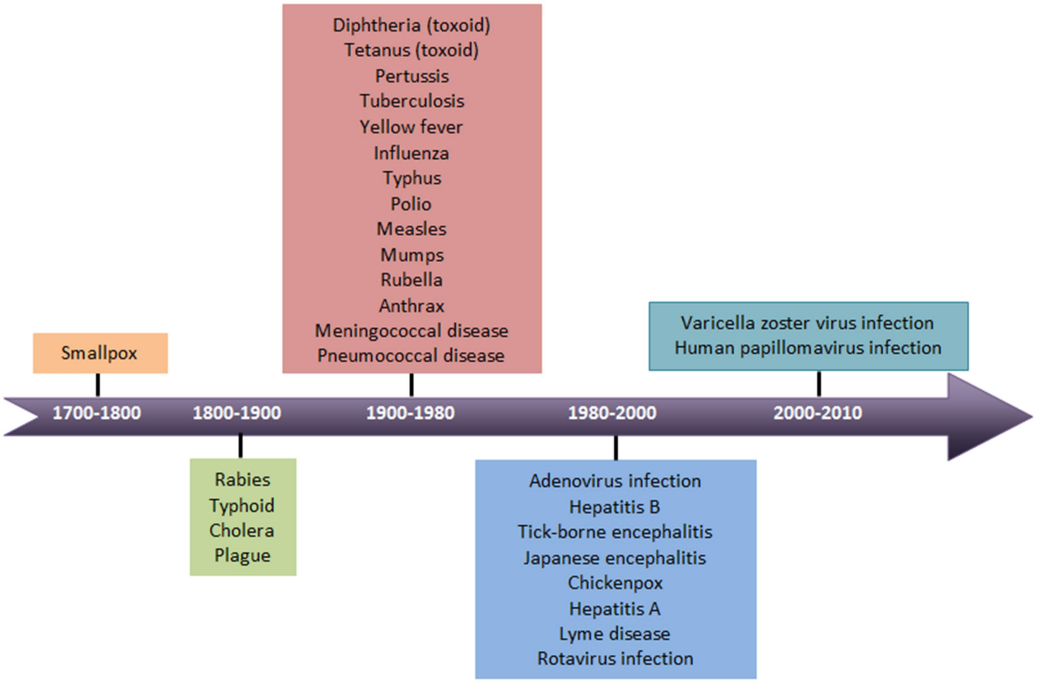


Figure 1.7. Timeline of vaccine development (Adapted from Plotkin and Plotkin, 2011).

To overcome these limitations, new approaches have been developed for the design of 'third generation' vaccines during the last thirty years. Synthetic vaccines, structural vaccinology and reverse vaccinology are some of the new approaches to produce more effective vaccines in a rapid fashion. Unlike the first generation vaccines which are produced from attenuated or killed pathogens, synthetic vaccines contain only some parts of pathogens such as peptides or carbohydrates. In addition, recombinant DNA technologies can be used to produce antigens in the laboratory (De Gregorio and Rappuoli, 2014). In structural vaccinology, X-ray crystallography, transmission electron microscopy and nuclear magnetic resonance spectroscopy are mostly used techniques for determination of protein structures. Computational analyses are also used to predict epitope sites of proteins, to design scaffolds, and to use in vaccine production (Cozzi *et al*., 2013). Another approach called reverse vaccinology is based upon the era of genomics where the genomes of most pathogens have been sequenced. After genome sequencing, protein-coding genes are compared with the sequences of known antigens available in the databases through bioinformatics analyses. Following the selection of potential sequences as potential antigens, selected sequences are cloned and expressed in *Escherichia coli* and used to immunize mice. Then, final candidates are selected based on the results of immunization experiments. This approach was firstly applied to Meningococcus B (MenB) because traditional vaccine approaches were not useful due to highly variable antigens of the pathogen (Sette and Rappuoli, 2010).

As mentioned above, different methods have been developed during vaccine production, so that various vaccine types including attenuated, inactivated, subunit, toxoid, conjugate, DNA and recombinant vector vaccines have emerged (HHS, 2017). Attenuated vaccines are prepared with pathogens weakened under laboratory conditions through several passages in non-human hosts; therefore

they cannot replicate in human hosts efficiently. Attenuation is more effective in viruses than bacteria due to less number of genes in viral pathogens. Vaccines against mumps, influenza and rotavirus can be listed as live, attenuated vaccines. These vaccines have some significant drawbacks although they have such advantages as high efficiency due to their resemblance to natural infections and efficiency even with two doses. Pathogens in attenuated vaccines are alive and they can gain their virulent properties through natural mutations or interactions with other pathogens even though it is very unlikely. Also, these vaccines cannot be used in people who have weakened or damaged immune systems. Moreover, shipping overseas requires transportation in cold environment (Plotkin and Plotkin, 2013). To overcome these disadvantages, inactivated vaccines have been developed by killing pathogens using radiation, heat or chemicals such as formalin or formaldehyde. These vaccines do not require cold environment for transportation and can be stored in freeze-dried form easily. Moreover, reversion to a virulent form is not possible due to killing pathogens. Despite being more stable and safer, inactivated vaccines provide short term protection against pathogens and induce weak immune response compared to attenuated vaccines. Also, remaining only one living cell after treatment could be dangerous (Plotkin and Plotkin, 2013). Whole cell pertussis vaccine prepared via killing *Bordetella pertussis* is an example of inactivated vaccines. Unlike live and killed vaccines, subunit and conjugate vaccines contain only essential antigenic molecules of pathogens, not whole organism and these molecules can be produced in the laboratories through recombinant DNA technologies (HHS, 2017). aP vaccines are subunit vaccines containing more than one virulence factors of the pathogen (van Twillert *et al*., 2015). In conjugate vaccines, an antigen is coated with polysaccharides which assist the immune system to react. Currently, there are conjugate vaccines against *Haemophilus influenza*, *Neisseria meningitidis* and pneumococcal disease (Grijalva *et al*., 2007; Palmu *et al*., 2013; Daugla *et al*., 2014). Toxoid vaccines

contain only bacterial toxins which are inactivated by treating with formaldehyde or formalin; these 'detoxified' toxins are called toxoids. Diphtheria and tetanus toxins are detoxified and used in a combination vaccine called DTaP (Diptheria, Tetanus, acellular Pertussis). DNA vaccines are made using plasmids containing gene sequences coding desired antigens and they are based on in situ production of these antigens (WHO, 2017b). Currently, there is no available DNA vaccine, however experimental studies are in progress (Sarwar *et al*., 2014). Unlike DNA vaccines, vector is not a plasmid, but a bacterium or a virus as in the case of recombinant vector vaccines. These organisms are engineered to become harmless and to produce desired antigens in infected host cells for induction of immune system like in a natural infection. DNA and recombinant vector vaccines can be solutions to epidemics where a rapid response is needed (HHS, 2017). Despite the presence of various processes for vaccine development, there are still ongoing studies to discover more effective approaches (De Gregorio and Rappuoli, 2014).

1.4.2. Impact of Proteomic and Immunoproteomic Technologies on Vaccine Antigen Discovery

Besides many traditional and modern techniques, protemic and immunoproteomic studies have provided enormous contributions to discovery of novel antigens in many pathogens (Rodriguez-Ortega *et al*., 2006; Altındiş *et al*., 2009; Yang *et al*., 2011; Choi *et al*., 2012; Dennehy and McClean, 2012; Altındiş, 2013). Proteomics reveals total protein content of a tissue, cell line or organism under various conditions along with a complete three-dimensional (3-D) map indicating where proteins are located (Graves and Haystead, 2002). Among many types of proteomic approaches, immunoproteomics specifically helps to identify proteins that stimulate an immune response by combining 2-D blots with immunoblotting using host serum collected after infection or

immunization. The most important advantage of immunoproteomic approach is the identification of final expressed proteins which are completely processed and post-translationally modified by the pathogen, and their localization (Dennehy and McClean, 2012). A recent immunoproteomic analysis of secreted proteins from *Streptococcus pneumoniae* revealed strongly immunogenic proteins along with its virulence factors and they identified a novel secreted antigen called Gsp-781 (Choi *et al*., 2012). In another study, Rodriguez-Ortega *et al*. (2006) performed proteomic analysis of surface proteins from group A *Streptococcus* M23_DSM2071 strain by combining the approach with Fluorescence-activated Cell Sorting (FACS) analysis. They identified 17 proteins, 14 of which were successfully expressed in *E. coli* for protection studies in mice and characterized one novel protective antigen, Spy0416 which confers protection against a lethal dose of M23_DSM2071 strain. In an earlier study, Yang *et al*. (2011) recently performed an immunoprotemic analysis of *Brucella melitensis* to detect immunogenic proteins in soluble proteins of the pathogen by using antiserum from infected patients and goats. Protection studies in mice demonstrated that riboflavin synthase alpha chain (RS-α) induced IFN-α and IL-2 production along with partial protection against *B*. *melitensis* infection, suggesting that it could be a promising vaccine candidate. In an immunoprotemics study on *Mycoplasma bovis*, 39 proteins were identified, 32 of which were newly detected. Among them, MbovP579 was identified as a specific and conserved antigen that could be used as a vaccine antigen and a promising diagnostic marker (Khan *et al*., 2016). In addition to identification of vaccine candidates and biomarkers, proteomic approaches can be used to detect possible biological pathways and processes associated with drug resistance by comparing with susceptible ones, as in case of *E*. *coli* and *Klebsiella pneumoniae* (Magalhaes *et al*., 2016).

Dicovery of complete *B. pertussis* genome extensively contributed to genomics, transcriptomics and proteomics studies related to the pathogen (Parkhill *et al*., 2003, Tefon *et al*., 2013). While proteomics studies enlighten many aspects of *B. pertussis* pathogenesis such as biofilm development and survival in human macrophages (Serra *et al*., 2008; Lamberti *et al*., 2016), immunoproteomic analysis provides contributions to discover novel antigens that can be used in third generation of aP vaccines (Vidakovics *et al*., 2007; Altındiş *et al*., 2009, Tefon *et al*., 2011; de Gouw *et al*., 2014a; de Gouw *et al*., 2014b). The first proteomics study of *B. pertussis* was conducted under iron-excess or iron-starvation conditions (Vidakovics *et al*., 2007). 39 proteins with differential expression were identified in *B. pertussis* and the authors proposed that low-iron-induced proteins identified in the study might have association with increased virulence. Moreover, they demonstrated that at least one of these proteins, expressed only under iron starvation, is quite immunogenic in infected patients. In another study conducted in our laboratory, total soluble proteins of *B. pertussis* were used for immunoproteomic analysis. 25 immunogenic proteins were identified by using sera collected from infected mice, 21 of which were demonstrated to be the novel antigens for *B. pertussis* (Altındiş *et al*., 2009). In addition, our proteomics group also performed surfaceome analysis from *B. pertussis* and they compared expression status of identified proteins between the vaccine strain Tohama I and a local isolate namely Saadet (Tefon *et al*., 2011). Among the identified proteins, immunoproteomics study revealed 11 immunogenic proteins along with differential expression profiles. Outer membrane protein Q (OmpQ) and iron superoxide dismutase (FeSOD) were identified in these two proteomics studies. In a recent study, a proteomic analysis of *B. pertussis* biofilm content was performed and 11 proteins were found ≥ three-fold more abundant in biofilm (de Gouw *et al*., 2014a). Moreover, they also investigated protective activity of the most abundant protein, BipA, in

mice. Immunization with BipA induced opsonization of the pathogen and reduced bacterial colonization in the lungs.

As it is seen, proteomics and immunoproteomics studies can pave the way for discovery of novel vaccine antigens along with unveiling many other aspects of the pathogens and pathogenesis.

1.4.3. Adjuvants

Adjuvant which means 'to help' in Latin is a substance that enhances efficiency of vaccines through induction of robust immune response (Vogel, 1998). Alexander Glenny discovered the immune-enhancing effects of aluminum salts which have been used in human vaccines for more than 85 years (Glenny and Pope, 1925). Since the discovery of adjuvants, they have been included in vaccine formulations; now there are currently more than 30 licensed vaccines containing adjuvants (Figure 1.8). It is thought that adjuvants function as Pathogen-Associated Molecular Patterns (PAMPs), which are mostly missing in purified vaccine components, to trigger the innate immunity. Although addition of adjuvants into vaccines can enhance immune responses, some vaccines such as attenuated ones do not require their addition. Also, some inactivated vaccines do not need adjuvants as a result of their success in induction of innate immunity due to the presence of various antigens (di Pasquale *et al*., 2015).

Adjuvants display their effects in various ways. They can help antigens localize in the lymph nodes, therefore T cells' recognition effectively takes place, resulting in high T cell activity and increased clearance of pathogens. Also, some adjuvants act as a depot releasing antigens in a long time period, which promotes longer exposure of antigens to immune cells and confers longer protection. Local reactions at the injection site can be induced by adjuvants;

therefore, more cytokines are released to stimulate high number of immune cells, leading to a strong immune response. Moreover, some may stimulate the secretion of pro-inflammatory chemokines which, in turn, confer greater immune response. Finally, some adjuvants can be recognized by Toll-like receptors (TLRs), resulting in enhanced innate immune response (Schijns, 2000).

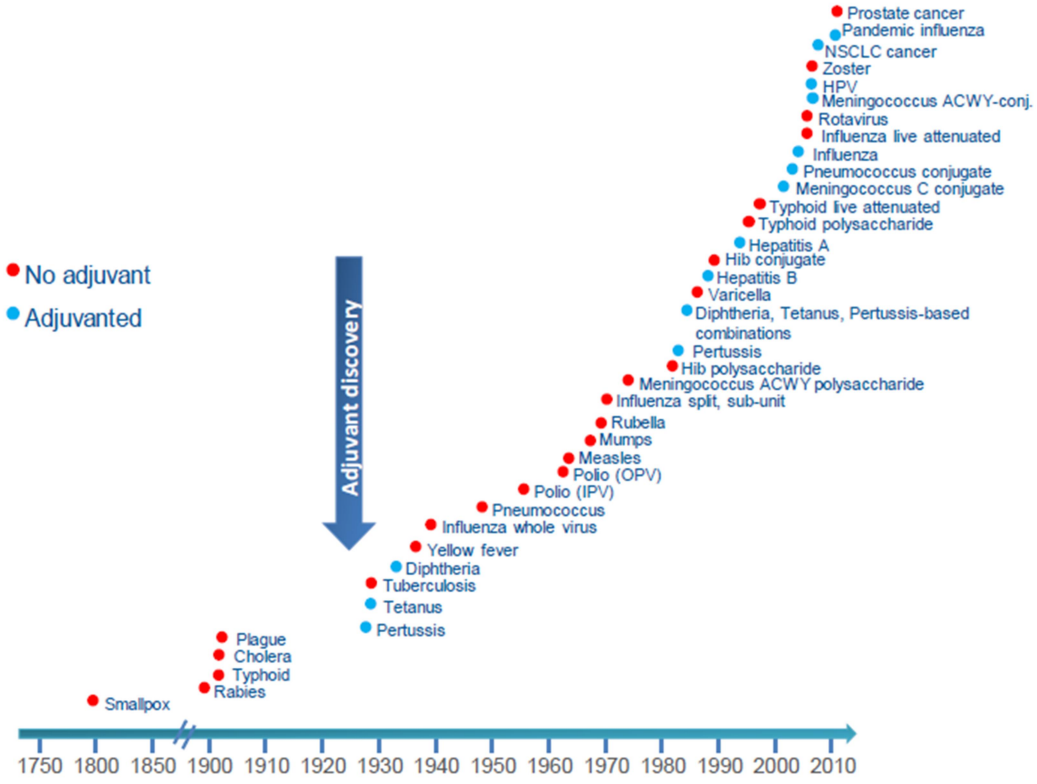


Figure 1.8. Some of the licensed vaccines with or without adjuvant (di Pasquale *et al*., 2015).

1.4.3.1. Adjuvant Types Used in Licensed Human Vaccines

Despite testing various adjuvants, only some of them are suitable to be used in human vaccines in terms of their reactogenicity and effectiveness (Table 1.2).

Table 1.2. Adjuvants used in licensed human vaccines (Lee and Nguyen, 2015).

Adjuvant (year licensed)	Class	Function
Alum (1926)	Mineral Salt	Improves humoral immune response and antigen stability. Antigens are adsorbed to the surface. Th2 type immune responses.
MF59 (1997)	Oil-in-water emulsion	Improves cell-mediated and humoral immunity. Used in influenza vaccines. Antigen delivery system.
Virosomes (2000)	Liposome	Improves cell-mediated and humoral immunity. A virosome is the reconstituted membrane of an envelope virus. The vaccines for influenza and Hepatitis A are approved products.
AS04 (2005)	Alum-adsorbed TLR4 agonist	Improves cell-mediated and humoral immunity. Combination of aluminum adjuvant with monophosphoryl lipid A (MPL) co-adsorbed. Used for HPV and HBV vaccine.
AS03 (2009)	Oil-in-water emulsion	Improves cell-mediated and humoral immunity. Used in influenza vaccine during 2009 H1N1 pandemic.

Aluminum salts (‘alum’) have been the first adjuvant used in human for more than 85 years. Until recent years, it was the only adjuvant licensed for the use in human vaccines due to the toxic effects of other tested molecules. It had been

proposed that alum's action mechanism is based on 'depot effect' which provides the continuous release of antigens from the infection site (Glenny and Pope, 1925). Although it contributes to the production of antibodies for a long period and is non-toxic, alum mainly stimulates Th2 type immune response (production of IgG1, IL-4, IL-5, IgE and activation of eosinophils) which is not sufficient for the clearance of pathogens some of which are able to survive intracellularly such as *B*. *pertussis* (Lindblad, 2004; Reed *et al*., 2009). Adjuvant system 03 (AS03) and MF59 belong to the oil-in-water emulsion group which is made of at least two immiscible liquids and both adjuvant contain squalene oil. MF59 was initially used in licensed influenza vaccines for the immunization of elders. Then, its safety and efficiency was proven in young children and infants. Unlike alum, MF59 does not function as a depot for antigen, but it enhances immune cell recruitment to the injection site, resulting in transfer of more antigens and adjuvants to the lymph nodes for presentation to other immune cells. MF59 induces local cytokine secretion and can provide balanced IgG1:IgG2a response which is a result of both Th1 and Th2 type immune responses (O'Hagan *et al*., 2012). AS03 contains α-tocopherol as well as squalene oil and can induce both cellular and humoral immunity. In 2009, AS03 was used in licensed H1N1 and H5N1 pandemic vaccines. However, a potential relationship between AS03-formulated H1N1 vaccine and narcolepsy was discovered in recent studies (Morel *et al*., 2011; O'Hagan *et al*., 2012). Another adjuvant type is called virosome which is made of viral envelope containing viral glycoproteins and membrane lipids without any genetic material. A virosome can induce both cellular and humoral immune responses due to its ability to present antigens through both MHC I and II complexes. It has been licensed for influenza vaccines and it can confer long-lasting antibody responses (Lee and Nguyen, 2015). Lastly, AS04 is an adjuvant containing monophospholipid A (MPLA) adsorbed to alum. MPLA is a TLR4 agonist which has a role in production of pro-inflammatory chemokines and cytokines,

resulting in activation and recruitment of immune cells to the injection site. It induces IL-2 and IFN-γ production, leading to a strong Th1-type response which is significant for the clearance of *B. pertussis* (Mascart *et al*., 2003, Ross *et al*., 2013).

Although different adjuvants licensed for human vaccines are currently available, studies are still in progress for the development of more effective ones without toxic effects (Table 1.3).

Table 1.3. Adjuvants that are being tested (Lee and Nguyen, 2015).

Adjuvant	Class	Function	Clinical Phase
CpG	TLR9 agonist	Enhances antibody production, Th1 type response and CD8 T cell-mediated immunity.	Phase III
Flagellin	TLR5 agonist	Enhances antibody production, Th1 and Th2 type response.	Phase I
Polyl:C	TLR3 agonist	Enhances antibody production, Th1 type response and CD8 T cell-mediated immunity.	Phase I
AS01	Combination	Enhances antibody production, Th1 type response and CD8 T cell-mediated immunity. Include MPL, QS21 and liposomes.	Phase III
AS02	Combination	Enhances antibody production and Th1 type response. Include MPLA, QS21 and emulsion.	Phase III
ISCOMs and ISCOMMATRIX	Combination	Enhances antibody production, Th1 and Th2 type response and CD8 T cell-mediated immunity. Include saponin and phospholipid.	Phase II

1.4.4. Pertussis Vaccines and Vaccine-Induced Immunity

Pertussis is a vaccine-preventable disease and it had high mortality and morbidity rate in prevaccine era. Although two types of pertussis vaccines, whole cell and acellular, are currently available, the latter is more commonly used in many countries due to safety concern.

1.4.4.1. Whole Cell Pertussis Vaccine

The first vaccine developed against pertussis was an inactivated whole cell vaccine containing *B. pertussis* killed by a chemical agent or heat or by combination of both. It was tested on mice with intracerebral challenge protocol (Kendrick *et al*., 1947). After introduction of the whole cell pertussis vaccine, a combined version containing tetanus and diphtheria toxoids (DTwP) was established in 1990s and have entered into extensive use (Cherry, 2015). According to the immunological studies, DtwP confers protective immunity mainly through the activation of CD4+ Th1 cells, Th17 cells and the production of IFN-γ and tumor necrosis factor alpha (TNF-α) (Edwards, 2014; Edwards and Berbers, 2014). While IFN-γ primarily has a role in prevention of bacterial spread, TNF-α promotes phagocytosis of *B. pertussis* by macrophages (Kilgore *et al*., 2016). Besides mouse models, a new animal model, baboon model, was introduced for pertussis studies due to the ability of baboons to develop clinical pertussis (Warfel *et al*., 2012b). In addition to mouse models, the studies conducted with baboons also demonstrated induction of Th17 memory response by DTwP as well as Th1 type response (Warfel *et al*., 2016). Although the protection conferred by DTwP is quite effective with the duration up to 14 years, it has been already replaced with DTaP in many countries due to the reactogenicity of DTwP. While mild adverse effects such as low-grade fever, headache and local skin reactions in the injection site are commonly seen after

DTwP vaccination, some severe reactions including hypotonic-hyporesponsive episodes, febrile seizures and sudden infant death syndrome may occur rarely (Warfel and Edwards, 2015).

1.4.4.2. Acellular Pertussis Vaccines

Following the evaluation of reactogenicity of DTwP and increasing knowledge about the molecular structure of *B. pertussis*, scientists tried to develop new generation pertussis vaccines and the first DTaP was introduced in Japan by Sato *et al*. (1984). The initial DTaP vaccines were impure and composed of mostly FHA with small amount of inactivated PT and PRN or fimbrial proteins in some cases (Kilgore *et al*., 2016). Since then, various DTaP vaccines have been developed with 2 to 5 purified pertussis proteins including PT, PRN, FHA, Fim2 and 3 whose individual antigen concentration ranges from 2.5 to 2μg per single human dose. Despite its routine application, optimization of aP vaccine components has been challenging due to the lack of a standard evaluation test for protective efficacy. Thus, currently available six DTaP vaccines are produced by different manufacturers and they vary in terms of component amount, number of components and methods of purification (Edwards *et al*., 1995) All of marketed DTaP vaccines mainly contain diphtheria, tetanus and pertussis components in different composition (Table 1.4.). Moreover, Kinrix, Pediarix, Pentacel and Quadracel contain inactivated polioviruses and Pediarix also contains hepatitis B surface antigen while Pentacel contains *H. influenzae* antigen (PRP) additionally (FDA, 2017).

Table 1.4. Pertussis components of currently available DTaP vaccines. The values represent mcg per dose (FDA, 2017).

Vaccine	PT	FHA	PRN	Fim 2-3
Daptacel	10	5	3	5
Infanrix	25	25	8	-
Kinrix	25	25	8	-
Pediarix	25	25	8	-
Pentacel	20	20	3	5
Quadracel	20	20	3	5

The average duration of protection conferred by DTaP vaccines is about 4 to 7 years. The studies demonstrated that DTaP vaccines predominantly confer protection through the activation of Th2 type immune response which is not as effective as the Th1/Th17 responses to clear the pathogen from the host (Rowe *et al*., 2005; Edwards, 2014; Edwards and Berbers, 2014). Therefore, it is proposed that the lower duration of protection of DTaP vaccines can be related to the lack of Th1/Th17 immune responses. For this reason, development of TLR4 ligands as adjuvants such as MPLA for the induction of Th1 and Th17 responses along with Th2 response can be potential strategy to enhance the efficacy of DTaP vaccines (Ross *et al*., 2013)

Pertussis vaccination schedule is listed in Table 1.5 for infants and in Table 1.6 for children and adolescents (CDC, 2017b). Besides DTwP and DTaP vaccines, acellular pertussis vaccines for adults (Tdap) have been recently developed due to high occurrence rate of pertussis among older ages. Although both DTwP and DTaP confer a significant level of protection, waning immunity causes decrease

in protection over time (Wendelboe *et al*., 2005). It seems that over 70% of current pertussis cases arise due to waning immunity in vaccinated household members who may serve as a reservoir for unvaccinated infants and neonates (Edwards and Freeman, 2006; de Greeff *et al*., 2010). Therefore, vaccination of adults with Tdap is highly recommended to prevent transmission of the disease. Currently, there are two available Tdap vaccines, Adacel and Boostrix which are not approved for persons younger than 10 years. Adacel contains 2.5 mcg/dose PT, 5 mcg/dose FHA, 3 mcg/dose PRN and 5 mcg/dose Fim2-3 while Boostrix is composed of 8 mcg/dose PT, 8 mcg/dose FHA and 2.5 mcg/dose PRN (FDA, 2017). As it is seen, they have reduced antigen content which provides lower reactogenicity and high level of immunogenicity and safety when compared to pediatric pertussis vaccines (Gabutti *et al*., 2014). Despite the fact that Tdap protection level also decreases over time, immunization with Tdap is recommended at the ages 9 to 11 and a booster dose of Tdap is recommended every 5 to 10 years until longer duration of protection is developed (Boyce and Virk, 2015). Moreover, CDC highly recommends vaccination of pregnant women with Tdap (CDC, 2015).

Despite the high vaccination coverage, the re-emergence of pertussis has been a significant issue after the introduction of aP vaccines due to their lower efficacies than DTwP vaccines. Thus, the resurgence of pertussis is mostly attributed to some failure of aP vaccines. Although it has some contributions to the resurgence, there are still other factors that may be responsible for increase in pertussis cases (Cherry, 2015).

Table 1.5. Recommended vaccination schedule for infants (Adopted from CDC, 2017b).

Age / Vaccine	Birth	1 month	2 months	4 months	6 months	9 months	12 months	15-18 months
Hepatitis B	1st dose	2nd dose			3rd dose			
Rotavirus			1st dose	2nd dose	3rd dose			
DTaP			1st dose	2nd dose	3rd dose			4th dose
H. influenzae type b			1st dose	2nd dose	3rd dose		4th dose	
Pneumococcal			1st dose	2nd dose	3rd dose		4th dose	
Inactivated poliovirus			1st dose	2nd dose	3rd dose			
Influenza					Annual vaccination		1 or 2 doses	
Measles Mumps Rubella							1st dose	
Varicella							1st dose	
Hepatitis A							2-dose series	
Meningococcal			Vaccination for high-risk groups					

Table 1.6. Recommended vaccination schedule for children and adolescents (Adopted from CDC, 2017b).

Age / Vaccine	19-23 month	2-3 years	4-6 years	7-10 years	11-12 years	13-15 years	16 years	17-18 years
Hepatitis B								
Rotavirus								
DTaP			5th dose					
H. influenzae type b			Vaccination for high-risk groups					
Pneumococcal			Vaccination for high-risk groups					
Inactivated poliovirus			4th dose					
Influenza	Annual vaccination		1 or 2 doses		Annual vaccination		1 dose only	
Measles Mumps Rubella			2nd dose					
Varicella			2nd dose					
Hepatitis A	2-dose series	Vaccination for high-risk groups						
Meningococcal					1st dose		2nd dose	
Tdap					Tdap			

1.5. Resurgence of *Bordetella Pertussis*

It has been suggested that several factors may contribute to the resurgence of pertussis (Cherry, 2015). These factors can be grouped into four topics: (1) increased awareness and improved diagnosis; (2) differential effectiveness of current pertussis vaccines; (3) waning of vaccine-induced immunity; and (4) antigenic variations between circulating and vaccine strains of *B. pertussis* (Chiappini *et al*., 2013).

1.5.1. Increased Awareness and Improved Diagnosis

The re-emergence of pertussis emphasizes the significance of a standardized, specific and sensitive laboratory diagnosis the absence of which results in differences in pertussis case notifications between countries. Fortunately, the development of new diagnostic tools improves pertussis clinical diagnosis. Besides physical examinations of patients and cultural characterization of the pathogen, more established tests including ELISA, PCR and direct fluorescence antibody test are available for pertussis today and the use of these methods has effectively increased pertussis diagnosis. In ELISA, the measurement of IgG antibody levels against *B*. *pertussis* antigens provides quite useful results for all age groups (van der Zee *et al*., 2015). Commercial Real-Time and Multiplex PCR kits for pertussis are the other tools that can be used for the diagnosis of pertussis through the detection of the genes of pertussis antigens such as FHA and the mobile DNA fragments called Insertional Sequence (IS) elements, specifically *IS*481 and *IS*1001 (Koidl *et al*., 2007; Lanotte *et al*., 2011; Hassan *et al*., 2014). Therefore, the increase in detection of pertussis can contribute to the increased incidence of pertussis among populations.

1.5.2. Differential Effectiveness of Current Pertussis Vaccines

As stated above, current DTaP and Tdap vaccines are produced by different manufacturers, resulting in different antigen compositions which can have a role in early waning of immunity. A recent study demonstrated that the children vaccinated with a 3-component acellular pertussis vaccine represented a longer duration of anti-PT antibodies 5 years after the first dose when compared to the children immunized with a 2-component vaccine (Carollo *et al*., 2014). Even the vaccination choice between DTaP and DTwP can cause variation in protection. Klein *et al*. (2013) compared effectiveness of DTaP with DTwP vaccines in

teenagers and showed that the teenagers vaccinated with DTaP are less likely to be protected, leading to higher risk of pertussis occurrence.

Besides antigen composition, adjuvant choice is also a significant factor that limits the type of protection immunity in the case of pertussis. Alum-adjuvanted aP vaccines are more likely to stimulate Th1 type response which is not sufficient to clear the pathogen effectively, resulting in increased pertussis incidence (Higgs *et al*., 2012).

1.5.3. Waning of Vaccine-Induced Immunity

In all vaccines, waning immunity which is the loss of protective antibodies over time is an expected phenomenon at a certain year after vaccination and thus a booster dose is mostly required. Type of vaccines (acellular vs. whole-cell) and the nature of the pathogen are the major players in waning protective immunity. In the case of pertussis, early waning of immunity is observed in the immunization with aP vaccines, leading to the accumulation of the immunized persons who are still vulnerable to the disease which can cause the resurgence of pertussis especially among the adults. Also, lower Tdap vaccination rates among adolescents and adults pave the way for the pertussis re-emergence due to waning immunity (Tartof *et al*., 2013).

1.5.4. Antigenic Variations between Circulating and Vaccine Strains of *Bordetella pertussis*

Genetic variations in circulating strains of *B*. *pertussis* are estimated as a major factor in the re-emergence of pertussis (Mooi *et al*., 2001). aP vaccines contain few antigens compared to DTwP and variations in the genes encoding these antigens have been demonstrated in circulating strains isolated in different

countries including United States, France, Poland, Australia, Korea, Japan and the Netherlands (Tan *et al*., 2005). Variations seem to be a result of polymorphisms in the virulence factors of the pathogen that are used in the aP vaccine formulations. In a study conducted with Dutch patients by Mooi *et al*. (1998), gene sequence analysis revealed polymorphisms in the genes encoding PRN and PT S1 subunit. The studies show that the regions of PRN and PT affected by polymorphisms contain T-cell and B-cell epitopes which can affect level of immune responses against these antigens (Mooi *et al*., 1998; King *et al*., 2001). Variations in the same antigens have been also observed in Finland where the vaccine strains were different from the circulating strains in 1990s in terms of pertussis toxin and pertactin (Mooi *et al*., 1999). Moreover, polymorphism in the pertussis toxin promoter allele (now called ptxP3) in clinical isolates was associated with increased toxin production and selection towards the strains carrying this ptxP3 allele has been observed (Mooi *et al*., 2009; Lam *et al*., 2012). In Korea, frequency of ptxP3 strains that has emerged in 2009 increased to 100% in 2012 and this increase was in parallel to a rapid increase in pertussis cases in the country (Kim *et al*., 2014). The similar relationship between increased pertussis notifications and the emergence of ptxP3 strains have been also demonstrated in Iran, Australia and Finland (Mooi *et al*., 2009; Octavia *et al*., 2012; Sadeghpour *et al*., 2015). The variations in the circulating strains are mostly observed after introduction of vaccination, especially aP vaccines, suggesting a vaccine-induced adaptation of the pathogen. It was proposed that antigenic changes can affect antibody efficiencies and immunological memory against the disease. Moreover, high level of toxin production by *B*. *pertussis* can enhance suppression of immune system (Mooi *et al*., 2014).

1.6. Ongoing Studies on Newer Generation Pertussis Vaccines

New solutions addressing the limitations of the current aP vaccines have been focus of interest since the reports of pertussis resurgence. Although there are only a few studies conducted on whole cell pertussis vaccines, the majority of the studies aims to develop more effective aP vaccines (Feunou *et al*., 2010; Thorstensson *et al*., 2014). For this aim, two common approaches have been taken: i) discovery of new antigens from *B*. *pertussis* as potential components of newer generation aP vaccines and ii) development of new vaccine formulations with effective adjuvants.

The protective capacities of proteins different from the virulence factors have been searched in recent years. In a study conducted by Hayes *et al*. (2011), an antigenic protein, IRP1-3, expressed under iron starvation was evaluated in intranasal challenge murine model in terms of protective immunity. It was revealed that IRP1-3 strongly stimulated both IgG1 and IgG2a and production of IFN-γ in high amount. Moreover, IRP1-3 displayed a significant protection against the pathogen in challenge experiments. The same group also demonstrated the protective capacity of another antigenic protein, AfuA, which is present on the bacterial surface and conserved among circulating strains like IRP1-3 (Hayes *et al*., 2013). Another study showed that biofilm-derived membrane protein of *B*. *pertussis* called BipA induced efficient antibody-mediated opsonization of the pathogen and decreased lung colonization (de Gouw *et al*., 2014a). In addition to investigations with individual antigens, bacterial outer membrane vesicles (OMVs) are being studied as a new vaccine strategy. Currently, two OMV vaccines are available for serogroup B meningococcal disease whose components are from *Neisseria meningitidis* (Holst *et al*., 2009). Characterization of OMVs isolated from *B*. *pertussis* has demonstrated the presence of many immunogenic proteins in addition to the

virulence factors of the pathogen such as pertactin and pertussis toxin (Fernandez *et al*., 2013). Raeven *et al*. (2015) performed a study comparing the immunogenicity profile of OMVs with aP and DTwP vaccines in mouse model. They showed that OMV vaccine induced the highest level of antibody response including all IgG subtypes as well as mixed Th1-Th17-Th2 type responses when compared to aP and DTwP. In another study, they first engineered OMVs to reduce their endotoxicity through the expression of a lipid A 3-deacylase to render the LPS molecules non-endotoxic through deacylation of Lipid A and then they evaluated the protective capacity in intranasal challenge mouse models. The results showed that OMV vaccine cleared the pathogen from the lungs of mice more rapid than non-vaccinated mice (Asensio *et al*., 2011). Based on these studies, addition of new antigens into current aP vaccines or vaccines including OMVs can be a promising approach to confer more effective protection against *B*. *pertussis*, although further investigations are required.

Besides discovery of new antigens, studies are also being directed to the development of new adjuvants due to insufficient protection conferred by alum. TLR agonists such as MPLA and bacterial CpG oligonucleotides seem to be promising candidates as adjuvants. Garcia *et al*. (2011) developed a novel vaccine platform consisting of genetically detoxified pertussis toxin (PTd) with multiple adjuvant components including CpG oligodeoxynucleotides, polyphosphazenes, and cationic innate defense regulator peptides. Co-formulation with these immunomodulators increased the serum IgG2a and IgG1 antibody titers in adult mice when compared to immunization with each of the selected adjuvants or immunization with PTd antigen alone. When used in combination, these adjuvants were able to induce a superior IgG2a response in both adult and neonatal mice, when compared to antigen alone or commercial vaccines.

As indicated in Table 1.3, CpG oligonucleotides are recognized by TLR9 on B and dendritic cells, resulting in activation of Th1 responses and production of pro-inflammatory cytokines. It was shown that combination of PT, FHA and PRN with CpG motifs provided more efficient protection against the pathogen than the combination with alum. Moreover, vaccination with antigens adjuvanted with CpG induced strong production of IgG2a antibodies and antigen specific-IFN-γ (Ross *et al*., 2013). Also, PT, PRN, FHA antigens adjuvanted with both CpG and alum induced more nitric oxide production by macrophages and IFN- γ production by spleen cells when compared to the antigens containing only alum as adjuvant (Asokanathan *et al*., 2013). Another TLR agonist is MPLA that has a lower toxicity than LPS itself and manufacturers have started to use MPLA in some vaccines for viral infections since 2005. As discussed in Section 1.4.3.1, MPLA is recognized by TLR4 and stimulates immune responses similar to LPS, resulting in protection represented by natural infection (Mitchell and Casella, 2017).

1.7. Outer Membrane Protein Q, Putative Lipoprotein and Iron Superoxide Dismutase

Outer membrane proteins (Omps) are present in the outer membrane region of Gram-negative bacteria and they can act as potential vaccine candidates due to their quick recognition by the host immune system. It was shown that recombinant OmpA of *E. coli* induced high level of antibody production and reduced bacterial colonization, resulting in increased survival rate in mice. It also conferred cross-protection against *Salmonella* and *Shigella* infections (Guan *et al*., 2015). In another study conducted in our laboratory, OmpH of *Pasteurella multocida* was fused with *Pasteurella* lipoprotein E (PlpE) and their protective capacities were evaluated in mice models. 100% protection was conferred by PlpEC-OmpH fusion protein adjuvanted with oil-based CpG in

mice (Okay *et al*., 2012). Another fusion protein containing OmpF and OmpI from *Pseudomonas aeruginosa* was evaluated in terms of protection in immunocompromised mice and it provided a considerable efficacy in protection against *P. aeruginosa* infection (von Specht *et al*., 1995). OmpQ of *B*. *pertussis* was first characterized in 1995 by Finn *et al*. (1995) and identified as a strongly immunogenic protein in *B*. *pertussis* surface immunosurfaceome analysis performed in our laboratory (Tefon *et al*., 2011).

Bacterial lipoproteins perform various roles, including nutrient uptake, signal transduction, adhesion, conjugation, and sporulation, and participate in antibiotic resistance, transport (e.g. ABC transporter system), and extracytoplasmic folding of proteins (Kovacs-Simon *et al*., 2011). In the case of pathogens, lipoproteins have been shown to play a direct role in virulence-associated functions, such as adhesion to host cells, colonization, invasion, evasion of host defense, modulation of inflammatory processes, and translocation of virulence factors into host cells. Lipoproteins (Lpps) can act as potential vaccine candidates. The vaccines developed against *Neisseria meningitidis* serogroup B constitute the best examples to the use of Lpps as vaccine components. In alternative approaches to the development of a vaccine against *N*. *meningitidis* serogroup B, the reverse vaccinology approach identified many novel surface-exposed Lpps as genome-derived neisserial antigens (Pizza *et al*., 2000; Pajon *et al*., 2009). One of these, GNA1870, or rLP2086 (NMA0586), conserved across many of the serogroups is currently a component of a pentavalent vaccine, 5CVMB, for serogroup B meningitis (Masignani *et al*., 2003). LP2086 is a bacterial surface-exposed virulence factor that binds human factor H, the latter being the complement regulatory protein enhancing serum resistance (Madico *et al*., 2016). Its inclusion in the pentavalent vaccine is due to its profound effect on protecting the meningococcus from the immune system. The resulting recombinant factor H binding protein (fHBP) vaccine

(Trumenba® bivalent rLP2086) was recently approved in the United States in individuals aged 10-25 years (Vesikari *et al*., 2016). A study conducted with recombinant PlpE of *P. multocida* demonstrated its protective capacity in mice and chickens and cross-protectivity against different *P*. *multocida* strains was inferred from high sequence similarity (Wu *et al*., 2007). In a study conducted by Dwivedi *et al*. (2015), recombinant lipoprotein of *Clostridium perfringens* promoted protective immunity against this pathogen in a mouse gas gangrene model. Different putative lipoproteins were identified in surface proteome of *B*. *pertussis* (Tefon *et al*., 2011). A subsequent meta-reverse-vaccinology study conducted by Dr. Emrah Altındiş (unpublished; personel communication) on the basis of our whole immunoproteomics data revealed that putative lipoprotein, BP2919, stands as a potential vaccine candidate with its highest hits, i.e. prioritized in silico among the other Lpps identified as based on pan-genomic data.

Superoxide dismutases (SODs) belong to metalloenzyme family that has functions in prevention of oxidative damage to DNA through conversion of O_2^- into O_2 and H_2O_2 (Sheng *et al*., 2014). Several studies reported iron superoxide dismutase (FeSOD) as a virulence factor in other microorganisms including *Francisella tularensis* and *Legionella pneumophila* (Sadosky *et al*., 1994; Bakshi *et al*., 2006). Another study showed that SodB of *Campylobacter jejuni* has a potential protective activity against colonization of the pathogen in chickens (Chintoan-Uta *et al*., 2015). Moreover, Daifalla *et al*. (2011) assessed immunogenicity of iron superoxide dismutase B1 (SODB1) protein of *Leishmania donovani* combined with a TLR9 agonist or TLR4 agonist and it was shown that SODB1 induced specific cytokine and antibody production *in vitro*. Iron superoxide dismutase (FeSOD), cofactor of which is iron, is encoded by *sodB* gene and the lack of FeSOD negatively affects the rate of cell growth in *B*. *pertussis*. Also, the expression of two main virulence factors ACT and PRN

was not detectable in *sodB*-deleted mutants of *B*. *pertussis* strains (Khelef *et al*., 1996). Immunogenicity of FeSOD was independently demonstrated by our former immunoproteomics researchers in their total soluble and surface proteome studies on *B*. *pertussis* (Altındiş *et al*., 2009; Tefon *et al*., 2011).

1.8. The Present Study

As discussed extensively in former Sections, the discovery of new antigenic proteins and development of effective adjuvant systems are considered as the most promising approaches to overcome the problems inherent to current aP vaccines.

As explained in Section 1.7, OmpQ, putative Lpp and FeSOD proteins were selected for the present study as based on the results of immunoproteomics studies conducted in our laboratory. The present study aims to investigate humoral and cellular responses to OmpQ, putative Lpp and FeSOD proteins from *B*. *pertussis* and evaluate their protective efficacy in mouse intranasal challenge models.

CHAPTER 2

MATERIALS AND METHODS

2.1. *Bordetella pertussis* and *Escherichia coli* Strains

B. pertussis Tohama I, a standard strain for vaccine production, was used for genomic DNA isolation and *B. pertussis* Saadet strain, a local Turkish isolate, was used for mice challenge experiments. Cloning and expression studies were performed by using *E. coli* DH5α and BL21(DE3) strains, respectively (Table 2.1).

2.2. Recombinant Plasmids

pGEM®-T Easy (Promega) was used for cloning studies in *E. coli* DH5α while expression of recombinant proteins was performed with pET-28a (+) (Novagen) vector in *E. coli* BL21(DE3) strain (Figure 2.1 and 2.2).

2.3. Solutions, Buffers and Culture Media

Recipes for buffer and solution are given in the Appendix A while preparation and component of culture media are described in the Appendix B.

2.4. Chemicals and Enzymes

The enzymes and chemicals used in this study are listed in the Appendix C.

Table 2.1. Bacterial strains used in this study.

Strain	Characteristics	Source and Reference
B. pertussis Tohama I and Saadet	Tohama I: standard strain Saadet: local strain	Kindly provided by Dr. Erkan Özcengiz
E. coli DH5α	F' ϕd*lac*ZΔ(*lac*ZY A-argF)U169 *sup*E44λ^- *thi*-1 *gyr*A *rec*A1 *rel*A1 *end*A1 *hsd*R17	American Type Culture Collection; Hanahan (1983)
E. coli BL21(DE3)	F$^-$ *ompT gal dcm lon* *hsd*S_B(r_B^- m_B^-) λ(DE3 [*lac*I *lac*UV5-T7 gene 1 ind1 sam7 nin5])	Novagen, Merck (Germany)
BL21/pET28a::*ompQ*	BL21 with pET28a(+) carrying the *ompQ* ORF, Kan^r	This study
BL21/pET28a::*BP2919*	BL21 with pET28a(+) carrying the *BP2919* ORF, Kan^r	This study
BL21/pET28a::*sodB*	BL21 with pET28a(+) carrying the *sodB* ORF, Kan^r	This study

2.5. Maintenance of Bacterial Strains

Cohen-Wheeler solid medium containing active charcoal (Appendix B) was used to grow *B. pertussis* strains at 37^{0}C for 3 days (Sato *et al*., 1972). Luria-Bertani (LB) liquid and LB agar media (Appendix B) supplemented with 100µg/mL ampicillin and 30µg/mL kanamycin were used to grow *E. coli* DH5α and *E. coli* BL21(DE3), respectively. Bacterial samples from cultures were mixed with 50% glycerol stocks and they were kept at -80^{o}C.

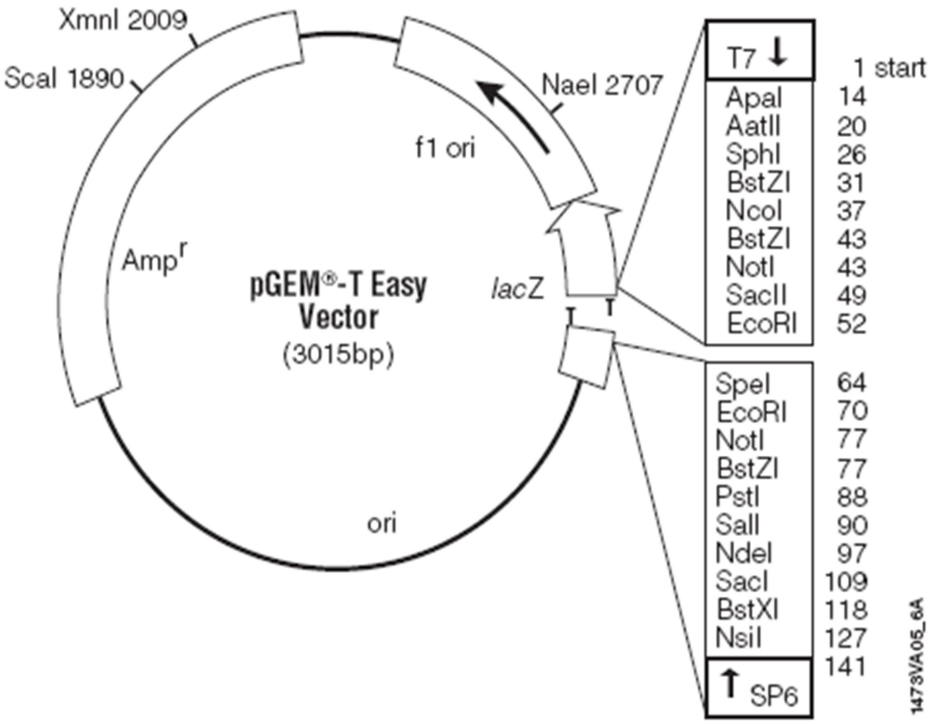


Figure 2.1. pGEMT-Easy vector map.

2.6. Isolation of Genomic DNA

The genomic DNA of *B. pertussis* Tohama I strain was isolated according to the protocol described by Storm *et al*. (2006). Colonies of *B. pertussis* grown on Cohen-Wheeler medium at 37ºC for 48 hours were dissolved in 120 µl TEN Buffer (Appendix A). After incubation at 100ºC for 10 min, the sample was centrifuged at 13000 rpm for 2 min and the supernatant was stored at +4ºC until use.

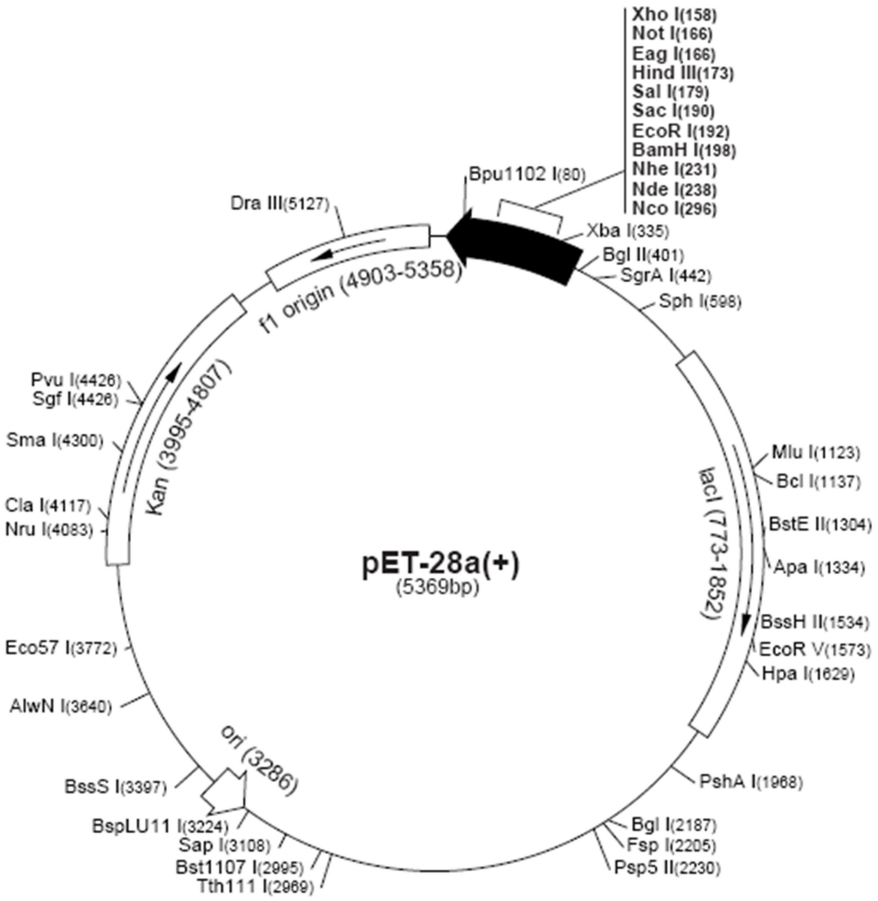


Figure 2.2. pET-28a(+) vector map.

2.7. Primers

The primers used in PCR amplification were designed according to the gene sequences obtained from National Center for Biotechnology Information (NCBI) database (Table 2.2.) and were purchased from IONTEK, Co. (Istanbul, Turkey). Primer design of *sodB* gene was performed by Aycan Apak (Yılmaz *et al*., 2016).

Table 2.2. Primers designed for *ompQ*, *BP2919* and *sodB* genes. Restriction enzyme cut sites were underlined.

Gene name	Primer	Oligonucleotide sequence	PCR product size
ompQ	Forward	5' GGATCCATGCGTCGTCTTCTCGTC 3'	1094 bp
	Reverse	5'AGATCTTCAGAAGCGCTGGGTCATTCC 3'	
BP2919	Forward	5' GGATCCGTGCCCCGAATCGCG 3'	744 bp
	Reverse	5' AGATCTTCAGCGGCGGGGCAAG 3'	
sodB	Forward	5' GGATCCATGGCACACACTCTT 3'	591 bp
	Reverse	5'AGATCTTTAGGCGAAATTCTTCG 3'	

2.8. Polymerase Chain Reaction (PCR) and Agarose Gel Electrophoresis

Condition and mixtures of PCR samples are given in Table 2.3 and 2.4. PCR amplification of *sodB* gene was performed by Aycan Apak (Yılmaz *et al*. 2016). After amplification, samples were run in 1% agarose gel prepared by 1X TAE buffer (Appendix A). 6X gel loading dye was used to load samples and 0.5 µg/ml ethidium bromide solution was prepared to stain the gel. Visualization of the DNA bands was performed with Vilber Lourmat Gel Imaging System (Vilber Lourmat, Marne-la-Valle´e, France). After size confirmation of the DNA band according to DNA marker, the bands were extracted from the gel with Qiagen Gel Extraction Kit (Qiagen Inc., Valencia, CA). Verification of the DNA sequences was carried out in RefGen Inc. using the chain termination method (Ankara, Turkey) and the BLAST search at NCBI website was used to compare the deduced nucleotide sequences.

Table 2.3. PCR condition for amplification of *ompQ* and *BP2919*.

		Temperature	Time
	Initial denaturation	94°C	3 min
40 cycles	Denaturation	94°C	1 min
	Annealing	60°C	1 min
	Extension	72°C	1 min
	Final extension	72°C	10 min

Table 2.4. PCR mixture for *ompQ* and *BP2919* genes.

Component	Control (total 50 µl)	Sample (total 50 µl)
Taq polymerase buffer 10X	5 µl	5 µl
$MgCl_2$ 25 µM	5 µl	5 µl
Forward primer 10 µM	2 µl	2 µl
Reverse primer 10 µM	2 µl	2 µl
DNTP mix 10 µM	1 µl	1 µl
DNA sample	0	2
dH_2O	34 µl	29.5 µl
DMSO	0	2.5
Taq polymerase	1 µl	1 µl

2.9. Ligation into pGEM®-T and pET-28a(+) Plasmids

A mixture containing 500 ng PCR products, 1 µl pGEM®-T Easy vector, 5 µl 2X ligation buffer, 1 µl T4 ligase and sterile distilled water to complete the volume to 10 µl was prepared for the ligation of the genes into pGEM®-T vector. Ligation mixture for pET-28a(+) expression vector contained 500 ng DNA sample (digested from pGEM®-T), 2 µl vector, 1 µl 10X ligation buffer, 1 µl T4 ligase and sterile dH_2O to complete the volume to 10 µl. Ligation reactions were implemented for 16h at 4ºC. Before ligation into pET-28a(+), the digested plasmid was treated with rAPid Alkaline Phosphatase Kit (Roche) according to the manufacturer's protocol. Ligation and transformation studies of *sodB* gene were performed by Aycan Apak (Yılmaz *et al*. 2016).

2.10. Bacterial Transformation Studies

2.10.1. Competent *Escherichia coli* DH5α and BL21(DE3) Cell Preparation with $RuCl_2$

100 µl -80ºC stocks of *E. coli* cells were inoculated on LB agar plates through streak plate technique. After overnight incubation, one colony was selected to inoculate into 10 ml LB medium. The culture was incubated at 37ºC for overnight. 3 ml of the seed culture was transferred into a fresh 200 ml LB medium. The culture was incubated at 37°C till OD_{580} is between 0.4 and 0.7. After incubation on ice for 15 min, centrifugation was performed at 3500 rpm at 4ºC for 5 min. The supernatant was discarded and cells were resuspended on ice in 20 ml Buffer 1 (Appendix A). They were centrifuged at 3500 rpm at 4ºC for 5 min and the supernatant was discarded. The pellet was resuspended in 8 ml Buffer 2 (Appendix A) and the suspension was stored as 100 µl aliquots at -80°C.

2.10.2. Bacterial Transformation

After incubation of 100 µl competent *E. coli* cells on ice for 10 min, 10 µl ligation product was combined with the cells and the mixture was gently mixed. Incubation of the mixture was carried out on ice for 20 min and then a heat shock was applied at 42°C for ~70 sec. The mixture was immediately put on ice to be incubated for 5 min. It was mixed with 900 µl LB and incubated for 80 min at 37°C at 100 rpm. After centrifugation at 3000 rpm for 10 min, the pellets were dissolved in 100 µl LB and the cells were inoculated onto LB agar plates with appropriate antibiotics. In ligation reaction for pGEM®-T, blue-white colony selection was performed on LB agar plates containing 100 mg/ml IPTG and 20 mg/mL X-gal as well as the appropriate antibiotic.

2.11. Isolation of Plasmid DNA and Restriction Enzyme Digestion

Manual plasmid isolation was carried out according to the protocol described by Hopwood *et al*. (1985) with some modifications. Transformed *E. coli* strains were inoculated onto LB agar plates containing appropriate antibiotics for 16-18 hours. A few colonies were selected to be resuspended in 100 µl STE solution by vortexing (Appendix A). After incubation for 10 min at room temperature, 60 µl lysis buffer (Appendix A) was added and heat application at 70°C was carried out for 10 min to denature genomic DNA. The tubes were cooled and 160 µl phenol-chloroform-isoamyl (25:24:1 v/v, Sigma, USA) was added into the mixture. The upper part of separated phases, after being centrifuged for 5 min at 13000 rpm, was taken and run on agarose gel electrophoresis.

For verification of ligation into the plasmids, the plasmid DNA was extracted from transformed *E. coli* strains with Qiagen Plasmid Purification Mini and Midi Kits (Qiagen Inc., Valencia, CA) according to the manufacturer.

Appropriate restriction enzymes were used to digest plasmids containing the gene of interest. Agarose gel electrophoresis was used for visualization and the genes were extracted from the gel with QIAquick Gel Extraction Kit when necessary (Qiagen Inc., Valencia, CA). Plasmid isolation and restriction enzyme digestion studies of *sodB* gene were carried out by Aycan Apak (Yılmaz *et al*. 2016).

2.12. Overexpression and Purification of the Recombinant Proteins

Overexpression of the recombinant proteins was performed according to the procedure described by Ayalew *et al.* (2008) with some modifications. 100 µl -80ºC stocks of transformed *E. coli* BL21(DE3) cells carrying pET-28a(+) with the genes were inoculated on LB agar plates containing 30 µg/ml kanamycin. After overnight incubation, a single colony was selected to be inoculated into 10 ml LB with kanamycin and incubated for 16-18 hours. 3 ml seed culture was transferred into two 150 ml fresh LB with kanamycin and incubation at 200 rpm at 37°C was carried out till OD_{600} was around 0.6. While one of the cultures remained as uninduced control, the other one was exposed to IPTG induction to a final concentration of 1 mM. After 5 hour-induction, 150 ml cultures were centrifuged at 13000 rpm for 10 min at 4ºC and the pellets were resuspended in 5 ml DSB (Appendix A). 2 cycles of freeze-thaw were applied and the samples were sonicated on ice for 5 times for 10 sec with 5 sec intervals. Following centrifugation at 13000 rpm for 15 min, the supernatants were taken and stored for purification.

Purification of the recombinant proteins was carried out with Protino® Ni-TED 2000 protein purification system. After equilibration of the His-tag columns with 4 ml DSB, the supernatants were added. Binding of polyhistidine parts of the tagged proteins to nickel ions allows selection of the recombinant proteins.

After passage of the supernatants through the columns, they were washed with DSB for three times. Elution of the proteins was achieved with 3 ml DEB addition (Appendix A). The optical densities of the samples were read at 280 nm and SDS-PAGE was applied to run the samples for confirmation.

The solution containing recombinant proteins was loaded into dialysis membrane previously wetted with sterile distilled water and placed into one liter dialysis buffer (Appendix A). After overnight incubation at 4°C, filter sterilization (0.2 µm filters) was made and protein concentration was determined.

2.13. Measurement of Protein Concentration

Protein concentrations were measured with modified Bradford assay (Ramagli and Rodrigez, 1985). 5X Bradford Reagent was diluted with distilled water and the solution was filtered through Whatman No.1 filter paper. A standard curve was constructed using Bovine Serum Albumin (BSA) (Table 2.5). For sample mixture, 20 µl of the supernatant, 400 µl of 1:5 diluted 5X Bradford Reagent and 1580 µl distilled water were mixed, incubated for 10 min at room temperature at dark and the absorbance of the solutions was measured at 595 nm.

Table 2.5. Mixture composition for preparation of standard curve.

Protein concentration	DEB + BSA*	dH_2O	Bradford Reagent
2 µg/ml	8 µl	1592 µl	400 µl
3 µg/ml	12 µl	1588 µl	400 µl
5 µg/ml	20 µl	1580 µl	400 µl
7,5 µg/ml	30 µl	1570 µl	400 µl
10 µg/ml	40 µl	1560 µl	400 µl

*Mixture= 100 µl DEB containing 100 µl BSA

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

12% separating gel (pH 8.8) and 4% stacking gel (pH 6.8) were prepared to run the protein samples on a vertical gel system (Bio-Rad, USA). Gel preparation was performed according to the protocol described in Bio-Rad Cell System manual (Table 2.6). The gels were run in 1X running buffer (Appendix A) at 16 mA/gel. Fixation treatment was applied using fixation buffer (Appendix A) for 1h and the gels were stained with Coomassie Brilliant Blue R-250 (Appendix A) for visualization (Neuhoff *et al.*, 1988).

Table 2.6. Composition of DSD-PAGE gel.

	Separating Gel (12%)	Stacking Gel (4%)
Acrylamide/bis	40.0 ml	1.3 ml
Distilled water	33.5 ml	6.1 ml
1.5 M Tris-HCl pH 8.8	25.0 ml	-
0.5 M Tris-HCl pH 6.8	-	2.5 ml
10 % (w/v) SDS	1.0 ml	100 µl
10 % Ammonium persulfate	500 µl	50 µl
TEMED	50 µl	10 µl
Total	100 ml	10 ml

2.15. *Bordetella pertussis* Antisera Preparation

B. *pertussis* Tohama I cells, grown on Cohen-Wheeler medium for three days, were suspended in 0.85% saline solution and diluted to $4x10^{10}$ bacteria/ml. After inactivation of the bacteria with incubation at 56ºC for 30 min, 10 female BALB/c mice were subcutaneously injected with 0.5 ml suspension/animal twice at two week intervals to develop antisera against *B*. *pertussis*. At day 14 after second injection, antisera was obtained and stored at -20ºC till used in Western Blot analyses.

2.16. Western Blot Analysis

Western blot analysis was performed according to the modified protocol of Towbin *et al*. (1979). Transfer of proteins to nitrocellulose membrane was carried out with a semi-dry horizontal blotting system (Cleaver Scientific Ltd, UK) at 1.5 mA/cm^2 for about 1 hour in transfer buffer (Appendix A). Protein

transfer was followed by blocking the membrane with 10% skim milk in TBS (Appendix A) for 2 hours at 37°C. The membrane was washed with 0.5% Tween-20 in TBS for 10 min and then incubated in 5% skim milk in TBS containing the diluted sera obtained from immunized mice at room temperature for 1 hour. After washing the membrane with 0.5% Tween-20 in TBS for 10 min, it was incubated in 5% skim milk in TBS containing anti-mouse IgG-alkaline phosphatase for 1 hour at room temperature. The membrane was then rinsed with TBS and AP Conjugate Substrate Kit (Bio-Rad, USA) was used to visualize the bands.

2.17. Mouse Experiments

3 weeks old female BALB/c mice purchased from Başkent University (Ankara, Turkey) were used in the study. For each experimental group, 15 mice were immunized.

All animal experiments were performed under the approval of the Ethics Committee on Animal Experimentation, Middle East Technical University, Ankara, Turkey (METU Etik-2015/10).

2.17.1. Vaccination of Mice

Each experiment set comprised 3 groups each with 15 mice. The Group 1 was vaccinated with PBS as a negative control, the Group 2 with 40 µg recombinant OmpQ, putative Lpp or FeSOD proteins plus MPLA adjuvant (Invivogen, USA), and the Group 3 with 40 µg/ml recombinant OmpQ, putative Lpp or FeSOD proteins adsorbed in Alhydrogel ($AlOH_3$ - alum) adjuvant. All injections were performed intraperitoneally at day 0 and day 21. All experiments were repeated for at least two times.

2.17.2. Enzyme-linked Immunosorbent Assay (ELISA) for IgG1 and IgG2a

Blood samples were collected from tail vein of vaccinated mice at day 20 before second injection and at day 30 before bacterial challenge. Test sera were obtained from the blood samples to carry out the measurement of humoral response in mice.

96-well microplates were coated with 4 µg/well of the recombinant protein in 200 µl Carbonate/Bicarbonate buffer (Appendix A). After incubation for overnight at 4ºC, the microplates were rinsed with washing solution (Appendix A) for three to four times. 50 µl blocking solution (Appendix A) was added into each well and incubated for 1 hour at 37ºC. After addition of 100 µl test sera into each well at different dilutions ranging from 1:100 to 1:102400 (when necessary) in blocking solution, the microplates were incubated for 1 hour at 37ºC and then rinsed with washing solution for three to four times. 100 µl alkaline phosphatase (AP) conjugated rat anti-mouse IgG1 or IgG2a (Southern Biotech, Cambridge, UK) diluted in blocking solution (1:2000) was added into the wells followed by incubation for 1 hour at 37ºC. The wells were rinsed with washing solution for three to four times and then p-nitrophenyl phosphate disodium salt (pNPP) (Thermo Scientific, MA, USA) was used to develop colorimetric change for detection at 405 nm.

2.17.3. Determination of Serum Level of Interleukin-10 (IL-10)

The sera obtained from the blood after second immunization were used to measure IL-10 levels in vaccinated mice with a Mouse IL-10 ELISA development kit (Mabtech, USA). The protocol described by the manufacturer was followed for the measurement.

2.17.4. Interferon-Gamma (IFN-γ) Assay

The spleens of three vaccinated mice from each group were dissected at day 30 (before bacterial challenge) and suspended in 5 ml RPMI 1640 medium supplemented with 1% penicillin/streptomycin and 10% Fetal Bovine Serum (FBS) (Biochrom, Cambourne, UK). The spleen cells (splenocytes) were obtained after homogenization with 70 mm nylon cell strainers (BD Bioscience, NJ, USA) and they were counted with a hemocytometer. The cells were diluted in RPMI 1640 medium at a concentration of $1x10^6$ cells/well and then distributed into each well of a 96-well microplate. After incubation in a CO_2 incubator at 37ºC for 1 hour, the cells were stimulated with PBS as a negative control, 30 mg/ml of OmpQ, putative Lpp or FeSOD and with 1 mg/ml Concanavalin A (Sigma, USA) as a positive control. The cell culture supernatants were collected at day 3 after induction and analyzed for IFN-γ titers with a Mouse IFN-γ ELISA development kit (Mabtech, USA). The manufacturer's protocol was followed to measure IFN-γ titers. Concanavalin A (ConA) was used as a positive control since it induces T-cell activation, in turn, leading to production of IFN-γ (Badillo-Godinez *et al*., 2015).

2.17.5. Bacterial Challenge of Mice

Vaccinated mice were intranasally challenged at day 31 with live *B. pertussis* Saadet strain which is more virulent than Tohama I. After inoculation on Cohen-Wheeler agar medium and incubation for 3 days at 37ºC, the bacterial colonies were collected and suspended in 0.85% saline solution (pH 7.0) supplemented with 1% casamino acid. 50 µl suspension containing $2.5x10^9$ CFU was administered to each nostril of the mice anesthetized with ketamine plus xylazine.

2.17.6. Evaluation of Bacterial Colonization in Mice Lungs

Four vaccinated mice from each group were dissected in sterile conditions to remove their lungs at day 5, 8 and 14, respectively, after bacterial challenge. The lungs were suspended in 0.85% saline solution supplemented with 1% casamino acid. After homogenization and serial dilution, 100 µl of each lung sample was inoculated on three Cohen-Wheeler agar plates containing cephalexin (40 mg/l) and the plates were incubated for 3-4 days at 37ºC. Colonies were counted and the $\log_{10}$ weighted mean numbers of CFU/lung were calculated for each day.

2.18. Statistical Analysis

One-way analysis of variance (ANOVA) was applied to determine statistical significances in IgG1, IgG2a, IL-10 and IFN-γ assays, and for evaluation of bacterial colonization. Tukey's range test was used for comparisons of data sets.

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Genomic DNA Isolation from *Bordetella pertussis*

The genomic DNA of *B. pertussis* Tohama I the genome sequence of which is available was isolated according to the protocol described by Storm *et al*. 2006 (Figure 3.1).

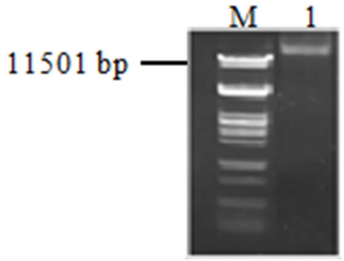


Figure 3.1. Genomic DNA isolation from *B*. *pertussis*. Lane 1: The genomic DNA, M: Lambda DNA/PstI, 24 Marker.

3.2. Amplification of Outer Membrane Protein Q (*ompQ*), Putative Lipoprotein (*BP2919*) and Iron Superoxide Dismutase (*sodB*) Genes

Isolated genomic DNA of *B*. *pertussis* was used to amplify *ompQ* and *BP2919* genes in PCR (Figure 3.2). Expected sizes for *ompQ* and *BP2919* were 1094 bp and 744 bp, respectively.

The PCR products of *ompQ* and *BP2919* were extracted from the agarose gel and they were cloned into pGEM®-T Easy vector (Figure 2.1). The amplification, cloning and transformation studies of *sodB* gene (591 bp) were performed by Aycan Apak (Yılmaz *et al*., 2016).

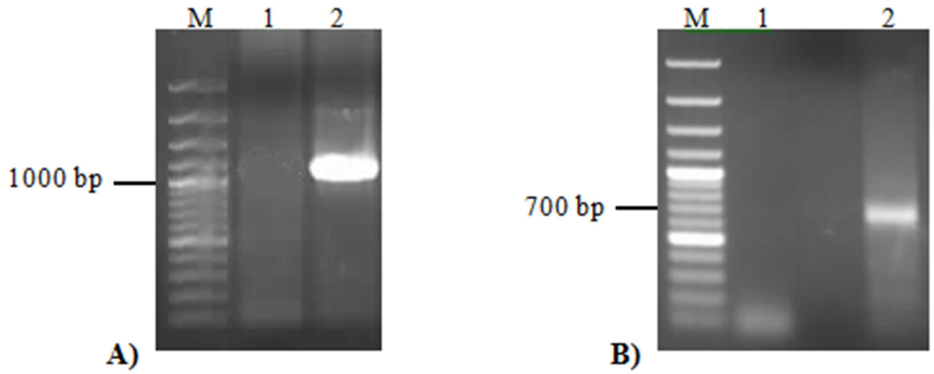


Figure 3.2. Amplification of A) *ompQ* (1094 bp) and B) *BP2919* (744 bp) genes from *B*. *pertussis* genomic DNA. Lane 1: Negative controls (no template), Lane 2: PCR products, M: CloneSizer 100 bp DNA Ladder.

3.3. Transformation of *Escherichia coli* DH5α with pGEM®-T Easy Vectors Carrying *ompQ* and *BP2919* Genes

After ligation of *ompQ* and *BP2919* genes into pGEM®-T Easy vector, *E. coli* DH5α competent cells were transformed with the recombinant plasmids. The recombinants were identified via blue-white selection on LA plates containing ampicillin, X-gal and IPTG. Manual plasmid isolation from white colonies was then performed to confirm the recombinants (Figure 3.3 and 3.4).

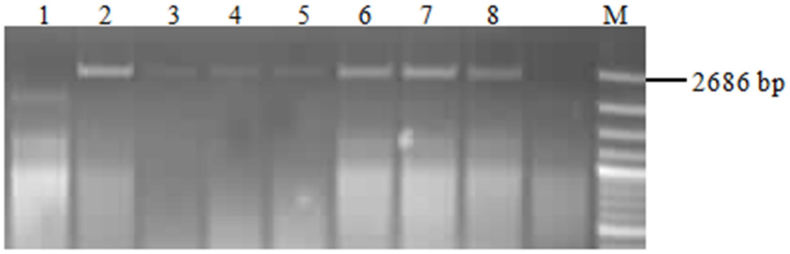


Figure 3.3. Manual plasmid isolation from white colonies. Lane 1: Empty pGEM®-T as control, Lane 2-8: pGEM®-T carrying *ompQ*, M: CloneSizer 100 bp DNA Ladder.

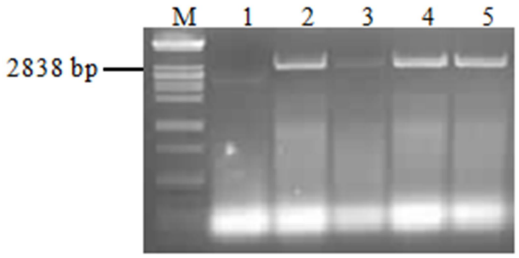


Figure 3.4. Manual plasmid isolation from white colonies. Lane 1: Empty pGEM®-T as control, Lane 2-5: pGEM®-T carrying *BP2919*, M: Lambda DNA/PstI, 24 Ladder.

3.4. Verification of *ompQ* and *BP2919* Cloning into pGEM®-T Easy Vector

Transformed *E. coli* DH5α cells were grown overnight and plasmid isolation was carried out. The plasmids were digested with *BamH*I and *Bgl*II restriction enzymes to confirm the cloning (Figure 3.5). In addition, sequence analyses of the clones were compared with the known sequences in NCBI database for the verification of recombination.

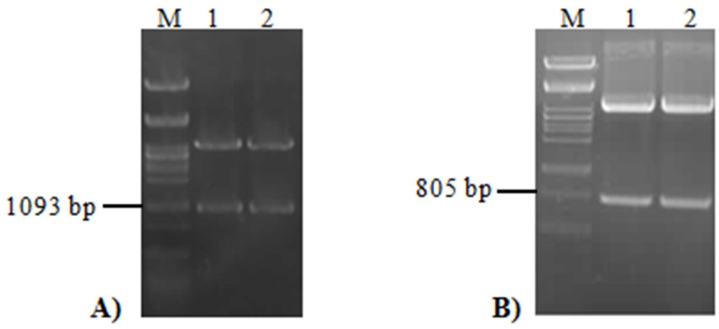


Figure 3.5. Confirmation of A) *ompQ* and B) *BP2919* cloning into pGEM®-T via restriction enzyme digestion. (A) Lane 1-2: *BamH*I and *Bgl*II digested pGEM®-T carrying *ompQ*, (B) Lane 1-2: *BamH*I and *Bgl*II digested pGEM®-T carrying *BP2919*, M: Lambda DNA/PstI, 24 Ladder.

3.5. Subcloning of *ompQ* and *BP2919* Genes into pET-28a(+) Vector and *Escherichia coli* BL21(DE3) Transformation with Recombinant Plasmids

ompQ and *BP2919* genes were subcloned into pET28a(+) expression vector which has an N-terminal His-tag sequence with an optional C-terminal His-tag and allows the expression of cloned genes under the T7 promoter control (Figure 2.2). pET-28a(+) was treated with alkaline phosphatase after digestion with *BamH*I producing two sticky ends for ligation of the genes of interest. *ompQ* and *BP2919* were digested from pGEM®-T vector with *BamH*I and *Bgl*II and ligated into *BamH*I-digested pET-28a(+) expression vector. *E. coli* BL21(DE3) competent cells were transformed with the ligation products and the transformed cells were inoculated into LA plates containing kanamycin.

3.6. Verification of *ompQ* and *BP2919* Subcloning into pET-28a(+) Expression Vector

Recombinant colonies were selected for the manual plasmid isolation (Figure 3.6 and 3.7) and the subcloning of *ompQ* and *BP2919* into pET-28a(+) was verified by the digestion of the recombinant plasmids with *BamH*I and *EcoR*I enzymes (Figure 3.8).

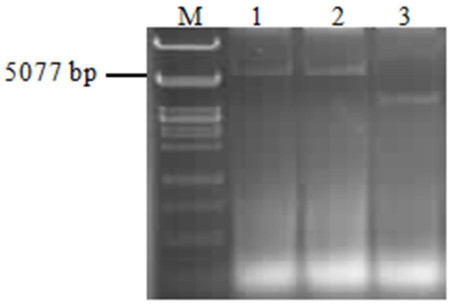


Figure 3.6. Manual plasmid isolation from recombinant colonies. Lane 1-2: pET-28a(+) with *ompQ*, Lane 3: Empty pET-28a(+) as control, M: Lambda DNA/PstI, 24 Ladder.

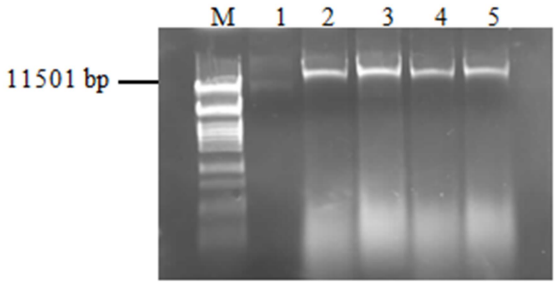


Figure 3.7. Manual plasmid isolation from recombinant colonies. Lane 1: Empty pET-28a(+) as control, Lane 2-5: pET-28a(+) with *BP2919*, M: Lambda DNA/PstI, 24 Ladder.

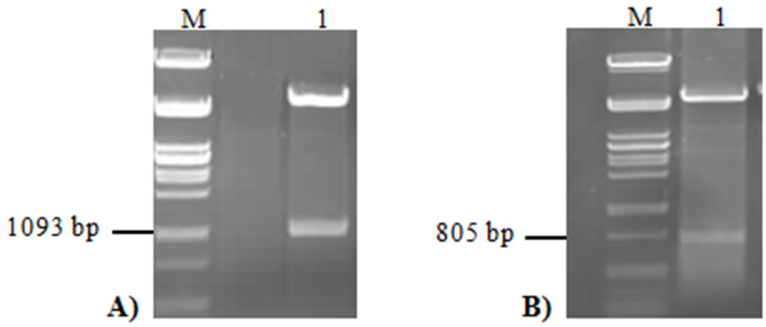


Figure 3.8. Confirmation of A) *ompQ* and B) *BP2919* subcloning into pET-28a(+) via restriction enzyme digestion. (A) Lane 1: *BamH*I and *EcoR*I digested pET-28a(+) with ompQ, (B) Lane 1: *BamH*I and *EcoR*I digested pET-28a(+) with BP2919, M: Lambda DNA/PstI, 24 Ladder.

3.7. Expression and Purification of Recombinant Proteins from *Escherichia coli* BL21(DE3) Transformed with pET-28a(+) Carrying *ompQ*, *BP2919* and *sodB*

E. coli BL21(DE3) cells containing the recombinant plasmids were inoculated into two different flasks of LB containing kanamycin. While one culture remained uninduced as a control, the other one was induced with IPTG to a final concentration of 1 mM for the expression of recombinant His-tagged outer membrane protein Q (OmpQ, ~39 kDa), putative lipoprotein (Lpp, ~25.5 kDa) and iron superoxide dismutase (FeSOD, ~23 kDa) (Figure 3.9, 3.10 and 3.11). After overexpression of the recombinant His-tagged proteins, they were purified with Protino® Ni-TED 2000 purification system which is designed as His-tag affinity chromatography. Then, dialysis was performed against dialysis buffer to minimize NaCl and urea concentrations for vaccine preparation and the samples were filter-sterilized. The His-tags of the recombinant proteins are small (0.84

kDa) and weakly immunogenic, and therefore they were not removed (Constans, 2002).

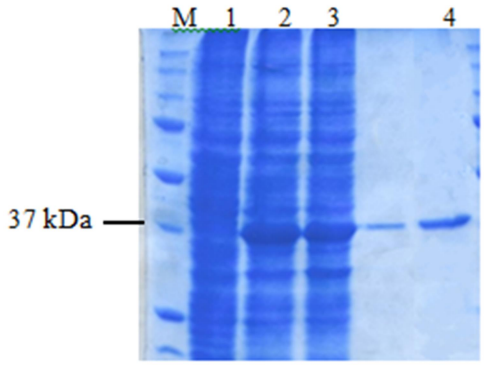


Figure 3.9. SDS-PAGE of recombinant OmpQ protein (~39 kDa). Lane 1: Uninduced sample as control, Lane 2-3: IPTG-induced sample, Lane 4: Purified recombinant OmpQ after dialysis, M: Precision Plus ProteinTM Unstained Standards, #161-0363.

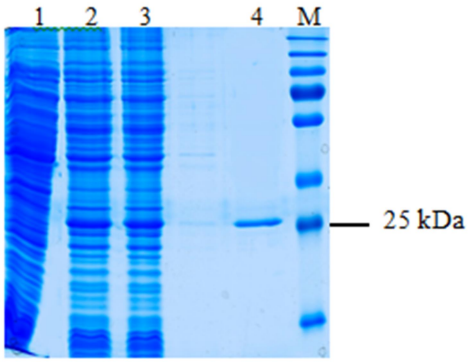


Figure 3.10. SDS-PAGE of recombinant putative Lpp protein (~25.5 kDa). Lane 1: Uninduced sample as control, Lane 2-3: IPTG-induced sample, Lane 4: Purified recombinant putative Lpp after dialysis, M: Pageruler Plus Prestained Protein Ladder, #SM1811.

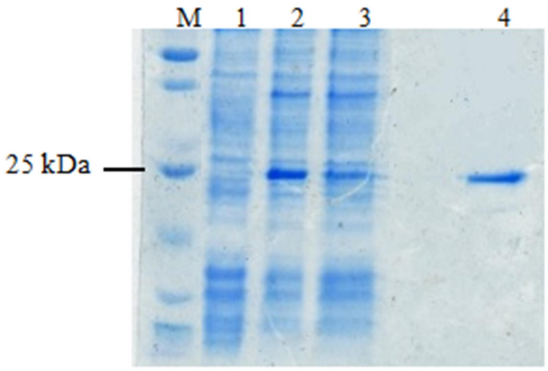


Figure 3.11. SDS-PAGE of recombinant FeSOD protein (~23 kDa). Lane 1: Uninduced sample as control, Lane 2-3: IPTG-induced sample, Lane 4: Purified recombinant FeSOD after dialysis, M: Precision Plus ProteinTM Unstained Standards, #161-0363.

3.8. Western Blotting of Recombinant OmpQ, Putative Lpp and FeSOD Proteins

In this study, the serum obtained from mice injected with *B*. *pertussis* Tohama I strain was used to verify the immunogenicity of recombinant OmpQ, putative Lpp and FeSOD proteins through Western blotting (Figure 3.12). The results revealed that all three recombinant proteins were immunogenic as expected.

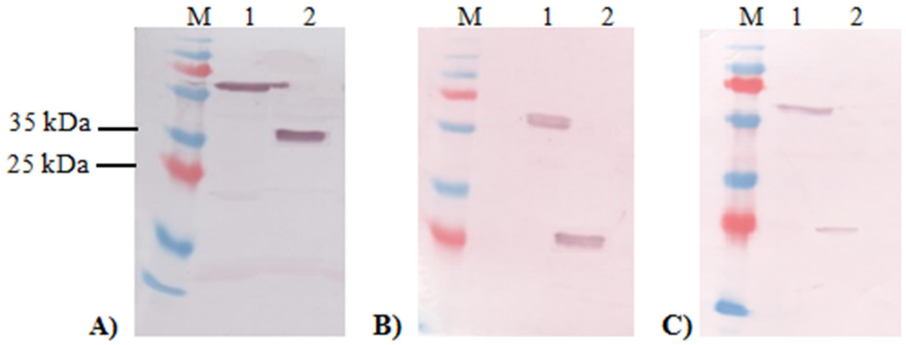


Figure 3.12. Immunogenicity analysis of the recombinant proteins via Western blotting. (A) Lane 1: Uninduced cell lysate as control, Lane 2: Purified His-tagged OmpQ, (B) Lane 1: Uninduced cell lysate as control, Lane 2: Purified His-tagged putative Lpp, (C) Lane 1: Uninduced cell lysate as control, Lane 2: Purified His-tagged FeSOD, M: Pageruler Plus Prestained Protein Ladder, #SM1811.

3.9. Challenge Experiments with Mice

Young female BALB/c mice were vaccinated with 40 µg recombinant OmpQ, putative Lpp or FeSOD proteins formulated with alum or MPLA at days 0 and 21. The sera were collected from the tail vein before second immunization and bacterial challenge to evaluate serum IgG1, IgG2a and IL-10 levels. At day 30, mice spleens were taken to obtain spleen cell culture for measurement of antigen-specific IFN-γ levels. Bacterial challenge of the mice was intranasally performed with $2.5x10^9$ CFU of *B. pertussis* Saadet cells through nostrils at day 31. Mice lungs were taken at day 5, 8 and 14 after bacterial challenge and the samples were inoculated into Cohen-Wheeler plates after homogenization to assess bacterial colonization and clearance.

3.9.1. Serum IgG1 and IgG2a Levels after Immunization of Mice with Recombinant OmpQ, Putative Lpp and FeSOD

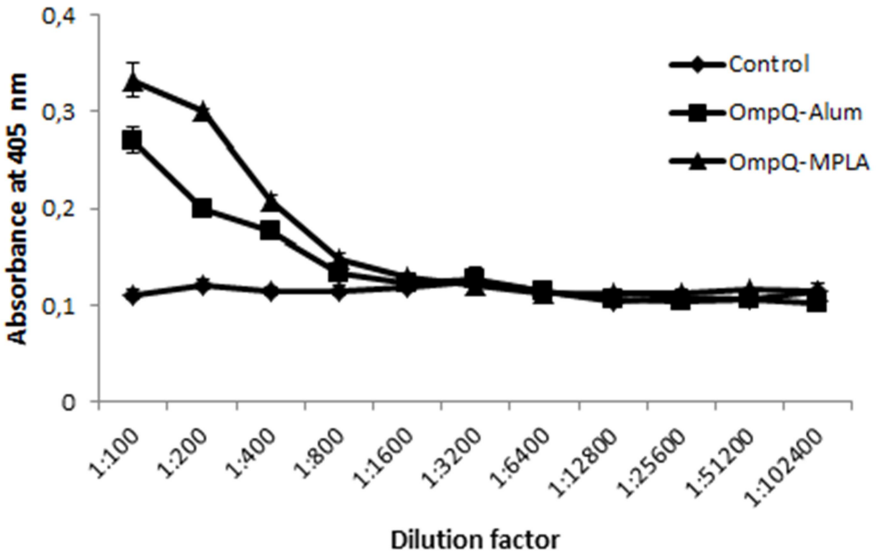
IgGs are essential in fight against pathogens although they are not sufficient alone to provide protective immunity. Still, they help host immune system to eliminate pathogens. In the case of *B*. *pertussis* infection, a balanced IgG1 and IgG2a titers in the sera are key factors in evaluation of the protective immunity against the pathogen (Hjelholt *et al*., 2013). IgG1 along with IgG3 has a role in neutralization of toxins and prevention of bacterial adherence in the respiratory tract while IgG2a is a key component involved in complement-mediated bacterial kill and opsonization (Allen and Mills, 2014). Moreover, IgG1 is associated with Th2-type response whereas IgG2a represents Th1-type immune response. After DTwP vaccination, dendritic cells activate naïve T cells; predominantly resulting in production of Th17 via activation of NOD-like receptors (NLRs) and Th1 cells through IL-12 expression of which is activated by TLRs. IFN-γ secreted by Th1 cells activates macrophages and IgG2a production from B cells which in turn enhance elimination of *B*. *pertussis* via phagocytosis and opsonization/complement fixation. On the other hand, as mentioned in Introduction, aP vaccination predominantly activates Th2 cell production along with Th17 cells and Th2 cells stimulate production of IgG1 which is mainly responsible for toxin neutralization and inhibition of adherence rather than elimination of the pathogen. Therefore, a balanced Th1:Th2 type response representing a mixed IgG1:IgG2a response is desired for more effective pertussis vaccines which can be established with new antigens and adjuvant systems (Higgs *et al*., 2012; Allen and Mills, 2014).

In the present study, IgG1 and IgG2a levels in the sera of mice immunized with the recombinant proteins were measured by ELISA to elucidate humoral response. To compare IgG1 and IgG2a levels in terms of adjuvants, antibody

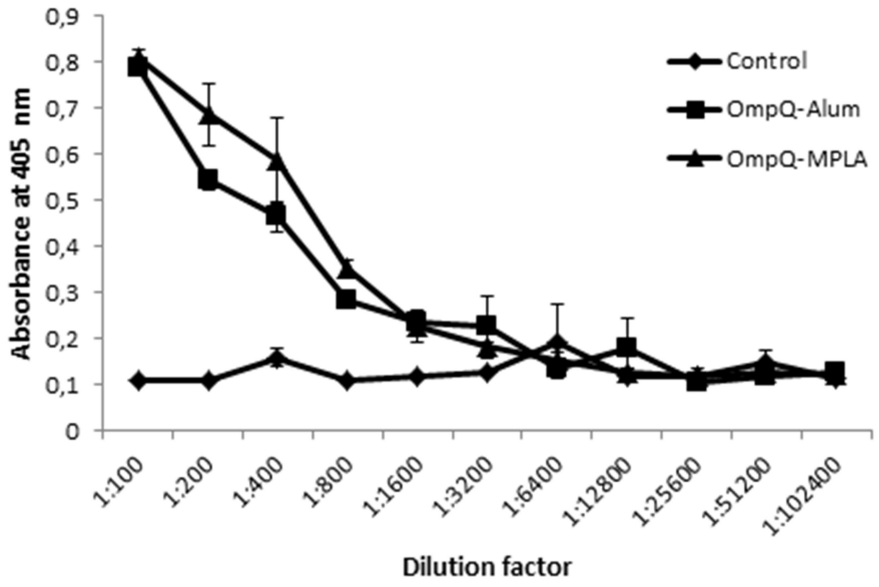
titers were calculated based on the reciprocal of maximum dilution at which an antibody signal was still obtained.

IgG1 and IgG2a levels induced by OmpQ-Alum and OmpQ-MPLA immunizations were represented in Figure 3.13 and 3.14, respectively. When first and second vaccinations were compared in both IgG1 and IgG2a dilution graphs, it was seen that both antibody levels elevated after second vaccination as it provides a booster dose for the host immune system. IgG1 levels induced by both OmpQ-Alum and OmpQ-MPLA were higher than control groups immunized with PBS (Figure 3.13). On the other hand, IgG2a levels were noticeably higher in OmpQ-MPLA than in OmpQ-Alum vaccination as expected due to the induction of IgG2a production by MPLA (Figure 3.14).

When IgG1 and IgG2a levels were compared as a function of adjuvants in OmpQ vaccination, a tendency to IgG1 elevation was obtained in OmpQ-Alum vaccination while a little increase in IgG2a level was observed with OmpQ-MPLA (Figure 3.15). The results were in accord with the literature in that alum predominantly induces IgG1 production while MPLA triggers IgG2a secretion.

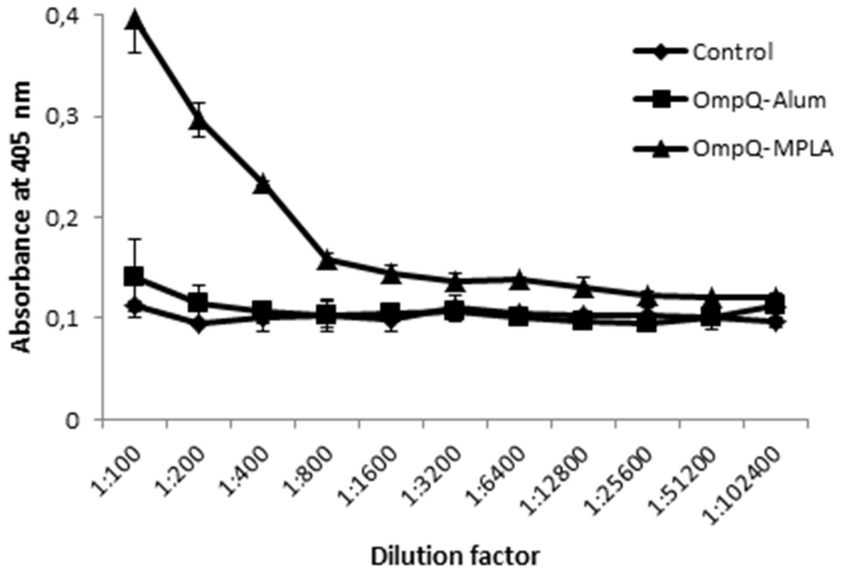


A)

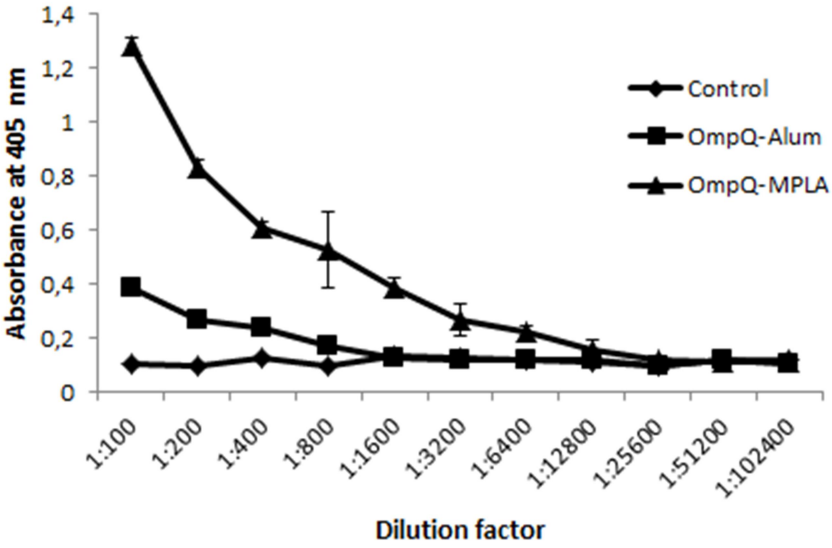


B)

Figure 3.13. IgG1 levels measured in the sera of mice after A) first and B) second vaccination with OmpQ adjuvanted with alum or MPLA. PBS was used as control.



A)



B)

Figure 3.14. IgG2a levels measured in the sera of mice after A) first and B) second vaccination with OmpQ adjuvanted with alum or MPLA. PBS was used as control.

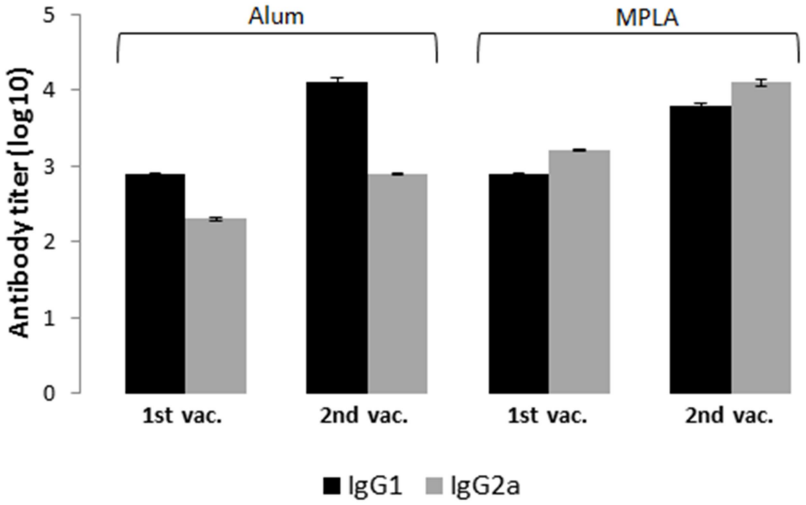
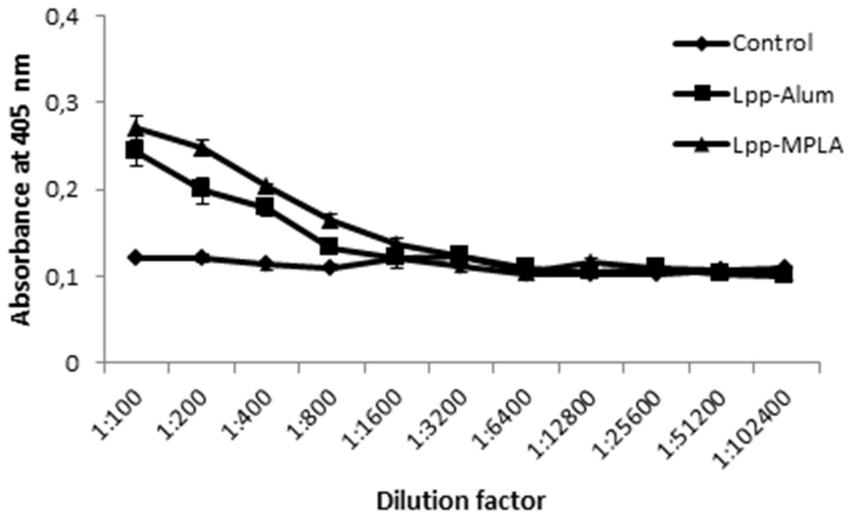


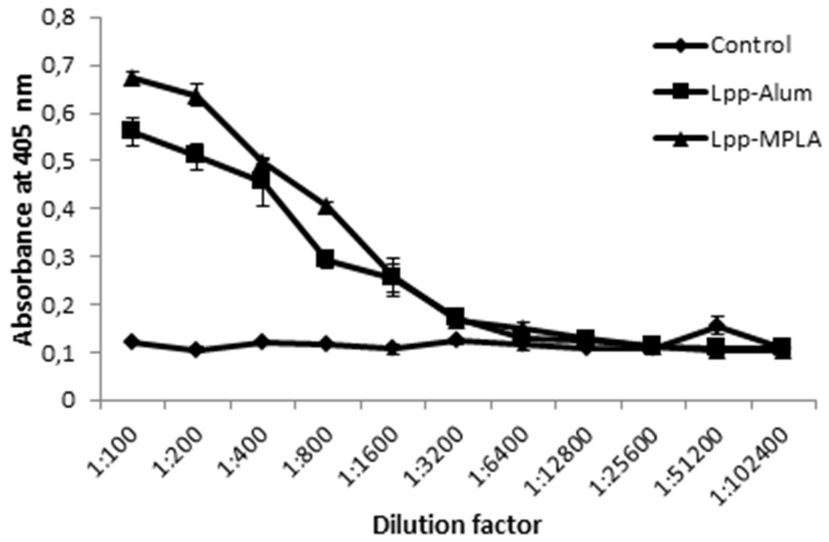
Figure 3.15. Comparison of IgG1 and IgG2a levels between the mice vaccinated with OmpQ-Alum and OmpQ-MPLA.

In the vaccinations with putative Lpp-Alum and Lpp-MPLA, a pattern similar to OmpQ vaccinations was obtained where antibody levels increased in second vaccinations when compared to first one and the vaccinated mice had higher antibody levels than control groups (Figure 3.16 and 3.17). However, antibody responses in putative Lpp vaccinations were not as strong as in OmpQ vaccinations, probably due to less immunogenicity of Lpp when compared to OmpQ.

When adjuvant effects were compared in putative Lpp vaccinations, IgG1 level was a little higher than IgG2a in the case of alum-adjuvanted vaccination while IgG2a was predominant upon MPLA-adjuvanted vaccination (Figure 3.18).

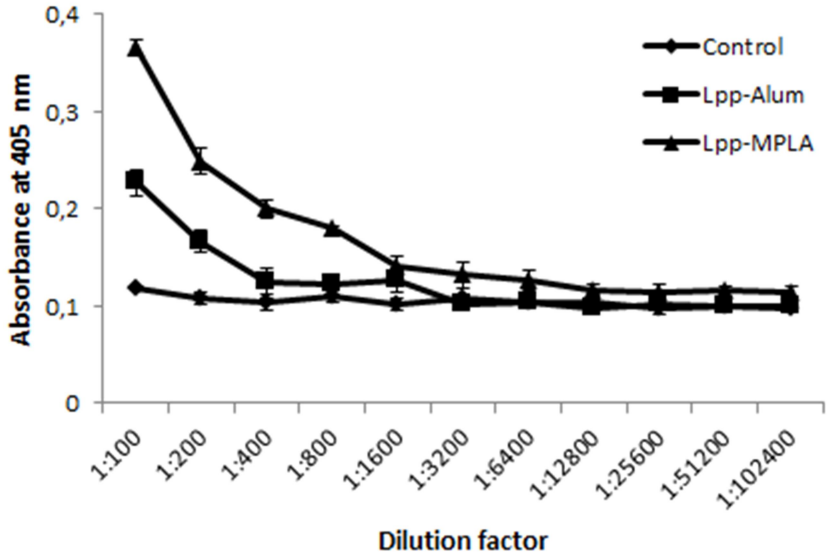


A)

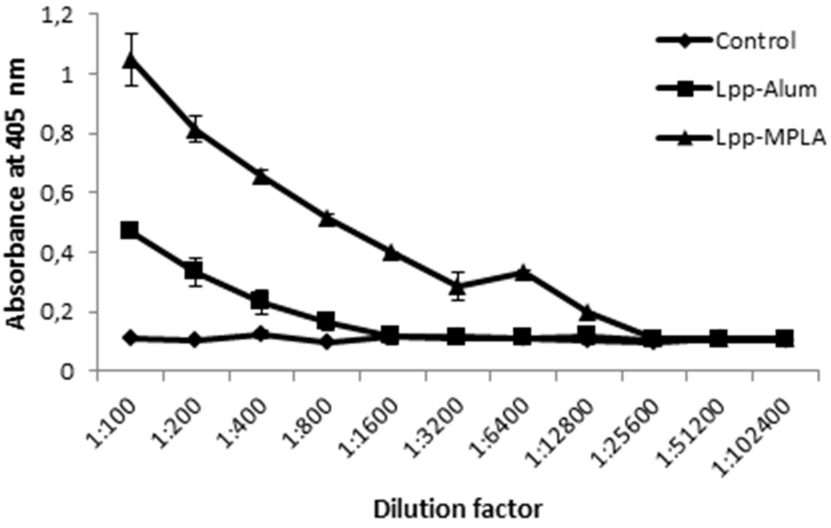


B)

Figure 3.16. IgG1 levels measured in the sera of mice after A) first and B) second vaccination with putative Lpp adjuvanted with alum or MPLA. PBS was used as control.



A)



B)

Figure 3.17. IgG2a levels measured in the sera of mice after A) first and B) second vaccination with putative Lpp adjuvanted with alum or MPLA. PBS was used as control.

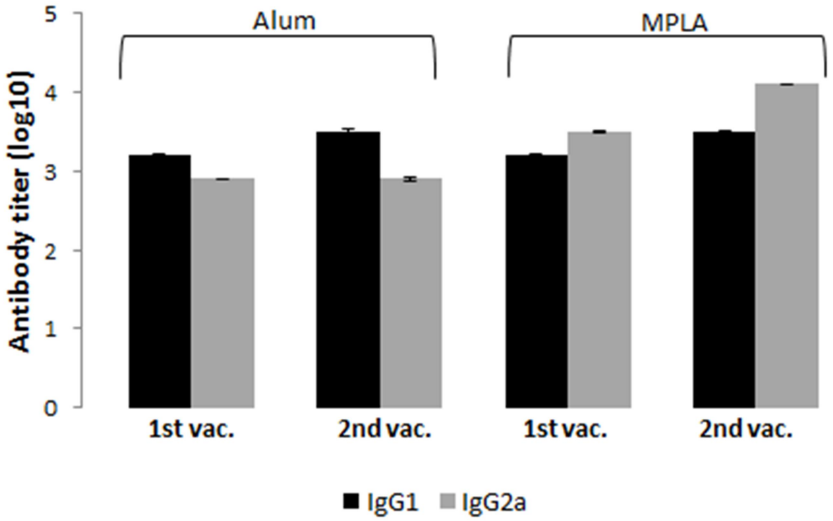
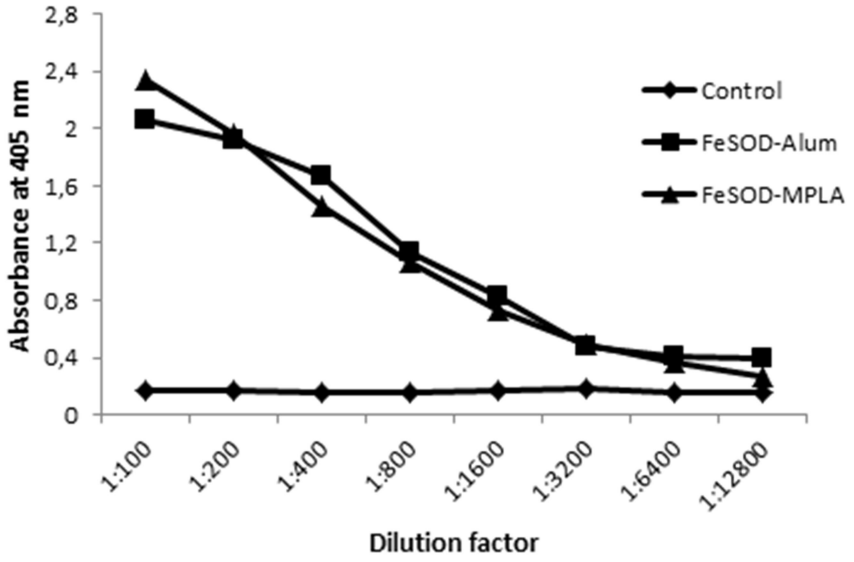


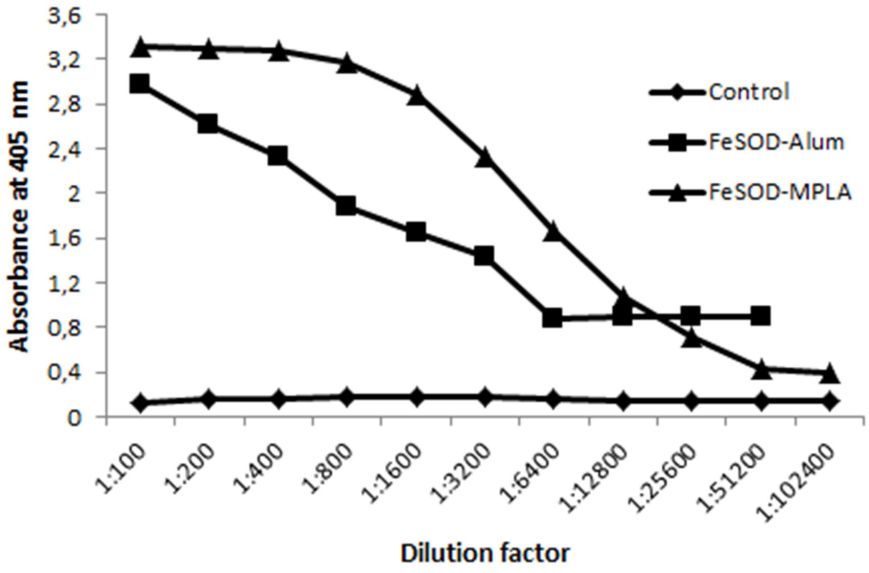
Figure 3.18. Comparison of IgG1 and IgG2a levels between the mice vaccinated with putative Lpp-Alum and Lpp-MPLA.

A similar pattern was observed with recombinant FeSOD vaccination (Figure 3.19 and 3.20). However, the antibody levels triggered by FeSOD were quite high when compared to putative Lpp and OmpQ which was indicative of a greater potential of FeSOD as an antigen in protection against pertussis.

Like recombinant OmpQ and putative Lpp, recombinant FeSOD vaccination with alum also induced more IgG1 production while MPLA-adjuvanted FeSOD provided a more balanced IgG1:IgG2a type response (Figure 3.21).

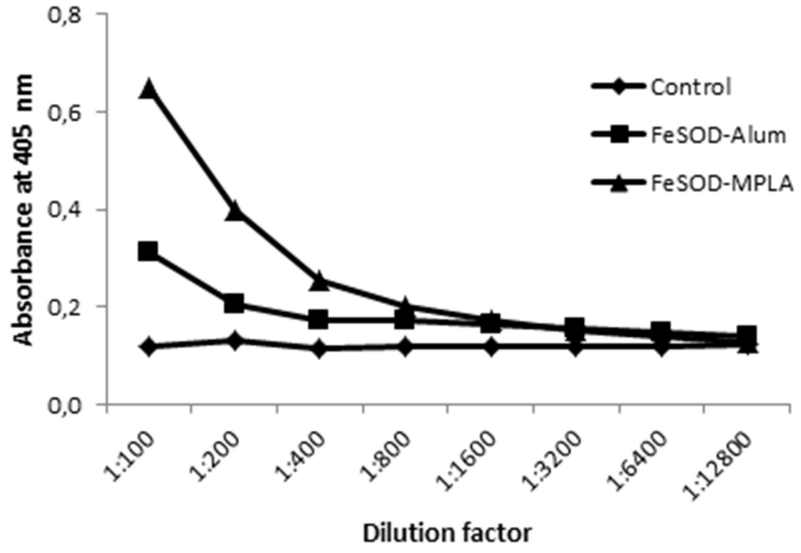


A)

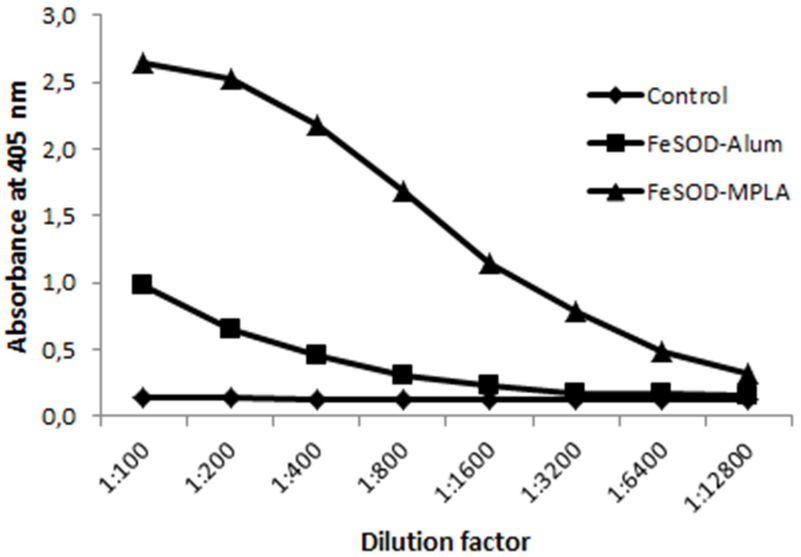


B)

Figure 3.19. IgG1 levels measured in the sera of mice after A) first and B) second vaccination with FeSOD adjuvanted with alum or MPLA. PBS was used as control.



A)



B)

Figure 3.20. IgG2a levels measured in the sera of mice after A) first and B) second vaccination with FeSOD adjuvanted with alum or MPLA. PBS was used as control.

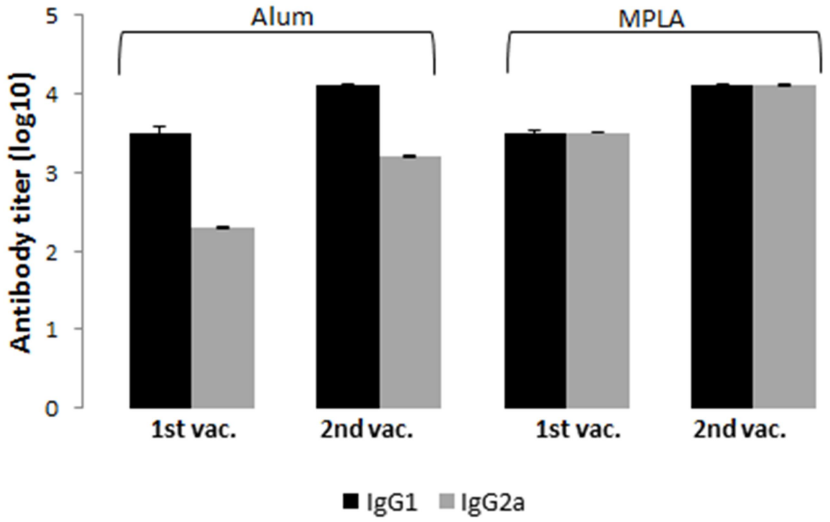


Figure 3.21. Comparison of IgG1 and IgG2a levels between the mice vaccinated with FeSOD-Alum and FeSOD-MPLA.

Taken together, the overall data related to antibody responses revealed that all three recombinant proteins stimulated much higher level of antibody responses in immunized mice than control groups. Moreover, MPLA-adjuvanted vaccinations had a little higher IgG2a production or a more balanced IgG1:IgG2a response when compared to alum which is as expected when known immune responses activated by these adjuvants are considered.

3.9.2. IFN-γ Levels in Mice Immunized with Recombinant OmpQ, Putative Lpp and FeSOD

For a long time, it has been known that *B. pertussis* is an extracellular pathogen and Th2-type immune response is sufficient to eliminate *B. pertussis* effectively (Bromberg *et al.*, 1991). After discovery of intracellular survival of *B. pertussis* in alveolar macrophages, it became more obvious that Th1-type response is also

required for effective clearance of the pathogen (Bromberg *et al*., 1991; Mills *et al*., 1993; Corbel *et al*., 1999). It is well-known that Th1 cells mediate immune responses against intracellular pathogens by activating neutrophils and macrophages through IFN-γ secretion (Sugai *et al*., 2005; Fedel *et al*., 2015). In this respect, level of IFN-γ as representative of Th1-type immune response was investigated.

IFN-γ is an important pro-inflammatory cytokine that is mainly produced by Th1 and NK cells in response to infection. Its secretion is essential to prevent bacterial spread in the host body and elimination of intracellular pathogens through the activation of phagocytic immune cells including macrophages and neutrophils (Allen and Mills, 2014). Intracellular pathogens are killed by reactive oxygen intermediates or nitric oxide secreted by IFN-γ-activated macrophages and neutrophils. Moreover, IFN-γ induces activation of complement-fixation antibodies and opsonization to eliminate pathogens through antibody class switching to IgG2a (Finkelman *et al*., 1988; Schoenborn and Wilson, 2007). Protective role of IFN-γ was reported in many studies conducted with various microorganisms including bacteria, viruses and parasites (Flynn *et al*., 1993; Sedegah *et al*., 1994; Suradhat *et al*., 2001). In the case of *B. pertussis* infection, IFN-γ plays a crucial role in clearance of the pathogen, especially from macrophages that it invades (Barnard *et al*., 1996; Barbic *et al*., 1997). In a study, a murine respiratory challenge model having disrupted IFN-γ receptor was characterized with a lethal infection due to systemic spread of *B. pertussis* from the respiratory tract and lungs to other organs such as liver (Mahon *et al*., 1997).

Spleen cell culture was established from mice vaccinated with the recombinant proteins and the supernatants after induction were used to measure IFN-γ levels

to elucidate Th1 type response in pertussis infection. Negative and positive controls were established with PBS and ConA induction, respectively.

Considering the results obtained from recombinant OmpQ and putative Lpp vaccinations, there were no significant differences between negative controls and vaccinated mice in terms of IFN-γ levels although a slight but not significant increase was observed in MPLA-adjuvanted formulations. The latter is an expected situation as MPLA induces IFN-γ production through Th1 cell activation (Figure 3.22 and 3.23). Also, this slight increase may explain high IgG2a levels in MPLA formulations because it is known that even low IFN-γ level is sufficient to induce class switching to IgG2a (Finkelman *et al*., 1988). Despite high antibody titer, when IFN-γ level is considered, it can be proposed that these two immunogenic proteins are not sufficient to stimulate a strong Th1-type response which is a part of cell-mediated immunity producing IFN-γ although they induce high level of antibody response but not enough by itself to fight against pathogens.

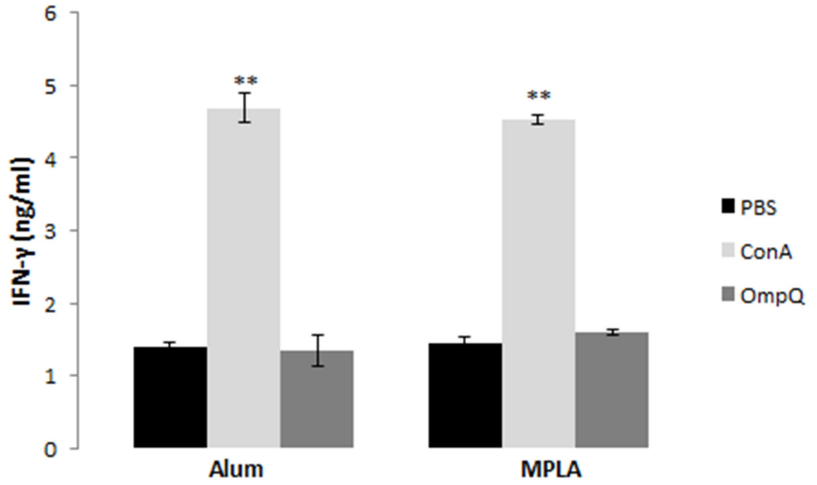


Figure 3.22. IFN-γ production by spleen cells of mice vaccinated with OmpQ-Alum and OmpQ-MPLA. PBS and ConA were used as negative and positive controls, respectively. The results are represented as mean ± SD (**p<0.01).

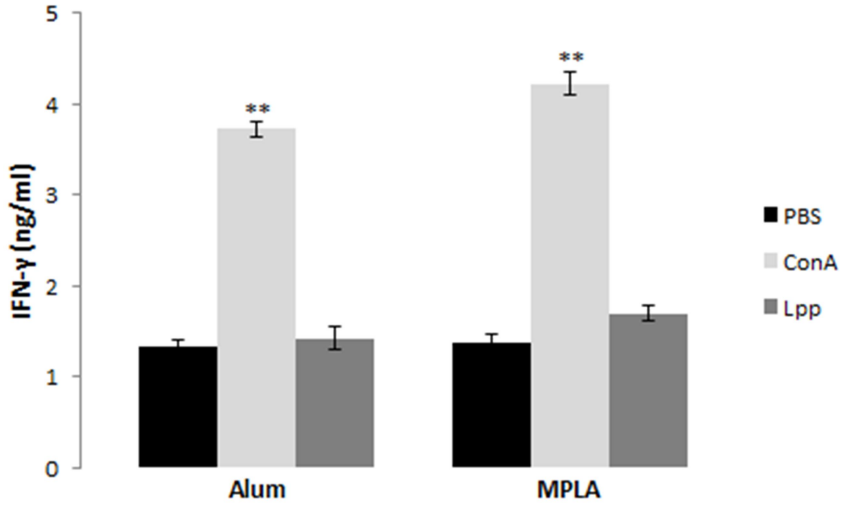


Figure 3.23. IFN-γ production by spleen cells of mice vaccinated with putative Lpp-Alum and putative Lpp-MPLA. PBS and ConA were used as negative and positive controls, respectively. The results are represented as mean ± SD (**p<0.01).

Unlike OmpQ and putative Lpp, IFN-γ level in spleen cells of mice immunized with FeSOD-MPLA was significantly elevated when compared to negative control and FeSOD-Alum vaccination (Figure 3.24). The data overall showed that FeSOD-MPLA can trigger Th1 type response while OmpQ-Alum, OmpQ-MPLA, putative Lpp-Alum, putative Lpp-MPLA and FeSOD-Alum cannot.

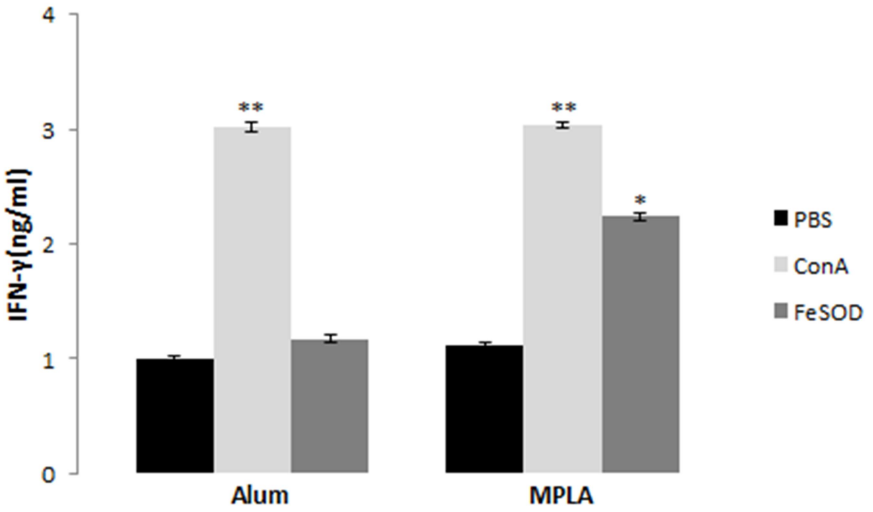


Figure 3.24. IFN-γ production by spleen cells of mice vaccinated with FeSOD-Alum and FeSOD-MPLA. PBS and ConA were used as negative and positive controls, respectively. The results are represented as mean ± SD (*$p<0.05$, **$p<0.01$).

3.9.3. Serum IL-10 Levels in Vaccinated Mice with Recombinant OmpQ, Putative Lpp and FeSOD

As known, cytokines have a significant role in immune responses after vaccination and natural infection (Higgs *et al*., 2012). While pro-inflammatory cytokines including IL-2, IFN-γ and IL-12 mainly induce activation and

recruitment of immune cells such as macrophages and neutrophils, immunoregulatory cytokines including IL-10 regulate functions of these cells and production of pro-inflammatory cytokines. IL-10 is produced upon activation of Th2 cells and IL-10 suppressive action provides protection against collateral damage mediated by pathogen-stimulated inflammatory responses (Fiorentino *et al*., 1991; Fillatreau *et al*., 2002; Higgins *et al*., 2003). A study conducted by Gazzinelli *et al*. (1996) revealed that IL-10 knockout mice were killed during acute infection by *Toxoplasma gondii* whereas mice able to produce IL-10 survived. It was proposed that mouse death was due to immune hyperactivity associated with high level of IFN-γ and IL-2, suggesting an important role of IL-10 in regulation of immune response. Similar results were also obtained by Suzuki *et al*. (2000) in IL-10 knockout mice.

IL-10 production reflects activation of Th2 type response in vaccine studies (Hayes *et al*., 2011). In a study conducted in a murine model, it was demonstrated that IL-10 level as well as IFN-γ level increased after second vaccination with a newly developed tri-component DtaP vaccine which conferred protection against *B*. *pertussis*. Increased IL-10 and IFN-γ levels reflected activation of both Th1 and Th2 type responses, respectively (Huh *et al*., 2017). Similarly, another study conducted with a newly discovered antigen in *B*. *pertussis* revealed high IL-10 production in vaccinated mice in addition to high level of IFN-γ, suggesting a Th1/Th2 mixed response (Hayes *et al*., 2011).

To elucidate whether recombinant OmpQ, putative Lpp and FeSOD trigger Th2 type response, IL-10 levels in the sera of mice vaccinated with the recombinant proteins adjuvanted with alum or MPLA were evaluated. In OmpQ-Alum or -MPLA and putative Lpp-Alum or -MPLA vaccinations, there were no significant differences between control groups and vaccinated mice (Figure 3.25 and 3.26). On the other hand, a significant increase in FeSOD-MPLA

vaccination was observed when compared to control group and FeSOD-Alum vaccination (Figure 3.27).

When both IL-10 and IFN-γ levels are considered, it can be suggested that FeSOD-MPLA can induce a mixed Th1 and Th2 type response which plays an important role in fighting with *B*. *pertussis*.

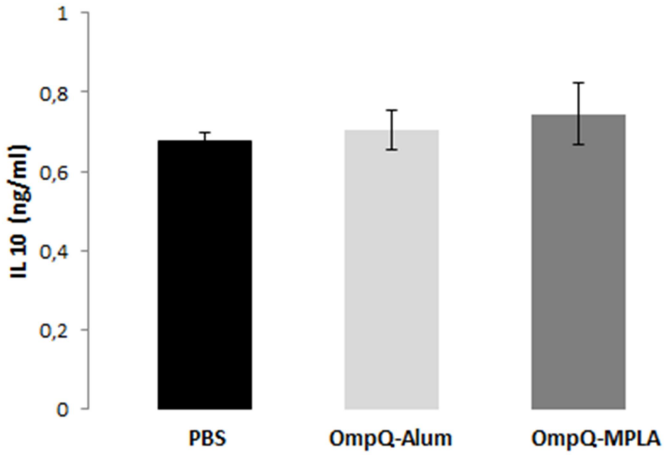


Figure 3.25. Serum IL-10 level in mice vaccinated with OmpQ-Alum and OmpQ-MPLA. The results are represented as mean ± SD.

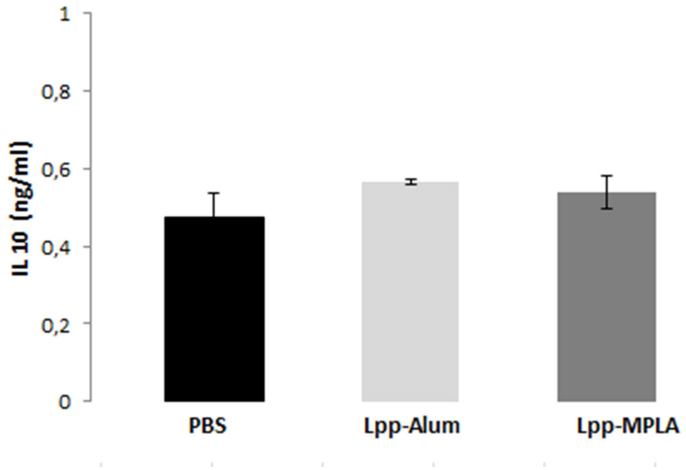


Figure 3.26. Serum IL-10 level in mice vaccinated with putative Lpp-Alum and Lpp-MPLA. The results are represented as mean ± SD.

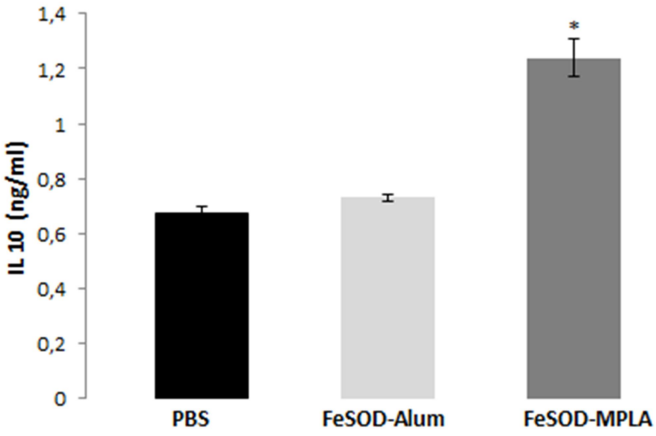


Figure 3.27. Serum IL-10 level in mice vaccinated with FeSOD-Alum and FeSOD-MPLA. The results are represented as mean ± SD (*$p<0.05$).

3.9.4. *Bordetella pertussis* Colonization in the Lungs of Mice Immunized with Recombinant OmpQ, Putative Lpp and FeSOD

It has been well-known that elimination of *B. pertussis* requires activation of cell-mediated immunity which mainly includes stimulation of Th1 cells and production of IFN-γ and IL-12. For clearance of this invasive pathogen from macrophages and lungs, antimicrobial effector molecules are produced by phagocytic cells activated by IFN-γ.

In protection studies related with *B. pertussis* infection, determination of bacterial colonization in the lungs of mice is an effective way to elucidate protective immunity against the pathogen because of the presence of a direct correlation between them (Kimura *et al*., 1990; Roduit *et al*., 2002; 1990; Marr *et al*., 2008; Hayes *et al*., 2011; Hayes *et al*., 2013). It has been suggested that aP vaccine prevents severe infections but is not efficient to inhibit bacterial colonization of the respiratory tract unlike natural infections or DTwP vaccination, constituting a rationale to improve current aP vaccines (Allen and Mills, 2014; Warfel *et al*., 2014).

When the results of bacterial colonization assays were analyzed, it was apperant that neither OmpQ nor putative Lpp by alone adjuvanted with alum or MPLA induced a significant decrease in bacterial colonization in the mice lungs although a slight but not significant decrease was present at day 14 upon OmpQ-MPLA immunization (Figure 3.28 and 3.29). The lack of a significant decrease in bacterial colonization might be related with the low level of IFN-γ as it is crucial for elimination of *B. pertussis* from the lungs. There are also examples in the literature for the proteins which were found to be highly immunogenic, but could not confer protection by alone against the relevant infection such as P55 of *Borrelia burgdorferi*, gpA of *Pneumocystis carinii* and human

metapneumovirus G protein (Feng *et al*., 1996; Gigliotti *et al*., 1998; Ryder *et al*., 2010).

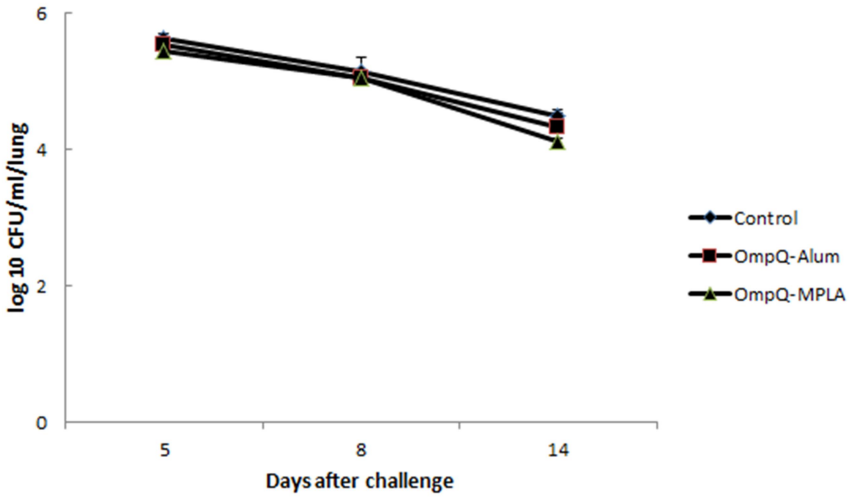


Figure 3.28. Mean numbers of CFU/mL/lung of groups of mice intraperitoneally vaccinated with OmpQ-Alum, OmpQ-MPLA and PBS as a negative control. The results are presented as mean CFU/mL/lung ±SD.

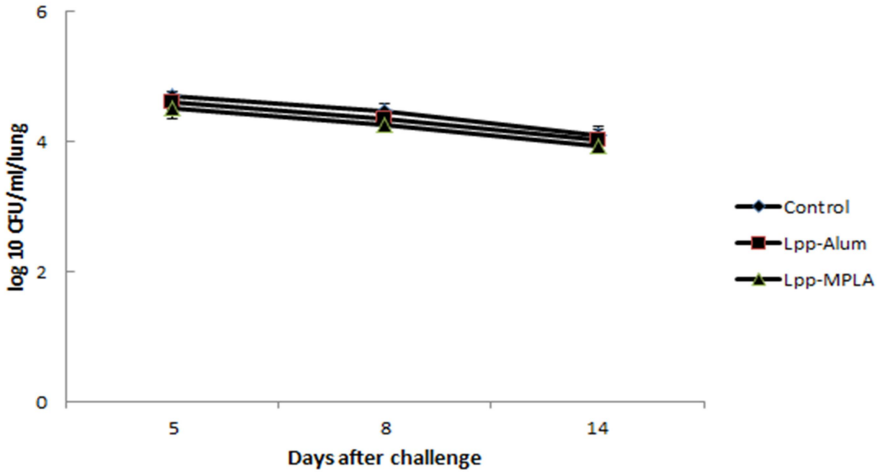


Figure 3.29. Mean numbers of CFU/mL/lung of groups of mice intraperitoneally vaccinated with putative Lpp-Alum, putative Lpp-MPLA and PBS as a negative control. The results are presented as mean CFU/mL/lung ±SD.

As stated earlier, current aP vaccines are well known to induce limited Th1-type immune response accounting for the need to novel vaccine formulations to induce protective immunity in infants in the earliest stages of life (Garcia *et al*., 2011). In one study, while the vaccination of mice with recombinant autotransporter protein BrkA of *B*. *pertussis* as the only *B*. *pertussis* antigen did not protect against colonization by *B*. *pertussis*, it significantly increased the efficacy of two-pertussis-component DTaP vaccine (PT, FHA) against *B*. *pertussis* in a sublethal intranasal murine respiratory challenge model (Marr *et al*., 2008). Its inclusion led to an antigen combination which was as efficacious in protecting mice as the commercial Infranrix^{TM} vaccine that also contains PT and FHA but PRN instead of rBrkA. The authors suggested that BrkA is a promising candidate antigen to improve existing aP vaccines for use in humans. Although we did not test the protective efficacy of recombinant OmpQ and/or Lpp BP2919 when combined to two-or more-pertussis-component DTaP

vaccine, our findings do not exclude the protective effectiveness of these proteins since high amounts of IgG2a antibodies (high IgG2a/IgG1 ratio) induced by OmpQ and Lpp is likely indicative of the existence of Th1-type of immune response (van der Berg *et al*., 2000). T-cell derived cytokines have distinct roles in immunoglobulin class swiching, with Th1 cytokines associated with IgG2a and Th2 with IgG1 (Barnard *et al*., 1996). IgG2a regulates immune responses to soluble antigens by targeting antigent to activating FcR_{γ} chain-containing receptors which enhances antibody as well as T cell responses (Getahun *et al*., 2004). A slight but not significant increase in IFN-γ levels observed in MPLA formulations may be related with high IgG2a levels in these formulations since it is known that class switching to IgG2a can be induced by even low IFN-γ level.

The result of bacterial colonization assay for FeSOD is presented in Figure 3.30. There was a significant reduction in bacterial colonization in FeSOD-MPLA vaccinations, possibly due to the high levels of IFN-γ production induced by FeSOD-MPLA immunization. Considering the high levels of IL-10 and IFN-γ, it can be suggested that FeSOD-MPLA triggered both Th1 and Th2 immune responses that promote elimination of the pathogen. No significant elimination with FeSOD-Alum vaccination, on the other hand, indicated that alum is not an ideal adjuvant for pertussis vaccinations.

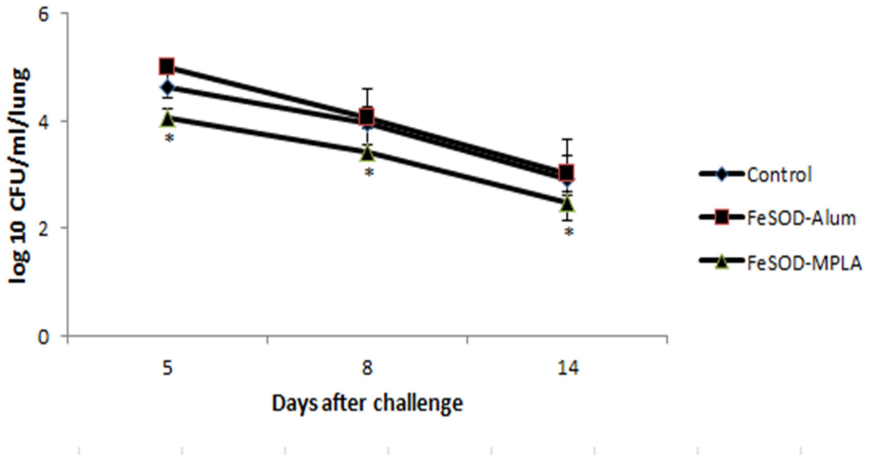


Figure 3.30. Mean numbers of CFU/mL/lung of groups of mice intraperitoneally vaccinated with FeSOD-Alum, FeSOD-MPLA and PBS as a negative control. The results are presented as mean CFU/mL/lung ±SD.

CHAPTER 4

CONCLUSIONS

- All formulations with recombinant proteins (OmpQ-Alum, OmpQ-MPLA, putative Lpp-Alum, putative Lpp-MPLA, FeSOD-Alum and FeSOD-MPLA) induced higher level of IgG1 and IgG2a responses in the sera of immunized mice than the control group immunized with PBS.

- Antibody responses were higher in second vaccinations when compared to first vaccinations in all cases. When antibody responses were compared in terms of adjuvants, it was shown that IgG2a response was predominant in MPLA formulations while IgG1 was the main type of antibody found in vaccinations with alum formulations.

- IgG1 and IgG2a levels triggered by FeSOD-Alum and FeSOD-MPLA were quite high when compared to putative Lpp and OmpQ. Moreover, FeSOD-MPLA induced a more balanced IgG1:IgG2a response.

- IFN-γ levels were measured to reveal the level of cell-mediated immune response against pertussis. There was no significant difference between control groups and mice immunized with recombinant OmpQ-Alum, OmpQ-MPLA, putative Lpp-Alum and putative Lpp-MPLA. However, a significant increase in IFN-γ level was observed in the case of FeSOD-MPLA vaccination while FeSOD-Alum did not induce IFN-γ

production, suggesting that FeSOD-MPLA is able to induce Th1 type response.

- As to the IL-10 levels, the results were similar to those obtained for IFN-γ levels. While OmpQ-Alum, OmpQ-MPLA, putative Lpp-Alum, putative Lpp-MPLA and FeSOD-Alum vaccinations induced no significant increase in IL-10, there was a significant increase in IL-10 level upon FeSOD-MPLA vaccination compared to control group, suggesting that FeSOD-MPLA can induce Th2 response besides Th1 response.

- Bacterial colonization assays demonstrated that there was no significant reduction in bacterial lung colonization upon the vaccinations with OmpQ-Alum, OmpQ-MPLA, putative Lpp-Alum, putative Lpp-MPLA and FeSOD-Alum although a slight but insignificant decrease was present in OmpQ-MPLA immunization at day 14. On the other hand, FeSOD-MPLA vaccination induced a significant reduction in lung colonization when compared to the control group, suggesting that activation of both Th1 and Th2 type immune response by FeSOD-MPLA promoted elimination of the pathogen.

- The lack of protection with recombinant OmpQ and Lpp formulations in our mice lung colonization model does not exclude a possible positive effect of these proteins on the efficacy of final vaccine when combined with PT only or PT plus other antigens. Moreover, OmpQ-MPLA and Lpp-MPLA in particular could induce high amounts of IgG2a antibodies (high IgG2a/IgG1 ratio) which is indicative of the existence of Th1-type immune response which is thought to result from a slight increase in the level of IFN-γ.

- The data, overall, indicated that FeSOD is a very promising candidate component and MPLA is a proper adjuvant for the development of third generation pertussis vaccines.

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APPENDIX A

COMPOSITIONS OF BUFFERS AND SOLUTIONS

TEN Buffer

40 mM Tris

1 mM EDTA

150 mM NaCl

TAE Buffer (50X)

Tris base	242 g
Glacial acetic acid	57.1 ml
0.5 M EDTA (pH 8.0)	100 ml

Loading Buffer (6X)

0,25% Bromophenol blue

0,25% Xylene cyanol FF

40% (w/v) Sucrose in water

Buffer 1

100 mM RuCl

30 mM KAc

10 mM $CaCl_2$

15% Glycerol

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

Buffer 2

75 mM $CaCl_2$

10 M RuCl

10 M MOPS

15% Glycerol

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

IPTG (Isopropyl-β-D-thiogalactoside)

IPTG	100 mg
Distilled water	1 ml

Filtered and stored at at -20° C

TBS (1X) (1000ml)

Tris	2.42 g
NaCl	29.2 g

STE Buffer

10.3% Sucrose

25 mM EDTA pH:8.0

25 mM Tris-HCl pH:8.0

2mg/ml Lyzozyme

Lysis Buffer

0.3 M NaOH

2% SDS (w/v)

PBS (1X) (1000ml)

NaCl	8 g
KH_2PO_4	0.2 g
Na_2HPO_4. $7H_2O$	2.17 g
KCl	0.2 g

Denaturing Solubilization Buffer (pH:8.0)

50 mM NaH_2PO_4

1 M NaCl

8 M Urea

Denaturing Elution Buffer (pH:8.0)

50 mM NaH_2PO_4

1 M NaCl

8 M Urea

250 mM Imidazole

Dialysis Buffer (pH:8.0)

50 mM NaH2PO4

500 mM NaCl

4 M Urea

Carbonate/Bicarbonate Buffer (1000ml, 0,05M, pH:9.6)

Na_2CO_3	1,59 g
$NaHCO_3$	3.88 g

Washing Solution (pH:7.2)

1X PBS

0.1% Tween 20

Blocking Buffer

4 % Bovine Serum Albumin, 5 % Sucrose in PBS

Running Buffer (5X) (1000ml, stored at 4°C)

Tris base	15 g
Glycine	72 g
SDS	5 g

Fixation Buffer

40 % Ethanol

10 % Acetic Acid

50 % dH2O

Coomassie Blue R-250 Staning

Coomassie blue R-250	0.25 g
Methanol	125 g
Glacial acetic acid	25 ml
dH_2O	100 ml

Transfer Buffer (1X)

20% Methanol

25 mM Tris

192 mM Glycine

0.037% SDS

APPENDIX B

COMPOSITIONS AND PREPARATION OF CULTURE MEDIA

Modified Cohen-Wheeler Medium for *Bordetella pertussis* (pH 7.2 - 7.5) (1000ml)

Bacto-agar	20 g
NaCl	2.5 g
KH_2PO_4	0.5 g
$MgCl_2.6H_2O$	0.4 g
Soluble starch	1.5 g
Yeast extract	2 g
Casamino acid	10 g
$CaCl_2$ (1%)	1 cc
$FeSO_4.7H_2O$ (0.5%)	2 cc
L-cystein	2.5 cc
$CuSO_4.5H_2O$	1 cc
Active coal	4 g
NaOH (26%)	for pH adjustment

- Mix all of them and autoclave

Luria Bertani (LB) Medium (1000ml)

Tryptone	10 g
Yeast Extract	5 g
NaCl	5 g

- Complete to 1000ml with distilled H_2O and autoclave

Luria Bertani (LB) Agar Medium (1000 ml)

Tryptone	10 g
Yeast Extract	5 g
$NaCl_2$	5 g
Agar	15 g

- Complete to 1000ml with distilled H_2O and autoclave

APPENDIX C

CHEMICALS AND THEIR SUPPLIERS

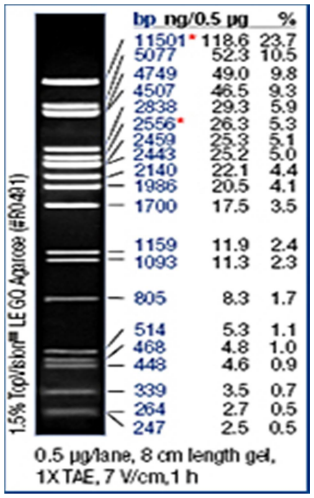
Chemicals	**Supplier**
Agar Bacteriological	Merck
Agarose	Prona
Ampicillin	Sigma
Bovine Serum Albumin	Sigma
Bromophenol Blue	Sigma
Casamino Acid	Sigma
Cephalexin	Sigma
Coomassie Brilliant Blue R-250	Sigma
dNTPs	Fermentas
Dimethylformamide	Merck
EDTA	AppliChem
Ethanol	Botafarma
Ethidium Bromide	Sigma
Glacial Acetic Acid	Merck
Glycerol	Merck
HCl	Merck
Imidazole	Merck
IPTG	Sigma
Kanamycin	Sigma
KH_2PO_4	Merck

KCl	Merck
L-cystein	Sigma
Ligase Buffer (2X)	Fermentas
Ligase Buffer (10X)	Fermentas
Luria Broth	Q-Biogene
Methanol	Merck
$MgCl_2.6H_2O$	Merck
NaCl	Merck
Na_2CO_3	Merck
$NaHCO_3$	Merck
NaH_2PO_4	Merck
NaOH	Merck
Phenol	Merck
Rubidium chloride	Merck
SDS	Merck
Skim Milk	Fluka
Sucrose	Merck
Tris-base	Merck
Urea	Fluka
X-gal	Fermentas
Yeast Extract	Difco

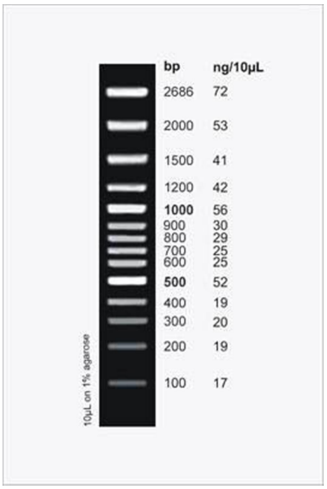
Enzymes	**Supplier**
*BamH*I	Fermentas
*Bgl*II	Fermentas
*EcoR*I	Fermentas
Lysozyme	AppliChem
T4 DNA Ligase	Fermentas
Tag DNA Polymerase	Fermentas

APPENDIX D

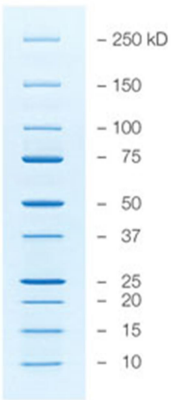
MARKERS



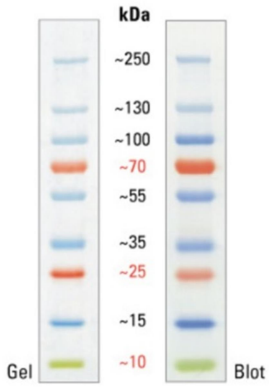
Lambda DNA/*Pst*I Marker (Fermentas #SM0361)



CloneSizer 100 bp DNA Ladder (Norgen Biotet, Cat. 11600)



Precision Plus Protein Unstained Protein Standards (Bio-rad, #1610363)



PageRuler Plus Prestained Protein Ladder (ThermoFisher, #SM1811)

VITA

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PERSONAL INFORMATION

Date of Birth	2 April 1985
Born in	Ankara/ Turkey
Gender	Female

DEGREES

2011 - 2017	Ph.D., Department of Biological Sciences, METU
2008 - 2011	M.Sc., Department of Biological Sciences, METU
2003 - 2008	B.Sc., Department of Biological Sciences, METU

WORK EXPERIENCES

06/2017 - Present	Research/Teaching Assistant, Amasya University, Turkey
02/2011- 06/2017	Research/Teaching Assistant, METU, Turkey
2013 - 2015	Advisor Assistant, iGEM-METU Team, Turkey
2007	Internship, Emory University, Atlanta, USA

2007	Internship, Ulus State Hospital, Ankara, Turkey
2006	Internship, Ahmet Andiçen Oncology Hospital, Ankara, Turkey

SKILLS

Highly experienced:

- Microbiological techniques including aseptic techniques, colony counting, inoculation of samples into agar plates and broth, preparation and sterilization of culture media and laboratory equipment, staining microorganisms, biochemical characterization and microscopic examination of microorganisms, maintenance and storage of bacterial cultures.
- Molecular biology techniques including PCR, primer design, DNA and plasmid isolation, restriction enzyme digestion, gene cloning, bacterial transformation, agarose gel electrophoresis, SDS-PAGE, ELISA, cytokine assay, Western blotting, recombinant protein expression and purification.
- Teaching and organizational skills and excellent communication skills with students gained through the experiences in microbiology laboratory and molecular genetics laboratory.
- Writing and management of projects for Scientific and Technological Research Council of Turkey

Experienced:

- Mouse dissecting and injection, microscopy.

Familiar:

- Proteome, immunoproteome and cell culture.

THESES:

- **M.Sc. Thesis:** Immune responses against the recombinant FimX and putative peptidyl-prolyl cis-trans isomerase from *Bordetella pertussis*

- **Ph.D. Thesis:** Assessment of immune protective capacities of the recombinant outer membrane protein Q (OMPQ), putative Lipoprotein and Iron Superoxide Dismutase (FeSOD) from *Bordetella pertussis*

PUBLICATIONS:

- Akcay U. C., Ercan O., Kavas M., Yildiz L., Yilmaz C., Oktem H. A., Yucel M. (2010). Drought-induced oxidative damage and antioxidant responses in peanut (*Arachis hypogaea* L.) seedlings. Plant Growth Regul. Volume 61, 21-28.
- Yılmaz Ç., Aycan A., Özcengiz E., Özcengiz G. (2016) Immunogenicity and protective efficacy of recombinant Iron Superoxide Dismutase protein from *Bordetella pertussis* in mice models. Microbiology and Immunology, 60(11), 717-724.
- Yılmaz Ç., Özcengiz G. (2017). Antibiotics: pharmacokinetics, toxicity, resistance and multidrug efflux pumps. Biochemical Pharmacology, 133, 43-62.

CONGRESS ABSTRACTS, WORKSHOPS, SEMINARS:

Poster presentation:

- Kuraklık stresinin yerfıstığı (*Arachis hypogea* L.) antioksidatif savunma sistemi üzerindeki etkisi. XXI. National Biochemistry Congress (29-31 October 2009, İstanbul, Turkey), Turkish Journal of Biochemistry, pp. 85.

- *Bordetella pertussis* Tohama I ve Saadet Suşuna Ait PPIase, POMP ve FIMX Genlerinin Klonlanması. XVI. National Biotechnology Congress (13-16 December 2009, Antalya, Turkey) Papers and Poster Abstract Book, pp. 71-72.
- Assessment of immune protective capacities of the recombinant Fimbrial Protein X (FimX), Glutamine Binding Periplasmic Protein (GlnBP) and Iron Superoxide Dismutase (FeSOD) from *Bordetella pertussis*. FEMS 2013 5. Congress of European Microbiologists (21-25 July 2013, Leipzig, Germany), Congress Book, p.307.
- Assessment of immune protective capacity of the recombinant Iron Superoxide Dismutase (FeSOD) from *Bordetella pertussis*. FEMS 2015 6. Congress of European Microbiologists (7-11 June 2015, Maastricht, The Netherlands), Congress Book, p.181.
- Bacilysin is a small, fine-tuning effector of mother cell compartment-specific σE and σK regulons in *Bacillus subtilis*. 19. International Conference on Bacilli & Gram-Positive Bacteria (11-15 June 2017, Berlin, Germany), GPC, Abstract book, P12.
- *Bordetella Pertussis*'e ait rekombinant dış membran protein Q, demir süperoksit dismutaz ve putatif lipoproteinin immün koruyucu kapasitelerinin değerlendirilmesi. 6. National Molecular Biology and Biotechnology Congress (5-7 October 2017, Adana, Turkey), Abstract book, p.49.

Participant:

- Workshop on the Story of Biomaterials from Design to Manufacturing, Participant (16 December 2013, METU, Ankara, Turkey).
- Meeting 'Nanotechnology & Tissue Engineering: Current Challenges and Future Prospects', Participant (20-21 December 2013, İstanbul Kultur University, İstanbul, Turkey).

- 8. Aykut Kence Evolution Conference, Participant (26-27 April 2014, METU, Ankara, Turkey).
- Workshop on Micro-Nanobiomaterials and Nanomedicine Applications, Participant (26 December 2014, METU, Turkey).
- 11. Aykut Kence Evolution Conference, Participant (18-19 February 2017, METU, Ankara, Turkey).

PROJECTS

- TÜBİTAK TBAG 112T916: Assessment of Immune Protective Capacities of the Recombinant Fimbrial Protein X (FimX), putative peptidyl prolyl cis-trans-isomerase (PPPIase) Glutamine Binding Periplasmic Protein (GlnBP), Putative Peptidolgycan Binding Protein, and Chaperone 10 (Hsp 10) from *Bordetella pertussis* (April 2013 – April 2015).
- Amgen Biotech Experience, Biotechnology Lectures for High School Students, Turkey Pilot Application Projects (September 2016 - January 2017).

SCHOLARSHIPS

- 2211-C TÜBİTAK doctoral fellowship program for primary research areas (2014-2017).
- TÜBİTAK TBAG 112T916: Assessment of Immune Protective Capacities of the Recombinant Fimbrial Protein X (FimX), putative peptidyl prolyl cis-trans-isomerase (PPPIase) Glutamine Binding Periplasmic Protein (GlnBP), Putative Peptidolgycan Binding Protein, and Chaperone 10 (Hsp 10) from *Bordetella pertussis* (2013-2014).

- TÜBİTAK TBAG 108T937: Molecular dynamics of heavy metal stress response in *Phanerochaete chrysosporium*: Time dependent cytosolic proteome, plasma membrane proteome and phosphoproteome (2009-2011).

AWARDS

- METU Scientific Publication Award, 2010, 2016

CERTIFICATE

- Use of Experimental Animals Certificate (2 December 2011, Gülhane Military Medical Academy, Turkey)